The diversity of silica-scaled protists

Thesis submitted for the degree of Doctor of Philosophy at the University of Oxford

Trinity Term 2013

Josephine Margaret Scoble

Green Templeton College

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ABSTRACT

This thesis investigates the diversity of two silica-scaled protist groups, Paraphysomonadida and Thaumatomonadida by light and electron microscopical observations and sequencing (rDNA) on novel clonal cultures. Despite these groups of protist dominating pelagic, littoral as well as inland freshwater and soil habitats, they are taxonomically poorly understood to the extent that any progress in ecological theory is hampered. Now that environmental DNA sequencing is being carried out faster than we can characterise protists from culture it is important that we understand how molecular and physical diversity match up, especially because so many protists are morphospecies.

Nearly one hundred isolates were cultured on which both morphological and molecular data was carried out in parallel to reveal around 50 new species of protist from eight different genera: two heterokont genera, *Paraphysomonas* and *Incisomonas* n. gen., and six cercozoan genera, *Thaumatomonas, Allas, Reckertia, Thaumatospina* n. gen., *Cowlomonas* n. gen., and *Scutellomonas* n. gen.

These data make major contributions to taxonomy and understanding aspects of protist diversity where previously morphological diversity was heavily biased towards over-generalized morphotypes. This thesis quickly showed that gross lumping of morphospecies was true of *Paraphysomonas*, for which many of the isolates cultured herein might have been regarded as one species (not more than 20). The many cultured isolates exhibited varied cell and scale morphology, and by sequencing (rDNA), it was possible to see the evolution of scale morphology map on to trees. This marriage of molecular and morphological data made it possible to view distinct groups of species that shared scale detail that might have otherwise been overlooked had either method been used alone.

This research has shed significant light on how scale morphology can be used as reliable taxonomic marker for protists, the insights of which can be applied to make taxonomic improvements to other silica-scaled protist groups.

Declarations of authorship and contributions.

Declaration of authorship

In accordance with University of Oxford's Notes of Guidance for Research Examinations (GSO.20a); *I declare that there are no parts of this thesis or its research herein that have been reproduced or accepted for another award or degree or diploma at any other university or learning institution. This thesis contains no other person's work except where stated in text.*

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Word count Break down of the word count for each section of the thesis is stated below and excludes references, figure legends and tables.

Total word count is **72,622**: Chapter 1 (5,443), Chapter 2 (3,773), Chapter 3 (3,872), Chapter 4 (34, 001), Chapter 5 (23,051), Chapter 6 (2,520).

Permission to submit a thesis made of partly or all of publications/manuscripts

On the understanding that 50% or more of the work for the publication/manuscripts must have been done by me. Permission granted by John Iles, Director of Graduate Studies, Zoology Dept. Email dated: 26th October 2011. Contribution percentages detailed below.

Contributions to each chapter **(given as percentages)**

Each chapter written in the main body of this thesis was the responsibility of myself (JMS), and oversaw by my supervisor, Tom Cavalier-Smith (TCS).

Chapter 1: Introduction (not written for publication). 100% JMS

Chapter 2: Methods (not written for publication). 100% JMS

Chapter 3: Published paper

> **Scoble, JM and Cavalier-Smith T.** Molecular and morphological diversity of *Paraphysomonas* (Chrysophyceae, order Paraphysomonadida). Nova Hedwigia. 142, 117-126. **90**% JMS, 10% TCS.
>
> This review article was an invited piece after I presented at the Eighth International Chrysophyte Symposium (Prague, 2012), and is part of a special edition of Nova Hedwigia. I wrote the complete article after which TCS made a few changes; proof-read and added a few sentences.

Chapter 4: Paper for publication

> **Scoble, JM and Cavalier-Smith, T.** Scale evolution, sequence phylogeny, and revised taxonomy of chrysomonads: 35 new *Paraphysomonas* species, and new genera *Clathromonas*, *Patellomonas*, and *Acanthomonas*. Protist. *Submitted*.

This chapter of work is the collection of all the data accrued for the *Paraphysomonas* isolates that JMS cultured, sequenced and analyzed in the electron microscope. JMS obtained all of the raw data and produced all of the figures and plates and described them. The molecular analysis and production of trees was overseen by TCS for which JMS and TCS collaborated on inclusion of certain groups of taxa and for which types of phylogenetic tree analysis to perform. For this part of the work I attribute **90**% JMS, 10% TCS. The written manuscript was initiated and the first draft written by JMS after which TCS started a discussion section to which JMS contributed and concluded. The written work was **65**% JMS, 35% TCS.

Chapter 5: Paper for publication

Scoble, JM and Cavalier-Smith, T. Scale evolution, sequence phylogeny, and taxonomy of thaumatomonad Cercozoa: 11 new species and new genera *Scutellomonas*, *Cowlomonas*, *Thaumatospina* and *Ovaloplaca*. European Journal of Protistology. *Submitted*.

All of the data for the thaumatomonad work is contained within this chapter for which JMS did 100% of the laboratory work. The molecular analysis and production of trees was overseen by TCS for which JMS and TCS collaborated on inclusion of certain groups of taxa and for which types of phylogenetic tree analysis to perform. For this part of the work I attribute **90**% JMS, 10% TCS. The written manuscript was initiated and the first draft written by JMS after which TCS added to each section. The written work was **80**% JMS, 20% TCS.

Chapter 6: General discussion and conclusion (not written for publication). 100% JMS

Signed:

Josephine Scoble

Date:

Dedication

To my parents.

Unconditional love travels well across the

miles and the years.

Acknowledgements

In Oxford: Mike Shaw for use of the TEM. The TCS lab; David Bass, Ema Chao, Ed Glücksman, Libby Snell, Cedric Berney, Anna Maria Fiore-Donno, Marieta González, Lex Howe, Rhodri Lewis, Cecilia Martinezperez, Akinori Yabuki – all co-lab members at one time or another. The Zoology Dept.; Peter Holland, Adrian Smith and Aris Katzourakis for insightful chats and encouragement. Seb Shimeld for letting me use his lab. José Lourenço, for; lunch, tea, squash games, and eternal friendship. Jordi Papps and Alistair Seddon for technical advice and support. Green Templeton College staff, especially the porters in particular, Kevin, and senior tutor, Chris Sauer. The students of GTC, especially the GCR committee 2009-2011, but most of all Kerry Mauchline, who got me through some of the toughest times of my life to date. Shauna Monkman, a new friend and nurse. Rebecca Tuck and Dr. Michelle Taylor for proof-reading. The university sports teams: OUSRC and OUWPC for giving me the chance to 'Beat the TABS!'

In Canada. Alastair Simpson, Sina Adl and Andrew Roger, for making my trip to Dalhousie, Halifax, Canada, so welcoming and comfortable. Everyone was so kind and helpful especially, Alastair with whom I discussed my work, Marin Kolisko for introducing me to PhyloBayes. To everyone else at 'Dal', Michelle Leger, Courtney Stairs, Aaron Heiss, Dan Gaston, Eleni Gentekaki and (husband Manoli), Gordon Lax, Matt Brown. Shehrebanoo 'Banoo' Malik Pightling, Gillian (and Adam) Gile, Susana Breglia and Claudio Slamovits. In Vancouver at UBC, Naoji Yubuki, thank you for your friendship and encouragement, you are an inspiration to me.

Funding bodies: Thank you to the BBSRC, for my 4-year stipend and travel allowance, to CIFAR for the funds to go to Dalhousie, NS, Canada. Green Templeton College, Oxford, for numerous travel funding and final year living expenses.

Finally, Tom Cavalier-Smith, thank you for inviting me to work with you on such a great project. It has been an honor and a delight to work with you, a man who has as much passion as a fresh and keen graduate learning about protists and evolution for the first time. I hope, like you, to keep that youthful passion with me always about science. You have been an exciting mentor, your faith in me was regularly shown, especially during a difficult year in the middle of my degree, a tough time for you also, your kindness, and understanding will always be remembered. I hope to make you proud long after this DPhil.

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- **B.** NOVEL CULTURED PROTISTS IDENTIFYING DEEP-BRANCHING ENVIRONMENTAL DNA CLADES OF CERCOZOA: NEW GENERA *TREMULA, MICROMETOPION, MINIMASSISTERIA, NUDIFILA, PEREGRINIA.* HOWE AT, BASS D, **SCOBLE JM**, LEWIS R, VICKERMAN K, ARNDT H, CAVALIER-SMITH T. (2011). **PROTIST**. 162, 332-372

Conference participation

TALKS AND POSTERS

2012

August. **Talk**. Eighth Chrysophyte Symposium. 'New insights into colourless scaly chrysophyte taxonomy; 'Butchering' the species-rich and morphologically diverse Paraphysomonas to make three new genera, *Clathromonas*, *Acanthomonas* and *Patellomonas*.' Charles University, Prague. Czech Republic.

2011

April. **Poster**. British Society of Protist Biology (BSPB) 'Diversity of *Thaumatomonas.*' Southampton, UK.

July. **Talk**. International Society of Protistology (ISoP) with Phycological Association of America (PSA), joint meeting. 'Remarkable genetic and silica-scale diversity in the colourless chrysomonad, *Paraphysomonas*: taxonomic and evolutionary implications.' Seattle at the University of Washington, USA.

July. **Talk**. European Congress of Protistology (ECoP).'Remarkable genetic and silica-scale diversity in the colourless chrysomonad, *Paraphysomonas*: taxonomic and evolutionary implications.' Free University of Berlin, Germany.

2010

May. **Talk**. Green Templeton College 2nd year DPhil presentations. The 'scale' of morphology and molecular diversity in free-living protists. Oxford, UK.

July. **Talk**. International Society of Evolutionary Protistology (ISEP). 'Biodiversity of thaumatomonads using rDNA sequencing and silica scale morphology.' Kanazawa, Japan.

July. **Talk**. International Society of Protistology (ISoP). 'Biodiversity of thaumatomonads using rDNA sequencing and silica scale morphology.' Canterbury, UK.

October. **Attendee**. Biological diversity in a changing world. The Royal Society, London. UK

October. **Talk**. University of Oxford Zoology Department Freshers' induction symposium. 'Biodiversity of thaumatomonads using rDNA sequencing and silica scale morphology.' Oxford. UK.

December. **Poster**. Young Systematists Forum. London Natural History Museum, UK. 'Morphology and phylogenetics of silica-scaled protists reveal increased diversity'

2009

December. **Poster**. Young Systematists Forum. 'Biodiversity of the silica-scaled chrysophyte, *Paraphysomonas*.' London Natural History Museum. UK

RESEARCH PLACEMENT

Research placement. 2011. May-June. Dr. Alastair Simpson's Laboratory at Dalhousie University. Halifax, Canada. During my stay I gave a departmental **talk** addressed to several Dalhousie laboratories; 'The diversity of a Chrysophyte genus; *Paraphysomonas.*'

CHAPTER ONE

General introduction

> *"…I look at the term species as one arbitrarily given for the sake of convenience, to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety (diversity), which is given to less distinct and more fluctuating forms. The term variety (diversity), again, in comparison with mere individual differences, is also applied arbitrarily, for convenience' sake"*
>
> (p. 45. Charles Darwin, *On the Origin of Species.*)

This thesis explores the diversity and improves the taxonomy of two silica-scale-bearing protist groups, Paraphysomonadida and Thaumatomonadida, by clonal culturing, light and electron microscopy, and molecular methods.

1. Background

Scales are present in many lines of the eukaryotic tree found in Unikonts in Amoebozoa (Kudryavtsev and Pawlowski 2013) and Corticates, in Hacrobia, (Heliozoa) (Cavalier-Smith and von der Heyden 2007) and in Harosa, Heterokonta (Scoble and Cavalier Smith 2013) and Rhizaria (Howe et al. 2011) as carbohydrate or mineralized (silicified and calcified) structures. Scales are a convenient morphological character used to identify cells to species-level providing good relic evidence of their ecological presence because they remain in sediments for months after the organism dies (Finlay and Clarke 1999b).

The scales observed in this thesis are made of silica and are formed within the cell inside silica deposition vesicles (SDV). Paraphysomonad SDVs are closely associated with the Golgi-body (Hibberd 1979; Manton and Leedale 1961; Preisig 1994; Preisig and Hibberd 1983), whereas thaumatomonad scales are formed in vesicles attached to the mitochondria (Karpov and Zhukov 1987; Preisig 1994; Swale and Belcher 1974). Silicon is present in the environment as silicic acid, H_2O_3Si. Silica-scale producing cells are required to take up silicic acid actively from the environment because the concentration is too low to support passive transport, and have evolved trans-membrane proteins with highly specialized interaction with silica. Interestingly, silica transporter–type genes (SIT) genes are proposed to have undergone lateral gene transfer between choanoflagellates and heterokonts (Marron et al. 2013). The biochemical importance of cells that utilize silicic acid has been highlighted by the hypothesis of a 'silica pump' (Dugdale and Wilkerson 1998), which theorizes that the export of new biological material, such as carbon, nitrogen, and phosphates, from the mixed upper layer of the ocean is intimately coupled with silica export, something exemplified in the diatom community (Yool and Tyrrell 2003).

Paraphysomonas (Chrysophyceae: Heterokonta) was chosen as a target genus for this study because: (1) it is one of the most ecologically important freshwater and marine heterotrophic/phagotrophic groups, and (2) has been used as a model for freshwater (Boenigk et al. 2005; Véra et al. 2001) and marine ecology (Andersson et al. 2013; Caron et al. 1999); (3) this genus is morphologically heterogenous with a broad range of scale structures so much so that it should be split into more genera (Preisig and Hibberd 1982a, 1982b); (4), the few published 18S rDNA seqeuncesa show the same species in multiple parts of the tree (Andersen et al. 1999; Boenigk et al. 2005;

Cavalier-Smith and von der Heyden 2007; Lefranc et al. 2005) and (5) *Paraphysomonas* is common in the environment so finding new isolates should be relatively easy. In particular, this genus was chosen because it was used by Finlay and Clarke (1999b) to argue for protists being cosmopolitan (Finlay and Clarke 1999b). However, my supervisor, Tom Cavalier-Smith (T. C-S) suspected Finlay and Clarke's conclusions were flawed due to their data being based on inadequate taxonomy and gross lumping of morphospecies; this postulation came from T. C-S's experience of working with one of the authors, Clarke, on heliozoan scales (Cavalier-Smith and von der Heyden 2007). Therefore, improving the taxonomy of *Paraphysomonas* would benefit several fields of protistology.

A second silica-scaled protist group was chosen, *Thaumatomonas*, to see how the results compare to *Paraphysomonas*; they are in different classes, and phyla and infrakingdoms. Initially Heliozoa was chosen as the second target group because Sophie von der Heyden's (former doctoral student of T. C-S) had started designing primers and already produced some scale and molecular data (Cavalier-Smith and von der Heyden 2007), but also because Finlay and Fenchel (Finlay and Fenchel 2004; Finlay and Maberley 2000) used heliozoans, although much more cursorily, in their argument for ubiquity. In addition to isolating a few heliozoan cultures, I also isolated some cercozoans, which contributed to a paper in which I undertook the preliminary work for the second major experimental component of this thesis, Thaumatomonadida (given in Howe et al. 2011 - Appendix A). For that publication, I isolated novel strains of *Rhogostoma*, sequenced several of the species described therein, and undertook electron microscopy of scales of *Peregrinia* and *Reckertia* as well as contributing to the manuscript with figures and literature searches of original descriptions to help sort and

revise the taxonomy. After this contribution it was more practical to continue to work on the thaumatomonads, and being less well studied by sequencing than Heliozoa there was more opportunity for making important improvements to their taxonomy and phylogeny, and there was a good chance discoveries would be made.

2. The existing taxonomy and knowledge of two silica-scaled heterotrophic protists, *Paraphysomonas* and *Thaumatomonas*

Paraphysomonads, heterokont algae, Ochrophyta (formerly Ochrista Cavalier-Smith 1986), and thaumatomonads, Cercozoa, are both within Chromista (Fig. 1) (Cavalier-Smith 2010b). Chromista (Cavalier-Smith 1981) is a kingdom of life that differ from other protists in a key event in which Chromista evolved from a single evolutionary event in which a red algae was enclosed by the phagocytic vacuole membrane of the host cell, which later fused with the nuclear envelope leaving the alga and its plastid inside the rough endoplasmic reticulum. These corticates (evolved cortical alveoli and ciliary hairs) reside under the subkingdom Harosa.

2 a. *Paraphysomonas*

Chrysophyceae Pascher, 1914, included a broad range of flagellate cells autotrophic, heterotrophic, phagocytic, uniciliate and biciliate, colonial and non-colonial protists with or without scales, and split into three orders according to their cilia, Chromulinales (one cilium), Isochrysidales (two equal cilia) and Ochromonadales (2 unequal cilia). Bourrelly (1957) revised Ochromonadales to include all Chrysophyceae with two cilia, indicating that the *Ochromonas* type of organiszation is the basic form from which a large proportion of the Chrysophyceae is derived

(Bourrelly 1957; Hibberd 1976, 1979). The taxonomy of Chrysophyceae was altered and refined further by Hibberd (1979) and, where Bourrelly considered mainly external features visible under the light microscope, Hibberd extended the knowledge, particularly of the second flagellum ultrastructural features. Hibberd (1979) concluded with a basic organization of the Chrysophyceae *sensu stricto* exemplified by features of *Ochromonas danica*: two cilia obliquely inserted, the longer cilium bearing mastigonemes; flagellar swelling; flagellar roots, a larger cross-banded root or rhizoplast extending from the basal bodies deep in to the cell; nucleus, pyriform; eyespot, single layer of pigmented lipoidal droplet at edge of chloroplast; large Golgi body; cell covering, chrysophycean cells mostly naked but loricate common; silica-scales are produced and some are un-patterned and un-mineralized of unknown origin within the cell. Isochrysophycidae (Haptophyceae) and choanoflagellates were removed from the Chrysophyceae thus leaving a largely homogeneous group (Hibberd 1979). These seminal revisions by Hibberd (1979) were made by recognizing the differences in ultrastructure within a previously large and heterogeneous group. There have been further removals of groups of organisms to make new classes outside of Chrysophyceae, most notably Chrysomerophyceae (Cavalier-Smith 1995) and Hypogyristea (the suffix –phyceae was inappropriate as many members of the class are non-algal) (Cavalier-Smith and Chao 2006). The re-inclusion of Synurales within Chrysophyceae was made by Cavalier-Smith and Chao (2006) after it was removed previously (Andersen 1987; Cavalier-Smith 1986). Molecular phylogenetic trees support the holophyly of Chrysophyceae (Cavalier-Smith 1996), albeit with weak support for the evolutionary relationships between the orders that lie within it (Andersen et al. 1999; Cavalier-Smith and Chao 1996, 2006; Kim et al. 2010; Riisberg et

al. 2009). I refer to Chrysophyceae within the phylogenetic tree data as 'holophyletic' meaning a complete group of organisms in a dataset with one common ancestor and all its descendants, as opposed to using 'monophyletic' that includes their last common ancestor but not necessarily all its descendants (Ashlock 1971; Cavalier-Smith 2010a).

Paraphysomonas has had a turbulent taxonomic history within Chrysophyceae. Some strains that I studied were previously called *Spumella*-like because by light microscopy alone they could not be distinguished from the scale-less chrysophyte *Spumella* (Boenigk et al. 2005). The scaly family Paraphysomonadaceae (Preisig and Hibberd 1983) included photosynthetic *Spiniferomonas* and colony-forming *Chrysosphaerella.* The diversity of these '*Spumella*-like' cells is grossly underestimated, and molecular evidence has shown that they are polyphyletic across Ochromonadales and Paraphysomonadales (Boenigk et al. 2005). The taxonomic boundaries and relationships between orders are often debated and have had confused taxonomy, e.g., *Polylepidomonas vacuolata* was originally placed within *Paraphysomonas* even though it was photosynthetic and did not have spine scales (Thomsen et al. 1981). Also, there was controversy whether or not *Spiniferomonas* (Takahashi 1973) was distinct from *Paraphysomonas* (Nicholls 1985; Preisig and Hibberd 1982b; Siver 1987) because *Spiniferomonas* was isolated and found to be photosynthetic, like the type species, and other times not. Phylogenetic analysis shows that *Chrysosphaerella* is unrelated to *Paraphysomonas* (del Campo and Massana 2011). The placement of the class Chrysophyceae within the Heterokonta infrakingdom is outlined in Table 1.

Paraphysomonas is a speciose and highly polymorphic genus containing around

56 species; the scales of each species vary greatly in size and shape. The type species, *P vestita* (Stokes 1888), has spine scales but just 11 of the 56 known species have spine scales, with the remaining 45 having more complex woven and basket structures. Molecular data for this genus is limited with only five species being represented in 18S rDNA databases, four of which are spine scaled. Phylogenetic trees using 18S rDNA consistently showed *P. butcheri,* the only non-spine scaled (basket-scaled) *Paraphysomonas* species, to be the deepest branch of a holophyletic *Paraphysomonas* clade (Andersen 2007; Boenigk et al. 2005; Caron et al. 1999; Cavalier-Smith and Chao 2006; Lefranc et al. 2005; Richards et al. 2005), but other species, supposedly belonging to *P. foraminifera* and *P. vestita,* appeared to be polyphyletic. Also, it is clear that a sequence named '*P. butcheri*' published by Rice et al. (Rice et al. 1997) was from an isolate that closely resembles the type specimen (Pennick and Clarke 1972) because their electron microscopy show basket scales very similar to the type, however the plate scales are dissimilar. The cells and spine scales of the species of *Paraphysomonas* shown in Rice et al. (1997), *P. foraminifera, P. vestita, and P. imperforata,* do not completely match the type descriptions, especially in the case of *P. foraminifera*, where a concentric ring in-between four concentric rows of perforations should be present. (For a more in-depth review of *Paraphysomonas* see Chapter 3; a review, Scoble and Cavalier-Smith 2013).

Paraphysomonas scale data was used as evidence for ubiquity of protist morphospecies (Finlay and Clarke 1999b). A Cumbrian lake in the UK, Priest Pot – "a test bed for ubiquity/biogeography", is said to hold 78% of known *Paraphysomonas* species found in 0.1 cm^3 of lake sediment. It was noted that the most commonly

reported species within their sample, *P*. *vestita* and *P. imperforata*, were also the most commonly reported species globally. Their data was said to represent a relative pattern of global commonness for each of the species in the genus; less common in the lake meant less common globally. They stated that there was little diversity within the genus so the rates of speciation would be dampened if *Paraphysomonas* were allowed to move freely on a global scale, and that is why there was modest species richness (Finlay and Clarke 1999a). Furthermore, this study assumed the authors of their 73-paper literature survey all correctly identified the *Paraphysomonas* species described within. Finlay and Clarke (1999a) also imposed over-generalised criteria for identification by discounting size and variation in spine shape, and thus, in applying over-generalised criteria for the identification of scales (e.g., *P. vestita* = any scale with a thickened margin and tapering spine) this would naturally increase the rate at which you would find a morphotype. There is great variation in size of spine length reported for *P. vestita,* from 1.2 µm to 10 µm and the spine shape varies (Manton and Leedale 1961; Petronio and Rivera 2010). The same broad variation of morphospecies as seen for *P. vestita* is also seen in the literature for *P. imperforata* (Thomsen 1975). There are distinct scale-structures in the type specimen of *P. imperforata*; spine tip, baseplate shape and margin, but these characteristics were deemed not useful for distinguishing between this species and *P. vestita* (Thomsen 1975). There are examples of '*P. imperforata'* scales (originally a marine species) that are ten times the size of the original description and from freshwater and soil environments (Kling and Kristiansen 1983; Takahashi 1976; Thomsen 1975). This oversight of spine scale detail has possibly led to scores of species not being described but lumped inappropriately within *P. imperforata*. Gross lumping of morphospecies can be likely applied to all the

commonest *Paraphysomonas* species because the limits of scale variation within species have not been yet shown or commented on.

2 b. *Thaumatomonas*.

Thaumatomonads belong within the phylum Cercozoa (originally named 'Rhizopoda') (Cavalier-Smith 1998) and the subphylum Filosa (infraphylum Monadofilosa), which excludes lobose amoebae which are in a separate phylum Amoebozoa (Cavalier-Smith 1996/7). Cercozoa together with Foraminfera are 'core Rhizaria' and both phyla carry one or two polyubiquitin amino acid insertions that other eukaryotes do not have, and as a taxonomic group are well-known by being unified as a monophyletic assemblage on gene trees (SSU, alpha-tubulin, beta-tubulin and actin) (Archibald and Keeling 2004; Archibald et al. 2003; Bass et al. 2005; Cavalier-Smith 1998; Cavalier-Smith et al. 2008; Keeling 2001). The notable feature of Cercozoa is the diversity of body forms within the group: phototrophic and heterotrophic flagellates (gliding and swimming), parasitic, amoeboflagellates, and amoebae (Howe et al. 2011). This group often finds itself home to organisms of previous uncertain placement (Bass et al. 2009; Burki et al. 2002; Chantangsi et al. 2008) for example, Ebriids; with their long fossil record and internal siliceous skeleton, they have been associated with silicoflagellates, dinoflagellates and radiolarians but now found to have 18S rDNA associated with well-characterized cercozoans, *Cryothecomonas* and *Protaspa* (Hoppenrath and Leander 2006). In addition, the discovery of *Auranticordis quadriverberis* with its four cilia and putative endosymbionts has given more diversity to Cercozoa (Chantangsi et al. 2008).

Cercozoa group together a variety of cell-types from a broad range of environments (freshwater, marine, brackish and anoxic) and in particular the 18S rDNA diversity of flagellates cercozoans has been limited by uncharacterized environmental DNA (eDNA) sequences (Bass and Cavalier-Smith 2004; López-García et al. 2001; Moon-van der Staay et al. 2001; Stoeck and Epstein 2003), furthermore, eDNA primers specific to Cercozoa have amplified many new uncharacterized clades (Bass and Cavalier-Smith 2004). However, increasingly there are more cultured and thoroughly characterized cercozoan cells whose 18S rDNA sequences have helped place these eDNA sequences and infer more about evolutionary relationships between clades (Brabender et al. 2012; Chantangsi et al. 2008; Hoppenrath and Leander 2006; Howe et al. 2011; Ota et al. 2012; Shiratori et al. 2012; Wylezich et al. 2007).

The genus *Thaumatomonas* De Saedeleer (1931) belongs to the family Thaumatomonadidae Hollande (1952) within the order Thaumatomonadida Shirkina (1987), which together with Euglyphida group together as class Imbricatea (Cavalier-Smith and Chao 2003). Classes Imbricatea and Thecofilosea are each consistently holophyletic as well as sister to one another (Howe et al. 2011). Imbricatea have two orders that make scales; Thaumatomonadida and Euglyphida, but the class was redefined to also include the non-scaly orders Spongomonadida and Marimonadida (Howe et al. 2011).

The first naked thaumatomonad was recently characterized and sequenced and is phylogenetically sister to the scaled thaumatomonad *Peregrinia clavideferens* (Shiratori et al. 2012) indicating that there has been at least one evolutionary loss of scales within the order. Thaumatomonadidae was recently revised to include five

genera (Howe et al. 2011): *Thaumatomastix* Lauterborn (1899), *Reckertia* Conrad (1920), *Allas* Sandon (1927), *Thaumatomonas* De Saedeleer (1931), *Hyaloselene* Skuja (1956). *Rhizaspis* and *Protaspa,* formerly included (Howe et al. 2011) are now placed in a separate order Cryomonadida within the class Thecofilosea. Whilst thaumatomonads and other cercozoans are found in both freshwater and marine environmental habitats, and are believed to represent between 5 - 10% of flagellate abundance in benthic habitats (Al-Qassab et al. 2002; Arndt et al. 2000; Bass and Cavalier-Smith 2004; Wylezich et al. 2007), *Thaumatomonas* has never been recorded outside freshwater or soil, whereas *Thaumatomastix* are found in both (Nicholls 2012). There are only six *Thaumatomonas* species, *T. lauterborni* (De Saedeleer 1931)*, T. seravini* (Mylnikov and Karpov 1993), *T. zhukovi* (Mylnikov and Mylnikov 2003)*, T. coloniensis* (Wylezich et al. 2007), *T. oxoniensis* and *T. vancouveri* (Howe et al. 2011), so in contrast to *Paraphysomonas, Thaumatomonas* is not as speciose. *Thaumatomonas vancouveri* was described from a fixed culture and electron microscope (EM) sections, and *T. oxoniensis* from live cultures. Both these species have scales most similar to *T. coloniensis;* two-tiered oval plate scale with between three to five end-struts and an upper tier with lateral perforations. There were obvious 18S rDNA nucleotide differences between these three species as well as the ITS2 rDNA secondary structure (a rRNA folding method) confirming two compensatory base changes between *T. oxoniensis* and *T. vancouveri* (Coleman 2007), which deemed them different species (Howe et al. 2011).

There exist fourteen *Thaumatomonas* 18S rDNA sequences from cultured isolates, which represent five out of the six known species. However, a mislabeling of

sequences or cultures by different groups of researchers has led to confusion in *Thaumatomonas* phylogenies resulting in *T. seravini* being shown as polyphyletic despite the same clone having apparently been sequenced (AY496044 and AF411259) (Cavalier-Smith and Chao 2003; Ekelund et al. 2004; Wylezich et al. 2007). In addition, a '*Thaumatomastix* sp.' AF411261 (ATCC50250) was misidentified and is now confirmed to be a *Thaumatomonas* species, and sister clade containing thaumatomonad *Peregrinia* (DQ211593) was misidentified as *Gyromitus* sp. HFCC94 (Howe et al. 2011; Wylezich et al. 2007). Current 18S rDNA phylogenies of *Thaumatomonas* have not been able to fully resolve the mix-up of *Thaumatomonas* sequences or the paraphyly of *Thaumatomonas* sister to *Allas* (Shiratori et al. 2012). There is just one *Reckertia* sequence available (Howe et al. 2011) and a few *Thaumatomastix* sequences, which are paraphyletic (Chantangsi et al. 2010; Ota et al. 2012) so these data are not enough to fully resolve the evolutionary relationship between these genera of Thaumatomonadida, so more sequences and characterization of cultured isolates must be done. It is suggested that additional ITS rDNA sequencing would be a useful additional marker for resolving the relationship of thaumatomonads, added to which siliceous scales are an important character for delineation of species, with the proviso that these scales could be subject to environmental influences (Wylezich et al. 2007).

Differences in scale morphology clearly separate thaumatomonad genera and although *Thaumatomastix* with spine and plate scales was separated from *Reckertia*, which only had plate scales (Howe et al. 2011), *Thaumatomastix* is still a polymorphic (and molecularly paraphyletic) genus. Moreover, *Thaumatomonas* and *Allas* do not

have scales on their anterior cilium whereas *Reckertia* and some *Thaumatomastix* do, but these tiny cilium scales are only visible in the EM (Howe et al. 2011). There is evidently further taxonomic refinement of these genera that could be done.

3. Diversity

Protists are diverse in size, shape and function, and Cercozoa is a group noted for its vast array of cell types. Heterokonts too are a diverse group of flagellates, and along with Cercozoa, are some of the commonest protists in marine and freshwater habitats resulting in them being some of the most important ecological components of many microbial food webs (Arndt et al. 2000; Azam et al. 1983; Boenigk et al. 2001; Ekebom et al. 1995/6; Lee and Patterson 1998; Patterson and Lee 2000). The diversity of protists has been well reported using various methods of culturing, flow cytometry, different types of light and electron microscopy, and eDNA and culture gene sequencing analyses (Massana et al. 2009; Moreira and López-García 2002; Patterson and Simpson 1996; Preisig and Hibberd 1982a, 1982b). These techniques have highlighted the tip of the proverbial iceberg with regard to how much we know of the diversity of protists in that there are many cryptic species complexes. Cryptic species are a veritable 'black-box' of protists with ecological functions within yet undefined ecological niches. With particular attention to cercozoan and heterokont groups, I will introduce some aspects of protist diversity relevant to this thesis.

3a. Cryptic species, a gateway to protist diversity

The diversity of protists appears 'hidden' by morphospecies because of groups not having many distinguishable morphological characters; there are many un-

described species hiding behind a morphological façade that have not yet been recognized as units with tangible taxonomic differences (Cairns 1993). The biological diversity of protists, in general, is grossly underestimated by use of simple microscopical methods so cryptic species have been reported in abundance and are a well-recognized part of protist species complexes (Esteban et al. 2006), including chrysophytes (Fenchel 2005), and Cercozoa (Kühn et al. 2000).

There are so many species complexes within well known and abundant protists groups, especially through eDNA studies where, in spite of PCR bias (Richards and Bass 2005), group-specific primers still reveal large diversity through clades unidentified sequences (Bass and Cavalier-Smith 2004; Moon-van der Staay et al. 2001; Moreira and López-García 2002; von der Heyden and Cavalier-Smith 2005). This unexpected molecular diversity highlights how much we need to understand cryptic species and group them into units that are more meaningful.

3b. Environmental sequencing and ecological diversity

Analysis based only of eDNA has its drawbacks; there is no morphological data and clades often consist of just one sequence, so no robust inference of its relationship to known clades of morphology can be determined. PCR chimeras are of concern as they yield false sequences, which if left in analyses can be interpreted as novel lineages but despite this, many novel phylogenetic clades group within known eukaryotic lineages (Epstein and Lopez-Garcia 2007; Richards and Bass 2005). Anoxic environments hold rich microbial communities and yield many novel sequences (Edgcomb et al. 2002; Stoeck and Epstein 2003; Zuendorf et al. 2006). Extreme environments are also of particular interest to protistologists because of the

conditions in which these protists thrive being close to the earliest ecosystems found in the planet's history. Organisms which live in these conditions could therefore hold some phylogenetic clues to early branching organisms (Reysenbach and Cady 2001) both for Archaea (Eme et al. 2013) and for protists (Takishita et al. 2012; Takishita et al. 2007).

It is often suggested that protists are cosmopolitan because they supposedly have wide-ecological tolerances (Atkins et al. 2000; Finlay et al. 2002; Patterson and Lee 2000); however, molecular and ecological evidence has shown that there are distinct ecotypes within environmental sequences of morphospecies. For example, in bodonids, there is evidence for *Neobodo designis* having marked divergence between freshwater and marine strains and extensive diversity of all morphospecies (von der Heyden and Cavalier-Smith 2005). Cercozoans have been shown to be confined to oligomesotrophic and eutrophic ecosystems of freshwater lakes (Lefranc et al. 2005). Lefranc et al. (2005) studied small eukaryotes (≤ 5um filter), cloning sequences from three lakes by their trophic status and found that while some groups were present in at least two of the lakes in different ecosystems, some were specific to one lake and present in all its ecosystems, and all the communities they studied varied from lake to lake.

Revealing the identity of environmental clades is a major objective in microbial biodiversity especially of those found in extreme environments as they might represent some of the deepest historical lineages (Kolodziej and Stoeck 2007). The 12 MArine STrameopile (MAST) eDNA only clades, identified from a seasonal ecological marine study in the Mediterranean from which clone libraries and cultured isolates

were sequenced, displayed an impressive genetic diversity among the heterokonts, and is a well-known example of how the diversity of such common protists is only just surfacing (Massana et al. 2004; Massana et al. 2002). The addition of eDNA sequences within the heterokont tree has helped to stabilize internal, although taxonomically unidentified, clades (Massana et al. 2004). After nearly a decade, new environmental sequences are still being added to the heterokont tree (del Campo and Massana 2011), and a recent survey of High Arctic Lakes revealed that in all three of their methods, 50 – 70% of the biomass was of chrysophytes as was 25 - 50% of their sequences (Charvet et al. 2011). This is just one example of how targeted environmental sequencing can illuminate genetic diversity of protist groups; the same having been done for Alveolates and Cercozoa (Bass and Cavalier-Smith 2004; Groisillier et al. 2006; Weber et al. 2012). There is obviously still plenty of room for the discovery of further genetic diversity and novel lineages with high-throughput sequencing and group-specific primers and enrichment culturing.

A singular morphospecies could be found to compromise many cryptic species complexes, which can be related to genetic divergence and ecophysiology. It may be found that there are distinct groups of cryptic species that represent different evolutionary peaks where the organism has adapted to certain habitats; where types of prey and sediment/pore size are of importance (von der Heyden and Cavalier-Smith 2005). Ecological studies using uncultured or filtered samples have attempted to unveil trophic functions of eDNA clades; 18S rDNA phylotypes have displayed habitat specificity and ecophysiological delimitation in chrysophytes and other protist groups (Boenigk et al. 2005; Kim et al. 2004; Pfandl et al. 2009). Tests for correlations between molecular distance and ecophysiological adaptation have found that the *Spumella*-like

organisms are composed of different ecotypes and genotypes adding to the cryptic species notion that morphotype is not homogeneous but, as *Spumella*-like cells, are composed of several ecotypes and genotypes in paraphyletic parts of the heterokont tree (Boenigk 2008; Boenigk et al. 2007; Pfandl et al. 2009).

I had originally hoped I would be able to do environmental DNA sampling and sequencing as an independent test of diversity from culturing for both groups of silica-scaled protists, but I obtained so much new data from culturing (many new species) the environmental DNA investigation would have to be done later. I felt it was more important to focus on using my new isolates to improve taxonomy. Even so, the 18S rDNA data from my new cultures will be invaluable basis for future targeted eDNA studies.

3c. Culturing

Culturing protists for characterization has never been so important in light of increased eDNA contributions to databases. However, a disparity between cultured and environmental sequence data is evident from little or no overlap between cultured and eDNA clades (von der Heyden and Cavalier-Smith 2005). Increased efforts are being made to close the gap between eDNA and sequences from cultured isolates by improving culturing methods. Culture bias is a major challenge faced by microbiologists trying to culture new protists, especially when aiming to understand complex ecological niches, and these efforts are eclipsed by the faster pace of eDNA data accumulation revealing multiple clades of uncharacterized protists. Del Campo (2012) characterized one of the smallest known marine flagellates (nanoflagellates are hard to culture), *Minorisa minuta*, just 1.0 - 2.1 µm in body length, by mimicking *in situ*

conditions with natural bacterial (food) assemblages and selection of cells using serial dilution and flow cytometry. Not only did they isolate novel protists by this method but strains belonging to eDNA-only lineages. There is a slow and steady increase in the literature of the number of cultured protists affiliated with these environmental-only clades and during my culturing efforts of targeting smaller *Paraphysomonas* cells, I made a serial dilution that isolated a novel heterokont, *Incisomonas marina*, which identified a part of the environmental MAST-3 clade (Cavalier-Smith and Scoble 2013). It would seem that even with heavy biases in culturing techniques adapting one's culturing protocol, even slightly, is a positive step toward isolating novel cells and sequences. Biases in culturing can yield planktonic cells, such as common *Paraphysomonas imperforata*, which are rare in the natural assemblage but dominant in enriched cultures (del Campo and Massana 2011; Jürgens et al. 2008). Therefore, cultured protists do not necessarily represent the most common in the environment, which again shows us how delicate the ecological balance is between microbes.

3d. Species

This short section discusses some molecular tools that have been used to delimit organisms to the species-level, and the debate about species.

As I have mentioned above, morphological characteristics are used to differentiate organisms from one another, usually very efficiently at higher taxonomic levels but sometimes not as discriminatory at the lower levels resulting in many cryptic species. In addition, ecology and pathology are a few ways in which species designation can arise, but there are different types of molecular methods in use aside from the standard small ribosomal subunit SSU 18S rDNA gene sequenced for

phylogenies. Mitochondrial genes (mtDNA) are commonly used in taxonomy because of their universal presence throughout eukaryotic cell life making them useful comparative genes. The divergence of these mtDNA genes can be viewed in phylogenetic trees and will hopefully reflect evolutionary relationships of existing morphological groupings. The different rates of gene mutation in the mtDNA genes means that they can be used to different levels of taxonomic resolution; 28S rDNA gene is usually more divergent than 18S and 5.8S rDNA genes (Howe et al. 2011). The even more divergent non-coding internal transcriber spacer rDNA regions ITS1 and ITS2 have been of particular interest because they have been used to delimit morphologically cryptic species using methods that delimit sexual and non-sexual species (Coleman 2007). It is not easy to determine if sexual recombination occurs for many protists a combination of morphological, molecular, and ecological differences are used to define species. ITS2 rDNA secondary structure has shown that this molecule can discriminate between sexual species (Coleman 2007; Ruhl et al. 2010) and is used increasingly in protist taxonomy in support of delimiting species (Caisová et al. 2011; Stoeck et al. 2007; Xu et al. 2012). The possibility of a 'molecular taxonomy' for protists is unlikely due to its reliance primarily on the rate of mutation being steady for all organisms and molecular divergence rates are not the same for any one gene (Caron 2013; Pawlowski et al. 1997).

The debate surrounding the definition of a species could be considered more frustrating than trying to establish species from nature because of the preoccupation with trying to devise one species concept for all (Boenigk et al. 2012; Caron 2013; Fenchel 2005). It is difficult to compare low-end taxonomic diversity without having a standardized unit in which to measure, but a species is not a standard unit because

species are established using a variety of techniques. The basic aim of taxonomy at the species-level is to group individuals into ecological and evolutionary units, which can then represent units of physiological ability and genetic inheritance (Caron 2013). Classification and taxonomy has a utilitarian purpose of categorizing diversity in a practical manner (Adl et al. 2012). All protist taxonomy relies on morphology, yet morphology can hide convergent morphological evolution, ecophysiological, pathological, and many other meaningful characters (Doolittle 1994). These cryptic species complexes can then be usefully grouped without having to assign species status using broader molecular investigation; barcodes for certain functional groups (operational taxonomic units) (Countway et al. 2007; Stoeck et al. 2006). Thus, we can observe a species complex as a composition of meaningful units that are yet to be defined taxonomically. The debate on what species are might be more useful if we said what we want a species unit to tell us, but that depends on the type of organism; ecological, pathological etc. so the definition should be considered case-by-case (Boenigk et al. 2012).

Although my thesis is not addressing novel ways of delimiting protists species it uses standard molecular and microscopy methods to compare multiple isolates of morphologically cryptic species and in doing so I have revealed tangible morphological and molecular differences between closely related strains of protists that might have otherwise been lumped together. This shows how if standard methods are used meticulously they can reveal increased diversity and taxonomic resolution. The results in this thesis affect the claims made about biogeography and ubiquity, which are mentioned above, (Finlay and Clarke 1999b); even without sampling all accessible areas of the globe, arguments will remain about the identification of species; if we

cannot correctly identify species then how could we possibly make statements about ubiquity or endemism?

4. The aim of the thesis

Fundamentally, my work is aimed at redefining taxonomy for future research because until this is done one cannot undertake meaningful work on ecology, phylogeny, biogeography, and taxon-specific primers.

5. The objective of the thesis

To culture as many novel strains as possible from both taxon groups, paraphysomonad and thaumatomonad, from which sequence and light and electron microscope data will be used to infer scale evolution, and molecular and morphological diversity. These combined data will then aid in the reorganization of their taxonomy.

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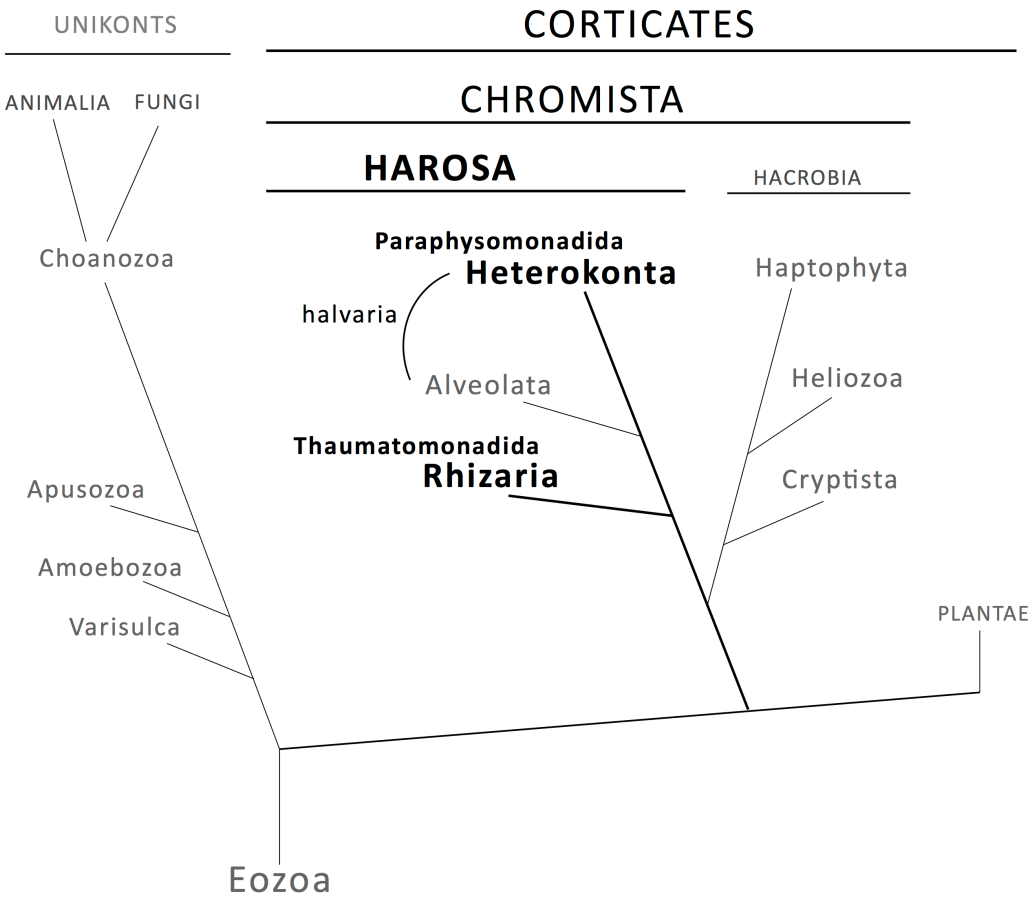


Figure 1. The main branches of the eukaryotic tree. Branches containing organisms studied in this thesis are in bold type; Kingdom Chromista and subkingdom, Harosa. Thaumatomonads belong within the phylum Cercozoa within the subkingdom Rhizaria. *Paraphysomonas* are within the class Chrysophyceae. Figure adapted from (Cavalier-Smith 2010b).

Table 1. The class Chrysophyceae within the kingdom Heterokonta. Asterisk denote Chrysophyceae lineage.

CHROMISTA (kingdom) (Cavalier-Smith 1981)

HAROSA (sub-kingdom) (Cavalier-Smith 2010b)

Heterokonta (Infra-kingdom) – sister to Alveolata (Cavalier-Smith 1986)

Gyrista (super-phylum) (Cavalier-Smith 1998)

Ochrophyta (phylum) – sister to Bigyra (phylum) (Cavalier-Smith 1997)

Phaeista* and Khakista (sub-phylum) – sister to Sagenista (sub-phylum) (Cavalier-Smith 1997)

Chrysophyceae* (class) (Cavalier-Smith and Chao 1996)

Paraphysomonadaceae (family) (Preisig and Hibberd 1983)

Genera: *Paraphysomonas*, *Spiniferomonas*, *Chrysosphaerella, Polylepidomonas*

CHAPTER TWO

Methods

This chapter details the culturing and fixing techniques developed during this thesis project, especially culture and grid preparation for transmission electron microscopy (TEM). The two main experimental chapters, Chapter 4 (paraphysomonads), and Chapter 5 (thaumatomonads) contain abridged versions of the methods. There are discussions throughout this chapter, on culturing at the end of section one, and molecular data at the end of section two, and a short overall discussion at the end.

1. Culturing

During the isolation process, all culture stages were kept at ambient temperature (laboratory temperature, about 22^{o}C). Once isolation was complete, all clonal cultures were stored at 15^{o}C to keep growth slow. Some cultures from tropical climates did not thrive at the storage temperature, which was evident when refreshing cultures every three months (low cell number and encystment), so these were kept at ambient temperature. Sterile plastic 96-well plates were used for serial dilution and sterile plastic Petri dishes (of various sizes) were used in up-scaling of the clones from the 96-well plates.

a) Source materials - soil, freshwater, and marine

An old soil sample collection was available to me the laboratory from previous research within the laboratory. They were stored in plastic boxes at ambient

temperature in and held samples from Brazil, China and Mexico (unknown collectors/years). Both target groups were known to encyst so I used this soil collection as source material and re-hydrated them to obtained clonal cultures. I also used fresh samples from freshwater and marine sites using 15 ml Falcon tubes, or when I ran out of tubes in the field I used empty drinks bottles. It was preferable to obtain some silt within the water samples because they seemed to yield more target protists. Grass/mud samples were collected by scraping (with a clean plastic bag) a pinch of surface soil and grass root; the bag was turned inside-out during the scrape, then outside-in to contain the sample.

b) Identification

The first *Paraphysomonas* species was described from a freshwater pond in America (Stokes 1888); a colourless (large ~20 μm) round cell with visible spine scales and two unequal cilia, and often stalked to the substratum. It was from this original description these strict criteria for isolation were set when selecting cells from culture. However, scale spines were not always clearly visible in the light microscope; all but one of the *Paraphysomonas* isolates had spine scales (one had basket scales) and a visible scale 'cuticle'. The cuticle was indication of the presence of scales even if I could not see the spines, so I was sure that I was selecting a scaled cell.

The thaumatomonad cells were selected by the following criteria; colourless dorso-ventrally flattened gliding cell producing pseudopodia and two cilia of unequal length. However, deviations from these criteria included visible spine scales (in light microscope) for *Thaumatomastix* isolates, and barely visible or no anterior cilium for the new thaumatomonad genus, *Scutellamonas.*

c) Clonal culturing

i) Enrichment of crude cultures

Approximately 1g of source material (solid and liquid) was placed in a plastic 90 mm Petri dish and freshwater or marine media was poured directly onto the sample to fill half the dish. A barley grain was added to enrich media and to encourage general growth of protists. Every twenty-four hours from enrichment, for up to three or four days, these enriched crude cultures were examined under light (phase) microscopy to check the condition of the culture and for the presence of target organisms. Serial dilution was performed upon these crude cultures when there was a good presence of protists, including even if the target protists were not seen as predators maybe keeping target protist numbers down.

ii) Serial dilution and single cell picking

Serial dilution was carried out from as early as 24 hours after enrichment depending on the amount of protist activity. Approximately 10 µl of crude culture (scraped from the bottom of the dish and fluid drawn during this motion) was serially diluted over eight series into a 96-well plate containing 200 µl of enriched media in each well. Six serial dilutions were made across the 96-well plate (two crude cultures per plate; ½ a plate each). After 24 - 48 hours each well was inspected via phase contrast light microscopy, and if target organisms were seen in any of these six serial dilutions the highest dilution well in which they were present would be used in the next serial dilution. The next stage of serial dilution has just four copies of a six-series dilution (¼ of the 96-well plate). From the first time a target organism was seen on its

own in a well, six more series of six serial dilutions were carried out (in fastest succession; 2 - 4 days between dilutions) to give better chances of a clonal culture. The highest dilution of the last series was used to inoculate fresh media into three small Petri dishes, each with a grain of barley and kept for further processing (as soon as possible). It was noted that inoculating (up-scaling) to standard-sized Petri dishes (9mm) from the 96-well plate well was not as successful as first inoculating to a larger well or smaller Petri dish. It was important to immediately rehydrate the well from which I was up-scaling after inoculating larger dishes, since up-scaling attempts did not always succeed.

Single picking was the standard isolation technique for thaumatomonad cultures from crude cultures or semi-crude cultures (first-stage serial dilution; contaminated thaumatomonad isolates). Single picked cells were washed four times before being put into a 96-well of fresh enriched media. The single pick 'wash' was a watch glass with fresh cooled media (cooled media would slow down the cell and stop it from floating away) in which the cell would be placed before being transferred to another watch glass. At least five single picks of one type of thaumatomonad cell were selected from a crude culture, or as many as possible, to be washed and left to multiply in 96-well plate wells. The single picked cells were left in the well to multiply before being inoculated into larger wells or plates for up-scaling.

Attempts to obtain *Paraphysomonas* cells via single picking was unsuccessful due to the cells swimming too fast and the stalked cells could not be easily picked off the substratum. Even though the stomatocysts of *Paraphysomonas* were easy to single

pick they were not often seen and one successful single pick took more than four months to excyst, which was not time effective.

iii) Media recipes

Marine; ASWP – Artificial Sea Water for Protists.

http://www.ccap.ac.uk/media/documents/ASWP.pdf

Freshwater; Volvic© (Danone). Straight from the bottle.

Barley Water; 200ml Volvic© microwaved with 10 g barley grain, 4 minutes (1 minute - 30" periods). Cooled and filtered through Whatman #1 filter paper and autoclaved (Autoclave at 15 psi for 15 minutes). The barley water was used to enrich media for serial dilution; 0.5 ml of barley water filtered through a syringe fitted with 0.45 µm syringe filter (Millipore®) and added to 200 ml fresh medium, this mix was dispensed into 96-well plates. Boiled barley grains were dried under U.V. light for 15 minutes and stored in 15 ml Falcon tubes at -20^{o}C for later use.

d) DNA extraction from clonal cultures

DNA extraction from clonal cultures was carried out as soon as possible after up-scaling the cloned isolate. DNA of the first bloom of cells was extracted using soil extraction kit UltraClean® (Mo Bio Laboratories).

e) Fixing cells for the electron microscope

i) TEM Grid and culture preparation for fixing

The fixing method was constantly being developed and refined over the course of the project and the final standard technique is outlined in Figure 1. Concentrating

the cells within the media was first tried by centrifugation at various speeds. As there was too much bacteria and detritus, a 'tilted filtration' protocol was developed, which was a more effective to get rid of bacterial cells and debris. Fresh cultures were preferable over older established cultures since they had less aggregate debris that would clog up the filter.

All of the TEM preparations were viewed on Formvar-coated copper grids (0.5%), which were kindly prepared by Mike Shaw (formerly of the William Dunn School of Pathology, Oxford.) This is also where I used the TEM machine (this chapter, section 3. b). I later experienced preparing grids myself when I visited Dahousie Univerisity in Halifax Nova Scotia Canada in May – June 2011.

Glutaraldehyde (50% EM grade) was added to the concentrated live cells, to a working concentration of 2.5 - 5%, and left to fix for ~1 hour before washing.

ii) Fixing versus not fixing

Applying live cells to the copper grid was not tried until the end of the project. I visited the Czech Republic for a conference (International Chrysophyte Symposium ICS-8, 2012) and discussed with other protistologists their fixing protocols. They single-picked whole cells of *Synura* and *Mallamonas* (colonies) and put them straight onto grids, let them dry, and washed the grid by a '30 second to 1 minute five series' wash; then put the dried grid face down on a drop of deionised water (figure 1, I). The grid is then left to dry and washed one more time by dipping the whole grid in deionised water. I did not single pick live cells but instead flushed some thaumatomonad cells with fresh media to clean debris and filtered the culture to concentrate the cells within suspension without pelleting (figure 1, A - B). I applied these live cells to the grid and

followed the rest of their protocol. The TEM results for live cells were successful in so far as I saw a lot of scales but not as satisfactory as if the cells were fixed (figure 2, G - N). Thaumatomonad cells (8-20 µm) are smaller than *Mallomonas* (50 - 100 µm) and *Synura* (~30 µm length) (and are not colonial), but more importantly, thaumatomonad scales, especially *Thaumatomonas*, are much smaller (≥ 5 x smaller) and so the scales were easily obscured by the contents of the lysed cell. The 30 "- 1 ' 5 series wash on the drops of deionized water worked very well and seemed to be beneficial in grid preparation for cultures that were difficult to clean by filter, but this was only tried two times with fixed cultures (Figure 2, M -N).

f) EM micrographs

The EM micrographs created by the microscope where saved as large tagged image file format (tiff) files and these were transferred to Adobe® Photoshop® for viewing and manipulating into smaller manageable files for observation and measuring. ImageJ, a java-based freeware was used to take accurate measurements of the scales.

g) Discussion

Paraphysomonas and *Thaumatomonas* were readily found in the environment and present in nearly every crude sample. The preliminary isolation results for *Paraphysomonas* indicated a 100% success rate for the spine scaled type. To obtain the other types of scales reported for this genus, it was necessary to revise the target size of the cell. In preliminary culturing efforts only the largest cells were being selected from between 8 - 20 µm diameter, but the majority of cells described and published for this genus had an average cell size around 5 µm diameter. Much smaller cells were selected in order to try and select *Paraphysomonas* cells with different scales types,

which resulted in the discovery of a basket scaled cell ('MD03') and other examples of novel spine scales ('Arb' and 'KU3').

This project pivoted on the success of isolating clonal target organisms and obtaining a range of morpho- and ribo-types. For *Paraphysomonas*-like cells almost 100% success rate was achieved for isolating the target organism, which was surprising since its scaleless counterpart, *Spumella*, looks so similar but without the scales. One *Spumella*, (CH3), was cultured, but this isolate looked obviously different from the other *Paraphysomonas* cultures with its bulbous and non-perfect circular cell body (Scoble and Cavalier Smith 2013), and so was targeted for isolation outside the rigid target criteria of cell type. It would appear that some *Paraphysomonas* cells can be unambiguously identified to the genus-level by light microscopy. The gliding cells of *Thaumatomonas* were also easy to identify by using rigid cell identification criteria, and other thaumatomonads*,* namely *Thaumatospina,* were also isolated by including other criteria such as visible spine scales. The speed of the gliding cells varied too and was effective as an additional criterion for isolating more examples of thaumatomonad cells, e.g. *Reckertia* and *Scutellamonas.*

Obtaining the first bloom of cells for DNA extraction whilst at the same time recording the live cells and fixing them was important but not always possible early on in the project. The method of fixing cells was not fully established until near the end of the four years of the project. Fixing techniques were always being improved and refined and modified for different culture conditions and types of cells. Even so, the cultures seemed to be stable and uncontaminated and when I repeated fixes for EM or recording light microscope data long after the original isolation and DNA extraction

was obtained these repeated data were congruent with earlier data of the same culture.

2. Molecular data

All novel molecular data was accrued from extracted DNA of clonal cultures (this chapter section 1d) on which PCR amplified rRNA genes for phylogenetic and other observations and analyses.

a) Novel sequence data: PCR, sequencing and contig assembly

i) Primers listed in Table 1

PCR programme: **Initial denaturation** 5 min, 95°C followed by 30 – 35 cycles of [**denaturation** 32 sec, 95°C; **annealing** all 36 sec, *Paraphysomonas* (18S) 62°C, Cerco (18S) 70°C, Cerco (ITS) 67°C; **extension** 2 min *Paraphysomonas* (18S) 72°C, 2 min Cerco (18S) 72°C, 3 min Cerco (ITS) 72°C]. **Final extension**, 7 min, 72°C. ∞ 4°C.

Sequencing programme: 96°C 1 min; x24 [96°C 10", 50°C 10", 60°C 4'] ∞10°C. (Applied Biosystems BigDye® Terminator Cycle Sequencing Kit).

The various stages of genetic data manipulation was carried out using free software (freeware) programs: Sequences were viewed by FinchTV (Geospiza®) (PC and MAC) and BLASTed (http://www.ncbi.nlm.nih.gov/BLAST) using the NCBI database to see if the sequences matched closely to target organisms. Sequence fragments were edited using Sequence Scanner v1.0 Applied Biosystems (Life Technologies™) (PC) and Contigs were assembled with BioEdit v7.1.8 (PC). The complete sequences were aligned using an online version of MAFFT (Katoh et al. 2009) hosted by Japanese servers. The setting used was 'Q-INS-I' because the secondary structure of rRNA is

considered. The alignments were edited using MacGDE v2.4 (Linton macgde.bio.cmich.edu) and an upgraded version (personally provided by Eric Linton, developer) for the latest Macintosh computer system OS X Lion 10.7.

b) Phylogenetic trees

i) RaxML version 7.0.4 (Stamatakis et al. 2005)

MacGDE was used to generate the Neighbour-Joining tree program.infile for RaxML using Phylip version 3.68 (Felsenstein http://evolution.genetics.washington.edu/phylip); DNADist, default settings to obtain an extended Phylip formatted file (extended file format allows taxon names longer than 12 characters). The ML algorithm was set to GTRMIX model, rapid bootstrap uses the GTRCAT model then switches to GTRGAMMA at the end to find the optimal tree with stable likelihood values and empirical base frequencies and 25 per site rate categories. (-f a) rapid bootstrap, (-x 12345) rapid bootstrap with random number seed.

ii) MrBayes v 3.1.2. 5 (Ronquist and Huelsenbeck 2003)

Five million generations of trees in two parallel chains were run, saving one in every 1,000 trees. GTR model with four categories co-varion and adgamma options. Number of trees to discard as 'burnin' was decided by viewing the convergence in a graph (Microsoft® Excel®), the number of generations at which the results converged (plateaued) bearing in mind 1 in every 1,000 trees were taken, e.g., if converged at 500,000 generations, 500 trees were removed.

c) ITS2 rDNA folding

The secondary structure of the ITS2 rDNA folds were all performed with freeware. Only thaumatomonad isolates were analysed because this group already has data published (Howe et al. 2011) and the 18S rDNA was not as resolving as it was for the paraphysomonads.

Firstly, the beginning and the end of the ITS2 rDNA region was determined using the online database and tool http://its2.bioapps.biozentrum.uni-wuerzburg.de/ (Koetschan et al. 2009). The resulting 'annotated' sequence was then folded by another online software MFold http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form (Zuker 2003). The default temperature (37^{o}C) was used and all portable document format (PDF) files illustrating the folds were downloaded. The fold with the lowest energy fold (closest to 0) was used in analysis; compared to other folds. Compensatory base changes (CBCs) and hemi CBCs were analyzed by eye.

d) Discussion

The novel sequences for each genus were put in their own alignment with other closely related sequences chosen from published rDNA trees (Bass and Cavalier-Smith 2004; Bass et al. 2009; Boenigk et al. 2005; Charvet et al. 2011; Howe et al. 2011; Kim et al. 2010; Lesaulnier et al. 2008; Massana et al. 2004; Massana et al. 2011; Richards et al. 2005; Shi et al. 2009; Tarbe et al. 2011). Additional searches for named or environmental sequences that were not in publications were found by BLASTing (NCBI) one sequence of each main clade and any from my new sequences. In this way, I hoped to have as many representative sequence taxa to give a stable tree and internal nodes, have strongly supported out-groups for both *Paraphysomonas* and *Thaumatomonas*, and attempt to identify unaffiliated environmental sequences/clades.

The 18S rDNA alignment for *Paraphysomonas* held 329 sequences (1654 positions) representing the division Ochrophyta, within Heterokonta for which Chrysophyceae were heavily represented. The results did not improve on the current published knowledge of the order or relationship of the genera within the Chyrsophyceae, however they did show how the 70+ *Paraphysomonas* sequences were grouped according to scale type and other morphological features observed in the light microscope. Details of the results and discussion are in Chapter 4.

The cercozoan alignment of 1785 nucleotide positions held 269 sequence taxa that extended to represent Monadofilosa. There were ten environmental sequences out of 46 thaumatomonad 18S rDNA sequences, the rest were cultured. A second alignment of 30 cultured and five eDNA thaumatomonad sequences was also made, which covered the ribosomal gene regions ITS1, 5.8S ITS2. Some of these data were then used for ITS2 rDNA secondary structure folding analysis. The folding analysis provided a functional insight about the levels of possible function divergence between closely related isolates. The thaumatomonad isolates appeared more cryptic at the 18S rDNA level, with less molecular divergence between closely related strains than the paraphysomonads. Thus viewing the ribosomal fold was another way of resolving the differences between these cryptic isolates. It would have been interesting to compare the ITS2 rDNA of closely related paraphysomonads strains to see if they had any CBCs but there simply was not enough time. The analysis of the CBCs was lengthy because although there are programs that perform these analyses (http://cbcanalyzer.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.php), I found inconsistencies in their results and up-dates to the software meant that I could no longer use them on my PC or Macintosh® computers. I received assistance from Pavel

Škaloud (Charles University, Prague) who has published work on protist ITS2 rDNA folding (Škaloud and Peksa 2010). He helped me to manipulate my ITS2 rDNA data using the latest upgraded versions of the ITS2 rDNA folding softwares but sadly, this remote assistance between Prague and Oxford was still too difficult to resolve the difficulties I had with the software updates and changes. However, the manual observations of the CBCs did reveal to me the slight differences in folds that I would have otherwise missed had I just analyzed the folds by software. An enlarged loop in a region where CBCs were common between isolates lowered the number of CBCs between two isolates with distinct morphological differences (Chapter 5).

3. Equipment

a) Light microscopes

i) Differential Interference Contrast (DIC) microscope; Nikon eclipse 80i, with x60 immersion lens

ii) Phase Contrast microscopes; Olympus IX70 (96-well plates and general observation of cultures) x10, x20, x40. Olympus CK40 (Single picking), x20 x40 lenses

b) Electron Microscope

i) FEI Tecnai 12

c) Thermocyclers

i) Techne touchgene gradient FTGRAD2D

ii) Biometra® Thermoblock T-1

d) Sequencing machine (out-sourced this protocol to the University of Oxford Zoology department sequencing service that used the following system)

i) Applied Biosystems 3730/3730xl DNA Analyzer (Life Technologies™). Capillary system using Applied Biosystems BigDye® Terminator v3.1 and v1.1 Cycle Sequencing Kits

4. General discussion

Between 70 – 75 *Paraphysomonas* isolates and around 50 thaumatomonad isolates were cultured and sequenced over the first two years of this project. Processing over 100 cultures for combined parallel data collection; rDNA sequencing, videoing live cells in DIC, and fixing cultures for the TEM, meant that I had to be very organised. I created culture description sheets for each isolate and dedicated a folder to each group; paraphysomonad and thaumatomonad. Microsoft® Excel® spread sheets were vital for keeping track of what had been sequenced, to see what sequences failed or succeeded, and what contigs had been made from which PCR amplicon. Once every three months I would spend a week maintaining these cultures to keep them alive.

The success of these methods that provided data in a short time were mainly two-fold. 1. The organisms were easy to find because they are common in the environment and they multiply fast, which is good for processing clean cultures; serial dilution could occur in quick succession so any contaminants were not given a chance to grow and pass down into the next serial dilution. Thus, the success of the simple culturing method meant that the second phase of the project, the molecular work, was straightforward. 2. Eukaryote-wide primers were used on the extracted DNA often obtaining a large single PCR band first time and a clean sequence read. Therefore, there was no need to create organism/group-specific primers to obtain sequences.

The method of culturing described here is very crude and biased towards obtaining cells that could easily withstand the same laboratory environment and culture conditions, yet even with these biases the results show molecular and morphological diversity (Chapters 4 and 5). If I needed to obtain more diversity within cultured isolates I might have changed the way in which I enriched the crude culture material. There are more gentle ways of encouraging protist growth from fresh crude material, either by adding fresh media via pipette to become more and more dilute thus avoiding osmotic shock that would harm more fragile cells. And, for the gliding thaumatomonad cells, I might have tried the coverslip method (Ekelund and Patterson 1997).

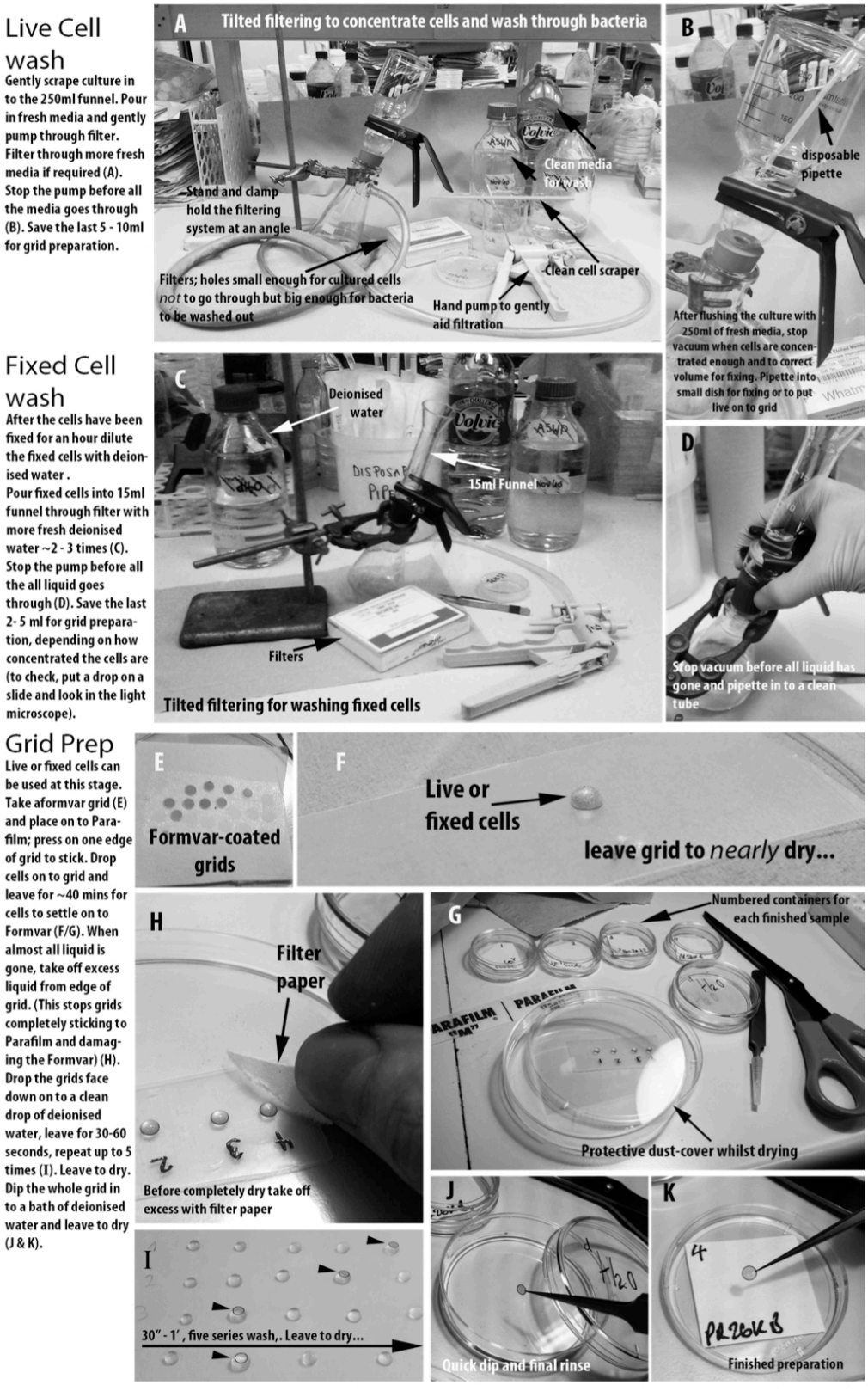


Figure 1. TEM preparation of live cultures: A general standardized protocol.
A - B, live cell wash. Cells are concentrated by 'tilted filtration'; fresh medium is used to flush out smaller particles and bacteria. The vacuum must be stopped before all the content filters through and this is why the tilt is important – for higher concentration of cells less liquid is required so tilting helps keep cells suspended in the liquid without being suddenly pulled through. C –D, the same system for washing fixed cells as live cells but using smaller volumes. E – K, preparation of grids using live and fixed cells. Additional washes (I – J) are effective especially for dirtier cultures that are difficult to filter-wash.

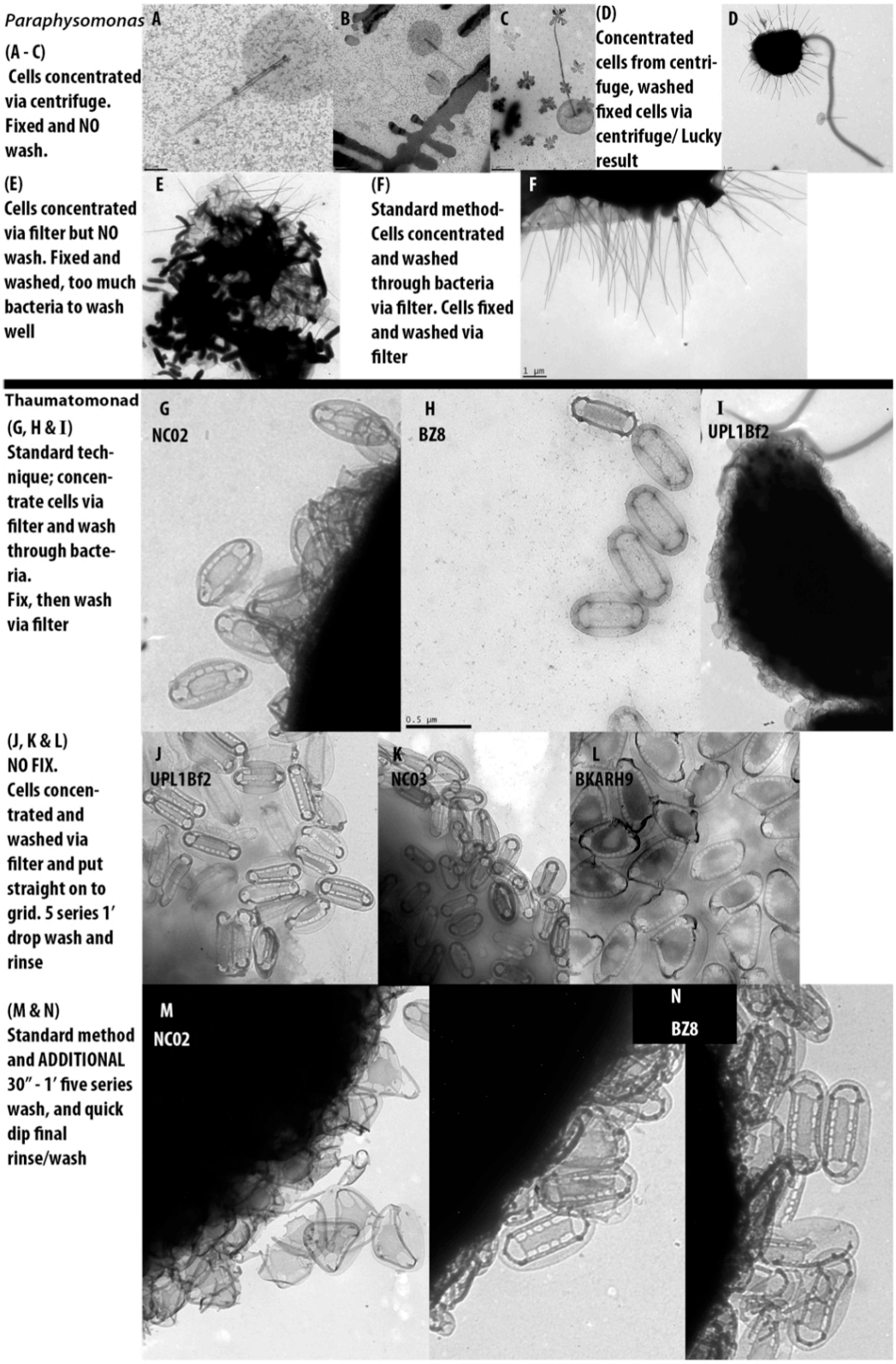


Figure 2. The development of fixing and grid preparation protocol. Early stages of protocol development (A – F) *Paraphysomonas* cells. Centrifugation to concentrate cells was problematic could hardly see anything on grid, and (E) without bacterial wash through. Standard method was developed (F); full filtration technique. Thaumatomonad results from standard technique (G – I) and with extra wash stage (M & N). Live cell preparation (J – L) was successful but not as good quality as fixed cell results. Strain codes for Thaumatomonads only shown because the results can be compared.

Specificity	Primer name	rDNA region	Sequence	Published in
+/- Euk	25F	18S	5' CAT ATG CTT GTC TCA AAG ATT AAG CCA 3'	(Bass and Cavalier-Smith 2004)
+/- Euk	Pre3NDF*	18S	5' CAG CAG GCG CGC AAA TTA CC 3'	(Karpov et al. 2006)
Cerco	1256R*	18S	5' GCA CCA CCA CCC AYA GAA TCA AGA AAG AWC TTC 3'	(Karpov et al. 2006)
Cerco	1259F	18S	5' GGT CCR GAC AYA GTR AGG ATT GAC AGA TTG AAG 3'	(Karpov et al. 2006)
+/- Euk	1801R	18S	5′ TGA TCC TTC TGC AGG TTC ACC T 3′	(Cavalier-Smith et al. 2008)
+/- Euk	PreBF*	18S	5' GTA GGT GAA CCT GCA GAA GGA TC 3'	Chao, E (not published)
+/- Euk	384F*	5.8S	5' YTB GAT GGT AGT GTA TTG GA 3'	(Dopheide et al. 2009)
+/- Euk	28SR1	28S	5' CAG CAG GCG CGC AAA TTA CC 3'	(Bass and Cavalier-Smith 2004)

Table 1. Eight primers were used of which two were cercozoan-specific 'Cerco'.
Four internal primers were used for sequencing (*). Primer pairs for *Paraphysomonas* PCR were eukaryotic-wide: (18S) 25F-1801R. Thaumatomonads 18S rDNA was sequenced with cercozoan-specific primer combination, 25F-1256R, and 25F-1801R for the whole 18S rDNA gene. Cercozoan-specific primer pair for the ITS1 and ITS2 rDNA regions: 1259F – 28SR1, and internal sequencing primers PreBF and 384F were used on this PCR fragment.

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CHAPTER THREE

Published paper

Review

This review article was part of a special edition of Nova Hedwigia, which was a collection of invited articles of those who presented at the Eighth International Chrysophyte Symposium (Prague, 2012). I wrote the article after which TCS made a few changes; proofread and added a few sentences. The written work was **90**% JMS, 10% TCS.

Nova Hedwigia, Beiheft 142, 117–126 **Article**

Stuttgart, September 2013

Molecular and morphological diversity of *Paraphysomonas* (Chrysophyceae, order Paraphysomonadida); a short review

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With 2 figures

Abstract: The chrysophyte genus *Paraphysomonas* has over 50 species, distinguished by a remarkably diverse array of scale morphology – from simple spine scales to complex basket or mesh-work scales. Only a few species are commonly found in environmental surveys, often by examining crude water and silt samples. Despite the commonness of those few species, only limited sequence data are publicly avail- able for known species. The few sequences that are available are not helpful in resolving the taxonomic or evolutionary relationship amongst all these morphologically diverse *Paraphysomonas* species, partly because different strains identified as the same species have such divergent sequences that misidentification must have been frequent, and partly because sequences are available for only two major scale types and four nominal species. There is much need for clonal cultures of *Paraphysomonas* on which sequencing and electron microscopy can be carried out in parallel, in order to shed light on the taxonomy of this 'genus'. This short review attempts to highlight mistakes in the literature that have led to the confusion of a few common *Paraphysomonas* species and thus propose new ways to scrutinize scale data by listing key spine-scale features, thus rejuvenating research on a common 'genus' and revealing its previously overlooked diversity. A more critical approach is expected to subdivide *Paraphysomonas* into several genera according to their contrasting scale morphologies.

Key words: silica scales, *Paraphysomonas vestita*, taxonomy, protist diversity, scale morphology

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1438-9134/2013/0142-0117 $ 2.5

Introduction

Silica-scaled chrysophytes are important phototrophs, heterotrophs or mixotrophs, are major contributors to the microbial loop of many freshwater and marine ecosystems, and are also found in hydrothermal vents and oxygen depleted and anoxic environments (Atkins et al. 2000, Stoeck et al. 2003, Zuendorf et al. 2006). Despite their ecological significance and commonness in the environment, this large and dynamic group of protists is still taxonomically misunderstood and confused (Cavalier-Smith & Chao 2006).

With improved and cheaper molecular surveying techniques we are now able to see an enormous molecular diversity among the Chrysophyceae, but large clades of unknown morphology are being discovered, because electron microscopic data are gained more slowly than DNA sequences, usually via silt washes or enriched crude cultures, often without any opportunity for molecular investigation. These two research methods have seldom been applied to the same strains, thus arguably contributing to taxonomic confusion and misplacement of some genera within the Chrysophyceae (Kristiansen & Tong 1989, Nicholls 1980, Takahashi & Shigemitsu 1984). For example, the photosynthetic *Chrysosphaerella* was placed in Paraphysomonadaceae (Preisig & Hibberd 1983), but sequence trees later showed it to be related not to *Paraphysomonas* (Andersen 2007, del Campo & Massana 2011) but to the uniciliate Chromulinales, yet it is still not properly assigned to another family. Traditionally Paraphysomonadaceae were included in Chromulinales despite having two cilia, but a separate order Paraphysomonadales was established for them after sequence trees showed they did not group with *Chromulina* (Cavalier-Smith et al. 1996). Because paraphysomonads are not algae (as defined by Cavalier-Smith 2007) we here follow Cavalier-Smith and Scoble (2013) in using the zoological spelling Paraphysomonadida for the order. Because of its deep divergence from photosynthetic chrysophytes Paraphysomonadida was placed in a separate exclusively non-photosynthetic chrysophyte subclass, Leucophycidae (Cavalier-Smith & Chao 1996).

Research data on the scales of Chrysophyceae sensu lato come in three, often disconnected, forms: 1. Extinct fossil evidence; scales found in rock and sediment cores have given data on how scale morphology has evolved over time as well as on the biogeography of some morphotypes (Siver & Wolfe 2009). 2. Scales of extant species; those from live cells in water samples have been used to describe most species, and those from dead cells in sediments have been used to test biogeographical theories (Finlay & Clarke 1999); both yield ecological and distributional data. 3. Gene sequences derived from environmental DNA surveys give us ecological snapshots (living or latent), which often culminate in entirely unidentified clades of chrysophytes (Massana et al. 2011). Whilst all of these have provided large amounts of data, it is not easy to correlate the latter two approaches with each other because we do not have examples of how genetic diversity matches morphological diversity. The full worth of our current data has not been as well exploited as in many other protist groups because of the rarity of studies that combine electron microscope and molecular data on the same cultures.

Below we provide a brief history of *Paraphysomonas*, a large heterogeneous group of common and well-known scaly and colourless chrysophytes, showing how generalizations of cell type and scale type have led to taxonomic confusion within the genus. We explain that published molecular rDNA evidence has not yet really aided in making sense of the genus owing to a number of misidentifications and lack of sequences. We then go on to emphasize that clonal cultures are needed to elucidate their hidden diversity through combining electron microscopic (EM) and sequencing methods to gain a deeper resolution and better understanding of the taxonomy of *Paraphysomonas*.

A brief history of *Paraphysomonas*

Morphology: Overgeneralization of scale types led to misidentification of 'common species' and underestimations of morphological diversity

The type species, *Paraphysomonas vestita*, was described in the light microscope (LM) by Stokes (1885), originally as *Physomonas vestita*, the generic name being changed later to *Paraphysomonas* as *Physomonas* was preoccupied. This large (~15 μm) round freshwater cell had visible 'radiating bodies' projecting all around the colourless cell body and two unequal cilia. Later, Korshikov published an intimate LM study of *P. vestita* (cell dimensions and habitat matched Stokes') and showed that the radiating bodies were silica scales and drew a picture of some detached nail-like scales from a fixed specimen he viewed under the LM (Korshikov 1929). It was Korshikov's small drawing that made Houwink believe that he had seen *P. vestita* scales for the first time in the EM, when he was doing a bacterial study (Houwink 1952). Houwink saw that his scale was like Korshikov's 'nails with relatively large flat heads'; however, Houwink's scale had a spine around a third shorter than the one described by Korshikov (~10 μm), making it likely that it was really an undescribed species. The literature became familiarized with EM pictures of '*P. vestita*' scales with key publications by Manton & Leedale (1961) and Balonov (1972). Manton and Leedale's study revealed a small diversity of scale size within their sample but the scale form was relatively uniform. From that time it became usual to conceive *P. vestita* scales as having the following generalized form: round base plate with a thickened margin and a central tapering spine. Any species reported by EM from fixed environmental whole-mount samples with this general type of scale was identified as '*P. vestita*' even though cell and scale dimensions and detailed scale structure varied considerably from one study to another (see references below). In the absence of evidence from cultures on the degree of such variability within a clone or sequence, an excessively broad interpretation of the applicability of the *P. vestita* name held sway.

Two new marine species of spine-scaled *Paraphysomonas*, *P. imperforata* and *P. foraminifera*, were described by Lucas (1967); EM and LM descriptions of these small marine flagellate cells showed

that they were covered in small spine scales with spines no longer than 1.5 µm long and base plates no wider than 1.2 µm. Both species differed from Manton and Leedale's freshwater *P. vestita* by having smaller scales lacking a thickened margin on the base plate; their scales also had a dense annulus in the mid-region of the base plate (interpreted as a slight ridge) not seen in *P. vestita*. They differed from each other in that *P. imperforata* had a plain base plate, whereas the base plate of *P. foraminifera* was perforated. These marine species were also about three times smaller in cell diameter than Stokes' original freshwater cells. However, despite this first description of *P. imperforata* being based on both EM and LM data, there have been numerous false reports of this species in the literature (actually having noticeably different scales) brought about by the same degree of overgeneralization of scale type as for *P. vestita* (Cronberg & Kristiansen 1980, Kling & Kristiansen 1983, Kristiansen 1989, Preisig & Hibberd 1982a, Takahashi 1976, Wujek & Saha 1995). It seems that '*P. imperforata*' came to refer to an excessively broad and variable morphotype distinguished solely by having scales of a plain baseplate with an annulus and a central spine; potentially distinctive features such as spine and base plate dimensions and whether the spine tapers or not were disregarded. For example, one early EM chrysophyte study, just eight years after Lucas' novel description, was a marine survey of *Paraphysomonas* under natural conditions (Thomsen 1975). This survey described and showed EM micrographs of '*P. imperforata*' scales that are quite different in size from Lucas' type description; some have a thinner apical tip as seen in Fig. 8 of the paper, (more like *P. antarctica* (Takahashi 1987)), blunt tip (Fig. 9) (like *P. vestita truncata* (Preisig & Hibberd 1982a)), non-tapering spine (Figs 8 & 9) and even some without spines (Fig. 12). Thomsen stated that the variation in scale size is considerably wider than was reported in the type description (Lucas 1967). Yet he did not consider the possibility that the EM data were from more than one species and perhaps four different novel species of *Paraphysomonas*. This overgeneralization of scale form continued in further studies, making the same assumption that the range of scale morphology seen within a single species could be rather broad. These assumptions of broad diversity seemed to assign a new attribute for the '*P. imperforata*' spine tip (Fig. 19 in Takahashi 1976); i.e., an angular pointed tip, which was not stated in the original description and, arguably, not seen in the original type EM micrographs. From these publications, '*P. imperforata*' eventually included examples that were ten times the size of the original, had non-tapering and tapering spines ending in a truncate, thin apical tip, and acute sharply-pointed tips arising centrally from a plain base plate that is oval or round and with or without an annulus! The only constant feature was a negative character: that they not have a thickened margin on the base plate. The type species of this genus, *P. vestita*, is the only *Paraphysomonas* species described without an EM scale being originally attributed to it; yet a tapering spine scale with a thickened margin baseplate has been treated as the sufficiently distinguishing morphological character for this 'species'. Of course, its validity depends on whether Manton and Leedale's strain was the same species as Stokes', which it was assumed to be simply because no other species had been described at that time. This assumption was never critically examined after other species with nail-like scales were examined, most of which were described without information on the appearance of the cells in the LM that is the only

possible basis for comparison with Stokes'. Perhaps because that assumption was never tested and no further critical morphological studies were made on living cultures of *P. vestita*, the 'vestita' morphotypes that have been reported ultrastructurally from fixed environmental samples in numerous publications show huge differences in size as well as subtle differences in base-plate form (ovalness, degrees of thickened mar- gin and radial spokes) and spine shape (length, degrees of tapering, and tip shape) (Dürrschmidt & Cronberg 1989, Lee & Takahashi 1993, Lim et al. 2001, Petronio & Rivera 2010, Rice et al. 1997, Vyverman & Cronberg 1993).
It is therefore unsurprising that the most commonly reported 'species' of *Paraphysomonas* are *P. imperforata* and *P. vestita* (Cronberg & Kristiansen 1980, Finlay & Clarke 1999, Preisig & Hibberd 1982a, Vørs et al. 1990). It is now clear from our studies of clonal cultures, to be reported elsewhere, that there are key features of spine scales that can be taken into account when identifying and describing new spe- cies, the combinations of which can be used diagnostically (Fig. 1). Using these key features, as well as size parameters (and ratio of spine length to base-plate width), to describe spine-scaled *Paraphysomonas*, will enable numerous novel spine-scaled species to be described. Only nine genuine *Paraphysomonas* species with spine scales have so far been described.

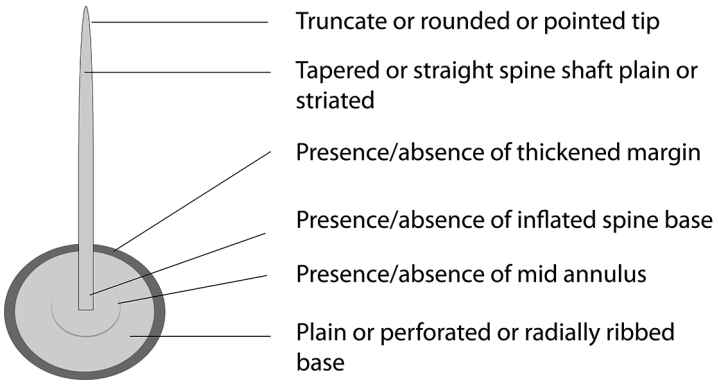


Fig. 1. Diagnostic features of *Paraphysomonas* species with spine scales. Combinations of features of the spine and base plate can be used to describe species, as can scale size and spine : base ratios.

The first basket-case

There are far more described *Paraphysomonas* species with basket- or lattice-shaped scales. Out of the 56 published species names there are 45 that do not have simple spine scales, most of which were described in the early 1980s (Fig. 2) (Preisig & Hibberd 1982b, Thomsen et al. 1981); very few have been described since. These scales are so different in shape from the spine scales that the species would be better placed in a different genus. That suggestion was first made by Lucas in 1968 (a year after he described *P. imperforata* and *P. foraminifera*) but is not yet adopted. Lucas described a small highly motile colourless flagellate with more complex scale structure, which he named *P. cribosa*, the first basket/lattice-scaled *Paraphysomonas* to be published (Lucas 1968). With such great divergence from the spine-scaled variety, Lucas said this very different scale form 'could be sufficient grounds for placing in a separate genus'. However, at this stage he left the newly discovered basket-scaled chrysophyte within *Paraphysomonas* because he felt that a clearer cellular and EM comparison between the two cell types should be made.

Misunderstanding and over-looking diversity of scale data

The implications for not truly understanding the diversity of a common protist 'genus' are especially evident with the biogeographical hypothesis 'Everything is Everywhere' (EiE), which claimed that microbes are virtually all cosmopolitan, unlike macroorganisms that are often restricted in distribution because of limited ability for global dispersal (Finlay 2002). Whilst this theory has not been easy to test, there is a growing consensus in favour of the alternative view that a substantial fraction of microbial species are not cosmopolitan but show moderate endemicity, their restricted geographic distributions having often been hidden by excessive taxonomic lumping in the past (Bass et al. 2007, Foissner 2008, Zufall et al. 2013). Finlay and Clarke's Cumbrian lake study (Esteban et al. 2012, Finlay & Clarke 1999) made a celebrity of *Paraphysomonas* as a common silica-scaled protist by using this genus as a model for looking at biogeography of protists. Their interpretation of the EM data, which included other data from a literature search, was made using the overgeneralized morphotype concept (i.e., that scale size and fine details did not matter) and so it was unsurprising that their conclusion was that all of these 'species' were found, biogeographically, everywhere. Grass is also found everywhere, but is not one species, just a generalized morphotype. Correctly interpreting scale and molecular data is important if we want to examine which broad morphospecies are genuinely single cosmopolitan species and which are simply crude aggregates of several distinct species with contrasting and more restricted distributions.

It is obvious from the literature over the past few decades that we have been dealing with overgeneralized morphotypes for 'common' *Paraphysomonas* species. That led to the small

amount of molecular data being misinterpreted or in some cases uninterpretable. With this in mind, molecular sequencing could help define the genetic relationships between the scale types so that we could see how diverse the spine-scaled *Paraphysomonas* really is, and also see how closely related the basket/lattice-scaled species are to the spine-scaled species.

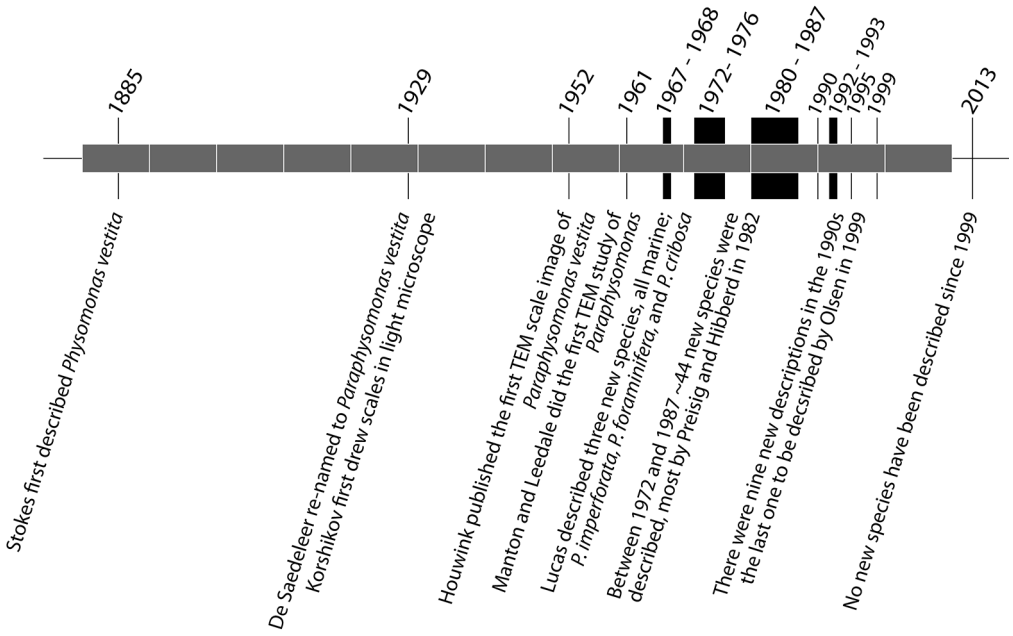


Fig. 2. Time-line of publications describing new species of *Paraphysomonas*.

Molecular diversity of *Paraphysomonas*

To date there have been very few (18S rDNA, some only partial) sequences of named *Paraphysomonas* sequences publicly available (NCBI database), *P. imperforata* (×5 – EF432518, EF432519, Z29680, AF109324, AF109323), *P. vestita* (×4 – GU220392, FJ886745, Z28335, AF109325), *P. foraminifera* (×4 – Z33646 & Z38025; the same strain, AF174376, AB022864), *P. bandaiensis* (×1 – AF109322) and *P. butcheri* (×2 – Z29679, AF109326). None of these stud- ies was made on authentic type strains so their utility depends on the cultures used being correctly identified. For most of them no EM evidence was published to validate the names given to the strains and there was no discussion of how or by whom the cells were identified. In most cases these strains are now dead so their true identity cannot be determined. In our view every strain for which such EM data were provided was misidentified through applying too broad a species concept. Only five named *Paraphysomonas* species are represented in genetic databases; four have spine scales and one species, *P. butcheri*, has basket/lattice scales. The position of *P. butcheri* within the chrysophyte molecular phylogeny is always as a sister to the base of the spine-scaled clade, often with lower but reasonable support than the strongly supported monophyly of the spine-scaled clade (Andersen 2007, Andersen et al. 1999, Boenigk et al. 2005, Caron et al. 1999, Cavalier-Smith & Chao 2006). Even with these few sequences we see that these species always fall together in the same part of the chrysophyte tree, and deeply divergent from all other chrysophytes. However, there are so few identified and named *Paraphysomonas* sequences that it is difficult to prove the closeness of the phylogenetic relationship of the basket-scale and spine- scaled species, especially as a sequence exists for only one of the many basket-scale species that have been described. Furthermore, identification of at least some spine-scaled *Paraphysomonas* must be erroneous because the phylogenetic trees show *P. vestita* and *P. foraminifera* in multiple places, some so radically different that they cannot be the same species even with the broadest of species concepts.

For named strains with available EM data we can also clearly see morphological scale differences from earlier descriptions. For *P. vestita*, 18S rDNA sequences and scale morphology both differ in Z28335 'SOTON1' (Rice et al. 1997) and GU220392 'J1' (Petronio & Rivera 2010). SOTON1 is a cell with spine scales that have a very long, barely tapering, spine with a round plain base plate with thickened margin. Compare this to strain J1, which is from a cell with spine scales relatively short compared to SOTON1 and having a strongly tapering spine with an inflated base that also comes from a plain round base plate with a thickened margin. This morphological divergence is reflected in the sequence divergence, which is so great that they cannot be the same species. From the phylogenetic trees we can deduce that there is probably also a gross misidentification of at least one other sequence, AB022864 'HT3', which is unlikely to be *P. foraminifera* but actually a '*vestita*' morphotype, as it does not branch with other '*foraminifera*' sequences, including one for which EM data was provided, unlike for AB022864, but far away from the sequences of *vestita*-like strains. In the absence of EM data we cannot formally rule out

the alternative possibility that its scale base-plates actually had holes; if that were true the presence of holes would cease to be a sufficient identifier for *P. foraminifera*. Either way, the tree is essentially uninterpretable at present because the EM and sequence data were not obtained from each organism, preventing such conflicts from being resolved.

There have also been a few sequences obtained from clonal cultures not yet formally identified as *Paraphysomonas*, but which probably are as they do sit with named *Paraphysomonas* sequences. These sequences were called '*Spumella*-like' as an umbrella term (Boenigk et al. 2005) because it was assumed that *Spumella* and *Paraphysomonas* cannot be separated by LM alone.

High throughput (next generation) sequencing is allowing addition of many new environmental sequences to the chrysophyte tree, revealing branches of *Paraphysomonas* sequences with unaffiliated scale morphology (Charvet et al. 2012, del Campo & Massana 2011, Richards et al. 2005, Tarbe et al. 2011) as well as sequences from other chrysophyte genera (Massana et al. 2004, Shi et al. 2009). This 'fleshing-out' of the tree improves the stability of the *Paraphysomonas* clade and the Chrysophyceae as a whole within Heterokonta.

Moving *Paraphysomonas* research forward

Chrysophytes have long been studied and used as model organisms in ecology (Boenigk 2008, Boenigk et al. 2006, Pfandl & Boenigk 2006), flagellar ultrastructure (Bradley 1966), life cycle (Yubuki et al. 2008), and in particular *Paraphysomonas* '*vestita*' has been used for studying direct effects of UV-B radiation on cells (Macaluso et al. 2009), growth inhibition, and flagellar motion (Christensen-Dalsgaard & Fenchel 2004, Lee 1978). Understanding the molecular and morphological diversity of all protists is important, not just for *Paraphysomonas*. We need to be able to evaluate the diversity of protists in order to better record and interpret field-data from the archives, now and in the future.

Many studies use either environmental sequencing or electron microscopy of crude environmental samples to look at diversity of silica-scaled protists, but without knowing how either data set relates to the other. We have now begun the task of isolating clonal cultures of spine scale and basket scale *Paraphysomonas* species and have been running molecular and EM analysis in parallel, which will eventually yield a much better idea of their diversity and over-all taxonomy. We expect soon to be better able to evaluate whether the differences of spine scale morphology are really linked to genetic diversity and determine how much variation is seen within a strain. Already new species are being described and catalogued according to the features outlined herein, which will advance research on this abundant protist group.

An improved taxonomy for *Paraphysomonas* will improve our understanding of ecological studies, especially those concerned with microbial food-webs, seasonality, and biogeography. Also, the benefit of knowing the genetic distance of silica-scaled protists like *Paraphysomo- nas* will give a more unified picture of their evolution and might also help us better interpret the

genetic diversity of scaleless protists, especially ones like *Spumella*, which branch in sev- eral parts of the chrysophyte tree and obviously had polyphyletic origins from algal ancestors entirely independently of *Paraphysomonas* (Cavalier-Smith & Chao 2006, Cavalier-Smith & Scoble 2013). Though *Spumella* were once included in Leucophycidae and Paraphysomonadida (Cavalier-Smith & Chao 1996), they are now firmly excluded (Cavalier-Smith & Chao 2006) and must be classified in at least three separate places within Ochromonadales, and at least two new genera established.

In conclusion, the combined ultrastructural and genetic study of clonal cultures of silica-scaled chrysophytes will overcome the limitations of studying either scale morphology or phylogeny alone and show which species described as *Paraphysomonas* really belong in the Paraphysomonadida and which belong in other orders. That will clarify the evolution and taxonomy of Chrysophyceae as a whole and pave the way for more detailed research.

Acknowledgements

Thanks to the International Chrysophyte Symposium Eight (ICS8) organizers for the invitation to present at the conference in Prague. JS thanks BBSRC for a DPhil studentship and TCS thanks NERC for a research grant.

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CHAPTER FOUR

Paper for publication:

paraphysomonads

This chapter of work is the collection of all the data accrued for the *Paraphysomonas* isolates that JMS cultured, sequenced and analyzed in the electron microscope. JMS obtained all of the raw data and produced all of the figures and plates and described them. The molecular analysis and production of trees was overseen by TCS for which JMS and TCS collaborated on inclusion of certain groups of taxa and for which types of phylogenetic tree analysis to perform. For this part of the work I attribute **90**% JMS, 10% TCS. The written manuscript was initiated and the first draft written by JMS after which TCS started a discussion section to which JMS contributed and concluded. The written work was **65**% JMS, 35% TCS.

This chapter is presented in the state as it was submitted to Protist rendered to the specifications of the rest of the thesis.

Scale evolution, sequence phylogeny, and revised taxonomy of chrysomonads: 34 new *Paraphysomonas* species, and new genera *Clathromonas*, *Patellomonas*, and *Acanthomonas*

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Heterotrophic chrysomonads of the genus *Paraphysomonas* are ubiquitous phagotrophs with diverse silica scale morphology. Over 50 named species have been described by electron microscopy from uncultured environmental samples. Sequence data exist for very few, but the literature reveals misidentification or lumping of most previously sequenced. For critically integrating scale and sequence data, 59 clonal cultures were studied light microscopically, by sequencing 18S rDNA, and recording scale morphology by transmission electron microscopy. We found strong congruence between variations in scale morphology and rDNA sequences, and unexpectedly deep genetic diversity, and describe 34 new species with spine scales in *Paraphysomonas* sensu stricto (Paraphysomonadidae). Species having base-plates with dense margins form three distinct clades; those with a simple margin only two. We place former *Paraphysomonas* species with markedly different scales in new genera: *Clathromonas*, belonging to a distinct clade, possibly sister to *Paraphysomonas*; *Patellomonas* (with *Clathromonas* forming Clathromonadidae fam. n. in Paraphysomonadida); and *Acanthomonas* (grouped with *Spiniferomonas* as Acanthomonadidae in Chromulinales). Molecular and morphological data are

mutually reinforcing; both are needed for evaluating paraphysomonad diversity and confirm excessive past lumping. New families for *Polylepidomonas* (Polylepidomonadaceae) and *Chrysosphaerella* (Chrysosphaerellaceae: Chromulinales) exclude these scaly phototrophs from Paraphysomonadida. We present evidence that chrysomonad scales evolved early.

Key words: 18S rDNA phylogeny; *Acanthomonas*; *Clathromonas*; heterokont; *Patellomonas*; scale ultrastructure.

Introduction

Colourless chrysomonads of the genera *Paraphysomonas* and *Spumella* are major phagotrophic components of freshwater and soil food webs, and *Paraphysomonas* is also widespread in marine environments (Charvet et al. 2011; del Campo and Massana 2011; Massana et al. 2004; Massana et al. 2006; Richards and Bass 2005). They are important feeders on bacteria that have received considerable experimental study (Jürgens et al. 1997; Lim et al. 1999; Pfandl et al. 2004; Simek et al. 1997; Zwirglmaier et al. 2009). However, it is becoming increasingly clear that the taxonomy of both 'genera' is unsatisfactory and that major revisions are needed. Ribosomal DNA phylogeny has shown that *Spumella* is certainly polyphyletic and that about five non-scaly chrysophyte lineages have independently lost photosynthesis and thus become *Spumella*-like in morphology (Boenigk et al. 2005; Boenigk 2008); eventually they must be divided into several genera. *Paraphysomonas* differs from *Spumella* by having numerous silica scales on its cell body; *Spumella* is naked, but it is easy to confuse these genera by light microscopy as scales cannot easily be seen in the light microscope for many species, and so many strains and their sequences are merely identified as '*Spumella*-like' (Boenigk et al. 2005). We focus here on the biodiversity and taxonomy of scaly heterotrophic chrysomonads formerly lumped as *Paraphysomonas* and show that more species than hitherto realised can be distinguished from *Spumella* in the light microscope.

Differences in scale morphology define ~56 *Paraphysomonas* species (Lucas 1967; Lucas 1968; Preisig and Hibberd 1982a; Preisig and Hibberd 1982b; Preisig and Hibberd 1983). The type species, *P. vestita*, is the only one not originally defined by scale morphology as it was discovered before electron microscopy (Stokes 1885 as

Physomonas vestita; De Saedeleer (1929) changed it to *Paraphysomonas vestita* because the type species (*Physomonas socialis*) was removed to another genus, *Monas*, now abandoned as a *nomen dubium* that roughly corresponds with *Spumella* (Boenigk 2008)). *Paraphysomonas vestita* spine scales were first drawn by Korshikov (1929) as 'nails with relatively large flat heads'. Houwink (1952), published the first electron micrographs of *Paraphysomonas* '*vestita'* spine scales, showing a circular base plate and long central pointed spine. Subsequent ultrastructural studies and environmental surveys of silica-scaled protists have shown a great variety of broadly similar, yet distinctly different, nail-like scales under the umbrella name *P. vestita* (Manton and Leedale 1961; Takahashi 1976; Cronberg and Kristiansen 1980; Thomsen et al. 1981; Santos and Leedale 1993; Bergesch et al. 2008; Petronio and Rivera 2010). It is unclear which, if any, of these structurally quite diverse scales are actually from *P vestita* or which represent undescribed species. Originally, 10 other nominal *Paraphysomonas* species have been described with more substantially different spine scales (8, retained within Paraphysomonadida, two having additional plate scales devoid of spines (not retained within Paraphysomonadida, one being a heliozoan and the other belonging to a different chrysophyte order). Hardly any *Paraphysomonas* species have been described from clonal cultures; nearly all are described from a few cells collected directly from the environment and dried on electron microscope grids. There is therefore almost no knowledge of the range of variation of their scales within a single strain, still less a single species, which has led to subsequent identification problems.

Ribosomal DNA sequences are available for only five named *Paraphysomonas* species. Unfortunately, existing trees show that some sequenced strains were misidentified. For three species (*P. vestita, P. imperforata* and *P. foraminifera*),

multiple sequences obtained by different authors are so different from each other that strains with the same name cannot reasonably be the same species (see the tree in del Campo et al. 2011). The position is most extreme for *P. vestita* and *P. foraminifera*, for each of which radically different sequences exist that are far apart on the trees; moreover one *P.* '*foraminifera*' sequence (AB022864) is almost the same as one *P.* '*vestita*' sequence (Z28335: Rice et al. 1997), just differing by *P. vestita* in having one inserted T (the *P. imperforata* sequence has three undetermined nucleotides). No ultrastructure was provided for most strains so their true identity cannot be judged and they are no longer available for study. Some sequences identified as *Paraphysomonas* have been published together with electron micrographs of scales (Caron et al. 1999; Rice et al. 1997); in all cases their detailed structure differs from that of the type strains, suggesting that none was correctly identified. The current picture of *Paraphysomonas* scale evolution is totally confused by these mistakes and the rarity of combined sequence and morphological data. It is also clear from environmental sequencing that more different sequences are already known in the *Paraphysomonas* spine-scale clade than the total number of named spine-scaled species, so the assertion that most *Paraphysomonas* species are already known (Finlay and Clarke 1999a) was overconfident.

A key problem is that the identity of the type species *P. vestita* is very loosely defined. Little is known from the original description beyond it being ~15 µm in diameter and having spines projecting from its body, but strains studied by electron microscopists under that name range in size from 8-26 µm and exhibit such a large range in detailed spine scale morphology and dimensions that they probably represent numerous different species. It has been claimed that *P. vestita* is the commonest and

most widespread *Paraphysomonas* (Finlay and Clarke 1999b). However, this claim could be a complete artefact of an excessively loose species definition arising from *P. vestita* not having originally been ultrastructurally defined. Insufficient precision in identification may also be true to some extent for the second commonest species *P. imperforata*, whose spine scales are relatively non-descript and differ obviously from those attributed to *P. vestita* only by lacking a dense margin on the base plate and from *P. foraminifera* by lacking holes on the base plate, i.e. *P. imperforata* is negatively defined. From the existing literature, not previously critically reviewed, it is clear that there are subtle and some more obvious differences in broadly similar scale types for both '*P. vestita*'-like and *P. imperforata*-like scales. To clarify these problems and put *Paraphysomonas* taxonomy on a sounder footing we studied 59 clonal cultures (mostly newly isolated) by light and electron microscopy and 18S rDNA sequencing and describe 34 new species with spine scales (including three new ones based on previously published work), and show how differences in scale morphology map onto the 18S rDNA tree.

In addition to the 8 previously known *Paraphysomonas* species with spine scales (i.e. *P. vestita, P. imperforata, P. foraminifera, P. bandaiensis, P. antarctica, P. circumforaminifera, P. porosa, P. oligocycla*) and *P. cylicophora*, whose scales we regard as modified spine scales, the 34 spine scale species described here make a total of 44 that we regard as constituting *Paraphysomonas sensu stricto* Table 3). All are relatively uniform morphologically. The large number of '*Paraphysomonas'* species described with very different scale types, most without spines and some with an open lattice, as in *P. butcheri* (Pennick and Clarke 1972) are here placed in other genera. Unlike species with spine scales, *P. butcheri* and most others with latticed scales have

two different scale types arranged in two layers: flat plate scales with perforations close to the plasma membrane and tiered crown scales outside them. Lucas (1968), in describing the first *Paraphysomonas* species with latticed rather than spine scales, thought it really deserved a separate genus, but unfortunately did not erect one. Others have suggested that the large array of '*Paraphysomonas*' species with ever more diverse open-mesh scales may deserve separation as a separate genus (Leadbeater 1972; Pennick and Clarke 1973; Takahashi 1976), but all have conservatively been left in *Paraphysomonas* making it excessively heterogeneous. 18S rDNA sequence trees show that *Paraphysomonas* with spine scales (*P. vestita, P. imperforata, P. foraminifera, P. bandaiensis*) are widely dispersed within a single large clade of environmental sequences, whereas '*P. butcheri*' with latticed scales is part of a smaller environmental clade, which on some trees groups weakly with the spine-scale clade and on some does not (Andersen 2007; Andersen et al. 1999; Richards et al. 2005). Thus it is unclear whether *Paraphysomonas* is monophyletic or polyphyletic. We have now isolated and sequenced a strain with scales indistinguishable from the original *P. butcheri* and describe as new species two strains studied earlier with different sequences and scale structures that it is now evident were previously misidentified as *P. butcheri* (Caron et al. 1999; Rice et al. 1997). As these *P. butcheri*-like strains have lattice scales that are structurally entirely unlike those of true *Paraphysomonas* with spine scales and either do not group with the spine-scale clade or do so only extremely weakly, we here assign them and all former *Paraphysomonas* species with latticed scales to a new genus *Clathromonas* with 28 named species, placed in a new family Clathromonadidae, but conservatively included with

Paraphysomonas in the existing order Paraphysomonadida (= Paraphysomonadales, but we use ICZN not IBN for this purely phagotrophic order as they are not algae).

The fact that *Paraphysomonas* scales can persist in sediments for several months after the cells have died allows them to be used for surveying their diversity in particular habitats, as well as their biogeography, without having to culture them (Esteban et al. 2012; Finlay and Clarke 1999a; Lee and Takahashi 1993; Santos and Leedale 1993; Saha and Wujek 1990). Whilst that approach to understanding their ecology has great potential, it is likely to be of severely limited value until their taxonomy has been radically improved. It is well known that conclusions about microbial ubiquity such as those deduced for *Paraphysomonas* from scale surveys (Finlay and Clarke 1999a,b) are greatly limited by the nature of the species concept applied to them: excessive lumping strongly biases conclusions towards ubiquity and away from limited geographic distribution that could become apparent with narrower and more discriminating species boundaries (Bass and Boenigk 2011; Boenigk et al. 2012; Patterson and Lee 2000). This has been clearly demonstrated for several protist groups, notably foraminifera and diatoms (Alverson 2008; Amato et al. 2007; Kucera and Darling 2002; Sarno et al. 2005). Data from our new cultures shows that there has been excessive lumping at the species level in both *Paraphysomonas* and *Clathromonas* and that these two genera are clearly very distinct and should not have been lumped together. But the degree of lumping has been even greater than that, as chrysomonads with three other broad classes of scales have also been lumped with and named as *Paraphysomonas*. We establish a new genus *Patellomonas* for 11 former *Paraphysomonas* species with bowl-shaped, non-lattice, plate scales only, and place it with *Clathromonas* in Clathromonadidae within Paraphysomonadida, but conclude

that numerous other former *Paraphysomonas* species with yet other scale morphotypes probably do not even belong in that order.

We critically review the literature on the other morphotypes and argue that their scales differ so greatly from those of *Paraphysomonas*, *Clathromonas,* and *Patellomonas* that they must be considered separate genera. Whether or not *Paraphysomonas* is distinct from *Spiniferomonas* (Takahashi 1973) has been controversial (Preisig and Hibberd 1982b; Nicholls 1985; Siva 1978). Preisig and Hibberd (1982a) transferred *Spiniferomonas bourrellyi* (Takahashi 1973) to *Paraphysomonas*, but its unbranched, pointed spine scales are very different from the nail-like spine scales of *Paraphysomonas* sensu stricto, and it has separate distinctive plate scales - unlike *Paraphysomonas*, so we retain *Spiniferomonas* as a separate genus to which we transfer six *Paraphysomonas* species established by Preisig and Hibberd (1982a) as well as *P. pileata* of Štefanová and Kalina (1992). However, we segregate those with winged and terminally branched spines to a new genus *Acanthomonas* that we group with *Spiniferomonas* as Acanthomonadidae. We also slightly realign the disputed boundary between *Spiniferomonas* and the largely colonial photosynthetic scaly genus *Chrysosphaerella*, previously placed in Paraphysomonadaceae (Preisig and Hibberd 1983) and establish a new family for it plus the new genus *Chrysospina* here segregated from it, as sequence trees had already shown that *Chrysosphaerella* is unrelated to *Paraphysomonas* (Andersen 2007; del Campo and Massana 2011) but related to the uniciliate Chromulinales. Our critical re-evaluation of scale diversity strongly suggests that Acanthomonadidae, for which no sequences are yet known, are more closely related to *Chrysophaerella* and *Chrysospina* than to *Paraphysomonas*, so we place them all in a new silica-scale-bearing suborder Chrysospineae in a revised

order Chromulinales. We also transfer one *Paraphysomonas* and one *Spiniferomonas* species originally misclassified as chrysomonads (Štefanová and Kalina 1992) to Heliozoa.

As numerous clades of chrysomonad DNA sequences of unknown phenotype have recently been discovered (Charvet et al. 2011; del Campo and Massana 2011) our trees include numerous representatives of all of them, in order to clarify their relationships to paraphysomonads, test the monophyly of Paraphysomonadida, and clarify their relationships with other chrysomonads. We include representatives of all major clades of cultured chrysophytes and all significant ochrophyte outgroups to provide the most comprehensive and arguably most reliably rooted chrysophyte tree to date. We found seven deeply branching clades of Chrysophyceae containing known organisms, plus either one or two huge environmental clades of unknown phenotype, though the relationships amongst these 8-9 major clades cannot be confidently determined from the 18S rDNA tree. Previously it had been shown that Ochromonadales was paraphyletic or polyphyletic (Cavalier-Smith and Chao 2006; Andersen 2007) and the boundaries between Ochromonadales, Chromulinales, and Paraphysomonadida were confused. We therefore now restrict Ochromonadales to the major clade that includes most *Ochromonas* strains and all four *Spumella* clades and have established a new non-scaly chrysomonad order Apoikiida for the other former ochromonadalean clade. Together with revisions of the boundaries between existing chrysophyte orders, this makes seven orders that now correspond precisely with the major sequence-defined clades of Chrysophyceae. We discuss how silica scale evolution maps onto this more taxonomically comprehensive chrysophyte tree.

Results

About 75 putative *Paraphysomonas* cultures were obtained from freshwater, soil, and marine environments. We sequenced 18S rDNA for phylogenetic analysis from 59 genetically distinct cultures, measured their cells and cilia in the light microscope, and took transmission electron micrographs of their scale structures using whole mounts. All but one of our cultures examined ultrastructurally had simple spine scales with a broad, almost flat usually circular unperforated base plate and relatively slender unbranched central spine broadly similar to those of *P. vestita*, as interpreted by Korshikov (1928) and Manton and Leedale (1961), and *P. imperforata* (Lucas 1967), yet showed a huge genetic diversity. We also found just one *Spumella* sp. (JQ967332 strain CH3). The relative dimensions and detailed structure of *Paraphysomonas* scales' base plate and spine varied systematically amongst the strains in ways that correlate with their position on the tree, enabling us to establish 35 new species with spine scales based on our new observations plus four more stemming from reinterpretation of the literature. The culture lacking spine scales had latticed plate and crown scales and was identified as *Paraphysomonas butcheri*, which is here transferred to the new genus *Clathromonas*. We present our sequence trees and explain our new subdivision of former *Paraphysomonas* into several genera before describing each new species in detail.

Phylogeny

Phylogenetic analyses used a very large alignment with 329 ochrophyte 18S rDNA sequences (1672 masked positions) and 239 chrysophyte sequences (1681 masked positions) in the hope that we could not only see where '*Paraphysomonas*' sequences

branch within Chrysophyceae but also clarify the uncertain relationships of the chrysophyte orders and positions of chrysophyte-related environmental DNA sequences. Figure 1 shows the large-scale structure of the tree: Chrysophyceae has 10 major clades, of which eight include known organisms and two only consist of exclusively environmental sequences of unknown phenotype. Six organismally defined clades are predominantly (or for Hydrurales entirely) algal (i.e. photosynthetic), whereas two comprise purely heterotrophic phagotrophs possessing scales: Paraphysomonadidae and Clathromonadidae. Paraphysomonadidae, Clathromonadidae, and environmental clade 1 form a robust clade (PP 0.97/BS 99%) (labelled Paraphysomonadida) in both Bayesian and maximum likelihood (ML) analyses, but the relative branching order of these three groups is essentially unresolved although both methods weakly place environmental clade 1, not Clathromonadidae, as sister to Paraphysomonadidae. The branching order of Paraphysomonadida and the other seven orders and environmental DNA clade 2 is also very poorly supported and inconsistent between the two methods. Thus single-gene analysis is inadequate to establish the basal branching order of Chrysophyceae, even though seven orders were consistently monophyletic by both methods, several with strong support; moreover, the purely photosynthetic, scale-bearing Synurales branches within Chrysophyceae and does not branch more deeply and is thus not sister to all the other branches. Clathromonadidae is a strongly supported clade in both Bayesian and ML trees. With this large taxon sample environmental clade 2 invariably groups strongly with Chrysophyceae, and Chrysophyceae are consistently sisters to Synchromales, with Picophagea apparently paraphyletic. Individual clades are collapsed in Figure 1 to emphasise the overall structure of the tree. The internal branching order of

Paraphysomonadidae is shown in Figure 2 and of Clathromonadidae and all other chrysophyte clades in Figure 3; the internal branching order of all ochrophyte outgroups is in Supplementary Figure S1.

Figure 2 shows that the large *Paraphysomonas* clade consists of four major subclades of distinctly different scale structure, treated here as subgenera, plus four sparsely represented environmental lineages of unknown scale structure (three with only one sequence) that branch outside them. Subgenera *Paraphysomonas* and *Hebetomonas* form a consistent strongly supported clade (Posterior Probability 0.79/ Bootstrap BS 81%); this joint clade is sister to subgenus *Acrospina*, but this relationship is strongly supported only on Bayesian trees (PP 1/ BS 38%). Three of the four deeply branching environmental lineages are specifically related to subgenera *Hebetomonas* and *Acrospina* and thus are likely to be of similar phenotype rather than being distinct lineages of novel phenotype; one marine clone DQ103782 is robustly sister to the *Paraphysomonas*/*Hebetomonas clade.* The largest clade (subgenus *Paraphysomonas*) includes twenty new named species that have spine scales with unperforated base plates with a marginal dense margin and relatively long, typically simply pointed, spines similar to those lumped in the literature as *P. vestita* from Houwink (1952) and Manton and Leedale (1961) onwards. Clearly, their genetic diversity is immensely greater than can reasonably be accommodated in a single species. This large clade has five speciose major subclades (A-E), whose relative branching order is robust and strongly supported by both methods; the three basal subclades (A-C) are all exclusively freshwater, suggesting that was the ancestral habitat for subgenus *Paraphysomonas*. The two derived subclades (D, E) with somewhat shorter spines are robustly sisters; subclade E, with two robust subclades, is exclusively from soil and subclade D has a

mixture of soil, freshwater and marine species. The two marine species in D previously identified as *P. vestita* are almost certainly misidentified (see discussion). They are genetically different from each other and both extremely distant from the third freshwater "*P. vestita*" (sub clade C), which might have been correctly identified but the authors did not specify which strain (PV10 or DB1) was used for transmission electron microscopy (TEM) (Lim et al, 2001), but their picture shows a scale with a spine of 2.9 µm and a base plate of 1.4 µm, which fits in with other scale dimensions measured in this study. In subclade D "*P. foraminifera*" is also likely to be misidentified, as *P. foraminifera* scale base plates lack a dense margin and are multi-perforated (Lucas 1967) unlike any of the 23 species in subgenus *Paraphysomonas* that have been both sequenced and studied ultrastructurally. Subclades B-E with very long branches all share numerous insertions in 18S rDNA absent from other *Paraphysomonas* (and chrysophytes), showing a correlation between extra rapid sequence substitution and extra molecular length; they share a common sequence signature AT (*P. vulgata vulgata* pos. 726-763) where all other Chrysophyceae in this alignment have TC. 18S rDNA sequence signatures were also found for the two smaller *Paraphysomonas* subgenera (see taxonomy section).

A robustly supported sister clade (PP 0.92/BS 79%) to the major long-spine, dense-margin clade (subgenus *Paraphysomonas*) is a small predominantly (and probably ancestrally) marine clade (subgenus *Hebetomonas*) with relatively small cells and dramatically smaller scales, whose shorter spines are always blunt-ended and emerge centrally from comparatively narrower base plates. The *Hebetomonas* clade consists of five marine environmental sequences and four new species (one only freshwater); the *P. hebes*/*hebetispina* subclade of four new species does not have a

dense base plate margin but sometimes a faint annular fold on the base plate that is not present in subgenus *Paraphysomonas* or *P. parahebes*. The *Hebetomonas* clade is consistently, but more weakly, sister to subgenus *Paraphysomonas*. The second most speciose clade (subgenus *Acrospina*) comprises species lacking a dense base-plate margin that may either be imperforate (most species, formerly lumped as *P. imperforata*) or with numerous holes (species formerly lumped as *P. foraminifera*). The *Acrospina* clade is predominantly and probably ancestrally marine, but has two substantial phyletically distinct freshwater subclades. Strains with a perforate base plate form a small subclade (PP 0.99/ BS 90%) within the predominantly and almost certainly ancestrally imperforate lineages. The *Acrospina* clade is predominantly short-spined, spines barely tapering with a short dull to rounded tip, but the deep-branching *P. acuminata*/*cuspidata* subclade (PP 1/ BS 96%) has characteristically long barely tapering spines with a short very pointed tip (as long as in subgenus *Paraphysomonas*). The fourth morphologically defined subclade (subgenus *Brevispina*) consists of freshwater or soil lineages (e.g. *P. ovalis*, *P. segmentata*) with small cells and scales, short spines, and dense base-plate margins. Thus three clades have dense margined base plates (subgenera *Paraphysomonas* and *Brevispina,* and *P. parahebes*) and two have plain base-plate margins (subgenus *Acrospina* and the main subclade of subgenus *Hebetomonas*). It is not possible to decide which of these alternate states is ancestral for *Paraphysomonas* sensu stricto.

To test whether the poorly supported basal topology of the chrysophyte tree is sensitive to taxon sampling amongst the ochrophyte outgroups we also ran chrysophyte trees after removing the most distant 90 outgroup taxa, i.e. all except *Picophagus flagellatus* and Synchromales, leaving 239 Chrysophyceae sequences. This

did not significantly affect the internal branching order of most chrysophyte clades (not shown) or the monophyly and separateness of both Paraphysomonadidae and Clathromonadidae, but it did disrupt the previously robust grouping of Paraphysomonadidae, environmental clade 1 (EC1), and Clathromonadidae, and caused environmental clade 2 (EC2) to split into two subclades (Fig. 4). Subclade EC2H remained at the base Chrysophyceae, where it is now joined by EC1 to form a new weakly supported joint clade, whereas EC2H, moved slightly to become weakly sister to Hydrurales (PP 0.25/BS 11%), which is no longer the deepest branching order of Chrysophyceae. This instability to outgroup taxon sampling means that we cannot really say whether EC2H is sister to EC2I (Fig. 1) or to EC1 (Fig. 4) or whether Paraphysomonadidae and Clathromonadidae are really mutually related as Figure 1 indicated. In either case there are only two major environmental clades. If the outgroups were to be removed entirely from Figure 4 and the tree were re-rooted as in Figure 1, the chrysophyte topology would be almost identical to that in Figure 1: the only difference would be the movement of EC2I, and reversed branching order of Synurales and Ochromonadales. Thus the apparently large difference in branching order of Figures 1 and 4 is deceptive, the main problem being not conflicting tree topology within Chrysophyceae but determining the position of its root.

We suspect that Figure 1 with its more extensive and balanced selection of outgroups may be closer to the truth for the position of the chrysophyte root and that the apparent rearrangement of its deepest branches in Figure 4 may arise from phylogenetically long-branch attraction of EC1 and Paraphysomonadidae towards the base of the tree by the remaining sparsely sampled picophagean outgroups; multigene trees are required to test this. If this interpretation is correct, Paraphysomonadida as

circumscribed here is probably holophyletic and probably includes EC1 and possibly also EC2H. In both Figures 1 and 4 Hibberdiales and Chromulinales are sisters and these two orders group with Ochromonadales and Synurales as a weakly supported four-order clade; the exclusion of both Paraphysomonadidae and Clathromonadidae from this reproducible clade is consistent with the exclusion of Paraphysomonadida from both Chromulinales and Ochromonadales as a distinct non-photosynthetic order (Cavalier-Smith et al. 1996). A sister relationship between Hibberdiales and Chromulinales is recovered by both methods in Figure 4, so in this respect ML and Bayesian trees agree with each other better than in Figure 1 with more distant outgroups, but they remain contradictory with respect to the possible sister relationship between Synurales and Ochromonadales sensu stricto.

Taxonomy: revised classification of Paraphysomonadida

This improved classification is partly based on our new ultrastructural data and partly on critical re-evaluation of existing literature. Formerly all chrysomonads with silica scales that do not belong in the relatively homogeneous order Synurales (Andersen 2007; Lavau et al. 1996) were lumped in one family, Paraphysomonadaceae (Preisig and Hibberd 1983; Preisig 1995), despite their huge variation in scale morphology and lack of any positive character to unite the whole family. Their scale morphology was far more diverse than that of the entire order Synurales and there has been no concrete evidence that all were mutually related, and some sequence evidence that they are not (Andersen 2007). Here we separate them into five families according to the major scale types (three of which occupy very distinct major clades on our sequence trees: Paraphysomonadidae, Clathromonadidae, and Chrysosphaerellaceae - the latter is photosynthetic and within Chromulinales in Figure 4). We also remove the majority of

the previously described *Paraphysomonas* species from that genus to make it morphologically homogeneous (all with nail-like scales as in Figure 2) and transfer them to the existing genus *Spiniferomonas* or to three new genera established below to embrace three contrasting scale types that are not nail-like. We preface the diagnoses of the new families and genera and new combinations for existing species by a highly condensed summary of their earlier taxonomic treatment:

The chrysomonad order Paraphysomonadales Cavalier-Smith, 1996 was established to include both *Paraphysomonas* and *Spumella* (Cavalier-Smith and Chao 1996), but the polyphyletic *Spumella* were transferred to Ochromonadales in the light of sequence evidence that all *Spumella* clades nest within photosynthetic Ochromonadales and none are related to *Paraphysomonas* (Cavalier-Smith and Chao 2006). Thus, Paraphysomonadales now includes just the family Paraphysomonadidae (=Paraphysomonadaceae) Preisig and Hibberd, 1983. This family is often included in Chromulinales, but as noted above our trees reproducibly confirm earlier sequence evidence that showed that is incorrect (Andersen 2007; Cavalier-Smith and Chao 2006), and that *Paraphysomonas* and *Clathromonas* are distinct deep- branching clades of Chrysophyceae, both genetically more distant from Chromulinales than is the photosynthetic scale-bearing Synurales. Thus Paraphysomonadales clearly merits its separate ordinal status; as it is totally heterotrophic and thus non-algal and is protozoan-like in its phagotrophic nutrition, we here treat it under ICZN as order Paraphysomonadida, following the recommendation that phagotrophic suprageneric chromist taxa that consist exclusively or almost exclusively of heterotrophs should be treated under the zoological code of nomenclature as they are not algae (Cavalier-Smith 2007). Especially following the inclusion of more purely phagotrophic phyla in

the kingdom Chromista (Cavalier-Smith 2010), the earlier convention of treating all Chromista nomenclaturally as plants (Cavalier-Smith 1981) must be discontinued.

Paraphysomonadidae originally included the heterotrophic *Paraphysomonas*, and the scaly photosynthetic *Chrysosphaerella* and *Polylepidomonas*, as well as *Spiniferomonas* as distinct genus despite Preisig and Hibberd (1983) having largely, but not entirely, merged *Spiniferomonas* into *Paraphysomonas.* Here we establish separate families Chrysosphaerellaceae and Polylepidomonadaceae for the first two genera as their scales differ greatly from each other and from those of *Paraphysomonas*. Because our and previous sequence trees show that the type species of *Chrysosphaerella* (*longispina*) belongs in Chromulinales (Fig. 4) and never groups with *Paraphysomonas*, we place it in Chromulinales not Paraphysomonadida. *Polylepidomonas vacuolata* Thomsen was originally described as a *Paraphysomonas* (Thomsen et al. 1981); Preisig and Hibberd (1993) made a new genus for it, as it is photosynthetic, unlike *Paraphysomonas*. The uniform *Polylepidomonas* scales are so unlike those of *Paraphysomonas* that we exclude it also from Paraphysomonadida. This and the exclusion for reasons given below of *Spiniferomonas* formalises the previous implicit exclusion of all photosynthetic genera when Paraphysomonadida was established (Cavalier-Smith and Chao 1996).

Our sequence trees showed that every *Paraphysomonas* strain with nail-like spine scales (those possessing a round or rarely oval base plate and single unbranched slender spine) is part of a very large and robust clade that does not contain any strains with contrasting scale types. We therefore restrict the genus *Paraphysomonas* to that morphotype. The photosynthetic *Polylepidomonas* with scales that are unique oval plates with elongated perforations is given a new family. We also exclude all

Spiniferomonas from Paraphysomonadida, not because some species have chloroplasts but because they possess both spine scales and also radically different plate scales that do not resemble spine scale bases, unlike any *Paraphysomonas*.

Family Paraphysomonadidae (=Paraphysomonadaceae) Preisig and Hibberd, 1983. **Revised diagnosis:** biciliate, non-amoeboid, unicellular, heterotrophic chrysomonads; cell body covered by numerous spine scales with a usually circular, rarely oval, base plate that is approximately orthogonal to a long thin central spine; spine unbranched, unwinged, many times narrower than base plate even at its base; base plate entire or with small perforations of varying distribution, but no large lacunae; spine length varies from just longer than to several times base-plate width; separate plate scales generally absent, but if present closely resemble the base plate of the spine scale, usually larger in diameter, but with the spine missing, and lack a distinctive morphology; slender posterior stalk anchors cell to substratum or trails behind swimming cell. Plastid: a colourless leucoplast without stigma. Contractile vacuole present in freshwater species. Posterior cilium lateral, much shorter than forward directed anterior cilium. There are four single nucleotide 18S rDNA signatures for family Paraphysomonadidae: A (position 1387); T (position 1465); C (position 1474); G (position 1476); all positions are for the reference strain 'Arb' *P. ovalis* (JQ967331) from the deepest clade. These nucleotide signatures exclude all other Chrysophyceae, except for position 1465 where one clone sequence 'Marine Biosope T3' (FJ537322) showed a G and all other chrysophytes had an A; this unusual difference could be a sequencing error. Sole and type genus *Paraphysomonas* De Saedeleer, 1929. Type species *P. vestita* (Stokes) De Saedeleer, 1929. We retain only 9 of the 56 existing

species in this genus (including *P. vestita*), which is now homogeneous with respect to scale type, but make 35 more in a later section. The other previously named species that remain in *Paraphysomonas* are: *P. imperforata* Lucas (1967) and *P. foraminifera* Lucas (1967), *P. bandaiensis* Takahashi (1976), *P. antarctica* Takahashi (1987), *P. porosa* Dürrschmidt and Cronberg (1989), *P. circumforaminifera* Wujek (1983), *P. oligocycla* Takahashi (1987), and *P. cylicophora* Leadbeater (1972) with scales with a solid base plate bearing a perforated goblet that we postulate may be a highly modified spine scale. There are therefore now 44 *Paraphysomonas* species plus many more that used to be included in the genus.

New subgenus *Paraphysomonas* De Saedeleer 1929. **Diagnosis:** round to slightly oval unperforated base plate with inflexion at edge, so edge appears denser by electron microscopy, inner annular pattern absent; central spine prominently tapers completely to a blunt or rounded tip or to a short oblique blunt tip (spine, on average 3.2 µm or more and the cell length typically 7 µm or more). Type species *Paraphysomonas* (formerly *Physomonas*) *vestita* Stokes, 1885.

New subgenus *Hebetomonas* Cavalier-Smith. **Diagnosis:** round imperforate base plate either inrolled at edge (appears denser by electron microscopy) or with inner annular pattern, but not both; central spine barely tapering, if at all, to a truncate or blunt tip (spine typically 1.4 µm or less. Cell small (typically 6.6 µm or less). Type species *Paraphysomonas hebetispina* Scoble and Cavalier-Smith. 18S rDNA sequence signature GGTTC at position 583-587 of *P. hebes* (JQ967320). **Etymol.** *hebes* L. blunt, referring to blunt ends of scale spines; *monas* Gk unit.

New subgenus *Acrospina* Cavalier-Smith. **Diagnosis:** round or oval base plate, not obviously inflexed at the edge, sometimes with inner annular pattern; base plate imperforate or perforated by many small holes; central spine non- or barely tapered, ending in a short rounded, pointed or acuminate end. Wide range of cell sizes from 3.6-9.5 µm, and spine lengths 0.79-5.4 µm. Long cilium typically more than 2.5 x cell length. Type species *Paraphysomonas acuminata* Scoble and Cavalier-Smith. **Etymol.** *acer*, *acr*- L. sharp; *spina* L. thorn, because of the sharp scale spines.

New subgenus *Brevispina* Cavalier-Smith. **Diagnosis:** round or oval unperforated base plate typically with dense inflexed edge, without inner annular pattern; central spine short (less than 1.5 µm), either non- barely tapering spine, sometimes segmented, ending in a blunt tip. 18S rDNA sequence signature CAAG at position corresponding to 583-587 of *P. segmenta* JQ967330). Type species *Paraphysomonas ovalis* Scoble and Cavalier-Smith. **Etymol.** *brevis* L. short; *spina* L. thorn, because of the short scale spines.

Family Clathromonadidae Cavalier-Smith fam. n. As in *Paraphysomonas*, cells stalked, non-photosynthetic, with leucoplast, without stigma, but differing in scale structure. Scales either non-perforated dishes with narrow margins or more often one or two types of open meshwork scales. Simple spine scales with entire bases absent, unlike most *Paraphysomonas;* perforated spine scales if present (rarely), never the sole scale type as in *Paraphysomonas*, but have an open mesh base plate, unlike the numerous small perforations of *Paraphysomonas foraminifera* as well as a meshwork broad base to the spine itself. Type genus ***Clathromonas* gen. n. Diagnosis:** usually with two types of scales: inner holey plate scales, round to oval, with large holes of varied shape

relative to intervening material; more complex three dimensional, basket-like scales built of a very open meshwork, of varied shapes, often present in addition to or instead of holey plate scales – these may be crown scales, chair-like or tower-like. In three species with dimorphic scales (*diademifera*, *ignivoma*, *undulata*) plate scales unperforated, somewhat like those of *Patellomonas*. **Etymol:** *clathri* L. lattice; *monas* Gk. unit. Type species *Clathromonas butcheri* comb. n. basionym *Paraphysomonas butcheri* (Pennick and Clarke 1972). Other included genus *Patellomonas* gen. n. (see below).

We make new combinations for 23 other existing species and for one subspecies here raised in rank to full species (*C. poteriophora*) as its scales are sufficiently different from *S. coronata* to indicate a substantial genetic difference in the light of our data for *Paraphysomonas*, so there are now 28 *Clathromonas* species; at least 10 are known to have leucoplasts:

Clathromonas cancellata comb. n. basionym *Paraphysomonas cancellata* (Preisig and Hibberd 1982b)

Clathromonas canistrum comb. n. basionym *Paraphysomonas canistrum* (Preisig and Hibberd 1982b) Leucoplast.

Clathromonas corbidifera comb. n. basionym *Paraphysomonas corbidifera* (Pennick and Clarke 1973)

Clathromonas coronata comb. n. basionym *Paraphysomonas coronata* Moestrup and Zimmerman in (Thomsen et al. 1981)

Clathromonas cribosa comb. n. basionym *Paraphysomonas cribosa* (Lucas 1968)

Clathromonas diademifera comb. n. basionym *Ochromonas diademifera* (Takahashi, 1972). Synonyms *Lepidochromonas diademifera* Kristiansen, 1980; *Paraphysomonas diademifera* (Preisig and Hibberd 1982a). Leucoplast.

Clathromonas eiffellii comb. n. basionym *Paraphysomonas eiffellii* Thomsen in (Thomsen et al. 1981)

Clathromonas elegantissima comb. n. basionym *Paraphysomonas elegantissima* (Kling and Kristiansen 1983)

Clathromonas faveolata comb. n. basionym *Paraphysomonas faveolata* (Rees et al. 1974)

Clathromonas homolepis comb. n. basionym *Paraphysomonas homolepis* (Preisig and Hibberd 1982b)

Clathromonas ignivoma comb. n. basionym *Paraphysomonas ignivoma* (Preisig and Hibberd 1982b) Leucoplast.

Clathromonas inconspicua comb. n. basionym *Paraphysomonas inconspicua* (Takahashi 1976). We do not accept its synonymization with *P. butcheri* (Preisig and Hibberd 1982b); though we agree that interpretation of crown scale structure is not easy (they appear to differ), its plate scales are very distinct, with a much greater contrast between the large holes and tiny ones than in *C. butcheri*. Moreover, *C. inconspicua* is from freshwater, not brackish like *C. butcheri* (however *P. butcheri* from Cambridgeshire freshwater ponds (Preisig and Hibberd 1982b) seems to have been correctly identified and is very similar to our brackish *C. butcheri* strain –see below). But we agree with Preisig and Hibberd (1982b) that *P. butcheri* of Takahashi (1976) was certainly misidentified (as was his *P. foraminifera*); however we do not agree with them that Takahashi's '*butcheri*' was *P. morchella*, as the small mesh holes of that species were much less evident; it may be an undescribed species somewhat similar to *P. morchella* with a less evident chair-back and fewer small holes.

Clathromonas manubriata comb. n. basionym *Paraphysomonas manubriata* (Preisig and Hibberd 1982b) stat nov. (Vørs et al. 1990)

Clathromonas morchella comb. n. basionym *Paraphysomonas morchella* (Preisig and Hibberd 1982b). Leucoplast.

Clathromonas poteriophora comb. n. basionym *Paraphysomonas poteriophora* Moestrup and Kristiansen in (Thomsen et al. 1981). We strongly disagree with its inclusion within *C. coronata* (Vørs et al. 1990), as their scales are very distinct. We think that figs 6-10 of Vørs et al. are not *coronata*, but a third, undescribed species more closely related to *coronata* than to *poteriophora*, and are not intermediate between *coronata* and *poteriophora* and do not justify their merger. Their claim that (Preisig and Hibberd 1982b) showed intermediates is also disputable; in our view, figs 19 I, L-O of

(Preisig and Hibberd 1982b) are neither *C. poteriophora*, nor intermediates between *poteriophora* and *coronata* as Vørs et al. apparently assumed, but a fourth species (undescribed) closer to *poteriophora* than to *coronata*.

Clathromonas quadrispina comb. n. basionym *Paraphysomonas quadrispina* Thomsen and Kristiansen in (Thomsen et al. 1981). Leucoplast.

Clathromonas runcinifera comb. n. basionym *Paraphysomonas runcinifera* (Preisig and Hibberd 1982b)

Clathromonas sideriophora comb. n. basionym *Paraphysomonas sideriophora* (Thomsen 1975)

Clathromonas sigillifera comb. n. basionym *Paraphysomonas sigillifera* Moestrup in (Thomsen et al. 1981)

Clathromonas stelligera comb. n. basionym *Paraphysomonas stelligera* (Preisig and Hibberd 1982b)

Clathromonas stephanolepis comb. n. basionym *Paraphysomonas stephanolepis* (Preisig and Hibberd 1982b). Leucoplast.

Clathromonas subquadrangularis comb. n. basionym *Paraphysomonas subquadrangularis* (Preisig and Hibberd 1982b). Leucoplast.

Clathromonas takahashii comb. n. basionym *Paraphysomonas takahashii* Cronberg and Kristiansen in (Thomsen et al. 1981)

Clathromonas subrotacea comb. n. basionym *Paraphysomonas subrotacea* Thomsen in (Thomsen et al. 1981). Leucoplast.

Clathromonas undulata comb. n. basionym *Paraphysomonas undulata* (Preisig and Hibberd 1982b). Leucoplast.

***Patellomonas* gen. n.** Scoble and Cavalier-Smith. **Diagnosis:** only one type of unperforated scale, usually bowl-shaped, often with surface textural differences between the narrow flat margin and concave central bowl; scales sometimes flat and undifferentiated; crown scales and spine scales absent. Type species *Patellomonas circumvallata* comb. n. basionym *Paraphysomonas circumvallata* Thomsen in

(Thomsen et al. 1981). **Etymol:** *patella* L. bowl; *monas* Gk unit. **Comment.** This genus is placed in Clathromonadidae because three *Clathromonas* species have somewhat similar non-meshwork plate scales. We establish new combinations for 5 other species and for a further 5 subspecies hereby raised in rank to full species:

Patellomonas amphiplana comb. n. basionym *Paraphysomonas amphiplana* (Wee and Wujek 1986; this species and *P. planus* have flat scales without a differentiated margin, so it is questionable whether they are related to the others)

Patellomonas atrema stat. n., comb. n. basionym *Paraphysomonas punctata atrema* (Preisig and Hibberd 1982b)

Patellomonas hexagona stat. n., comb. n. basionym *Paraphysomonas punctata hexagona* (Olsen et al. 1999)

Patellomonas limbata comb. n. basionym *Paraphysomonas limbata* (Preisig and Hibberd 1982b)

Patellomonas mediogranulata stat. n., comb. n. basionym *Paraphysomonas circumvallata mediogranulata* (Preisig and Hibberd 1982b)

Patellomonas microlepis stat. n., comb. n. basionym *Paraphysomonas punctata microlepis* (Preisig and Hibberd 1982b). Leucoplast.

Patellomonas planus comb. n. basionym *Paraphysomonas planus* (Wujek and Saha 1995)

Patellomonas punctata comb. n. basionym *Paraphysomonas punctata* Zimmerman in (Thomsen et al. 1981)

Patellomonas simplicior stat. n., comb. n. basionym *Paraphysomonas punctata simplicior* (Preisig and Hibberd, 1982b)

Patellomonas runciniferopsis comb. n. basionym *Paraphysomonas runciniferopsis* (Wujek and Saha 1995)

Taxonomy: Revision of Chromulinales to include scaly suborder Chrysospineae

Lauterborn (1890) established *Chrysosphaerella* for a photosynthetic colonial chrysophyte *C. longispina*, with very few spine scales, which electron microscopy later showed to have two distinctive angled flanges at the base. A heterogeneous mixture of species, some very similar to the type species, e.g. *C. brevispina* Korshikov (1942), and some very different, e.g. *C. salina* Birch-Andersen (1973), has been added to the genus. Some are non-photosynthetic and have scales more like those of *Thaumatomastix*, and have been removed from Chrysophyceae (*C. tripus* (Takahashi & Hara) Beech & Moestrup (1986)) and are now in phylum Cercozoa (Howe et al. 2011). Others have been transferred to and fro between *Chrysosphaerella* and the genus *Spiniferomonas*, about which there is controversy as to the presence or absence of chloroplasts. We take this good opportunity to sort out the confused taxonomy of these genera. Preisig and Hibberd (1983) transferred *Spiniferomonas* and *Chrysosphaerella* to Paraphysomonadaceae and, at the same time, described several new species of *Paraphysomonas,* which we think are related to *Spiniferomonas* and *Chrysosphaerella* and not *Paraphysomonas,* and therefore exclude them from our refined definitions of Paraphysomonadida, Paraphysomonadidae and *Paraphysomonas*. These now exclude former *Paraphysomonas* species that had both complex spine scales and oval plate scales, as do *Spiniferomonas* and *Chrysosphaerella*. In all except one of these excluded species the plate scales have an oval raised ridge on the dorsal side, set in from the outer margin of the scale, thus leaving a flat outer margin. Such plate scales with an oval ridge are not seen in Paraphysomonadida sensu stricto or in Synurales. We regard them as a synapomorphy for a new group of chrysomonads unrelated to those two orders and characterised by the presence of oval-ridged scales

and spine scales dissimilar from those of *Paraphysomonas*. Ribosomal RNA trees robustly show that *Chrysosphaerella longispina* is not related to *Paraphysomonas*, but is weakly but consistently sister to a robust non scale-bearing clade of uniciliates including the type species of *Chromulina*, *Chrysamoeba* and *Oikomonas* (Fig. 3) that must be regarded as the core of any revised order Chromulinales (Andersen 2007). Thus, species with ridged oval plate scales must be excluded from Paraphysomonadida as a new major group. In view of the sequence trees we chose not to make the new scaly higher taxon a separate order, but a new suborder of a refined order Chromulinales, which now excludes all taxa that do not group on trees with the core *Chromulina*/*Chrysamoeba*/*Oikomonas* clade that corresponds with Pascher's original suborder Chromulineae (Table 1):

Chrysospineae Cavalier-Smith subord. n. **Diagnosis:** chrysomonads with both silicified spine-scales and often more numerous oval plate scales; plate scales usually with raised distal oval ridge separated from scale edge by a broad margin; spine basally flared out or with one or two lateral flanges, sometimes with lateral wings and/or branching distally into two or three points; colourless or photosynthetic plastids; unicellular or colonial, never amoeboid. Long hairy anterior cilium and shorter lateral smooth cilium; centrioles roughly orthogonal. **Etymology:** name based on included genus *Chrysosphaerella*. Three families:

Family Chrysosphaerellaceae Cavalier-Smith fam. n. **Diagnosis**: colonial or unicellular phototrophs, with one or few extremely long spine scales; spine base with second lateral flange, angled relative to lower flange (not parallel), so spine tilts at an angle and is not radially symmetric (unlike *Spiniferomonas* and *Chrysospina*). Type genus *Chrysosphaerella* Lauterborn, 1890. Type species *C. longispina* Lauterborn, 1890 and

three others: *C. astrea* Dürrschmidt in Dürrschmidt and Croome, 1985; *C. brevispina* Korshikov, 1942; and *C. enigmata* (Nicholls) Kristiansen and Tong, 1989. Other nominate species are either thaumatomonad Cercozoa or here treated as *Spiniferomonas* or placed in a new genus:

Chrysospina Cavalier-Smith gen. n. unicellular biciliate chrysomonads with two types of silica scales: 8-13 hollow spine scales with trumpet-shaped flared base, tapering to a usually notched but unbranched tip; circular ribbon-like ridge present on base plate surrounding the spine but somewhat closer to the scale margin than to the spine; inner layer of oval plate scales with scalloped oval ridge; chloroplasts present; phagotrophy unknown. Type species *Chrysospina solitaria* Cavalier-Smith comb. n. basionym *Chrysosphaerella solitaria* Preisig and Takahashi, 1978. Other species: *Chrysospina coronacircumspina* comb. n. basionym *Chrysosphaerella coronacircumspina* Wujek et al., 1977 (synonym *Spiniferomonas coronacircumspina* Nicholls, 1984). **Comment:** we do not agree that *Chrysosphaerella/Spiniferomonas coronacircumspina* of Nicholls (1984b,c) are the same species as *C. solitaria*; their spines are thicker, shorter (<10 μm), straight and largely un-notched, not 14-24 μm often curved and clearly terminally notched; the pale scales have distinctly fewer scallops – indeed their number is so different in figs 5 and 6 of Nicholls (1984) that even they may be different species. *Chrysosphaerella coronacircumspina* of Řezáčová and Škaloud (2004) is quite different; pictured spine scales are more like *C. solitaria* and plate scales more like those of Nicholls (1981, 1983). We suspect that there may have been as much lumping of subtly different '*Chrysosphaerella*' species as we found for *Paraphysomonas*. We make *Chrysospina* the type of new family **Chrysospinaceae** Cavalier-Smith with diagnosis as for the genus.

Family Acanthomonadidae Cavalier-Smith**. Diagnosis:** biciliate chrysomonads having two types of silica scales: hollow spine scales with trumpet-shaped flared base or narrow basal flange; inner layer of oval plate scales. Type genus ***Acanthomonas*** gen. n. **Diagnosis:** biciliate heterotrophic chrysomonads having two types of silica scales: thick hollow spine scales with trumpet-shaped flared base, spine with single pointed tip with lateral flanges terminating in thorn like points or terminally bifurcate or trifurcate with thorn-like side branches; inner layer of oval plate scales, usually with projecting distal oval ridge bordered by a flat outer margin; chloroplasts unknown. **Etymol:** *acantho* Gk. thorned; *monas* Gk. unit. We describe no new species but make five new combinations. Type species *Acanthomonas caelifrica* comb. n. basionym *Paraphysomonas caelifrica* (Preisig and Hibberd 1982a).

Subgenus *Acanthomonas* Cavalier-Smith**. Diagnosis:** spine scales flanged with long central point and two thorn-like lateral points, not bifurcated; plate scales with obvious broad margin and central zone demarcated by a raised oval ridge, with or without distal pegs. Includes *A. caelifrica* and *Acanthomonas acantholepis* **comb. n.** Basionym *Paraphysomonas acantholepis* (Preisig and Hibberd 1982a).

Subgenus *Furcomonas* Cavalier-Smith subgen. n**. Diagnosis:** spine scales distally flaring out and bifurcated; plate scales with or without distal ridge. Type species *Acanthomonas capreolata* comb. n. basionym *Paraphysomonas capreolata.* (Preisig and Hibberd 1982a). Other species: *Acanthomonas alata* comb. n. basionym *Spiniferomonas alata* Takahashi, 1973; *Acanthomonas takahashii* basionym *Spiniferomonas takahashii* Nicholls, 1981.

Comment. Unlike the other four species, the plate scales of *A. capreolata* are smooth, but as its spine scales resemble those of *A. alata* we consider all four species one genus. As there is no evidence that *Acanthomonas* has chloroplasts and as Preisig and Hibberd (Preisig and Hibberd 1982a) showed that one species does not, we have described *Acanthomonas* and Acanthomonadidae under ICZN. If most are photosynthetic it is unsurprising that we found no new ones as we focused solely on culturing colourless cells

Spiniferomonas Takahashi, 1973. Revised diagnosis: chrysomonads with numerous oval plate scales with one or sometimes two central hollows, and a much smaller number of long spine scales; spine scale bases with single basal flange (unlike *Chrysosphaerella*), narrower than the plate scales, distally tapering and with pointed (or very slightly notched) tips (unlike *Acanthomonas*); spine often with lateral hole near base. Contractile vacuoles. Some species with chloroplasts and/or stomatocysts; some species with leucoplasts.

Type species *Spiniferomonas bourrellyi* (Takahashi 1973; Preisig and Hibberd 1982a) (synonym *Paraphysomonas bourellyi* (Takahashi 1973; Preisig and Hibberd 1982a) has slightly notched spine tips, all others with simple point. Includes all the other species described by Takahashi 1973, except *S. alata,* which we have transferred to *Acanthomonas* because of the wings and lateral spines on its spine scale. We add 6 new combinations of former *Paraphysomonas* species and a former *Chrysosphaerella*.

Spiniferomonas annulata comb. n. Basionym *Chrysosphaerella annulata* (Kristiansen and Tong 1989).

Spiniferomonas campanulata comb. n. Basionym *Paraphysomonas campanulata* (Nicholls 1984)

Spiniferomonas corynephora comb. n. Basionym *Paraphysomonas corynephora* (Preisig and Hibberd 1982)

Spiniferomonas gladiata comb. n. Basionym *Paraphysomonas gladiata* (Preisig and Hibberd 1982)

Spiniferomonas pileata comb. n. Basionym *Paraphysomonas pileata* (Štefanová and Kalina 1992)

Spiniferomonas sediculosa comb. n. Basionym *Paraphysomonas sediculosa* (Nicholls 1984)

Spiniferomonas spinapunctata comb. n. Basionym *Paraphysomonas spinapunctata* (Wujek and Gardinier 1985)

Comment. *S. bourellyi* was depicted with parietal chloroplasts (Takahashi 1973). There has been much controversy over whether *Spiniferomonas bourellyi* has chloroplasts or not (Preisig and Hibberd 1982a; Nicholls 1985). Several other species (e.g. *S. triangularis*: Siver 1988; *S trioralis*, *S. cornuta*: Preisig and Hibberd 1983) have chloroplasts, but *S. corynephora* definitely has a leucoplast. We consider that the

controversy arises because the cells studied by Preisig and Hibberd (1982a, 1983) are probably not *S. bourellyi*, but a mixture of species with spine scales more like *S. conica* and plate scales more like *S. bourellyi*. We do not accept the merger of *S. conica* and *S. andersonii* into *S. bourellyi* (Nicholls 1981), which Preisig and Hibberd (1982a, 1983) followed. The difficulty of assigning Nicholl's scale types from Ontario to these three species probably means that they were mixtures of yet other undescribed species, not evidence that *S. bourellyi* is highly polymorphic as Nicholls (1981) assumed. We consider *Acanthomonas* and *Spiniferomonas* as more closely related to each other than to other genera because their plate and spine scales are much more similar to each other than to those of *Paraphysomonas* or *Clathromonas* or even *Chrysosphaerella*. Statements that the scales of *Spiniferomonas* are similar to those of *Chrysosphaerella* (e.g. Takahashi, 1973) are somewhat exaggerated. Nicholls (1981) suggested that the scales of unicellular *Spiniferomonas* and the two colonial species of *Chrysosphaerella* form a continuum, with his *Spiniferomonas coronacircumspina*, and *enigmata* being morphologically intermediate and used unicellularity versus coloniality to demarcate the two genera, but others assigned the two latter solitary species to *Chrysosphaerella* (Wujek and Christiansen, 1977; Kristiansen and Tong 1989) We disagree in part with both views. In our view the presence of a separate flange above the base plate but angled to it, not parallel, sharply distinguishes the asymmetric spine scales of *Chrysosphaerella* from the radially symmetric spines of *Spiniferomonas*, and consider them distinct enough to be in separate families. We agree with Kristiansen and Tong (1989) in placing *S. enigmata* in *Chrysosphaerella* and not using clonality, which may be easier to evolve than a really distinctive scale structure as the generic criterion. However, we regard the symmetric corona around the base of the spine in *S. coronacircumspina* (Wujek and Christiansen) Nicholls, 1984 as a misleading and superficial similarity to the asymmetric upper flange of *Chrysosphaerella* and thus agree with Nicholls in treating it as a *Spiniferomonas*. We would however agree that *Acanthomonas* and *Spiniferomonas* scales form more of a continuum, as *Spiniferomonas* as here delimited has species with winged spines with basal flanges that resemble *Acanthomonas* as well as the original types with basally flared unwinged trumpet shaped scales. Sequencing is needed to show whether species with the former type should also be transferred from *Spiniferomonas* to *Acanthomonas*. As *Chrysosphaerella* is related to Chromulinales on sequence trees, if *Spiniferomonas* is related to *Chrysosphaerella* it cannot be a member of Paraphysomonadidae so transferring *S. bourellyi* to *Paraphysomonas* would definitely be incorrect.

Spiniferomonas septispina Nicholls, 1981 does belong in *Spiniferomonas* as do several other species, but *S. pectinata* Štefanová and Kalina (1992) is obviously not a *Spiniferomonas* as its spine scales strongly differ from both types included above. In fact, its non-radially symmetric spine scales with basal wings and plate scales with central line, strongly show that it is a new species of heliozoan closely related to *Pterocystis canadensis* (Cavalier-Smith and von der Heyden 2007), so we transfer it to *Pterocystis*. In fact, all three new scaly 'chrysophyte' species of Štefanová and Kalina (1992) were seriously misidentified and misclassified. *Spiniferomonas* (*Paraphysomonas*) *pileata* was wrongly placed in *Paraphysomonas* and *Paraphysomonas erinacea* actually belongs to *Choanocystis* in Heliozoa, as shown by its characteristic spine scale with asymmetric deeply notched base (never seen in *Paraphysomonas* or other chrysophytes, but the defining characteristic of *Choanocystis* (Cavalier-Smith and von der Heyden 2007) and its large oval plate scales with central blob. As it is apparently a new species with pointed spine scales, we make a new combination for it. Both heliozoa were stated to be motile but no micrographs showed cilia, so there is no reason to consider them heterokonts rather than heliozoa as their scales conclusively indicate. '*Spiniferomonas coronacircumspina*' of Štefanová and Kalina (1992) was also misidentified; all features of its spine scales are much closer to *Chrysosphaerella enigmata*, but as its distal flange is broader than the basal one we suspect that it may be an undescribed closely related species - we identify it as *Chrysosphaerella* aff. *enigmata.*

New combinations for Heliozoa here excluded from Chrysophyceae

Pterocystis pectinata Cavalier-Smith comb. n. basionym *Spiniferomonas pectinata* Štefanová and Kalina (1992).

Choanocystis erinacea Cavalier-Smith comb. n. basionym *Paraphysomonas erinacea* Štefanová and Kalina (1992).

Taxonomy: new order Apoikiida Cavalier-Smith. **Diagnosis:** flagellates or rhizopodial, non-ciliate and loricate chrysomonads without scales; generally phagotrophic, but most also photosynthetic; if non-photosynthetic then with a well-defined anterior groove, unlike *Spumella* in Ochromonadales. Includes the common ancestor of *Apoikia* and *Lagynion* and all its descendants, i.e. three existing mainly loricate photosynthetic

families (Table 1) and the new heterotrophic **family Apoikiidae** Cavalier-Smith:

Diagnosis: heterotrophic phagotrophs with long anterior hairy cilium and short posterior cilium lacking a basal swelling and lying mainly in anterior grove or depression; planktonic, colonial swimmers, numerous cells partially embedded within mass of mucilage, showing collective motility; cilia lack paraxonemal rod; leucoplast within periplastid membrane and nuclear envelope at anterior end beside Golgi; no eyespot. Type genus *Apoikia* Kim et al. (2010).

Taxonomy: new *Paraphysomonas* species and strain descriptions.

All new isolates described below are colourless biciliate cells with tubular hairs on the long undulating cilium (LC) and a smooth shorter largely passive cilium (SC). They all swim with the cilia leading and bear a stalk that is used to attach to the substrate in the sessile (feeding) stage. All new species described herein had spine scales and imperforate base plates. These shared characters are not repeated in the diagnoses. Except where stated otherwise all base plates are rounded. Cell length (CL) measurements and estimates of cilium length were made on live cells; mean cell length is given first followed in brackets by the range and number of cells measured. The diameter of scale base-plates was measured across the widest point. Spine-length to plate-width ratios (S/P ratios) are also given, being important in distinguishing species. For spines that are basally thicker we sometimes give spine-base widths (not to be confused with the far greater width of the whole base-plate). Average values are given followed by the range in parentheses. In some strains the spines of the scales are visible individually in the light microscope (LM), mainly in those with unusually thick spines, but in most they are not. Even when one cannot see spines, the base plates may collectively form a visible layer in some strains seen as a dense line outside the

main cell surface, which we refer to as a cuticle since its LM visibility or not is a constant feature for each strain. The descriptions below are grouped similarly to their positions on the tree (Fig. 2), which also usually places those with more similar scales closer to each other. In addition to 34 completely new species, we raise one existing subspecies to full species rank. When we designate type sequences, strains and illustrations (or any two) all are to be regarded as part of a syntype for reasons explained by Cavalier-Smith and Chao (2010).

We cannot precisely compare new species with the type species *P. vestita* because its scale type is unknown. As the discussion explains more fully, cultures previously identified as '*P. vestita*' have been repeatedly studied ultrastructurally since Houwink (1952) and Manton and Leedale (1961), but their scale structure differs as greatly as many species described here that have radically different sequences, so we cannot know which if any are really the same species as Stokes' *P. vestita*. Either no data were given to enable identification to be checked (e.g. Houwink 1952) or those given strongly suggest that the organism studied was not *P. vestita* but an undescribed species (e.g. Manton and Leedale 1961). Ideally we would have liked to establish a neotype to end that confusion, but no isolates was sufficiently similar (by light microscopy) to Stokes'. Therefore it is unlikely that any new species described here for which we give LM data can be *vestita*. We formally raise subspecies *Paraphysomonas vestita truncata* (Preisig and Hibberd 1982a) to a full species, as we consider that its spine scales are distinct enough from other electron microscopically studied strains to merit that, its spines being too short for *P. vestita*: ***Paraphysomonas truncata*** Preisig and Hibberd, 1982a stat. n.; their diagnosis and type applies.

Subgenus *Brevispina*: two new species

Paraphysomonas ovalis sp. n. Type Figure 5A-F. **Diagnosis:** CL 5.2 µm (4.1-6.4; N=17); LC 1.5-2 x CL; SC 0.75 x CL. LF beats constantly. Attached cells round, sometimes flattened on one side. Swimming cells elongate to pyriform, sometimes round, swim in slow spiral and direct trajectories. Stalked cell close to substratum or attached to detritus. One type of spine scale with oval to irregular base plate. Spine 1.5 µm (1.1-1.9) tapers gently to rounded tip, slightly flared out at very base; base plate 0.8 µm (0.7-0.95) with prominent thickened margin caused by inflexion. S/P ratio 1.9 (range 1.4-2.4). Type strain **ARB**: CCAP 935/15. (2010; Arboretum, Oxfordshire, UK. JMS). Soil. Type 18S rDNA sequence GenBank JQ967331. **Etymology:** *ovalis* L. oval. **Comment:** *P. ovalis* is most similar to *P. bandaiensis*, *truncata*, and *porosa*; all have a base-plate with thickened margin. *P. ovalis* differs from them all by its base-plate being oval to irregular, not regularly circular; it is also unperforated, unlike *P. porosa*. *P. ovalis* has a rounded spine tip; that of *P. truncata* is truncated. *P. bandaiensis* spine tip is also rounded but its shaft is non-tapered, unlike *P. ovalis*. Unlike in most *Paraphysomonas* species, *P. ovalis* cells attached to the bottom of the Petri dish in young sparse cultures are not randomly arranged, but occupy local patches within which cells are solitary and well separated but separated from similar patches by extensive empty areas. This local patchiness might arise from daughter cells moving only short distances after they separate before attaching, making a local patch a single recently multiplied clone. Probably species that do not show this patchiness swim for much longer before settling and so become randomly distributed spatially. We refer below to a few other strains with similar patchiness as 'patchy settling'.

Paraphysomonas segmenta sp. n. Type Figure 5G-I. **Diagnosis:** CL 6.0 µm (5-7.3: N=22); LC 2 x CL; SC 0.5 x CL. LC sometimes static. Round to oval cell attached via short stalk to substratum or detritus. Swimming cell oval to pyriform, stalk often trailing. One type of spine scale, spine 0.65 µm (0.52-0.73) usually in two non-tapering segments, distal half thinner than proximal half, ending in a rounded tip, sometimes more prominently flared at base than in *ovalis*. Round to oval base plate 0.44 µm (0.40-0.53) with prominently thickened inflexion at margin. S/P ratio 1.4 (1.3-1.7). Type strain: **KU3b2**. (Keele University, Staffordshire, UK. JMS). Freshwater, pond water. 18S rDNA sequence GenBank JQ967330. **Etymol:** *segmenta* L. segmented. **Comment:** Occasionally exhibit patchy settling. Cells sometimes appear indented or flat at the base of cilia. Sessile cells occasionally jerk once and are then still. A small

'pinch' at the base of the cell, where the stalk is attached, sometimes gives a teardrop appearance, especially when LC beats strongly. *P. segmenta* resembles *P. bandaiensis*, *P. truncata*, and *P. porosa* in its base plate with a thickened margin caused by an inflexion. It is most similar to *P. bandaiensis,* which also has very small scales with a larger base-plate inflexion but *P. segmenta* spines lack the lateral striation of *P. truncata* (Takahashi 1976). The non-tapered distal segment of *P. segmenta* spines ends in a rounded tip, unlike the truncated end of *P. truncata*. *P. bandaiensis* non-tapered spines have rounded tips, but its scales are much smaller; the spine is nearly a third smaller than in *P. segmenta*, base plate diameter nearly half. *P. bandaiensis* cell was 1 µm smaller and its LC over twice as long, but comparison may not be meaningful as Takahashi did not state whether he measured live cells or TEM preparations where they would be smaller through shrinkage during drying onto the grid. A shorter environmental sequence **A42** (AY642748: Lefranc et al 2005) from a freshwater lake has just two nucleotide differences and a deletion, the latter probably a sequencing error because it is in a well-conserved region near the end. These two sequences share some very distinct signatures, so we call A42 *P.* aff. *segmenta*.

Subgenus *Acrospina*: five new species

Paraphysomonas acuminata sp. n. Type Figure 6A-B. **Diagnosis:** CL 9.0 µm (6.4-10: N=22); LC 2.5-3 x CL; SC 0.75-1 x CL. Round bright cell commonly attached to substratum/detritus. Swimming cells common and slow. LC long, moves constantly; but often slows greatly, then undulating asymmetrically. Oval to pyriform cells absent. Scales and spines conspicuous in LM. One form of spine scale covers cell. Spine 5.2 µm (4.2-6.7), non-tapering to barely tapering ending in strongly oblique short pointed tip. Spine base width; 0.191 µm (0.132-0.228), spine tip width; 0.133 (0.094-0.158). Oval to rounded and irregular-shaped base plate 1.9 µm (1.5-2.1), no/barely visible inflexion at margin, commonly with broad medium density annulus midway on base plate. S/P ratio 2.8 (2.4-3.2). Type strain **PML6A** CCAP 935/18. (Port Meadow, Oxford, UK. JMS). Freshwater lake. 18S rDNA sequence GenBank JQ967329. **Etymol.** *Acumen* L. point. **Comment.** Spines barely taper and the upper range of thickness of the tip reaches beyond the minimum thickness of the base; slight tapering is detectable, but hardly noticed. Isolate '**CCL3C**' (Oxford, UK. JMS) is very close to *P. acuminata* (possibly identical, but as three nucleotides were ambiguous - 'n' we call it aff. *P. acuminata*). CL 8.2 (8.6-9.1 n=4). The cells look very similar, as do the scales; spine 5.4 µm (4.5-6.9), base plate 2.1 µm (1.7-2.2) 18S rDNA sequence GenBank JQ967328. An additional isolate,

WA20KP (aff. *P. acuminata*, from Austria. JB), with just two definite nucleotide differences, is a similar size and has the same spine scale morphology with similar dimensions CL 9.5 µm (7.3-11.4: N= 5); LC x 3.5 CL, SC x 0.75 CL. Spine 5.3 µm (4.1-6.5), base plate 2 µm (1.9-2.1), S/P ratio 2.6 (2.0-3.3). Spine base width; 0.157 µm (0.14-0.18), spine tip width; 0.091 (0.07-0.13). 18S rDNA sequence GenBank JQ967327. We do not give it a separate name as its scales are indistinguishable.

Paraphysomonas cuspidata sp. n. Type Figure 6F-G. **Diagnosis:** CL 9.2 µm (8.2-10.5 N=16); LC 2.5-3 x CL; SC 0.5 - 0.75 x CL. Long LC, undulates often asymmetrically fast to gentle sometimes almost to a stop. Round to oval cells with obvious spines especially at high magnification. Slow swimming stage common, common foraging at substratum. Swimming cell often pyriform with trailing stalk. Scales visible in LM. One form of spine scale 4.7 µm (4.2-5.0), barely tapering spine to a short oblique point starting from spine-shaft base width 0.17 µm (0.15-0.20), tip width 0.089 µm (0.07-0.10). Spine positioned centrally from oval to irregular base plate 1.6 µm (1.2-2.1) no inflexion at edge, common mid-point annulus. S/P ratio 3.0 (2.2-3.4). Type strain **PR26KB**. (Freshwater, Austria. JB). 18S rDNA sequence GenBank JQ967326. **Etymol.** *cuspis* L. pointed. **Comment.** *P. cuspidata* is most similar to *P. acuminata* (similar cell sizes and spine scale morphology) and forms a clade with it and two morphologically similar strains (Fig. 2). Their spines are distinct; the spine tip of *P. acuminata* is much more oblique and slightly shorter than in *P. cuspidata*, which is typically few hundred nanometres longer. The long cilium in both species (up to 3x cell diameter) sharply differentiates them from other new species; *P. acuminata* typically has longer LC than *P. cuspidata* and 18S rDNA sequences are distinct.

Paraphysomonas mikadiforma sp. n. Type Figure 6H-I. **Diagnosis:** CL 7.0 µm (6.4-8.2 N=20); LC 3.5-5.0 x CL; SC 1 x CL. Stalked cell very round. LC can be very long, appearing like a dark hair. Asymmetric undulation, fast to slow. Trailing stalk with detritus common. Swimming cell common, often elongate or pyriform. One form of spine scale, visible in LM. Spine 5.2 µm (3.8-5.9), non-tapering to barely tapering spine with small oblique pointed tip. Spine base width 0.13 µm (0.1-0.16), tip width 0.094 µm (0.053-0.123). Base plate 2.1 µm (2-2.3) irregular oval to round, common midpoint annulus, no inflexion at margin. S/P ratio 2.5 (1.8-3.0). Type strain **JBM02.** (Lake Mondsee, Austria. JB). Freshwater. 18S rDNA sequence GenBank JQ967325. **Etymol.** *mikado* popular generic Japanese game of pick-up-sticks; spine resembles sticks. **Comment.** JBM02 is a mixed *Paraphysomonas* culture. *P. mikadiforma* is identified

through its close phylogenetic relationship with *P. cuspidata,* sp. n. and *P. acuminata* sp. n., which both have similar scale morphology and cellular characteristics, particularly the roundness of the cell and LC being > 2.5 x CL. The other *Paraphysomonas* in the culture exhibits a tapering spine with a strong inflexion at the margin of the base plate, spine length 4.3 µm and base plate 1.25 µm (two observed), closely resembling *P. crocotilla*. (Figure 10, A-F). Other colourless cells in the culture were more ovoid in shape and had shorter LC, perhaps twice CL. A very closely related published sequence, AY651096, strain JBM06 (Boenigk et al. 2005), which has a few undetermined nucleotides, one in a variable region, different from *P. mikadiforma*, was also isolated from Lake Mondsee, and so is probably the same species because they share distinct signatures along the sequence. We regard AY651096, strain JBM06 as *P.* aff*. mikadiforma*.

Paraphysomonas lucasi sp. n. Type Figure 7A-B. **Diagnosis**: CL 3.6 µm (3.2-5.0 N=25); LC 2.5-3 x CL; SC 0.75 x CL. Small bright round to oval to irregular cell, commonly attached to substratum via short stalk, evenly spaced cells. Fast swimming cell common. LC often static, held in a curved kinked position. One type of spine scale. Spine 0.92 µm (0.8-1.2), barely tapers to slight shoulder, not always visible, usually somewhat below half-way up spine (variable), continues to barely taper until small oblique rounded tip. Spine base width; 0.042 µm (0.035-0.049), spine tip width; 0.017 µm (0.011-0.23). Spine protrudes centrally from round to oval base plate 0.7 µm (0.64-0.77); no visible inflexion at margin, annulus not observed. S/P ratio; 1.3 (1.1-1.9). Type strain **NC10-16**. (Wrightsville Beach salt marsh, North Carolina, USA. JMS). Marine. 18S rDNA sequence GenBank JQ967323. **Etymol.** *lucasi*, after I. A. N. Lucas for contributions to *Paraphysomonas* research. **Comments:** *P. lucasi* is similar to *P. imperforata* (Lucas 1967), but despite both being marine average cell size for *P. lucasi* is smaller and cilia lengths differ slightly. The scales of *P. lucasi* have, on average, marginally smaller base plates and no annulus; *P. imperforata* always exhibits an annulus (denser ring on the base plate centred on the spine base). Lucas described the spine as tapering, but it is unclear what Lucas meant by 'tapering to a point' - his figures do not make it clear whether the tip was sharp or blunt, or rounded like *P. lucasi*, so we cannot precisely compare scale morphologies. Below we describe two marine isolates more similar than *P. lucasi* to the original *P. imperforata*, which differ from it so subtly that they must either be the same species or

extremely closely related. One (CCAP 935/14) was previously identified as *P. imperforata*, probably correctly, and has 3 nt 18S rDNA differences to our new strain below:

Paraphysomonas* aff. *imperforata Figure 7C-D. CL 4.7 µm (3.2-6.8 N=44); LC 2.5 x CL; SC 0.75-1 x CL. Small bright round to irregular cell commonly attached to substratum via short stalk, evenly spaced cells. Swimming cell fast and often elongate just as common as sessile. LC is often seen static, held curved and kinked. SC is conspicuous. One type of spine scale. Spine barely tapers, sometimes showing 'shoulder' along shaft with rounded tip. Spine protrudes from round to oval base plate. Spine 0.79 µm (0.65-0.89), spine base width; 0.042 µm (0.035-0.048), spine tip width; 0.020 µm (0.012-0.30). Base plate 0.71 µm (0.65-0.88) inflexion sometimes visible at margin mostly absent, annulus sometimes seen. S/P ratio 1.1 (0.9-1.2). Strain **EP1**. (Porlock harbour, Exmoor National Park. UK. JMS). Marine, muddy flats. Its 18S rDNA (1669 nt) was not included in Figure 2 as it is identical to four others except for a few probable sequence errors.

The four included sequences (EF432518 (C1), EF432519 (D1, AF109323 (VS1) AF109324 (SR3)) are probably all actually identical except for a few sequencing errors that misleadingly prevent their grouping on the tree. Our strain is similar to the original description of *P. imperforata*: both are marine cells of similar size and cilia lengths. Lucas did not mention a static LC, common in this isolate. The biggest difference between the scales is that this strain has a slightly smaller scale base plate that only sometimes exhibits an annulus (always present in *P. imperforata*). Lucas described the spine as tapering, but it is unclear if it was to a point or rounded or if there was a segmented element to the tapering (shoulder); his figure D suggests that the basal half may be less tapering than the distal half (as usually in ours); its apparent greater thickness is probably attributable to metal shadowing. As we cannot be totally sure it is equivalent to Lucas' organism, and the frequent absence of annulus suggests it may not be, we call it aff. *imperforata*. The single scale of '*P. imperforata*' in Caron et al. (1999) is morphologically indistinguishable from those of EP1 and has a base plate diameter of 0.88 µm; but it was not stated which of the three different imperforata strains they sequenced it came from. Both those in GenBank (VS1. SR3) are in our tree.

Strain CCAP 935/13 *P. imperforata* (coll. 1983 Fenchel) from Aarhus Bay, Denmark, resembles very closely Lucas's original description; its scales are extremely close in size, although, as in EP1, an annulus

is not always present (Figure 7, E-F). CL 4.5 µm (3.2-6.3 N=23); LC 3-3.5 x CL; SC 1 x CL. It has slightly longer cilia, but behaves similarly to EP1. The 6 sequences identified as *P. imperforata* in Figure 2 are clearly all related, but are part of a 9-sequence clade (present on both Bayesian and ML trees). Inspection of the alignment suggests that they probably represent at least 4 distinct species with *P. imperforata*-like scales; probably all are marine though for two there is no published information. The sequence of the CCAP strain is clearly distinct from the four probably indistinguishable strains mentioned in last paragraph. But the partial sequence Z29680 (Rice et al. 1997) is identical in its region of overlap with all the other five sequences labelled *imperforata* so its position on the tree is obviously arbitrary (Figure 1). Its scale spine length was similar to the CCAP strain not to EP1. As Table 2 indicates, scale spine and base plate dimensions differ between CCAP 935/13 and EP1, which are consistent with the genetic differences; both suggest that they are probably different species. Though data are too limited to be sure, for now we regard the CCAP strain as the same species as Lucas'. Probably the cluster of five species with sequences represents new species but until more data are available we call them all *P.* aff. *imperforata*. Note that marine environmental sequence from an anaerobic habitat (AY180017) that is sister to the *imperforata*-like clade on the Bayesian (but not ML) tree is wrongly annotated as a diatom in GenBank (Stoeck and Epstein 2003).

Paraphysomonas perforata sp. n. Type illustration figure 1C of Rice et al (1997). **Diagnosis:** One type of spine scale with perforated base plate (0.73 µm) with uninterrupted disordered perforation over the entire base except for a small unperforated non-inflexed margin. Central, non-tapering spine with rounded tip (1.1 µm). Original strain isolated by S. Tong Southampton Water, U.K. (Rice et al. 1997). Type sequence GenBank Z38025; SOTON A. **Etymol:** Perforated base plate. **Comment**: The original *P. foraminifera* Lucas (1967) spine scale was larger on average that *P. perforata,* this new species not even reaching the lower ranges of the *P. foraminifera* scale measurements (spine 1.46 µm - 1.63 µm; base plate 0.97 µm - 1.12 µm). The very short (326 nt) *P. foraminifera* sequence (GenBank Z33646; confusingly stated to come from the same strain SOTON A as their *imperforata*) (Rice et al. 1997) is in our tree (Figure 1). Furthermore, a closely related strain at the base of this small *P. perforata* clade is TPC2, isolated from a hydrothermal vent by Atkins et al (2000) who saw no scales and could not identify the species, yet submitted the sequence to GenBank in 2000 as *P. foraminifera* (AF174376) probably

because of its 99.6% sequence similarity the SSU of *P. 'foraminifera*' (Z38025), now *P. perforata*. There are 8 differences between these strains, so it would be reasonable to recognise them as different species; this strain might even be a real *P. foraminifera* Lucas (1967), but without scale data we cannot identify it or make a new species; but it should no longer be called *P. foraminifera*. It is more likely that scales of TCP2 were lost in preparation than it is really is a naked *Paraphysomonas*.

Subgenus *Hebetomonas*: four new species

Paraphysomonas hebes sp. n. Type Figure 8A-E. **Diagnosis**: CL 4.7 µm (3.2-5.9 N=37); LC 2.5-3 x CL; SC 0.5-1 x CL. LC beats constantly at various speeds. Small cell round, oval sometimes appearing irregular, stalked close to substratum often swimming with trailing stalk and detritus. Cell often congregates with other cells. Cuticle of silica scale base plate just visible at x60 in LM. One type of spine scale, spine 1.4 µm (1.3-5.6) varies in length and barely tapers to a truncate tip, base width of spine; 0.034 µm (0.028-0.043), width of spine tip; 0.018 µm (0.012-0.024). Spine often curved or bent and is smooth with no bulges or inflation. Round to oval base plate 0.6 µm (0.5-0.7), barely noticeable, gentle inflexion. S/P ratio 2.4 (2.0-2.8). Type strain **Ind1:** CCAP 935/17. (India, Goan beach. JMS). Marine surf. **Etymol.** *hebes* L. blunt: 18S rDNA sequence GenBank JQ967320. **Comments:** *Paraphysomonas hebes* differs from all formerly described *Paraphysomonas* as the only species with a slightly tapering spine and truncate tip. The only published one with a truncate tip is *Paraphysomonas truncata* (Preisig and Hibberd 1982a), which had a non-tapering striated spine with a prominent thickened rim to the base plate, and also an annular fold. No annular fold was seen in this isolate but *P. hebetispina* sp. n., a marine close relative, has some scales with an annulus (see below). *P. truncata* and *P. hebes* sp. n. are likely close relatives, especially since cell size (4-6 µm and 3.2-5.9 µm, respectively), overlaps and spine scale dimensions are similar.

Paraphysomonas hebetispina sp. n. Type Figure 8G-M. **Diagnosis**: CL 5.3 µm (3.2-5.9 N=37); LC 2 x CL; SC 0.5 x CL. Small oval to round cells with undulating LC moving fast to static, often twitching whole cell with fast ciliary movement. Sometimes seen in water column in groups joined together via short stalk and detritus. No scale evidence in LM except bacteria being stuck in position away from cell and faint cuticle halo. One form of spine scale; spine 1.2 µm (0. 9-2.2) slender, barely tapers to blunt or slightly rounded tip. Spine base width 0.032 µm (0.023-0.037), spine tip width 0.02 µm (0.015-0.028). Base plate

width 0.5 µm (0.39-0.61) sometimes shows faint concentric annular fold and gentle inflexion at rim. S/P ratio 2.5 (1.6-3.7). Type strain **NC10-20**. (Drainage ditch, North Carolina, Cape Fear River, Wilmington. JMS). Brackish - treated as marine. **Etymol.** *hebes* L. blunt: 18S rDNA sequence GenBank JQ967321. **Comments:** Scale spine tips vary from rounded to flatly truncate; some spines appears slightly thicker than others. Annulus observed, but not for all scales. This species differs from *P. hebes* in SSU (17 nt differences) and presence of annular fold in the base plate of the scales and tip variation, but scale dimensions are very similar.

Paraphysomonas limna sp. n. Type Figure 8F. **Diagnosis**: CL 6.6 µm (6.4-6.8 N=7); LC 2 x CL; SC 0.75 x CL. Small round to irregular-shaped bright cell, most on substratum. Occasional jerky movement from strong LC beat. Scale presence not obvious in LM except occasional faint cuticle. Type 18S rDNA sequence GenBank JQ967322. Strain **PML2A-e2** culture dead. (Port Meadow, Oxford, UK. JMS). Freshwater still stream. **Etymol.** *limna* Gk Lake. **Comments.** The culture died before it could be observed in the electron microscope. However it is distinct from the other two species in being isolated from freshwater and having 5 unique nucleotide differences in 18S rDNA. When the culture was alive it was difficult to observe cilia at x40 unless one agitated the dish to move cells, perhaps because of their orientation; they can easily be overlooked.

Paraphysomonas parahebes sp. n. Type figure: Fig. 1A, B of Caron et al. (1999). **Diagnosis**: One type of spine scale covers the cell; spine 1 µm, barely tapering to a rounded or blunt tip, the base centrally positioned on a round base plate with inflexion at margin, 0.6 µm. (strain not stated from which of two; HFlag, WH1). Type sequence GenBank AF109322. **Etymol**: *para* Gk. Beside *hebes* L. blunt. **Comments**. This cell misidentified as *P. bandaiensis* had very different scales from the original *P. bandiaensis* Takahashi (1976), which had an extremely dense base-plate margin and spine 0.3 µm equalling the base plate diameter, 0.3 µm. The spine scale shown in Caron et al., (1999) is more than twice as large and has a base plate with much more delicately inflexed margin, similar to the *Hebetomonas* clade to which it is sister. Therefore no 18S rDNA sequence of a genuine *P. bandaiensis* is available to see where its very small scales fit on the tree; they might be in the *Hebetomonas* clade like the misidentified strain, but it is also possible that *P. bandaiensis*-like scales are more like those in subgenus *Brevispina* as the cells are of

similar dimensions and the scales very small with a prominent inflexed margin; however the long cilium is shorter in known *Brevispina* species than in *P. bandaiensis* Takahashi (1976).

Subgenus *Paraphysomonas*: 23 new species

The first two plus several morphologically uncharacterised lineages form the most divergent short-branch clade of subgenus *Paraphysomonas* on Figure 2:

Paraphysomonas hemiradia sp. n. Type Figure 9A-E. **Diagnosis:** CL 9.9 µm (7.3-14.1: N=27); LC 2 x CL; SC 0.5-0.75 x CL. Bright round to oval cell with obvious halo, spines sometimes visible in LM. Plastic, usually stalked cell. Swimming cell oval, elongate to pyriform often with trailing stalk and detritus. One type of spine scale; spine often bent; 4.6 µm (3.6-5.8) varying length and thickness, commonly has broad base 0.2 µm (0.07-0.3) and basal inflation/bulge, tapering to small oblique dull point, 0.046 µm (0.028-0.063). Round to oval base plate 1.8 µm (1.5-2), strong inflexion at rim. S/P ratio 2.6 (2-2.9). Base of spine flares onto plate usually giving prominent transverse crease across centre. Type strain **AU30KV** (Lake Augstsee, Austria. JB). Freshwater lake. Type 18S rDNA partial sequence GenBank JQ967318. **Etymol:** *hemi* Gk. half, *radius* L. radial. **Comments:** *P. hemiradia* fed upon a very small chrysophyte (*Oikomonas*-like) contaminant. We are unsure whether the radial spoke effect of *P. hemiradia* is partially a creasing artefact during TEM preparation or a permanent structure, but in either case its reproducibility must reflect a basic difference from other species. The scales may cope with the large cell being very plastic by the large base plate really being concave or convex over the cell as it changes shape; the cell makes pyriform as well as elongate shapes. The silica could be semi-plastic too, the structure accounting for the radical cell movement. No other named species have such prominent radial 'spokes', but they are evident in unnamed micrographs (figs 10-11 of Řezáčová and Škaloud 2004); they are absent in the next, closely related species:

Paraphysomonas uniformis sp. n. Type Figure 9F-G. **Diagnosis.** CL 11.6 µm (6.8-16.4: N=31); LC x 1.5 - 2 CL, SC x 0.5 CL. SC difficult to see in LM. Extremely plastic cell, round to oval and pyriform when swimming, often with trailing stalk and attached detritus. Mostly attached to substratum, swimmers common. Scales visible in LM: obvious cuticle but spines not so clear. One type of spine scale, spine 4.5 µm (4-5.3), often slightly curved, tapering usually smooth and uninterrupted; gently tapered crooked to straight spine with small oblique blunt point. Spine base width 0.116 µm (0.088-0.153), spine tip width;

0.054 µm (0.042-0.071). Round to oval base plate 1.8 µm (1.5-1.9) with thin inflexed margin. S/P ratio 2.6 (2.2-2.9). Base of spine sometimes inflated and has transverse crease across centre. Type strain **WA28KT (**Wallersee, Austria. JB Freshwater lake.). Type sequence GenBank JQ967317. **Etymol:** *uniformis* L. uniform. **Comment.** Cells can become large in culture, especially when preying upon smaller *Paraphysomonas*, large bacterial clusters and very small (*Oikomonas*-like) contaminant. The size and shape of the scales are relatively uniform compared to other species described here. *P. hemiradia* (above) also has long spine scales from a large plastic cell, but the scales are obviously different because *P. uniformis* has a narrower spine base as well as tip and no radial ribs. Some aberrant scales of *P. hemiradia* resemble *P. uniformis*. Another strain we studied '**WA32KAG**' (from lake Wallersee, Austria. JB) with smaller cells, 8.1 µm (6.4-10.5), 18S rDNA partial sequence **GenBank** JQ967319, and 'uncultured eukaryotic picoplankton clone **P1.35**', **GenBank** AY64271 (Lake Pavin in the Central Massif, France) have identical 18S rDNA sequences to WA28KT except for two ambiguous nucleotides; therefore we treat both as *P. aff. uniformis*. The partial sequences *P. hemiradia* (931 nt) and *P.* aff *uniformis* (WA32KAG) (1417 nt) account differ from them by one nucleotide substitution and an ambiguity.

The next four species (plus morphologically similar or uncharacterised lineages) form the long-branch vulgar clade that is exclusively from freshwater (Fig. 2).

Paraphysomonas vulgaris sp. n. Type Figure 10C-D. **Diagnosis.** CL 9.0 µm (7.7-12.7: N= 26); LC x 1.5-2 CL, SC x 0.5-0.75 CL. Round to oval bright and plastic cell with conspicuous scale cuticle in LM, sometimes spines visible. Commonly attached to detritus or substratum, often swimming with trailing stalk and detritus. Cell swims smoothly through water column. One type of spine scale; spine 3.9 µm (3.1-4.5), gently tapering from wide, usually bulbous, base to oblique dull pointed tip. Spine base width 0.2 µm (0.014-0.31), spine tip width 0.063 µm (0.052-0.068). Base plate 2.1 µm (1.8-2.2), oval to round with thickened inflexed rim; base plate exhibits transverse crease across centre. S/P ratio 1.8 (1.6-2.0). Type strain **PML2B**. (Non-flowing stream. Port Meadow, Oxford. UK. JMS). Freshwater.18S rDNA sequence GenBank JQ967314. **Etymol:** *vulgaris* L. common. **Comments:** The culture contained an obvious non-*Paraphysomonas* contaminant; non-photosynthetic colony-forming flagellated cone-shaped chrysophyte, on which the target organism is thought to have fed, as well as the bacteria and other smaller *Paraphysomonas*. The transverse crease is visible on the base-plate when the spine has fallen

over to one side during specimen preparation; as this crease is typically absent when the spine has broken off it is probably an artifactual, albeit reproducible, distortion of a basically symmetric baseplate. Two other freshwater isolates, '**SW02**', (Zurich, CH. River Stahl near meeting with River Limnat, Switzerland. JMS), and '**PML2Af2**' (non-flowing stream, Oxford, UK. JMS) have just two nt differences in 18S rDNA from *P. vulgaris* and identical sequences to each other and may be another closely related species, but we designate them both simply as *P.* aff*. vulgaris* as our data are somewhat incomplete for each, so we cannot be sure that they are morphologically identical to each other. Strain SW02 was dead before cell measurements could be taken, but TEM (Fig. 9A-B,) shows very similar scales: spine 3.3 µm (1.2-5.4) gently tapering from commonly wide slightly bulbous base to oblique dull pointed tip; spine often crooked or slightly bent. Base plate 1.8 µm (1.3-2.3), round to oval with thickened inflexed rim with transverse crease across centre. S/P ratio 1.8 (0.9-2.7). Isolate PML2Af2 CL 10.6 µm (7.7-18.2), conspicuous scale cuticle on a plastic cell, has no TEM data. All these isolates exhibit plasticity and an obvious scale cuticle. An isolate not from this study, 'Monas' sp TON-O (AB168053), is sister to SW02 in this clade.

Paraphysomonas vulgatana sp. n. Type Figure 10E-F. **Diagnosis:** CL 11.3 µm (8.2-12.7: N= 17); LC x 2 CL, SC x 0.5 CL. Large plastic oval to round cell often bright. Smooth meandering swimmers, oval to pyriform. Scale cuticle visible in LM. Trailing stalk and detritus, common. Scale morphology unknown although expected to be similar to other members of the 'vulgar' clade. Type strain **W03**. (Stream up from beach, Nolton Haven, Wales, UK. JMS). Freshwater. 18S rDNA sequence **GenBank** JQ967316. **Etymol:** *vulgaris* L. common. **Comments:** Plasticity of this cell can be impressive, reaching very large sizes; one can often see other smaller *Paraphysomonas* cells inside a larger cell. *P. vulgatana* branches near the base of the 'vulgar' subclade (Fig. 2) that has the most isolates from this study (nine other isolates had identical SSU to others in the clade), from which this species has up to a dozen nt differences.

Paraphysomonas cambrispina sp. n. Type Figure 10G-H. **Diagnosis:** CL 9.0 µm (7.3-11.4: N=20); LC x 2 CL, SC x 0.5-0.75 CL. SC obvious in LM. Plastic oval to round cell, sometimes bright. Pyriform when swimming, slowly, with stalk often trailing behind. Obvious scale cuticle in LM. One form of spine scale; spine 2.7 µm (2.0-3.1), tapering sometimes curved to an oblique dull tip. Base plate 1.2 µm (0.97-1.67) with dense inflexion at rim. S/P ratio 2.3 (1.8-2.7). Type strain **WI34KN**. (Austria. JB). Freshwater lake.

Diagnosis: 18S rDNA sequence **GenBank** JQ967316. **Etymol:** *camber* L. curved. **Comment.** This species is sister to all the other members of the 'vulgar' phylogenetic clade, including *P. vulgatana*, *P. vulgaris*, *P. vulgana* and *P. vulgata*, all having scales with tapering spines ending in a small oblique dull-pointed tip; but unlike the others there is no obvious transverse crease on the base plate.

Paraphysomonas vulgata* subspecies *vulgata sp. and subsp. n. Type Figure 11A-B. **Diagnosis:** CL 10.4 µm diameter (8.2-12.3: N=24); LC 1.5-2 x CL; SC 0.5 x CL. Round to oval cell often stalked to substratum/detritus. Swimming cell often elongate/pyriform. Cuticle of base plate visible in LM, spines inconspicuous. Plastic cell, especially when ingesting bacterial aggregates. One type of spine scale; spine 2.4 µm (1.8-3.1), commonly inflated at base ~0.35 µm wide, above which spine sometimes appears crooked or bent, gently tapering to oblique dull point. Oval to round/irregular base plate, obvious inflection at rim, base plate 1.9 µm (1.7-2.2), S/P ratio 1.2 (1-1.6). Small base-plate crease beside spine base frequent. Type strain **PML4B.** (Wetland temporary pond, Port Meadow, Oxfordshire, UK. JMS). Freshwater. 18S rDNA sequence **GenBank** JQ967311. **Etymol.** *vulgaris* L. common. **Comments:** There is no genetic difference in the SSU of *P. vulgata vulgata* and *P. vulgata elongata* (below) but the scales of *P. vulgata elongata* are, as the name suggests, more elongate. As well as this notable difference, the average cell size of *P. vulgata vulgata* is larger, whereas the range of cell lengths for *P. vulgata elongata* is notably smaller. Eight other isolates, including a subspecies, *P. vulgata elongata*, had identical 18S rDNA: '**SW03**' (Lake, Konstanz, Switzerland. JMS), '**Ind7**' (Stream, tropical India. JMS), '**CCL3A**' (Stream, Oxford, UK. JMS), '**PML5B**' (River, Oxford, UK. JMS), '**GMDL3-g4**' (Seasonal pond, Shropshire, UK. JMS) '**W04**' (Weir, Pembrokeshire, UK. JMS), '**W07**' (Stream leading to sea, Pembrokeshire, UK. JMS), all now identified as *P. vulgata* but most not assignable to subspecies without scale pictures. Isolate Ind7, has TEM data that matches more closely to subspecies *P. vulgata vulgata*, spine 2.7 µm (2-4.1), base plate 1.8 µm (1.3-2.1) S/P ratio 1.5 (1.1-2.2). Ind7 appears to have scales more similar in size to *P. vulgata vulgata*, suggesting that this subspecies is found in both temperate and tropical habitats; it is likely that the other isolates with the same SSU will have scales more or less like one or other subspecies. Two sequences, one from this study '**BZ5b'** (JQ967312) with just two nucleotide differences from *P. vulgata*, and a freshwater lake isolate 'Monas' TON-0 (AB168053), published only in the GenBank database, differs by just one nucleotide substitution and deletion, so we have deemed both *P.* aff. *vulgata.*

Paraphysomonas vulgata* subspecies *elongata subsp. n. Type Figure 11C-D. **Diagnosis:** CL 8.8 µm diameter (6.8-10 N=34); LC 2 x CL; SC 0.5 x CL. LC beats constantly. Round to oval plastic cell. Swimming cell round to oval and elongate to pyriform, slow swimming. Scale cuticle visible in LM, spines not always visible. Cell bears one type of spine scale; spine longer than in *P. vulgata vulgata*: 3.6 µm (2.3-4.7) tapers to an oblique dull tip, often has slightly inflated base bulging to 0.42 µm up the shaft often bent. Oval to round/irregular base plate with strong inflexed rim, base 1.9 µm (1.1-2.1). S/P ratio 1.9 (1.4-2.5). Transverse crease common on base plate. Type strain **PML8.** (Water from grassy floodplain, Port Meadow, Oxfordshire, UK. JMS). Freshwater. 18S rDNA sequence identical to *P. vulgata vulgata* GenBank JQ967311. **Etymol.** *elongata* L. elongated.

The next five species and less well-characterised lineages form the second major long-branch freshwater clade (Fig. 2):

Paraphysomonas variosa sp. n. Type Figure 12A-F. **Diagnosis**: CL 8.3 µm (7.3-9.1: N = 14); LC 1.5 x CL; SC 0.5 x CL. LC constant motion although slows to an asymmetrical undulation. Difficult to see LC and SC on stationary cells in LM because of orientation of cell. Round to oval cell, commonly attached to substratum or detritus. Swimming cells usually oval to elongate, swimming high in water column. One type of spine scale; spine 2.9 µm (1.4 - 3.7), tapering to blunt tip. Base width of spine 0.14 µm (0.08 - 0.24); tip width 0.027 µm (0.015 - 0.049). Round to oval base plate 1.2 µm (1 - 1.5); S/P ratio 2.5 (1.6-3.7), conspicuous to barely visible inflection at rim. Type strain **Ind5**. (Freshwater, India. JMS). 18S rDNA sequence, GenBank JQ967296. **Etymol:** *variosa* L. various. **Comment:** The species name refers to the variety of length and breadths of the spine, not the width of the base plate, which is very uniform. The extremely varied length of the spines and width of the base of the spine gives the scales a very non-uniform appearance and highly variable S/P ratio. *P. variosa*'s spine tip is most similar to *P. stylata* in that some have a more prominent pinched tip. One strain from our study with one nucleotide difference from *P. variosa* is strain **EP4** from freshwater in Somerset, UK (GenBank JQ967297). No microscope data was collected for this strain but we assign it as *P.* aff. *variosa* based on its sequence similarity.

Paraphysomonas caroni sp. n. Type Figure 12G-K. **Diagnosis**: CL 7.3 µm (5.9-9.6: N = 48); LC1.5-2 x CL; SC 0.5 x CL. Round to oval cell with cuticle sometimes visible on larger cells. Often trailing stalk when swimming. One type of spined scale covering cell with spine 1.9 µm (1.3-2.1) tapering to a short oblique

blunt pointed tip. Round base plate with obvious dense inflexed margin 1.0 µm (0.9-1.1); S/P ratio 1.9 (1.4-2.3). Type strain. **CH2**. (Marsh in zoo, Beijing, China. JMS). Freshwater.18S rDNA sequence, GenBank JQ967292. **Etymol:** named after D. A. Caron for his contribution to *Paraphysomonas* research. **Comment:** This was a mixed culture; *P. caroni* and notably smaller Chrysophyte flagellate on which it fed, shown in the Fig12K. Two cloned culture sequences '**PV10'** (AF109325 - second isolation from CCAP 935/14 '*P. vestita*'. Freshwater) and isolate '**TGS6'** (AB616676 - Miyagi, Sendai-shi, Japan Hirose River. Freshwater) have identical 18S rDNA sequences and differ in sequence from CH2 by just two nts. Isolate '**JBAS37'** (AY651094 Nepal, Kathmandu. Freshwater), has three different nts. These additional strains are regarded as *P.* aff. *caroni*.

Paraphysomonas mantoni sp. n. Type Figure 12M-L. We did not include morphology in the diagnosis, which is based only on the unique DNA sequence we obtained, as we have some concern that the culture might be mixed because of its unusually wide variation in cell size, though this worry may be groundless. Nonetheless, we describe the range seen: CL 6.8- 20.5 µm; modal and median value, 8.6. Average, 9.7 µm: N=31. LC, 1-4, smaller cells ≥8.6 µm appeared to have longer LC from 2.5-4 x CL. Spine scales taper to a dull point (Fig. 12M) or else to a small oblique tip (Fig. 12N). Oval base plate with a thickened inflexed rim. Cells with longer cilia seem to exhibit more prominent spines in LM as well as obvious cuticle. Large cells very plastic with large vacuoles, scale presence not obvious. Type strain **BZ5a** (Freshwater, Brazil. JMS). **Diagnosis:** Type 18S rDNA sequence GenBank JQ967295. **Etymol:** named after I. Manton for her contribution to *Paraphysomonas* research. **Comment.** The culture was difficult to evaluate because of the unusual diversity of chrysomonad cell sizes. TEM was done on two separate occasions, over a year apart, and yielded the same variation of spine scales, so the presence of two spine tip morphologies seems to be stable. the type in case it is a mixture of two closely related strains of different cell size but with very similar but fractionally different scales. We are unsure whether the two forms of the spine tip are different views of the same thing, true intrastrain variation or a sign that the culture is mixed. We did not have time to re-clone the cells and repeat the electron microscopy a third time or re-sequence fresh single-cell isolates, which could, in principle, be done to test the latter possibility. The fact that the next species, which we isolated twice and which is genetically so close to *P. mantoni* that they cannot be distinguished on the tree, but whose 18S rDNA sequence differs in 11

respects, is also more variable in the light microscope than most strains (but less so than BZ5a) makes it possible that both species are intrinsically unusually variable and that BZ5a is not mixed.

Paraphysomonas balonovi sp. n. Type Figure 12O**. Diagnosis**: CL 10.1 µm (8.2 - 13.6: N = 22); LC 1.5-2.0 x CL; SC 0.5-0.75 x CL. Large plastic oval to round cell, sometimes bright, sometimes becoming pyriform or very thin and elongate when swimming. Scale cuticle visible. No TEM data for this species. Type strain **Bassen**. (Bassenthwaite, Cumbria, UK. JS). Freshwater. 18S rDNA sequence, GenBank JQ967294. **Etymol:** *balanovi*, named after I. M. Balonov for his contribution to *Paraphysomonas* research. **Comment:** This culture shows a range of size and shapes of cells. Its sequence places in a large freshwater clade next to *P. mantoni*. The tree shows very slight genetic distance between *P. mantoni* and *P. balonovi* despite there being eight indels between them (which would not affect the analysis), as well as very distinct 3 nt differences, which is ground for making them separate species. Another isolate, '**SW04**' (Lake of the four cantons (Vierwald Stattersee), Luzern, Switzerland. JMS) CL 10.6 µm (8.6 - 14.6; N=23), here called *P.* aff. *balonovi*, has just one indel and nt difference from *P. balonovi*.

Paraphysomonas petronia sp. n. Type illustration Fig2A of Petronio and Rivera (2010). **Diagnosis**: One type of spine scale covers cell; spine 2.9 µm (2.5-3.0), tapering to small oblique dull tip, centrally protruding from round to oval base plate with thickened inflexed margin 1.8 µm (1.6-2.0). Type strain **J1** (Laguna de Bay, Philippines) (Petronio and Rivera 2010). Freshwater. Type 18S rDNA sequence: GenBank GU220392. **Etymol:** named after first author of the paper in which this specimen was first described, JAG Petronio. **Comment:** this species has a unique large 137 nt insert (position 938-1075) not seen in any other *Paraphysomonas*; when blasted against GenBank a match was only made to *Paraphysomonas*. The insert matches closely, but not exactly, an adjacent part of this species' sequence, so is probably a fairly recent internal duplication.

The next six species form a long-branch subclade with a mixture of species from soil, freshwater, and marine habitats (Fig. 2) with relatively long spines:

Paraphysomonas crocotilla sp. n. Type Figure 13A-F. **Diagnosis:** CL 8 µm (6.8 - 9.1: N=20) LC 1.5 x CL; SC 0.5 x CL. Round to oval and elongate cells, swim relatively fast up and down water column. One form of spine scale; spine 3.7 µm (2.8 - 4.6) slender, sometimes slightly curved, gently tapers to a blunt tip. Spine base usually slightly inflated, width 0.13 µm (0.103 - 0.188), spine tip width 0.017 µm (0.011 -

0.021), larger inflation at the base rare (0.3 µm). Base-plate 1.2 µm (1.0-1.6 µm), round with delicate but conspicuous inflection at rim. S/P ratio 3.1 (2.5-3.8). Type strain **UPL1B**. (University Parks, Oxford, UK. JMS). Soil from mole-hill under tree. 18S rDNA sequence, GenBank JQ967308. **Etymol:** *crocotilla* L. slim. **Comment:** Some early micrographs suggest that this species sometimes also has spineless scales; one round to oval measured 1.4 µm (Fig. 13B). Such spineless scales were not observed when TEM was repeated. Stomatocysts in live culture, but culture mixed with another non-*Paraphysomonas* swimming flagellate. *P. crocotilla* is most similar to *P. dimorpha* (which belongs to the same subclade) because of the likely occasional presence of a spineless scale in addition to the spine scales. *P. crocotilla* is, on average a larger cell than *P. dimorpha*. However, their cell length ranges partially overlap. They also share a similar S/P ratio (~3.0) and scale bases have similar sizes, but *P. crocotilla* averages a longer spine by 0.5 µm. The spineless scale measured for *P. crocotilla* is larger than the average size of its spine scale bases, as noted below for *P. dimorpha*. As the spine of *Paraphysomonas* scales probably grows by evaginating from a primary disc (Hibberd 1979; Lee 1978), a developmental failure of such evagination might well result in a disc with the same amount of starting material ending up as a larger plain disc. *P. spiculosa* has similar spine scales to *P. crocotilla*. However, the spine is shorter and the tip ends in a smaller point than *P. spiculosa,* which has a subtly rounded tip; thus both would have the same gradation of tapering, except that *P. spiculosa* generally with a longer spine would appear thinner. Morphological differences between these soil species are relatively slight compared with their very large 18S rDNA divergence. P. crocotilla fed on an very small Chrysophyte (*Oikomonas*-like) contaminant.

Paraphysomonas dimorpha sp. n. Type Figure 13, H-N. **Diagnosis:** CL 6.7 µm (5-8.6: N=22); LC 1.5-2 x CL; SC 0.5-7.5 x CL. LC moves constantly at varying rates, sometime slowing almost to a stop, exhibiting slow undulation. Cell bright, round to oval, sometimes pyriform when swimming; thick scale cuticle conspicuous in LM. Stalked cell close to substratum/detritus. Two forms of scale cover cell surface; spine scales with round base plate and inflexed rim; spine 3.2 µm (2.1-4.6) tapers gently slightly curved to a blunt end and often starts from base with slight bulge; base plate 1.1 µm (1.0-1.2 µm), S/P ratio 3.0 (2.0-4.2). Spineless scales round to slightly oval, 1.3 (1.1-1.6 µm), sometimes have central short line (120 nm). Cell sometimes covered in one scale type but often covered in both forms. Type strain **CA01** CCAP 935/16. (Leaves, grass and mud. Monterey Bay, California, USA. JMS). Freshwater. 18S rDNA sequence,

GenBank JQ967310. **Etymol:** *dimorpha* L. Two forms because of two scale types. **Comments:** A smaller cell body, thought to be a contaminant was also present in the culture Figure 13H, but perhaps dormant cells preparing to encyst although cysts not readily observed. Spine length varies greatly but the base plate is consistently ~1.1 µm. The spineless scales are more varied in size and can be more oval as well as exhibiting an obvious short line in the centre (not from a spine that has broken off, because there would then be a hole from where the wide base of the spine had protruded). The regularity of the spine scale base plate measurements, plus the fact that the spineless scales are usually larger together also make confusion with base plates of broken spine scales unlikely. For fuller comparison with similar species, see *P. crocotilla* above. Another isolate, '**Mike**' (lawn mud/grass, Staffordshire, UK. JMS), with the same 18S rDNA sequence was found from soil, showing it to be geographically widespread. This other culture was a very mixed and so not processed for TEM or DIC measurements.

Paraphysomonas solis sp. n. Type Figure 14A-B. **Diagnosis:** CL 8.3 µm (6.4-11.4: N=32); LC 1.5-2 x CL; SC 7.5 x CL. Bright oval to round cell, vary in size. Often oval when swimming. One type of spine scale; spine 2.6 µm (1.5-4.4) tapers strongly to a dull point, base plate 1.1 µm (0.8-1.3) oval to round with a varying thickened inflexion at rim. Type strain **GMCCL6**. (Wet mud from end of stream, Christ Church Parks, Oxford, UK. JMS). Freshwater. 18S rDNA sequence, GenBank JQ967309. **Etymol:** *solum* L. soil. **Comment:** Difficult to interpret culture because of 'granule'-like bodies ~1 µm in live culture as well as the TEM showing small very round scaleless chrysophytes up to 5 µm in diameter. All these smaller cells were not included in the measurements for cell length. Even though the smaller cells, believed to be a food for *P. solis*, the granules could be a life stage of this *Paraphysomonas* species.

Paraphysomonas stylata sp. n. Type Figure 14C-F. **Diagnosis:** CL 6.9 µm (5.5-8.2 N=21); LC 2.5 x CL; SC 0.5-0.75 x CL. LC beats constantly at various speeds. Bright round to oval cell. Commonly attached to substratum and floating debris by stalk of various lengths. Swimming cells round to oval and elongate, sometimes pyriform with stalk trailing. Cuticle of scale base plates visible in LM. One type of scale; spine 3.6 µm (2.4-5.5) tapering to pinched tip. Spine base width, 0.079 µm (0.06-0.14). Round to oval base plate 1.3 µm (1.0-1.6) with inflected rim. Transverse central crease on base plate often seen. S/P ratio 2.8 (1.7-3.7). Type strain: **W02.** (Wet beach sand, Pembrokeshire, UK. JMS). Marine. 18S rDNA sequence GenBank JQ967307. **Etymol:** *stylus* Gk instrument for writing. **Comment:** If not studied carefully, the *P.*

stylata spine tip could be confused with the oblique tips seen in the 'vulgar' clade, but the blunt end of *P. stylata* is broader when un-pinched, exhibiting little change or interruption in the overall tapering of the spine. *P. stylata* is most similar to freshwater *P. stylaris*, its sister on Figure 2 that has much longer spines: see below.

Paraphysomonas longispina sp. n. Type Figure 15A-B. **Diagnosis:** CL 6.9 µm (6.8-13.6: N=46); LC 2-2.5 x CL; SC 0.75-0.5 x CL. Plastic cell with large vacuoles, often bright oval to round. Readily ingests cells of own kind. Scale cuticle visible in LM. One form of spine scale; spine 5.7 µm (2.2-7.3) tapering to a dull point, often slightly curved along whole length. Base plate 1.4 µm (0.9-1.7), round to oval with prominent delicate inflexed rim. S/P ratio 4.0 (2-5.3). Type strain **MEX3** (Calzadas River, Calzadas Coatzacoalcos, Veracruz. Mexico. JMS). Freshwater. 18S rDNA sequence, GenBank JQ967305. **Etymol:** *longus* L. long, *spina* L. backbone/spine. **Comments:** The very base of spines is sometimes inflated or cone-shaped and compressed in TEM preparation; often saw base plates with detached spines showing 'pinched' remnant of where the spine started. Aberrant scale forms were observed, usually the spine was malformed and not fully formed. Three other isolates from this study have the same 18S rDNA sequence, the only case where we found identical 18S rRNA sequences from both marine and freshwater isolates: **CA03**, from a fresh water outlet to sea (stream) - not observed in the TEM, **MEX1** (marine sample, Coatzacoalcos, Veracruz, Gulf of Mexico. JMS), and **VA02**, (Brackish waters, Lee Mont, Virginia, Chesapeake Bay. USA. JMS) treated as marine, not observed in the TEM. The mix of these four samples being marine and 'fresh' water close to the sea may indicate that these cells are tolerant of salinity changes or brackish water specialists; but as we did not study ITS sequences we cannot rule out the possibility that those from marine or freshwater are separate closely related species with identical 18S rDNA. All are from coastal regions on the American continent. There is only one TEM picture of a MEX1 scale, which resembles *P. longispina* (MEX3), but MEX1 has a shorter spine, and the cell is also slightly smaller; CL, 7.7 µm (6.4-8.6: N=29), but for now we treat these three (MEX1, CA03, VA02) all as *P. longispina*. The *P. longispina* rDNA has 1 nt different from marine strain '*vestita*' SOTON1 (Z28335), isolated from Southampton waters, UK, for which a TEM scale had dimensions within the *P. longispina* range, spine ~ 6.2 µm, base plate ~1.2 µm, and closely similar morphology but without an inflated spine

base (Rice et al. 1999 fig. 1A). We regard it as *P.* aff. *longispina*, misidentified as *P. vestita,* which had longer and thicker spines easily seen by LM and was from fresh water.

Paraphysomonas stylaris sp. n. Type Figure 15E-F. **Diagnosis:** CL 9.4 µm (7.7-12.3: N=14); LC 2.0-2.5 x CL; SC 0.75 x CL. Plastic oval to round cell. Scale cuticle and nucleus conspicuous in LM. Swimming cells elongated and oval. One form of spine scale; spine 5.4 µm (3.8-7.2), gently tapering completely to a tip, spine base width 0.082 µm (0.06-0.12). Oval to round base plate with delicate thickened inflexed margin, 1.3 µm (0.9-1.7). S/P ratio 4.0 (3-6.8). Type strain: **PML5D**. (from water flooded over grassy riverbank, Port Meadow, Oxford. UK. JMS). Freshwater. 18S rDNA sequence GenBank JQ967306. **Etymol:** *stylus* Gk instrument for writing. **Comments:** Base plate sizes vary a lot as does spine length (giving large S/P ratio range), as in *P. longispina*. However, 18S rDNA of *P. stylaris* is much closer to *P. stylata*; only 4 nts differ. These two closely related species have two significant nt differences and two less so, given variation across the entire *Paraphysomonas* alignment. Despite their genetic closeness, subtle differences in morphology exist: tapering is stronger and comes to a more slender end in *P stylaris* than in marine *P. stylata*, which commonly comes to a pinched tip; their spine base widths match closely. It is difficult to interpret the tip of *P. stylaris* because the TEMs are not clear enough to perfectly deduce these subtle difference in tip shape.

The next five species form a robust clade (Fig. 2) that is exclusively from soil and has a tendency to form shrunken smaller cells in culture. The last three readily encyst as stomatocysts of distinctive morphologies:

Paraphysomonas sinensis sp. n. Type Figure 16A-E. **Diagnosis:** CL 9.8 µm (7.7-13.6) N=25); LC 2.0 x CL; SC 0.5-0.75 x CL. Round to oval large plastic cell. Cuticle of scales just visible in LM. Short stalk, sometimes thicker nearer the cell end, Scales heterogeneous, commonly spine scales with rounded base-plate and inflexed rim; base-plate width 1.3 µm (1.1-1.5); spine length 2.62 µm (1.8-3.4) S/P ratio 2.1 (1.5-2.7). Spineless larger plate scales present, sometime with a central stub or stump, usually much larger than base-plate of spined scales, 2.1 µm (1.5-2.7). Aberrant scale forms common; mostly spine is hyper-inflated and/or obscurely shaped. Large spineless scales commonly have marks/scarring in the centre. Strain: **CH9**. (Soil, Yunnan, China. JMS). Freshwater. 18S rDNA sequence GenBank JQ967303. **Etymol:** *sinensis* L. Chinese. **Comment:** This culture is difficult to maintain and strange smaller dormant-

looking cells were observed ~5 µm, perhaps a life stage. The scales are unusually non-uniform; the larger spineless plate scales could be malformed spine scales, but unlike in *Paraphysomonas crocotilla* (see above) there also appear to be intermediates with shorter, stubbier spines than typical spine-scales. Only pictures of the dormant-looking cells were captured in the light microscope (Fig 16A) before the culture died.

Paraphysomonas tropica sp. n. Type Figure 16F. **Diagnosis:** CL 7.6 µm (6.8-8.2 N=19); LC 2.5 x CL; SC 0.75 x CL. Oval to round cells with scale cuticle visible in LM. Swimming cell oval to elongate. No TEM data. Type strain: **BZ1**. (Soil, Brazil. JMS). Freshwater. 18S rDNA sequence GenBank JQ967304. **Etymol:** *tropica* of tropical origin. **Comment:** The cell is, on average, >2 µm smaller than *P. sinensis* with slightly longer LC. 18S rDNA of *P. braziliensis* and *P. sinensis* (sisters on Fig. 2) differ by over a dozen nts.

Paraphysomonas terricola sp. n. Type Figure 17A-B. **Diagnosis:** CL 9.7 µm (7.3-10.9 N = 11); LC 1.5-2.0 x CL: SC 0.5 x CL. Oval to round cells, sometimes elongate when swimming, often stalk trailing. Scale cuticle, sometimes spines, visible in LM. Plugged stomatocysts common (6.8-10 µm); refractile collar around stoma relatively tall (3.7-4.4 µm), almost as high as its basal width, slightly undulating (less straight than in *edaphica*) and with thicker margin. One scale form. Spine 3.2 µm (2.2-5.4) tapering to a subtle rounded tip from a sometimes inflated spine base, spine base width 0.083 µm (0.05-0.12), spine tip width 0.021 µm (0.02-0.03). Oval to round base plate, 1.0 µm (0.8-1.3) with obvious, sometimes delicate inflected rim. Type strain: **GMBGL1**. (Soil, Botanical Gardens, Oxford, UK. JMS). Freshwater. 18S rDNA sequence GenBank JQ967301. **Etymol:** *terra* L. earth *cola* inhabit. **Comment:** Another strain from our study, **GMSTL1,** from soil in Sandon Staffordshire, UK had exactly the same SSU (indicated in parenthesis Fig. 2), as did **GMAL1**, also from soil, isolated from a golf course in Audlem, Cheshire, UK (GenBank JQ967300). Some light microscope observations were recorded including the presence of plugged stomatocysts with a long collar as well as pyriform and oval swimming cell from but sadly no footage of the culture was recorded or scales observed in the TEM since the culture died. This strain differs by one nucleotide (and one undetermined nt) from *P. terricola*. Both strains exhibit very distinct insertions, more than 6, all along the length of the SSU sequence; we call GMAL1 *P.* aff. *terricola*.

Paraphysomonas spiculosa sp. n. Type Figure 17C-E. **Diagnosis:** CL 8.4 µm (6.4 - 12.3: N = 36); LC 2.0 x CL: SC 0.5 x CL. LC in constant motion, slows to symmetrical undulation. Round to oval cell, attached to

substrate/detritus; swimming cells oval to elongate, trail stalk and detritus. Plugged stomatocysts common; similar size to *P. terricola* but collar much shorter (~2.1 µm, about half the height of *terricola*), curved in side view (unlike *edaphica*), width ~5.2 µm. One form of spine scale; spine 3.2 µm (2.4 - 3.6) tapers to subtly rounded tip, spine base width 0.11 µm (0.088 - 0.118), spine tip width 0.027 µm (0.022 - 0.029). Round to oval base plate 1.3 µm (1-1.8) delicate conspicuous inflexed rim. S/P ratio 2.8 (1.33-3.6). Type strain **BZ8** CCAP 935/19. (Soil and leaf litter, Brazil. JMS). 18S rDNA sequence, GenBank JQ967298. **Etymol**: *spiculosa*; Stokes, when he first described the radial projections of *P. vestita* called them 'spicules'. **Comment:** The closest relatives are *P. indica* and *edaphica*:

Paraphysomonas indica sp. n. **Diagnosis:** distinguished from all other species by its 18S rDNA sequence with at least 5 nts different from closest relatives *P. spiculosa and edaphica*: Type sequence GenBank JQ967299. Large cells, but no data for cell dimensions or scales as the strain died before measurements or TEM could be done: strain **IND4**. (Soil, India. JMS). **Etymol:** *indica* L. from India. **Comment:** It was difficult to maintain the culture, as with many soil strains, which became overgrown by another smaller chrysomonad, possibly a *Paraphysomonas*, measuring ~5.5 µm. *P. edaphica* (below) is by 5 significantly different nucleotides all present in highly diverse regions of the molecule that were not included in the mask. *P. indica* and *P. spiculosa* are arbitrarily positioned together on the tree because they share two similarities that are included in the mask.

Paraphysomonas edaphica sp. n. Type Figure 17F. **Diagnosis:** CL 9.1 µm (9.1-9.1 N=1); LC 1.5 x CL; SC 0.5-0.75 x CL. Round to oval cell. Cuticle visible in LM. Plugged stomatocysts (diameter ~8 µm) common; collar around stoma straight sided, height ~3.1 µm, 4.4 µm diameter at base. Swimming cell often elongated with trailing stalk and detritus. Type strain: **CH6**. (Soil, Beijing, China. JMS). 18S rDNA sequence GenBank JQ967302. **Etymol:** *edaphos* Gk ground. **Comment:** Difficult to interpret culture because of 'granule'-like bodies which could be dormant or starved cells, similarly to *P. solis* (see above), another isolate from soil in a fairly distant sister clade. Only a few cells were observed and measured in LM. There is no TEM data to describe scales, however; 18S rDNA is distinct in three areas, from its closest molecular relatives, with two sets of two deletions (between positions 119-147) and two other nt differences at positions 59 and 1361.

New species of *Clathromonas*

We designated *C. butcheri* as the type of *Clathromonas* because it was the only species that we were able to culture ourselves and thus obtain DNA sequences and define its phylogenetic position. Two sequences attributed previously to *C.* (*Paraphysomonas*) *butcheri* (Rice et al. 1997; Caron et al. 1999) cannot be from that species as the micrographs of scales fortunately included in the reports of the sequencing show distinct morphological differences from the original culture (Pennick and Clarke 1972) that died before DNA sequencing was invented. The two sequences also differ significantly from each other and from our strain, but all three are part of the same clathromonad subclade (Fig. 3). As the scales of those two earlier strains also differ from all other described species we make them new species, but first describe our new isolate to demonstrate that it is indistinguishable from the original *P. butcheri.*

We isolated an arguably genuine ***Clathromonas butcheri*** (Strain **MD03**, CCAP 936/1) in 2010 from brackish waters in Chesapeake Bay, Queenstown, Maryland, USA. 18S sequence GenBank JQ967291 (Figure 18). **Description:** CL 3.3 μm (2.7 – 6.3: N=14); LC 1.5 – 2.0 x CL. SC 0.5 – 0.75 x CL. LC beats constantly. Round, often bright cell stalked close to substratum/detritus. Jerky movement common from LC temporarily changing/stopping movement. SC is clearly seen in the LM. Swimming stage common with trailing stalk, often high in water column, pyriform cell observed. Swimming motion slow and cell body rotates vaguely in situ with LC flailing outward. Two forms of scale; mesh plate and basket. Plate scales 0.7 x 0.6 μm (0.52-0.87 x 0.40-0.72), bear 11-16 holes in the outer ring and 9-13 on the inner ring and a central area of irregularly placed holes the centre of the plate scale. Sometimes cell predominantly has more of one scale type or the other, or in equal amounts. **Comment:** Images from the original description (Pennick and Clarke 1972) strongly show the LC hairs because of shadowing with gold-palladium whereas our sample was not metal-shadowed so the hairs were less conspicuous. Otherwise our new strain of *Clathromonas butcheri* matches nearly exactly the original description

down to the brackish habitat. However, the original description gives cell size of *C. butcheri* as smaller with proportionally longer cilia (via multiples of cell diameter) than our strain. This difference is probably an artefact because Pennick and Clarke measured dead, fixed cells and there must have been shrinkage in their preparation compared with living cells, as there was in ours which showed the exact dimensions as Pennick and Clarke's when fixed. The description of the scales and number of holes in the outer ring of the plate scales are the same in both strains; a wider range of number of holes in the inner ring (9-13, compared to 10-12), a negligible difference probably arising from a difference in the number of scales measured. The basket scales look are extremely similar, though the strut dimensions are hard to compare as we not know at what points their measurements started and ended, but the range of sizes of our struts are happily close to theirs (0.24-0.31 µm) compared to their one measurement (0.24 µm). The basket scales of our strain have the same structure and size (0.5 µm width) as the original description but we see more 6-strutted than 5-strutted basket scales, the reverse of the original. Even so, we consider MD03 an authentic live strain of *C. butcheri.* Its sequence is very different from the two other, supposedly '*butcheri*', strains which we now make new species:

Clathromonas tongi sp. n. **Diagnosis:** Two forms of scale; plate scale oval to round open mesh 0.55 µm (0.35-0.77, N=4), with 5-9 outer holes of fairly regular size, not 11-16 as in *C. butcheri*. Internal holes are fewer than in *C. butcheri*: ~2-6. Sizes of plate scales vary a lot. Basket scale 0.69 µm (0.71-0.67: N=2), open mesh very angular shape, no curves 5-6 struts 0.28 µm (0.23-0.31). Type illustration: fig. 1d in Rice et al. (1997); all measurements taken from it. Type 18S rDNA sequence GenBank Z29679. Original strain: **SOTONA** (Southampton Water, England, UK. Marine) no longer available. **Etymol:** named after S. M. Tong who isolated the organism. **Comments:** This species is morphologically distinct from *C. caroni* in scale dimensions, and outer plate scale hole numbers do not overlap with the range for *C. butcheri*; the

basket scales are less angular. *C. tongi* has similar dimensions to *C. butcheri* but has plate scales that are more diverse in size and have many fewer holes. Even though the sequence is partial, just 328 nucleotides, this region unambiguously places it within the '*butcheri*' clade (along with the TEM data) and distinguishes it from close relatives.

Clathromonas caroni sp. n. CL, at least 3.3 µm LC = x2 CL (N=1, Lim et al, 2001). Two scale forms: open mesh plate and basket scales; all descriptions based on Caron et al. (1999) and Lim et al. (2001). Plate scale, oval to round, length 0.92 µm (0.67-1.25); peripheral ring of holes (8-12) sometimes with smaller perforations at joins as well as irregular holes in centre. One plate scale with inner ring of ~9 holes, but seven plate scales showed irregular size and shape of central holes. Basket scale (one) 0.9 µm wide, five struts with curved upper tier on oval to round ring, strut ~ 0.16 µm. Unclear number of holes in upper tier, but appear irregular in shape and size, similar to holes in plate scales. Type illustrations; Fig 1f from Caron et al. (1999) and Fig 3a,b from Lim at al. (2001). Type strain: **DB4** (Patuxent River, Maryland, USA). Marine. Type 18S rDNA sequence GenBank AF109326. **Etymol:** *caroni* after D. A. Caron, author of both papers on this species. **Comments:** The single basket scale looks more like *C. inconspicua* (Takahashi, 1976), but the plate scales are clearly different and sometimes resemble the top tier of the basket scale in *C. corbidifera* (Pennick and Clarke, 1973). The basket scale is larger than *C. butcheri* and has shorter struts. The variety of hole-shapes and sizes is similar between the species but scales are bigger than in *C. butcheri* and plate scale holes fewer on average, though ranges overlap.

Discussion

A major conclusion is that *Paraphysomonas* was formerly a polyphyletic heterogeneous repository for all non-photosynthetic scaly chrysophytes irrespective of their vastly differing scale types and is far too diverse morphologically and genetically to be accepted as a single genus. Former *Paraphysomonas* species are now dispersed among six genera, each with relatively uniform types of siliceous scales, which are as different from each other as any of them are from the silica scales of Synurales. Secondly, there are far more species of *Paraphysomonas* sensu stricto (essentially

those with only spine scales) than previously assumed (Finlay and Clarke 1999b). We have increased the number from 9 to 45 and there are already >30 more environmental DNA sequences on Figure 2 for *Paraphysomonas* sensu stricto distinct enough to constitute separate species, so there must be nearly 80 genuine *Paraphysomonas* species, and probably many more – perhaps several hundred as there is no reason to think that sampling is anywhere near saturation. Thus excessive taxonomic lumping has probably underestimated the number of spine-scaled *Paraphysomonas* species by at least tenfold. Nearly all the new species we described would previously have been lumped in just two species categories: *Paraphysomonas vestita* and *imperforata*. No wonder those two 'species' were thought to be the most frequently encountered and geographically ubiquitous (Finlay and Clarke 1999b). They were not single species but swarms of separate species, some at least as genetically different from each other as the complete range of variation within the entire order Synurales. Figure 19 compares 15 of our 35 new species that would probably have been lumped as *P. vestita* (the 10 species with a dense rim around the base plate) or *P. imperforata* (the five species with plain base-plate rims) in past whole mount TEM environmental surveys of *Paraphysomonas*. Yet these species differ obviously in cell size and shape and in scale size and proportions and in spine tip structure and many are far distant from each other on the sequence tree (Fig. 2). Lee (1987) even argued for treating all as one species on the grounds that one strain can live in both marine and freshwater environments; the common assumption of free movement between these habitats for *Paraphysomonas* generally seems to be false: Figure 2 shows that marine and freshwater lineages seem to be phylogenetically rather stable in habitat preference; only one 18S rDNA genotype of the 82 *Paraphysomonas* sensu stricto in

Figure 2 was found in both habitats. As only six genotypes were found more than once (one nine and another four times) there must still be gross under sampling of *Paraphysomonas* and well over 100 species must exist.

Likewise Paraphysomonadaceae of Preisig and Hibberd (1983) was a heterogeneous assemblage of all scaly chrysophytes with an *Ochromonas*-like rather than a *Synura*-like body plan, which we have now split into six families, two exclusively heterotrophic and four largely or exclusively photosynthetic (Table 1). As we specifically targeted heterotrophic cells for culturing it is unsurprising that we obtained representatives only of the two purely heterotrophic scaly families, Paraphysomonadidae and Clathromonadidae, which constitute Paraphysomonadida as here revised.

It is often stated that most species of *Paraphysomonas* are indistinguishable in the light microscope from *Spumella*, a phylogenetically heterogeneous array of non-scaly heterotrophic chrysomonads abundant and diverse in freshwater. As some grow like weeds in culture we were initially concerned that isolating colourless chrysomonads at random would yield cultures that would mostly turn out to be *Spumella* (irrelevant to this project) only after the effort of purifying and sequencing them or examining them by TEM. As many *Spumella* cells are elongated or irregular (perhaps because not so constrained by scales) in shape and some are very small, we initially focused on culturing large (≥ ~ 7 μm) completely round colourless cells with two visible cilia (one long, one short: to avoid *Oikomonas* which lacks the short one), and with a stalked stage. To our surprise this very successfully biased the results against *Spumella*, despite not being able to visualise or clearly see scales in the light microscope. This mode of selection has almost certainly given us a biased sample of

paraphysomonad diversity - against smaller, unstalked cells or irregular-shaped ones, or any (if such exist) without a short cilium, and could be one reason why we obtained so many *Paraphysomonas* and only one strain of *Clathromonas*; the *Clathromonas* was found only later in the project when we targeted smaller cells to see if this would yield other scale types. The rarity of Clathromonadidae in our cultures might in part be because they are harder to culture under our conditions, but that is pure conjecture. In future it should be possible to obtain many more clathromonad cultures for combined TEM/sequencing studies similar to ours for *Paraphysomonas*, since Preisig and Hibberd (1982a,b) obtained several uniprotist cultures of six species that we assign to *Clathromonas*. Sequences are now desirable for *Patellomonas* and a greater morphological diversity of *Clathromonas* to test the unity of Clathromonadidae.

Large-scale evolution within Chrysophyceae

We have placed three of the essentially photosynthetic scaly families in a new chromulinid suborder Chrysospineae because they have both spine scales (which are unlike those of *Paraphysomonas*) and plate scales that differ from those of Clathromonadidae and Synurales (Table 1). The only sequence (EF185316 – *Chrysophaerella* sp. *?longispina*) available for Chrysospineae groups consistently with classical photosynthetic Chromulinales, and never with either paraphysomonad family. Given the relative homogeneity of the chrysospinid scales and their marked dissimilarity from either the spine scales of *Paraphysomonas* or the latticed scales of *Clathromonas*, we consider that Chrysopineae is probably monophyletic. But sequences for the two acanthomonad genera are needed to test this and verify that all the former *Paraphysomonas* species that we have transferred to *Spiniferomonas* and *Acanthomonas* are really related to *Chrysosphaerella*, as their scale morphology

indicates. A sequence is needed also for the photosynthetic *Chrysolepidomonas*, another former *Paraphysomonas* with such distinctive scales that it cannot presently be assigned to either Paraphysomonadida or Chrysospineae.

Our trees agree with earlier ones (Andersen 2007; Cavalier-Smith and Chao 2006) in three respects: (1) resolution at the base of the Chrysophyceae is very low; (2) *Paraphysomonas* is a very distinct clade from both the scaly Synurales and non-scaly Hibberdiales; and (3) Ochromonadales as previously constituted consists of two major clades, one including all the *Spumella*s and most *Ochromonas*, and one that includes *Lagynion*, *Chrysosphaera* and *Chrysosaccus*. We have now shown that the latter also includes the purely heterotrophic phagotroph *Apoikia* (Kim et al. 2010) for which we have established the new order, Apoikiida. By excluding Apoikiida from Ochromonadales sensu stricto and placing Chrysothallaceae within Hydrurales, the six orders Chromulinales, Ochromonadales, Apoikiida, Hibberdiales, Synurales, and Hydrurales, each correspond to a distinct monophyletic branch on the chrysophyte tree. Only for Paraphysomonadida is there some doubt about its monophyly; if EC1 is phagotrophic and has scales, and thus belongs to Paraphysomonadida, it may be holophyletic. Paraphysomonadida including EC1 is strongly holophyletic on trees with an extensive balanced set of outgroups (Fig. 1), but appears not to be on trees rooted just on Picophagea (Fig. 4), which we argued above may be a rooting artifact caused by a restricted unrepresentative outgroup combined with some long-branch taxa being attracted towards it. Carefully rooted multigene trees are needed to test its monophyly further. Thus seven or eight phylogenetically distinct clades on the chrysophyte sequence tree have phenotypically identified representatives, which correspond with the seven orders whose revised classification is in Table 1.

Our trees also show fewer radically distinct clades comprising only environmental sequences than previously thought (del Campo et al. 2011; Charvet et al. 2011). On the MrBayes tree of Figure 1 there are two such clades; environmental clade 1 and 2 (EC1, EC2), but on the ML tree of Figure 1 and by both methods on Figure 4 EC2 is split, one half grouping with EC1 in Figure 4. It is not known whether EC1 and 2 are photosynthetic or not, but as they are so speciose establishing their phenotype is very important for understanding deep phylogeny of Chrysophyceae. In the absence of such knowledge, and if Paraphysomonadida is holophyletic, it is the only purely heterotrophic and phagotrophic order within Chrysophyceae and photosynthesis was lost once in the last common ancestor of Paraphysomonadidae and Clathromonadidae. Hydrurales, Synurales, and Hibberdiales on present knowledge are purely photosynthetic, non-phagotrophic algae, whereas Chromulinales, Apoikiida, and Ochromonadales all have a mixture of often phagotrophic algae and of purely phagotrophic non-algal protists (*Oikomonas*, *Apoikia* and *Spumella* respectively). As *Spiniferomonas* has both photosynthetic and heterotrophic species (references above), if it belongs in Chromulinales, then Chromulinales lost photosynthesis at least twice. As there are four distinct *Spumella* clades Ochromonadales did so at least four times. Thus, there were at least eight distinct losses of photosynthesis in Chrysophyceae and the class should not be thought of as typically algal. Clearly it was ancestrally phagotrophic and phagotrophy and photosynthesis were each lost more than once. The absence of heterotrophy in the probably non-phagotrophic orders suggests that retention of phagotrophy facilitated the multiple independent losses of photosynthesis and the reversion to a more protozoan way of feeding. It is also evident from our trees

that there were several independent losses of the posterior cilium and at least two independent losses of both cilia in chrysophytes.

With respect to scales, Synurales, Paraphysomonadales, and Chrysospineae are three phylogenetically distinct clades that uniformly bear scales. As they diverge very close to the base of the chrysophyte tree and it is unlikely that scales evolved three times independently we suggest that they share a photophagotrophic common ancestor with at least siliceous plate scales. If so our sequence trees imply that scales were lost by the other two suborders of Chromulinales: Chromulineae and Cyclonexineae. We suggest that the ancestor of Ochromonadales also had silica scales and that silicification and/or scales were lost by ancestors of most extant Ochromonadales, but that non-silicified scales of some Chrysolepidomonadaceae share a common ancestor with the silicified scales of the other three orders. The number of other silica scale losses that need be invoked during chrysophyte history depends on the basal branching topology of the class, which is currently unclear. However, as Hydrurales, which have no scales and also probably no phagotrophy, are consistently the most early diverging clade on the tree (albeit in some cases without strong statistical support), we suggest that this is probably correct and that silica scales first evolved in the common ancestor of all the other six orders, here grouped together as new subclass Chrysomonadia. If that is correct, then scales were lost by Hibberdiales and Apoikiida, and first evolved in the ancestral chrysomonadian and were not lost by Hydrurales. Most Hydrurales have a distinctive multicellular body form stuck onto stones or rocks; possibly that was the ancestral body plan for the whole hyduralean clade and *Ochromonas*-like strains that group within it are secondary reversions to unicellularity. By segregating Hydrurales as a distinct subclass and grouping the other

six orders as Chrysomonadia, we can retain both the algal name Chrysophyceae for the whole class and the protozoan name chrysomonad for the subclass that includes all phagotrophic heterotrophs such as *Paraphysomonas*, *Spumella*, *Oikomonas*, and *Apoikia*. As discussed previously (Cavalier-Smith and Chao 2006), it is no longer desirable to treat Synurales as a separate class or even subclass. Their distinctive cell organization is a secondary consequence of reorientation of their centrioles to make them parallel unlike the near orthogonal arrangement in ancestral Chrysophyceae and heterokonts generally, and the loss of chlorophyll c1 and phagotrophy are evolutionarily relatively trivial. Consistent grouping of Synurales as just one of several chrysophyte orders reinforces ranking them no higher than order (Cavalier-Smith and Chao 2006), not as a separate class (Andersen 1987; Cavalier-Smith 1986) or subclass (Cavalier-Smith and Chao 1996).

Major environmental clades

Previously del Campo et al. (2011) found three major environmental DNA clades of putative chrysophytes of unknown phenotype: clades G (corresponding to our EC1) H (corresponding to our EC2H) and I (corresponding to our EC2I). Their RaxML analysis grouped clades G and H as sisters to each other and clade G/H as sister to *Paraphysomonas* sensu stricto (their clade F1), with *Clathromonas* (their clade F2) sister to that joint clade, all without BS support. Our analyses of both datasets found the same three well-supported environmental clades, but neither grouped EC2H with EC1 and they were differently grouped in each, and both differed from the topology of del Campo et al. (2011), whose tree (their fig. 3) included some chrysophyte sequences

not in ours (but also excluded many more on our tree) but was rooted on only a single outgroup, species (the eustigmatophye *Nannochloropsis*), making its rooting even more problematic and leaving the chrysophyte nature of clades G-I open to some doubt. Our extensive outgroup analysis unambiguously confirms that all are chrysophytes, though if EC2 is the deepest branch, we cannot be sure whether stomatocysts evolved before or after its divergence (Fig. 1). As noted above, the position of subclade EC2H is uncertain and sensitive to taxon composition of outgroups – it may be sister to EC2I (Fig. 1 with well sampled outgroups) or to EC1 (Fig. 4 with only sparsely sampled but more closely related outgroups) or to Hydrurales (clade D in del Campo et al. 2011). Both EC1 and EC2H include freshwater and marine clones (typically clustering separately) and some longer branching divergent sequences, which could account for the unstable position and splitting of EC2. Interestingly marine subclades have substantially longer branches and thus faster rDNA evolution than freshwater ones; clade EC2I is entirely marine and thus includes no short-branch representatives, which may help explain why its position is unstable – grouping with EC2H on Figure 1 and with EC1 in del Campo et al. (2011).

Charvet et al. (2011) found two clusters of chrysophyte environmental DNA sequences from High Arctic lakes that they could not assign to an existing order, even suggesting that cluster I might be outside and sister to Chrysophyceae. Our more comprehensively sampling (Fig. 3) shows that is incorrect and that both clusters branch reliably within specific chrysophyte orders. Cluster I is a deep branch within Chromulinales and II belongs in Apoikiida. Their misinterpretation arose from the tree being rooted on a too distant single (dinoflagellate) outgroup and not having any non-chrysophyte ochrophyte sequences. Their 'novel clade' Environ1 is not sister to

Paraphysomonas as on their sparsely sampled tree but part of EC2H (Fig. 3). Their clade Environ2 is part of one of the Chromulinales novel clades. Their clade Environ3 is part of EC1Ga and Environ4 part of EC1. We therefore conclude that only the three major environmental clades of del Campo et al. cannot confidently be assigned to a specific order. If the topology of either our Bayesian tree in Figure 1 or of del Campo et al. (2011) figure 3 were correct, then there would really only be two major phenotypically undetermined chrysophyte clades.

We hypothesise that Figure 1 with diverse balanced outgroups is less likely to be subject to idiosyncratic misrooting than Figure 3, and that EC1 may therefore be relatively closely related to Paraphysomonadidae and Clathromonadidae (also found by del Campo et al. 2011), and so may also consist of scaly heterotrophic biflagellate phagotrophs; perhaps it corresponds with or includes the third paraphysomonad genus (*Patellomonas*) of our classification (Table 1). If our classification is correct, EC1 is less likely to be Acanthomonadaceae, predicted to group with *Chrysosphaerella* within Chromulinales; as Figure 3 indicates, there are three very distinct multispecies environmental subclades within Chromulinales any of which might correspond to Acanthomonadaceae or Chrysospinaceae – one is sister to *Chrysophaerella* so quite likely to have scales also. The deep-branching position and instability of clade EC2 makes it harder to predict its phenotype; though we cannot exclude the possibility that it includes species with scales, the most parsimonious interpretation assuming the Bayesian tree of Figure 1 to be correct is that scales evolved after EC2 and Hydrurales diverged, so a reasonable guess is that EC2 cells are naked phagotrophs.

Light microscopy reveals differences within *Paraphysomonas*

Very few *Paraphysomonas* had previously been studied live in the light microscope (Lucas, 1967, 1968; Leadbeater, 1972; Pennick and Clarke, 1972, 1973; Rees et al, 1974); most measurements of previously established *Paraphysomonas* species were on fixed material likely to have shrunk in preparation. Therefore cellular features visible in the light microscope have not previously been used to help distinguish species. Our results show that though not sufficiently detailed to be diagnostic for individual species, variations in cell size map sensibly onto the molecular tree as does long cilium length (x CL). Cell size and cilium length can be characteristic of a set of species, not individual ones. These features may help rule out certain species during initial identification.

With respect to cilium length one can recognise three *Paraphysomonas* clades. First, subgenus *Acrospina* having non-inflexed margin on the scale base plate with round or oblique pointed spine tips (*P. imperforata, P. lucasi, P. mikadiforma, P. cuspidata, P. acuminata*) never has a LC shorter than 2.5x CL. Secondly, *P. tropica*; thirdly and three species belonging to a larger thickened margin base plate scale clade with completely tapering spines, namely *P. longispina, P. stylaris* and *P. stylata* (a subclade of subclade D of subgenus *Paraphysomonas*) have slightly longer LC than the rest in their clade (Table 2). All other species described here have a shorter long cilium (≤ 2.0 x CL). Stokes' *P. vestita* had LC 2X CL like most of our isolates. However, none of our cells exactly match his other cellular descriptions.

Cell size is indicative of groups of species; formerly misidentified *P. foraminifera and P. imperforata* sequences (subgenus *Acrospina*), all have cells below 5 µm, as well

as the new closely related species *P. lucasi*. Other small cells we studied form two distinct groups too, *P. ovalis* and *P. segmenta* in subgenus *Brevispina,* and *P. hebes*, *P. hebetispina* and *P. limna are a subclade of* subgenus *Hebetomonas*; all these species have average cell sizes between 4.7 - 6.6 µm. The 'vulgar' subclade of subgenus *Paraphysomonas* has some of the largest cells in this study, averaging 11.3 µm. So, average cell size, as well as scale dimensions and features like presence or absence of concentric annulus and spine tip shape, can sometimes rule out certain species.

Cell behaviour can also be indicative of certain species. Some smaller, shorter stalked, species tend to grow as loose patches on the substratum, fixed by a short stalk; some of these exhibit a twitching motion, particularly *P. ovalis*, whilst others like *P.* aff. *imperforata* (EP1) and *P. imperforata* (CCAP 935/13) and *P. lucasi* readily have static LC in a kinked/curved ciliary position. Long cilium motion in the larger-celled species with very long LC is distinct from other species in that the LC looks like a dark hair; motion too can be erratic. Other behavioural characteristics could have been described, especially swimming style, but were not noted in this study.

Electron microscopy shows diversity of *Paraphysomonas* spine scales

All new *Paraphysomonas* species described here have spine scales, none of those from our new cultures with holes or perforations in their bases. Previously there were only three species of non-holey spine-scaled *Paraphysomonas* (not including *P. vestita*): *P. imperforata*, *P. bandaiensis*, and *P. antarctica*. We have added 35 new species of non-holey spine-scaled *Paraphysomonas* Not only are they all genetically different, sometimes only slightly, they can additionally be distinguished by morphological features of their spine scales, whose evolutionary and taxonomic significance had

previously been almost entirely overlooked. Features of the spine scale useful for identification include:

1. Presence or absence of an inflated/bulbous base to the spine. 2. Degree of tapering of the spine. 3. Shape of the spine tip: tapered to a blunt tip, rounded tip, oblique sharp, blunt or pinched. 4. Shape of base plate: oval, round or irregular. 5. Presence or absence of inflexion at rim of base plate, which appears in most whole mount micrographs as a dense border of thickness characteristic for each species. 6. Occurrence, or not, of an annulus midway on the base plate. (The annulus is thought to be a slightly raised mid-way point across the plate (Lucas 1968), and for some species it does not seem to be a persistent character, e.g., *P.* aff. *imperforata* strain EP1 and, *P imperforata*, strain CCAP 935/14.) 7. Crease at the base of the spine present or not, thought to be relic of EM preparation. 8. Presence or absence of radial ribs on the base plate. 9. Size of the scale: length of spine, width of base plate, and S/P ratio. All of these characters are good diagnostic features. One main feature split the genus into two major groups, as shown on figure 2; the presence or absence of an inflexion at the edge of the base plate. However, there appear to be varying degrees of inflexion, so some species have more subtle inflexions than others. In particular, one group of new species has a distinct spine scale with a truncate/rounded spine tip and circular base plate with a non-inflexed to slightly inflexed margin, *P. hebes*, and *P. hebetispina*. These species seem to bridge the gap between the two otherwise distinct groups.

Within these two defined groups, according to the presence or absence of base plate inflexion, spine characters further define subsets (Table 2). There are inflexed rim species in this study that all have a small oblique dull spine point. No spines ending in an oblique dull tip are seen in any *Paraphysomonas* species with non-inflexed rim.

Conversely, there are non-inflexed rimmed species that have a sharp oblique tip that do not appear in the thickened rim group. Another distinct group within the thickened rim scale group are the ones with completely tapering spines ending in either a rounded or blunt tip. Hibberd's study of a strain of *P. bandaiensis* illustrates the inflexion very well (Hibberd 1979). In the case of *P. segmenta*, the inflexion appears to be very wide whereas others, like *P. hemiradia* could be a more compact curl giving a thinner edge. It would be interesting looked at these in more detail here in future studies, e.g. through TEM sections.

The base plate of the spine scale is usually round to oval with a few species showing more irregularity in roundness (*P. acuminata, P. ovalis* and *P. cuspidata*). Variations in shape were more apparent in some species than others. Radial ribs on the base plate, as seen for *P. hemiradia*, had been noted before in the literature (Takahashi 1976; Preisig and Hibberd 1982a; Eloranta 1989), but it is unclear if such radial ribs are a consequence of artifactual regular wrinkling during EM preparation or a natural rigid structure; but their consistent occurrence in very few species only means that they must reflect an underling structural difference from the generality of species. It was impractical also to do scanning electron microscopy (SEM) for so many species, and we chose not to for any as some studies have shown that SEM can be less useful and even confuse identification of silica-scaled chrysophytes (Boo et al. 2010; Kynčlová et al. 2010).

The diversity of scale morphology within a species has been shown to vary. *P. variosa* is named so because of the unusually large range of sizes and thicknesses observed amongst the scales. One must take care when attempting to identify a single spine scale as it could belong to an organism that makes more than one type of scale.

This highlights the importance of having more than once scale to identify *Paraphysomonas* to species-level. *P. bimorpha* is the only *Paraphysomonas* so far known to have two qualitatively different types of scale: spine and plate. Our results showed that some *P. bimorpha* cells had only spineless scales or mostly spine scales, perhaps indicating a life cycle stage of the organism. Varying ratios of different scale types in one species is also seen in *Clathromonas butcheri*. In this study it was seen, as in the original observations of (Pennick and Clarke 1973) how some cells had just plate or basket scales or a mixture of both. Even though the scale morphology for these two species is extremely different (crown/basket versus spine) the same phenomena of having cells bearing more or less of one type of scale type might indicate that this could be a commoner occurrence in the scaly chrysophytes.

Unfortunately, we did not isolate a *P. foraminifera* strain so the evolution of *Paraphysomonas* with perforated base plates remains confused, especially since two of the three sequences in GenBank labelled '*P. foraminfera*' are so far apart that one cannot possibly both be like the original species. One of them was accompanied by a micrograph that clearly shows that it was misidentified (Rice et al. 1997); its spine is only two thirds as long as *P. foraminifera* and tapers only near its tip not along most of its length and the base plate holes are more regular and relatively larger than the intervening trabeculae in *P. foraminifera*. We therefore made it a new species, *P. perforata*. The other sequence AB022864 from strain MBI-HT3 has no associated electron micrograph; the strain is unavailable. It might be a genuine *P. foraminifera*, but if it was grown in freshwater medium DY-IV as stated (Andersen et al. 1999), it was probably not actually *P. foraminifera*, which was marine (Lucas 1967), so we placed its name in inverted commas on Fig. 2. As all neighbouring strains to HT3 are freshwater

vestita-like species, it is most likely that the true *P. foraminifera* will turn out to be related to the marine isolate from Rice et al., not to HT3. If HT3 actually has a perforated scale base plate then, such perforations must have evolved at least twice in ancestrally unperforated *Paraphysomonas* lineages.

Our study highlights the importance of studying small differences in scale structure when identifying *Paraphysomonas* or describing new species. Neglect of such differences led to their previous excessive lumping into very few species: predominantly just '*vestita*' with a dense base plate border and *imperforata* lacking it, by far the two most frequently encountered 'species' (Finlay and Clarke 1999b); we have shown that this difference characterises two huge species-rich groups that differ genetically as much as do whole families in many eukaryotes. These crudely differentiated morphotypes cannot be real species; the '*vestita*-like' morphotype is a clade, corresponding with the entire new subgenus *Paraphysomonas* and the *imperforata* one with the majority of the new subgenus *Acrospina* is an ancestral group that also incudes several species formerly lumped as *P. foraminifera*. This makes all previous records of the distribution and ecological preferences of *P. vestita* and *P. imperforata* meaningless at the species level; they pertain only to the whole subgenus *Paraphysomonas* and a major subsection of subgenus *Acrospina* respectively and thus are too crude to be useful for most purposes. Because his marine strain (supposedly of *P. vestita*, but probably not) could grow in freshwater Lucas (1978) even suggested that *P. vestita* and *imperforata* might be the same species. However species with holey base plates like *P. foraminifera* and *perforata* appear to be strictly marine and have not been reported in freshwater or soil.

Evolution of chrysomonad scales

Attempts have been made from morphology alone to make morphological groupings of the different scales of *Paraphysomonas* and in doing so to make hypotheses about their evolution (Thomsen et al. 1981; Preisig and Hibberd 1982b). Thomsen et al. 1981 proposed four major groups, A-D, which is a prescient foretaste of the major generic distinctions we have now made. Their group C of spine scales corresponded closely with our *Paraphysomonas*, except for the inclusion also of what is now *Clathromonas takahashii*, which has spine scales that unlike those of *Paraphysomonas* sensu stricto exhibit a meshwork structure in their base plate (and even the base of the spine) that closely resembles that of the scales of the non-spiny *Clathromonas* species and is very different from the perforated base plate of *P. foraminifera*. Their group C corresponds with *Clathromonas* except for our inclusion also of *C. takahashii* from group A and of *C. subrotacea* from group A. *C. subrotacea* lacks crown or tower scales present in many *Clathromonas*, and has only meshwork plate scales. Therefore, Thomsen et al. put it in a group A of four species with only plate scales. However, their group A was a heterogeneous assemblage that is likely to be polyphyletic. Two species with plate scales devoid of large holes (*circumvallata* and *punctata*) form the core of our new genus *Patellomonas* characterised by bowl-shaped plate scales often with distinctive margins, now with 10 species differing by the detailed sculpturing of a basically common scale type. The two species with large holes (photosynthetic *vacuolata* and heterotrophic *subrotacea*) might be considered to be mutually related as both have a flat strip-like margin to the scale that is broader than and different from the more rounded rod-like margins of most *Clathromonas* plate scales, and therefore more resemble the inner struts of the lattice. However, a variety of broader strip-like distinctive margins are found in some later-described *Clathromonas* plate scales (e.g.

ignivoma, *runcinifera*, *undulata*) as well as in the crown scales of *C. morchella* and *diademifera* (Preisig and Hibberd 1982b). Because of this and the generally flatter appearance of the struts in both *subrotacea* and *homolepis*, we have placed *subrotacea* in the completely heterotrophic genus *Clathromonas* which always has at least one type of latticed open meshwork scales rather than grouping it with the photosynthetic *vacuolata*, whose scales are fundamentally flat plates perforated by unique linear holes and thus unlike any others in either Paraphysomonadida or Chrysospineae.

Thomsen et al. (1981) suggested that the *C. subrotacea* scale type could be ancestral to all other *Clathromonas* latticed scales even though they did not consider it itself to be a meshwork scale. That remains a possibility, but the discovery that *C. undulata* and *C. diademifera* have entirely non-perforated plate scales in as well as meshwork scales (Preisig Hibberd 1982b) made it possible that the putative evolutionary link between entire plate scales only as in *Patellomonas* was via an entire plate scale intermediate rather than a meshwork one as Thomsen et al. assumed. The existence of such species with both entirely plate scales and other meshwork plate scales (Pavel Škaloud pers. comm.; Figure F. http://chrysophytes.eu/paraphysomonas) is why we placed *Patellomonas* in Clathromonadidae.

Thomsen et al. also suggested that the *circumvallata* type of *Patellomonas* scale might be ancestral to spine scales of *Paraphysomonas* sensu stricto, via spine scales with inrolled margins on the base-plate (inflexion) then believed to characterize *P. vestita*. That also was not unreasonable. However, from our tree it seems likely that spine scales with inrolled margins may not be ancestral for *Paraphysomonas*. Moreover, *Patellomonas* with simpler scales more similar to the simpler

Paraphysomonas base plates (i.e. *Patellomonas amphiplana* and *planus*) are now known and make more plausible candidate intermediates between *Patellomonas* and *Paraphysomonas*.

Hibberd (1979) showed that *P. bandaiensis* spine scales develop in membrane-bounded scale vesicles abutting and probably adhering to the smooth side of a partly rough, partly smooth endoplasmic reticulum cisterna. It appears that *Paraphysomonas* spines arise as essentially hollow finger-like membrane protrusions from the centre of a usually circular basal membrane cisterna within which the base plate is assembled. It is reasonable to suggest that the ancestral chrysomonad scale was a simple entire circular or oval base plate that became differentiated in more complex ways in different lineages. We suggest that this first evolved and became silicified in the common ancestor of all Chrysomonadia after it diverged from Hydrurales, the deepest chrysophyte branch on our MrBayes tree (Fig. 1). Our tree with its poorly resolved base makes it likely that all the other six orders diverged very soon after the origin of scales and that there has been no recent evolutionary transition between them or between the contrasting scale types represented by Paraphysomonadida, Synurales, and Chrysospineae. The trees suggest that the divergence between the radically different spine scales of *Paraphysomonas* and *Clathromonas* was almost as early.

On this view the spine scales in *Paraphysomonas* and Chrysospineae evolved independently from a plate scale ancestor. In *Paraphysomonas* the ancestral scale evolved a central protrusion with a lumen so narrow that throughout almost its entire length the walls of the spine were effectively touching and there was no differentiation into more than one scale type. The presence of plate scales in addition to spine scales in only two species, *P. bimorpha* and *P. tropica*, which do not group together and

neither of which is deep branching, is clearly not the ancestral state but the result of independent reversions to that state by developmental aberrations. Presumably their ancestors had mutations in one or more genes that established the spine-forming process, and therefore now fails in a proportion of cases. Hibberd's (1979) micrographs show a thick surface coat over the whole cytosolic surface of the spine part to the developing scale vesicle but not over the base plate portion. We suggest that this coat may be clathrin and that the spine-secreting membrane is effectively a coated pit whose length and width would control the shape of the spine assembled within it. The fact that the aberrant minority plate scales of both *P. bimorpha* and *tropica* invariably have a larger diameter than spine scale bases in the same cell is consistent with our thesis that they are developmentally aborted spine scales, not a truly separate scale type. Their greater diameter necessarily follows if the scale cisterna primordium has the same initial size. In normal spine development evagination of a given sized primordium by the central coated pit would necessarily make the cisterna narrower than its initial size; if the evagination is aborted for any reason, the spineless plate will necessarily be wider as the scale proteins will be assembled to fill the whole initial width of the cisterna. One might expect that in species with highly variable spine scales (e.g. *P. variosa, P. sinensis*) those with longer spines would on average have narrower base plates and thus a greater S/P ratio as the spine gets longer. Plotting scale spine length against S/P ratio as a scatter graph (Sup. Fig. S2) verifies this predicted relationship for these two spine scale types. This hypothesis may therefore usefully inform future investigation of the mechanisms of silica scale formation.

All Chrysospineae by contrast have both basally strongly flared spine scales and flat spine scales. Therefore, ancestrally the original flat plate scale vesicle must have

duplicated into two forms, one that never evolved such a coated pit and thus remained a plate scale and one that did and secreted spine scales. The flared base of the spine in Chrysospineae means that the membrane evagination must initially be much broader (and then strongly taper distally), which is consistent with spine formation by scale membrane evagination having evolved independently in *Paraphysomonas* and Chrysospineae. An additional shaping force would be needed to modify the scale vesicle shape to make the corona of *Chrysospina* and the upper tilted flange of *Chrysosphaerella* and the terminal branching of *Acanthomonas* (the latter analogous to distal modifications in *P. cylicophora* discussed below). Including *Spiniferomonas* within *Paraphysomonas* (Preisig and Hibberd 1982b, 1983) was clearly erroneous; the spine scales of Chrysospineae and *Paraphysomonas* are only superficially and convergently similar.

P. cylicophora has a radial base-plate similar to *P. hemiradia*, but has a perforated cone instead of a spine, making it difficult to categorise; both Thomsen et al. (1981) and Preisig and Hibberd (1982b) placed it in its own group in total isolation from all others. Both the resemblance of its radially strutted base plate to that of *P. hemiradia*, which both spine structure and sequence show that it is an authentic *Paraphysomonas*, and developmental considerations cause us to include it in *Paraphysomonas* despite its unique goblet-like inverted cone. The membranous central finger of a developing spine-scale vesicle with a narrow mouth as in typical *Paraphysomonas* could be simply modified to form a goblet by becoming distally inflated and then invaginated, all of which could be controlled by modifications to the coat, perhaps with the help of additional cytoskeletal elements to help mould it. The perforations in the cone could arise secondarily in the same way as the perforations of

the base-plate of *P. foraminifera*. It is less easy to see how it could have evolved from a latticed scale like that of *Clathromonas*; the narrow neck at the base of the goblet is best regarded as the retention of a synapomorphy that evolved only in the last common ancestor of all *Paraphysomonas*, as it is not found in any other Chrysomonadia.

Now that we know that many *Clathromonas* have only meshwork plate scales there is not a sufficiently sharp distinction between *C. subrotacea* and species with crown scales to merit their placement in separate genera. It is possible that *Clathromonas* species with only plate scales include some whose ancestors never had crown scales and some that did and which subsequently lost them. More sequences are needed to test this, but until they are available it would be premature to subdivide *Clathromonas* further, though that might one day be desirable as their scale morphology is very diverse despite always including some meshwork scales. We regard the scales of *C. takahashii* as pseudospine scales that evolved from latticed plate scale ancestors by the evolution of a central spine vesicle membrane protrusion independently of both *Paraphysomonas* and Chrysospineae. The fact that its scales are extremely variable, some having only a scarcely visible rudiment of a spine and some with long basally fenestrated spines (Thomsen et al. 1981) suggests that these spines may have evolved relatively recently from a *Clathromonas* having only uniform plate scales. The developmental programme for making the membrane invagination seems not yet to have become established as it is in most *Paraphysomonas* and Chrysospineae.

***Paraphysomonas vestita* identification problem**

We have shown that there are a very large number of genetically often extremely different species of *Paraphysomonas* having nail-like scales with a non-perforated entire round base plate with a dense margin and relatively long more or less pointed central spine. Previously all such species would have been lumped together as *Paraphysomonas vestita* despite the different dimensions and many small differences in morphology of their scales that we have discovered. Are any of our strains *P. vestita* as described by Stokes (1885) under the name *Physomonas vestita*? As it was described without electron microscopic evidence for its scale morphology, we can only go by features visible in the light microscope: cell size, shape, and cilium, stalk, and spine lengths. Stokes only observed sessile and stalked cells taken from shallow ponds and streams in New Jersey, USA; they were 1/1666 inch in diameter, which is ~15.2 µm, not 11 µm as incorrectly stated by Manton and Leedale (1961), who made the first comprehensive electron microscope study of a *Paraphysomonas* in a mixed protist culture, but only studied non-sessile cells. Stokes did not say whether his measurement included or excluded the spines, but as the anterior cilium was said to be twice body diameter he must have excluded the spines for that to be true (assuming that his drawing is accurate). His drawing depicts the spines as approximately 81% of the length of the shorter cilium, said to be 'one-fourth' the length of the longer one. These figures are likely to be approximations to simple fractions, not precise measurement, and the recurring decimals in his diameter seems likely to result from a calculation from a starting measurement with perhaps only one significant figure so could be rather approximate. But taking them literally makes the *P. vestita* long cilium ~30 µm, the short cilium ~7.6 µm, and the stalk ~61 µm. The fact that the stalk in the drawing is actually ~3.4-3.6 X the body diameter (width slightly

less than length) not 4X gives an idea of the likely rounding approximations in his descriptions/drawings. From the spine length/cell diameters in the 1885 drawing, the spines would be ~6.3- 7.5 µm long, but if we compared them instead with the short cilium and assumed that its length was accurately shown, we would get a figure of ~9.4 µm. In Stokes (1888) the text description is identical except for omitting some detail concerning contractile vacuoles, but the figure is redrawn with proportionally shorter posterior cilium (closer to proportions in text) and a somewhat less circular cell; from it similar calculations would indicate spine lengths in range 4.9-6.4 µm); because of the discrepancy between the spine-length/PC ratio in the two figures we base our *vestita* spine length estimates on cell diameter/spine lengths, not spine length/PC length. Given the excessively broad lumping as *P. vestita* of different genetically unrelated strains that our trees reveal, and the comparably excessive lumping of strains with substantially different scale morphology discussed below, the concept of what is a *P. vestita* has clearly been far too vague and excessively elastic in the past, making it highly desirable to establish a neotype in order to stabilise nomenclature and make it more precise.

If that were to be done the type strain should be from fresh water (ideally from the USA as was Stokes's, rather than Europe) and should have a sessile cell size range of about 13-17 µm with a mean close to 15 µm and spines ~5-7.5 µm that are very conspicuous in the light microscope. We have not made a neotype primarily because, as Table 2 shows, none of our strains has a mean diameter as great as 15 µm, and those with the largest most conspicuous spines were not amongst those with the largest cells. Our largest strain is *P. uniformis* with mean diameter 11.6 µm but its

spines are only 4.5 µm, so it is not *P. vestita*. The freshwater strain of Manton and Leedale (1961) was 12-20 µm and its spines 2-10 µm, so might have been *P. vestita*, though its extreme variation in spine lengths makes us doubt that. Manton and Leedale's cilium measurements also differ from Stokes' in that the LC is longer (40-45 µm not 30 µm). A better candidate for *P. vestita* is that carefully studied by Korshikov (1929), who first showed the nail-like morphology and siliceous nature of the scales, though as his strain and that of Manton and Leedale (1961) had just one contractile vacuole, not two like Stokes', and had a shorter short cilium and longer long cilium, we cannot be sure of that.

Bec et al. (2010) figure a *Paraphysomonas* identified as *vestita* that is 22 µm in diameter and with much more conspicuous spine scales under DIC than most of those described here. Thus some other authors have found larger strains more like Stokes' *P. vestita* than any we isolated. Stokes did not say what his strain ate, but that of Manton and Leedale (1961) fed on a variety of smaller microorganisms but especially the haptophyte *Chrysochromulina*. Korshikov's strain ate the large photosynthetic bacterium *Chromatium okeni*. If *P. vestita* prefers photosynthetic prey, that might explain why we did not find it. Though seven of our cultures, all from the subgenus *Paraphysomonas*, were contaminated with smaller *Oikomonas*-like chrysophytes (or granule cells), none contained eukaryotic algae, and our strains must have subsisted solely on heterotrophic bacteria (or on these contaminating '*Oikomonas*') or as saprotrophs. If smaller *Paraphysomonas* can survive on a diet of heterotrophic bacteria but larger ones require a diet including eukaryotes or photosynthetic organisms, our culturing method would have prevented our isolating them and explain why we did

not isolate *P. vestita* or any other similarly large species that may exist but have not yet been described. We cannot exclude the possibility that some of our new species can feed on eukaryotes, or that if they did their cells would be larger, but would suggest that a broader range of food organisms should be used in future in an effort to obtain *P. vestita* for sequencing and probably a different set of species from those we found. Not supplying the right food could be why we isolated so few *Clathromonas* species and no *Patellomonas* or *Acanthomona*s.

Four 18S rDNA sequences are labelled *P. vestita* in GenBank, but all were probably from misidentified strains. AF109325 was from a strain recloned from a contaminated derivative of CCAP 935/14 isolated from the eutrophic freshwater pond Priest Pot (Caron et al. 1999); as its scale spines were approximately 3 µm long it cannot be *P. vestita*; This sequence and two '*Spumella*-like' (AB616676, AY651094) sequences share a large (55 nt) insert and just differ by a few presumed sequencing errors, we have made all of these *P.* aff. *caroni* because they are so close to our new more deeply branching species. A different but closely related sequence GU220392 from marine strain J1 with much thicker spines 3.0-4.3 µm (Petronio and Rivera 2010), is also not *P. vestita* and we made it new species *P. petronia*. NCBI GenBank sequence, Z28335 (Rice et al. 1999), is an extremely different sequence that is from a marine strain with spines ~ 6 µm long, but more like *P. vestita* (Manton and Leedale, 1961); however, because of the slenderness of its spines, lack of light microscope evidence of its cell and ciliary dimensions, and its marine habitat (Rice et al. 1997) we are sceptical of it actually being *P. vestita* (Stokes). Therefore, we made Z28335 *P.* aff. *longispina* because it is so close in sequence (just one nucleotide different) to our new species

that had similar scale dimensions. We isolated another freshwater and two marine isolates that shared the exact same 18S rDNA sequence as *P. longispina* making this species the first to be found in both freshwater and marine environments. The literature has often stated that *P. vestita* is able to inhabit both marine and freshwater environments, but this pertains only to the generalized morphotype that *P. vestita* has had up to now. Even so, it has not been shown if the same isolate can grow in both marine and freshwater environments, even if it does share the same 18S rDNA its ecological counterpart. One could argue that even with the same 18S rDNA sequence, there could be a species distinction based on their ecology, but we have not done so here. Until genuine fresh water *vestita*-like strains are cultured clonally and shown to be genetically and scale-morphologically indistinguishable from marine strains, it would be unwise to assume (as is sometimes done: Finlay and Clarke 1999a) that *P. vestita* can grow in seawater. As several species we described to have much thicker spines than Z28335 we suspect that of the spines of the cell from which this sequence was from would be less conspicuous in the light microscope (*P. longispina* had obvious scale cuticle but the spines were not obvious) than for *P. vestita*, and that freshwater strains will one day be isolated with more robust scales of similar length. A short fragment FJ886745 (348 nt), from a strain from the Marine Biology Laboratory of Copenhagen University (Bochdansky and Huang 2010) and therefore presumably marine, is nearly identical (one T insert) to both Z28335 (*P.* aff. *longispina*) and AB022864 (‘*P. foraminifera’* of Andersen et al. 1999, whose identity we called into question above, but which differs elsewhere in the molecule from *P.* aff. *longispina*). Because the AB022864 fragment is not associated with evidence for scale length, we cannot say whether it could be *vestita*, but is probably not. No evidence was published

that the 18S rDNA sequence was from a correctly identified strain, this also should not be treated as representing genuine *P. vestita*, given that both 18S rDNA and scale structures give evidence that almost every previously studied strain identified as *P. vestita* is a different species.

Scales seen in the three just cited TEM studies are clearly different from each other and from the possibly correctly identified '*P. vestita*' of Manton and Leedale; those of 10 further publications are all different from the aforementioned as well as from each other (Dürrschmidt and Croome 1985; Finlay and Clarke 1999a; Jacobsen 1985; Kristiansen 1989; Kristiansen 1992; Lee and Takahashi 1993; Preisig and Hibberd 1982a; Preisig and Hibberd 1982b; Stefanova and Kalina 1992; Takahashi 1976). From these examples alone the spine lengths range from 1.2 - 10 μm in length and would not have been visible in the light microscope because they are so small, and are therefore not examples of *P. vestita*, e.g., figure 1D of Finlay and Clarke (1999b) are only 2 μm long. Except for Manton and Leedale (1961), none of the many publications showing scales identified as *P. vestita* provides sufficient evidence, even from light microscopy, that the specimen is from a cell like the original *P. vestita*; the resemblance of these scales to those of Manton and Leedale's are of a greatly generalised likeness and too imprecise to be regarded as evidence that they were from the same species.

The *P. vestita*-like and *imperforata*-like spine-scale morphotypes

Both *P. uniformis*, possibly the closest of our strains to *P. vestita*, and the strain of Manton and Leedale (1961), whose cell size and scale dimensions overlap with those

of *P. vestita*, have scales with an entire (unperforated) base plate with a inflexed margin that appears as a dense boundary line in whole mount TEM and a long pointed central spine. Therefore the widespread assumption that the original *P. vestita* would have had similar scales is probably correct, though until a strain even more similar to Stokes' is studied genetically we cannot be sure of that. However, we have shown that this morphotype is not restricted to one genotype but is present across a major part of the *Paraphysomonas* clade and thus defines a large ancient group of species (the entire subgenus *Paraphysomonas*) that could include nearly half of all *Paraphysomonas* species. Thus these broad scale features are insufficient to identify scales, as being from *P. vestita*; to identify species it is necessary to use the detailed differences in scale morphology discussed above as well as DNA sequencing. Identifying species from individual scales in the absence of the cells that bear them (Finlay and Clarke 1999a,b) is likely to be particularly hazardous given the marked variation within one clone in a few of our new species and in the strain of Manton and Leedale (1961), and the presence of quite similar scales in some genetically different species. Past records of *P. vestita* are best regarded as of the whole subgenus *Paraphysomonas*, not any one species.

If variations in scale morphology are compared across the tree it is clear that in some regions of the tree small variations in scales correspond with small variations in sequences and that if proper note is made of such small differences scale morphology can often be similarly resolving to DNA sequences. We found one case where scale differences seem to be more resolving than sequences, but several instances where quite similar scales were present on rather distant lineages. Thus though there is often

a broad (and sometimes quite close) correlation between scale and genetic differences, this is not a precise correlation and sequences in general offer a more reliable picture of *Paraphysomonas* evolution and affinities that is less subject to convergent evolution (e.g. with respect to the appearance of the base plate margin and spine tips). What is clear is that in *Paraphysomonas* there are two very common generalised spine scale morphotypes, both with unperforated base plates and each shared by numerous lineages and several rarer more distinctive ones. The *vestita*-like morphotype (subgenus *Paraphysomonas*) has tapering spine scales with a thick-rimmed base-plate, whereas the *imperforata*-like morphotype (a major subclade of subgenus *Acrospina* probably including about half its species) lacks the thickened base plate rim. From our trees it is likely that *Paraphysomonas* ancestrally had scales with imperforate base plates. We cannot confidently deduce whether the base plate rim was originally plain as in *P. imperforata* and the rest of subgenus *Acrospina* and the major subclade of subgenus *Hebetomonas* or dense as in subgenera *Paraphysomonas* and *Brevispina* plus *P. parahebes*; but if the topology of Figure 2 were correct, assuming that a dense rim is ancestral would give only two origins of plain rims, whereas assuming plain rims were ancestral gives three independent origins of dense ones, and so is marginally less parsimonious. Though the difference in rim structure is relatively small, it is fairly conservative and numerous clearly related lineages share similar base plate margins. The margin is most dense and conspicuous in *P. bandaiensis*, which led to its being separated as a species even without sequence information.

P. imperforata (Lucas 1967) was a tiny (4.5 µm) marine strain with very small scales with plain-rimmed circular base plate with lightly distinguished inner annular

marking. Because its scales lack strongly distinctive characters, almost all unperforated spine scales without a thick rim have been identified as "*P. imperforata*", an excessive lumping like *P. vestita*. These over-generalisations let to the incorrect belief that "*P. imperforata"* scales have an added attribute, not in the original description, of an oblique sharply pointed tip (Preisig and Hibberd 1982a), as here described for *P. acuminata*, a species that would have been formerly lumped into *P. imperforata* despite the scale being more than ten times larger than Lucas's (1987) original description. By using genetically characterised clonal cultures we have shown how finer details of this morphotype, e.g. overall size, spine length and tip shape, can be used as to help define numerous species and should be considered in identification. Without sequence information as well as clonal cultures it would not have been possible to interpret the significance of the small differences in scale morphology we found and rectify the gross taxonomic lumping that predominated in the past. Our study raises the question whether other scale morphotypes of Paraphysomonadida previously treated as single species might actually contain several genetically distinct ones.

Biogeography and the environment

Our molecular results and taxonomic conclusions are highly relevant to earlier claims based on studying single scales temporarily preserved in mud from a single pond after cell death and lysis that *Paraphysomonas* species are mostly cosmopolitan and can disperse freely across the globe (Finlay and Clarke 1999a,b). That assertion was indirectly extrapolated from the fact that 32 morphospecies were identified from scales in the eutrophic pond Priest Pot, in Cumbria in the UK and the assumption that if

there are many non-cosmopolitan species in the world fewer should not have been found in Cumbria; as a corollary it was hypothesised that most of the world's *Paraphysomonas* had already been described. We have shown here that that corollary is certainly untrue. 32 of the 33 new species described here would all have been lumped by Finlay and Clarke as just three species: *P. vestita*, *imperforata* and *butcheri*, which are respectively the first, second, and fourth most widely reported globally in TEM whole mount surveys, and second, first, and 19th most common in Priest Pot. Figure 1 of Finlay and Clarke (1999b) showed examples of two of them, and also of the marine "*P. foraminifera*", somewhat misleadingly included even though they did NOT find it in Priest Pot; they do not say where any of these three samples came from, so the *P. butcheri*, which may be correctly identified, might (like "*P. foraminifera*") not have come from Priest Pot. As noted above, their "*P. vestita*" is probably a still undescribed species. The same is true for "*P. foraminifera*"; its base plate diameter (0.9 μm) is outside the range given by Lucas (0.97-1.12 μm). More importantly the pores are much more regularly circular than in *P. foraminifera*, and the broad mid-margin space between the fifth and six ring of pores (counting inwards) of *P. foraminifera* is absent; instead there is a noticeably different ring-like structure between the fourth and fifth rows not seen in *P. foraminifera*; finally its spine (1.21 μm) is well outside the range for *foraminifera* (1.46-1.63 μm).

Of the 32 '*Paraphysomonas*' species recorded in Priest Pot by Finlay and Clarke only four are actually *Paraphysomonas* according to our new taxonomy. Of these, records for *P. vestita* and *imperforata* are useless given the high degree of lumping and misidentification. One should not take this record of *P. imperforata* as evidence that

this marine species can exist in fresh water, as we have found many new freshwater species that would have been lumped with it (all those in subgenus *Acrospina* with imperforate scale base plates). Finlay and Clarke (1999b) asserted that '*P. vestita* and P. *imperforata* are known to be very tolerant of a wide range of conditions' but cited in support only the study of the marine strain that we argued above was misidentified as *P. vestita* (Lee 1987). The third species *P. circumforaminifera* is a relatively rarely recorded freshwater one that we did not find. The fourth *P. bandaiensis* (Takahashi 1976) is a freshwater species previously recorded from Cumbria (Hibberd 1979), which we also did not find. There were four distinctive *Paraphysomonas* that neither we nor Finlay and Clarke found: three are marine (temperate *P. foraminifera* and two from Antarctic sea ice: *P. oligocycla*, *P. antarctica*), so their absence from Priest Pot is unsurprising, and consistent with our finding that almost all strains of *Paraphysomonas* can be found in either marine or freshwater/soil habitats but not both. It is equally unsurprising that neither we nor Finlay and Clarke found the fourth species *P. porosa* from a tropical freshwater pool if it is a strictly tropical species. Therefore of the 9 previously known species of *Paraphysomonas* sensu stricto it is likely that only three freshwater ones (*vestita*, *circumforaminifera*, *bandaiensis*) occur in Priest Pot (but there are no published micrographs to support the correct identification of any of them) and that all four marine species (*foraminifera*, *oligocycla*, *antarctica*, and perhaps *imperforata*) are absent. As all three freshwater species were described from the Holarctic their likely presence in Priest Pot scarcely bears on the question of global protist dispersal. The apparent absence of *P. porosa* described from the tropics is more telling evidence that everything is not everywhere, as it would not have been excluded by salinity differences as *P. foraminifera* most clearly is. As Priest Pot did not

exist 10,000 years ago but was under an icecap it is has long been obvious that all species now found there are capable of easy dispersal within Europe at least in relatively short historical intervals. To assess whether *Paraphysomonas* species are restricted to particular climatic zones, e.g. temperate, tropical and Arctic/Antarctic, or are truly cosmopolitan, requires much more genetic and TEM sampling of clonal cultures (similar to ours) from the two latter regions and cannot be settled by further study in the temperate zone alone, still less of one geologically ephemeral pond. Moreover, to establish what fraction of the presently known 45 species of *Paraphysomonas* sensu stricto and the many-fold more that are probably still undescribed requires a greater focus on culturing and sequencing clones from other continents.

A similar claim for global ubiquity of diatoms (Finlay et al. 2002) has been comprehensively disproved by direct multifaceted evidence (including interstrain sexual compatibility and incompatibility) that many ubiquitous morphospecies are actually aggregates of several cryptic or semi-cryptic biological species that are genetically quite distinct (Amato et al. 2007; Poulíčková et al. 2010; Souffreau et al. 2012) and that diatom dispersal is not rapid but historically constrained with gene flow between populations sufficiently restricted (Rynearsen et al. 2009) to produce geographically localised genetic differentiation within cosmopolitan species (Evans et al. 2009; Vyverman et al. 2007) and species biodiversity patterns within genera that are better explained by historical and geographic constraints to dispersal (Vyverman et al. 2007) than by the theory of instantaneous global mixing (Finlay 2002). As that theory of global mixing and its corollary of no geographic endemicity have both been conclusively disproved for diatoms, whose biological species boundaries are better

studied than in any other protists, it is likely to be wrong for all protists, including paraphysomonads. There is steadily growing evidence that genetically well-defined protist species are subject to basically the same principles of historical biogeography as higher organisms (Evans et al. 2009; Vyverman et al. 2007) and that the idea that protists obey qualitatively different rules (Finlay et al. 2002, 2004; Fenchel et al. 2006) is erroneous.

One way protists do differ substantially from most animals and plants is that genetically diverse cryptic or pseudospecies are far more common than the classical sibling species of higher organisms, having been found in every genetically well studied group (e.g. Amato et al. 2007; Bass et al. 2007, 2009; Cavalier-Smith et al. 2008; Darling et al. 2004, 2007; Howe et al. 2009; Kucera and Darling 2002; von der Heyden and Cavalier-Smith 2005). Moreover, the degree of genetic divergence amongst such species is often also far greater than in most animal or plant sibling species. This arises because of the relative paucity of morphological characters suitable for defining protist compared with animal or plant species; consequently incorrect taxonomic lumping of numerous species into single morphospecies has been far greater, giving an erroneous conclusion of species ubiquity, a possibility long recognised (Patterson 2000) but now conclusively shown as the general rule. Excessive lumping will be worst in groups with extremely simple morphology, such as bodonids (von der Heyden and Cavalier-Smith 2005), glissomonads (Howe et al. 2009, 2011), and planomonads (Cavalier-Smith et al. 2008; Glücksman et al. 2012) in all of which apparently ubiquitous morphospecies like *Bodo designis*, '*Heteromita globosa*', and '*Ancyromonas sigmoides*' proved to consist of numerous highly divergent lineages, often as ancient as whole genera, families, or even orders of animals or plants. Thus perception of protist species ubiquity is a simple

artefact of inadequate taxonomy. There is growing evidence reviewed by Foissner (2006) and Bass and Boenigk (2011) that when taxonomy is done properly by sufficiently discriminating methods protists have a qualitatively similar mix of cosmopolitan and geographically restricted or endemic species as higher organisms. As in macroorganisms, the degree of endemicity of a protist taxon will depend partly on dispersal efficacy (for which their small size, cysts and large populations help protists more than some plants and animals, but no more than large organisms like ferns and puffball fungi with billions of microscopic spores, but which still exhibit historically constrained geographic patterns) and partly on the antiquity of divergences within the group in question, ancient or easily dispersed groups showing lower endemicity than recent or less vagile ones.

Though it might be true that there are proportionally fewer protist endemics than for macroorganisms (Foissner 2006), the high frequency of cryptic genetic variation recently discovered in all groups casts doubt on that. Even in animals the degree of cosmopolitanism of species has often been overestimated, especially in microscopic ones with few discriminating characters, where cryptic species were overlooked for the same reasons as in protists. Thus freshwater microzooplankton, e.g. crustaceans, were once considered cosmopolitan (Mayr 1963) until recent molecular studies comparable to those in protists revealed a prevalence of continental and regional endemism; as Xu et al. (2009) put it: 'Cosmopolitanism in freshwater zooplankton often appears to represent an artefact due to morphological stasis, extensive phenotypic plasticity, and poorly resolved taxonomy rather than biological reality.' This may also be true for *Paraphysomonas*: our new sequence data provide the tools to test this.

In diatoms, whose frustules provide superbly detailed light microscopic characters, genetic antiquity of the lumped morphospecies is (as one would expect) substantially less, but it is nonetheless found in all carefully studied cosmopolitan morphospecies (e.g. Sorhannus et al. 2011). However, the likely antiquity of the aggregated species complexes is probably generally less in diatoms than for groups like bodonids, glissomonads and planomonads that are largely devoid of detailed discriminatory microscopically visible details. Because diatom frustules fossilize well, the age of diatom species and genera can be fairly accurately estimated from fossils and correlated with sequence trees (Souffreau et al. 2011). Though their well-fossilized stomatocysts show chrysophytes to be almost as old as diatoms (at least early Cretaceous, about 130 My ago) they are insufficiently discriminating of lower-level taxa to date them; synurid scales go back at least to the mid-Eocene (>40 My ago: Siver et al. 2009), but those of paraphysomonads are too unstable to fossilize. Nonetheless as 18S rDNA trees suggest that Paraphysomonadida diverged in the same early rapid divergence as all other stomatocyst-bearing orders, they are probably not dramatically younger than stomatocysts. Estimating their age is complicated by the conflicting topology of Figures 1 and 4; if that in Figure 4 is correct they probably diverged at least 120 My ago, but if as we suspect Figure 1 is closer to the truth, they could be somewhat younger than other chrysophyte lineages, perhaps no more than 80 My old. Thus paraphysomonad nail-like scales have remained more or less the same morphologically for about 100± 20 My, with only the rather trivial variations discussed above. We estimate from the proportions of Figure 1 that the *imperforata*-like subclade of subgenus *Acrospina* may be roughly 35-50 My old. The accelerated evolution of subgenus *Paraphysomonas* rDNA makes estimating its age still more

hazardous, but it is likely to be no less. Thus morphological stasis of the two commonest morphotypes, *imperforata*-like and *vestita*-like, has probably persisted for several scores of millions of years – more that the likely age of most diatom morphospecies but substantially less than estimates for the *Bodo designis*, '*Heteromita globosa*', and '*Ancyromonas sigmoides'* morphotypes (von der Heyden and Cavalier-Smith 2005; Howe et al. 2009, 2011; Cavalier-Smith et al. 2008), this intermediate value being consistent with *Paraphysomonas* scales providing more discriminating characters for taxon subdivision than is available for these groups but fewer then in diatom frustules. These paraphysomonad age estimates, though crude, help reconcile the global ubiquity of protist morphotypes like that of subgenus *Paraphysomonas* or '*P. imperforata*-like' and thus the apparent cosmopolitanism of protist morphospecies correctly emphasized by Finlay (2002, 2004) and Patterson (2002), with the decisive evidence from diatoms (and to some degree other protists, notably Foraminifera where as for diatoms there is direct fossil evidence for divergence times, e.g. Aurahs et al. 2009; Darling et al. 2004, 2007) that protist dispersal is insufficiently fast for global genetic homogenization, and that genetic diversity at the biological species level and processes of allopatric speciation are not fundamentally different from that of higher organisms, contrary to Fenchel et al. (2006). It appears that protist dispersal is slow enough to allow genetic differentiation within a cosmopolitan biological species and allopatric speciation, but sufficiently fast for global homogenisation of morphotypes that show stasis over scores of millions of years.

Not only protist morphospecies but also genera, families and orders are probably often less finely divided than animal and plant taxa, so genera and families may often be substantially older than the time needed for global dispersal of most

protists. Therefore one would not expect geographically endemic families and orders in protists, as are common in recently evolved animal and plant groups (e.g. flowering plants, mammals), but rare in older ones (e.g. insects) whose origin predated the breakup of Pangaea. Thus, though Finlay and Fenchel's conclusions are probably largely incorrect for species, there would be some truth in them if applied instead to higher taxa. But the causes of the absence of endemic higher taxa are not because short-term evolutionary processes are different in protists from higher organisms, but mainly because (a) the sparsity of protist differentiative characters biases taxonomic ranking relative to genetic divergence, yielding lower ranks for comparable sequence differences, and (b) because most major groups of protists originated much earlier than mammals and angiosperms. Though protist long-distance dispersal is easier than for elephants, the large population sizes of protists do not favour global genetic homogenization over geographic differentiation as decisively as Finlay (2002) supposed, because the massive populations in recipient regions quantitatively hugely dominate the relatively rare immigrants, allowing geographic variants to persists despite considerable immigration, enabling genotypes to diverge faster than dispersal homogenizes them (De Meester et al. 2002). The possibility of global dispersal even of millions of cells will not prevent evolution of geographic patterns if genes diverge fast enough. Whether geographic genetic differentiation occurs depends not on the absolute level of dispersal, but the on the fraction of a population replaced per generation by immigrants compared with the fractional mutation rates and selection coefficients (Wright 1940, 1951). Therefore the fact that protist populations are often much larger than those of macroorganisms is irrelevant to the likelihood of allopatric speciation, because large populations increase the denominator and numerator of the

fractional immigration rate proportionally, so the idea that allopatric speciation is less likely in protists than animals or plants (Fenchel and Finlay 2006) is based on a simple mathematical error: thinking of absolute migration rates not fractional ones.

Applying standard population genetic ideas of mutation-selection and migration, selection and genetic drift interactions, emphasizes that it would be easier to detect restricted distribution in faster-evolving genes than in slow-evolving ones. Unfortunately limited time for this study prevented our obtaining faster evolving ITSs for our strains (Coleman 2007), which would in future allow testing whether the widely dispersed genotypes we found are truly identical or include more than one species. As closely related biological species can have identical 18S rDNA but different ITS (Lynn and Strüder-Krypke 2006; Chantangsi et al. 2008), some of our new species might actually be clusters of closely related sibling species, so we may have somewhat underestimated the number of different species. Nothing is currently known about sex in Paraphysomonadida and whether they have biological species or are clonal. However, as species even differing by very few 18S rDNA nucleotides generally had detectably different scales, whereas except for the one case where we established subspecies, independent isolates with exactly the same genotype had virtually indistinguishable scales, 18S rDNA identity is a sensible practical criterion for membership of the same nominal species; even if some 18S rDNA ribotypes consist of two or even three cryptic species this pragmatic and objective criterion will avoid the past excessive clustering of dozens into single crude morphospecies. Species estimates based on rDNA identity will be at least of the correct order of magnitude and could be accurate within a factor of two or three given more comprehensive sampling.

It is interesting to note that no environmental clones appear in the large long-branch subclade of subgenus *Paraphysomonas*, but there are environmental sequences in all short-branch clades. This is precisely what was found previously for Heliozoa (Cavalier-Smith and von der Heyden 2007) and likely to have the same explanation involving PCR bias. Environmental sequencing primers may have missed these clades of *Paraphysomonas* from general probing of environmental DNA because primers were not specific enough for these cells but it more likely arises because the long-branch clade has insertions making amplicons longer (as in long-branch Heliozoa). It is most unlikely that all 35 strains in this clade were so rare in all environments that rarity alone explains their absence from environmental DNA data, especially as they include the 'vulgar' clade that we isolated most often. That culturing reveals an entire clade of *Paraphysomonas* not been picked up via general environmental DNA cloning emphasizes the necessity of using both culturing and environmental PCR to assess protist biodiversity, as previously found in Cercozoa (Bass and Cavalier-Smith 2004; Howe et al. 2009) and Heliozoa (Cavalier-Smith and von der Heyden 2007). It would be valuable to create a primer specific to this clade for environmental probing to uncover the diversity of these organisms, especially because culturing can be so cumbersome and limited to cells that can live in laboratory conditions; that culturing itself can be biased is well known – Lim et al. (1999) found that *Paraphysomonas imperforata* is disproportionately represented in bacteria-rich marine enrichment cultures.

Present evidence for the 44 morphospecies found in this study is insufficient to decide whether any are cosmopolitan, but we found the same 18S rDNA genotype in multiple countries for several, suggesting that some at least may be very common and widespread and perhaps distributed world-wide at least in temperate zones. However,

it is important to stress another source of bias in interpreting biogeographic data on protists. In the past few hundred years human transport of soil (e.g. with plants or on shoes or vehicles) and water (e.g. bulk ship ballast) will have sharply increased the rate of global mixing of protists, so some cases of protist cosmopolitanism will be as anthropogenic as that of agricultural weeds or deliberately introduced species. With only geographically sparse records so far for specific *Paraphysomonas* genotypes we cannot say whether these few widely distributed samples reflect natural dispersal processes or are distorted by unwitting human transport of propagules. Denser genetic sampling across several continents is essential for clarifying *Paraphysomonas* biogeography, which remains an almost entirely open question.

Similar studies are needed for *Clathromonas*, which make up the majority of the paraphysomonads recorded in Priest Pot. As discussed above, *C. butcheri* originally from salt marsh pools has been subject to excessive lumping. We therefore doubt whether most (if any other than the original description) records for this species were correctly identified. We suspect that (as we found for *Paraphysomonas* sensu stricto) closely related but genetically distinct saline and freshwater species may exist. The same could be true of other Priest Pot *Clathromonas* originally recorded from marine habitats. We suspect that a substantial fraction of these records may be of still undescribed species. That was certainly true of the majority of the new species of Heliozoa described by Cavalier-Smith and von der Heyden (2007). Culturing/genetic/TEM studies on *Clathromonas* would test this surmise.

Figure 2 shows that habitat preferences for marine, freshwater and soil environments are phylogenetically quite strongly conserved in *Paraphysomonas*, i.e.

major subclades are habitat specific and rather few evolutionary shifts between marine and freshwater/soil are evident. Figure 3 shows this to be equally true of both major environmental DNA clades of chrysophytes. A strong phylogenetic conservatism with respect to occurrence in marine versus freshwater habitats has been previously found in numerous other protist groups (Cavalier-Smith and Chao 2012; Cavalier-Smith and von der Heyden 2007; Glücksman et al. 2012; von der Heyden et al. 2004 von der Heyden and Cavalier-Smith 2005), so it is unsurprising that this is also true of *Paraphysomonas*. It is clear that the largest subgenus *Paraphysomonas* was ancestrally freshwater (as its two deepest subclades exclusively are) and that later invasions of soil and marine environments were rather few. In the whole genus *Paraphysomonas* there appear to be relatively few such habitat switches, but it is harder to decide whether their ancestor was freshwater (somewhat more likely) or marine. Invasions of soil seem to be even rarer, with perhaps only one in subgenus *Paraphysomonas* (if we assume the freshwater habitats of *P. stylaris* is a secondary reversion of an ancestrally marine subgroup).

Conclusion

We have shown that in the past *Paraphysomonas* taxonomy has suffered from excessive lumping at both species and generic levels, and was therefore inadequate for making claims about the ubiquity or numbers of species within '*Paraphysomonas*' (Finlay and Clarke 1999a,b). Our restriction of the genus to species with nail-like scales, and demonstration that previously over-looked subtle differences and finer details in the morphology of such scales correlate with robust sequence phylogeny, provide a sounder basis for future studies of the biodiversity and biogeography of

Paraphysomonas sensu stricto. Similarly detailed studies are needed for *Clathromonas* and the other genera here segregated from *Paraphysomonas*: a greater culturing effort for them and for the two major environmental clades is essential for testing our revised chrysophyte classification and better understanding their large-scale evolution. Environmental DNA studies are also needed for all of them using group-specific primers to estimate the true biodiversity of the former *Paraphysomonas* morphotypes, which we suggest may collectively turn out to have hundreds of species globally once intensively studied from all continents jointly by culturing, sequencing, and electron microscopy.

Methods

Obtaining *Paraphysomonas* isolates. Clonal cultures of *Paraphysomonas* were obtained from soil, freshwater and marine environments. Ten to 20 g of soil, sand or sediment and water were taken from the environment and a few grams put into Petri dishes along with media (Artificial Salt Water for Protists (ASWP CCAP media recipes http://www.ccap.ac.uk/media/) or Volvic® for freshwater samples and were enriched with barley grain juice (tablespoon of barley grain in 100ml Volvic® bring to boil and filter water through 0.22 µm filter - put a few drops in the culture to encourage general growth of protists via bacterial food bloom) and left at ambient temperature for 48 h. These enriched cultures where examined by phase microscopy for the presence of *Paraphysomonas*-like cells; if present, 10 µl of the culture was used in serial dilution up to 8 times over a 96-well plate; 12 copies of each dilution. A further 48 h later, the 96 wells were checked for *Paraphysomonas*-like cells, further serial dilutions were performed at least another 4 times (every two days), and once a well was thought to contain a pure colony it was again serially diluted twice more to give more chance of a pure colony being selected. Cell selection was initially based on size and basic features; large (≥ ~ 7 µm) completely round cells with two visible cilia (one long one short), colourless and with a stalked stage. After these preliminary efforts reaping 100% spine-scaled *Paraphysomonas,* smaller cells were then targeted, adhering to the same criteria as before, which is when *P. lucasi*, *P.* aff. *imperforata* and *Clathromonas butcheri* were found.

Only round cells were chosen and often mainly stalked to the substratum. Eight strains (JBM01, JBM02, WA20KP, WI34KN, WA28KT, PR26KB, PR26KA and AU30KV) were kindly provided by Jens Boenigk.

DNA extraction. As soon as the new clonal culture was established one standard size 9 cm Petri dish of the culture was extracted using UltraClean® Soil DNA Isolation Kit. Whatman GF/F glass fibre (0.2 µm) filters were used in this process to filter the cells onto and these where then chopped up and put into the soil extraction bead tube of the extraction kit.

PCR and sequencing. The same eukaryote-wide primers, targeting the 18S rDNA gene, were used in PCR and sequencing: 25F (forward: 5'-CATATGCTTGTCTCAAAGATTAAGCCA-3'), 1801R (reverse: 5'-TGATCCTTCTGCAGGTTCACCT-3'); a third internal primer was used at the sequencing stage: 3NDF (forward: 5'-GGCAAGTCTGGTGCCAG-3'). PCR reactions were mixed in 25 µl (using Invitrogen™ reagents). Denaturation (5 min at 95°C) followed by 35 cycles: 95°C for 32 s; 60°C for 30 s; 72°C for 2 min. Final extension for 7 min at 72°C. 5 µl of the PCR product was subjected to 1% agarose gel electrophoresis, and after ethidium bromide staining viewed under UV. If there were multiple bands the correct band size PCR fragment (~1,800 kbp) was cut out and cleaned using a GE Healthcare GFX™ extraction kit. If there was a single band at the correct size, the remaining PCR reaction was cleaned using polyethylene glycol (PEG) for a 25 µl reaction; 25 µl PEG and 1µl of 3 µM NaCl is added to each reaction and mixed by vortex, kept 30 min at ambient temperature, and pelleted by centrifugation at 1500 g for 30 min. Supernatant was discarded, the pellet washed with 25 µl 70% EtOH, centrifuged again for 10 mins before removing supernatant. The pellet was left to dry before resuspending in deionised water, and storage at -20°C. Sequencing was performed using dye terminators and separated on an automated ABI-377 sequencer. Editing of fragments was via free program Sequence Scanner v. 1.0 (http://www.appliedbiosystems.com); contig assembly was by BioEdit, CAP Contig Assembly Program (Hall 1999).

Phylogenetic analysis. All new 18S sequence fragments were blasted (http://www.ncbi.nlm.nih.gov/BLAST) to determine whether they were from a *Paraphysomonas* culture or a non-scaly *Spumella* before spending time fixing cells for TEM. Unidentified environmental sequences related to *Paraphysomonas* were obtained from GenBank both by BLAST-based selection and from published work on chrysophytes (Richards et al. 2005; Shi et al. 2009; Charvet et al. 2011; del

Campo and Massana 2011; Tarbe et al. 2011). A very extensive alignment (available on request) was made of over 500 18S rDNA sequences manually with the help of MacGDE (http:// macgde.bio.cmich.edu) of chrysophytes and representatives of all major heterokont outgroups from which we selected two representative taxon samples for detailed analysis: an ochrophyte-wide alignment of 329 sequences and 1672 nucleotide positions and a smaller one restricted to 239 chrysophyte sequences plus four belonging to their closest outgroup Picophagea (1681 positions). Trees for each were calculated by RAxML v.7.0.4 (Stamatakis 2006) using the GTRGAMMAMIX model with 8 rate categories and by Mr Bayes (Ronquist and Huelsenbeck, 2003) and the covarion and adgamma options with 4 rate categories and 5 million generations (1 M generations discarded as burnin).

Fixation and transmission electron microscopy (TEM). Fresh cultures were used as they have less detritus in the sample; excess medium was filtered out before fixation. EM grade 25% glutaraldehyde was added directly to the filtered culture to a working concentration of 2.5%. The fix was washed after 1 h, rinsed with distilled water, again via filtration, to a final volume of ~2 µl. The entire sample was never allowed to pass completely through the filter; vacuum pressure was released before all old and new media was passed through, the remaining concentrated-with-cells fluid recovered via disposable pipette. The sample was allowed to settle before pipetting ~8 µl of the concentrate (from the bottom of the Eppendorf tube) onto a formvar-coated 200 mesh copper grid. The sample was allowed to practically dry-out, then washed in distilled water. Samples were viewed as unstained whole mounts with an FEI Tecnai 12 electron microscope.

Observations via light microscope. All cultures were recorded live using Sony HDV 1080 Handycam® via an adapter fitted to Nikon Eclipse 80i microscope and viewed using a differential interference contrast water immersion lens (NA 1.0). Cell measurements were all made by videoing live specimens and calibrating the measurements by a micrometer scale videoed using the same settings. Video footage was uploaded to computer using FinalCut Express HD 3.5.1 from which still images were exported and transferred to Adobe Photoshop CS4 11.0.2 to make plates.

Acknowledgements

JS was supported by a BBSRC studentship and TC-S thanks NERC for past research grants and fellowship support. We thank the Canadian Institute for Advanced Research Integrated Microbial Diversity Program

for a travel grant that allowed JS to visit the Simpson and Adl labs at Dalhousie University where some of the TEM data was obtained using the same TEM model as in Oxford. We thank Jens Boenigk for providing cultures.

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Table 1. Revised classification of Chrysophyceae Pascher, 1914

Subclass 1. Chrysomonadia Cavalier-Smith subcl. n.

Order 1. Paraphysomonadida Cavalier-Smith, 1996 (heterotrophic phagotrophs; scales; never rhizopodial)

Family 1. Paraphysomonadidae Preisig and Hibberd, 1983 em. (*Paraphysomonas*)

Family 2. Clathromonadidae fam. n. (*Clathromonas*, *Patellomonas*)

Order 2. Chromulinales Pascher, 1910 em. (nutrition mixed; with or without scales)

Suborder 1. Chromulineae Pascher, 1925 (single cilium; no scales; flagellates or rhizopodial)

Family 1. Chromulinaceae Engler, 1897 (*Chromulina*) (photosynthetic flagellates)

Family 2. Oikomonadidae Hartog, 1922 (much older than Oikomonadaceae Cavalier-Smith, 1995/6 (*Oikomonas*) (non-photosynthetic flagellates)

Family 3. Chrysamoebaceae Bourelly, 1957 (e.g. *Chrysamoeba*) (photosynthetic rhizopodial amoebae)

Suborder 2. Chrysospineae Cavalier-Smith subord. n. (2 cilia; spine and plate scales)

Family 1. Chrysosphaerellaceae Cavalier-Smith fam. n. (*Chrysosphaerella*) (photosynthetic, mostly colonial; spine scales with asymmetric bobbin-like base)

Family 2. Chrysospinaceae Cavalier-Smith fam. n. (*Chrysospina*) (photosynthetic; spine scale base with raised annulus on base plate)

Family 3. Acanthomonadaceae fam. n. (*Acanthomonas*, *Spiniferomonas*) (unicellular, photosynthetic or heterotrophic; spine scale base simply flared)

Suborder 3. Cyclonexineae Cavalier-Smith subord. n. (multicellular rings; phototrophs)

Family Cyclonexaceae Cavalier-Smith fam. n. (*Cyclonexis* Stokes)

Order 3. Apoikiida Cavalier-Smith ord. n. (nutrition mixed; no scales; flagellates or rhizopodial, non-ciliate and loricate)

Family 1. Stylococcaceae Lemmerman, 1899 (e.g. *Lagynion, Stylococcus*) (no cilia, branched rhizopodia)

Family 2. Chrysosaccaceae Bourelly, 1957 (e.g. *Chrysosaccus*)

Family 3. Chrysosphaeraceae Pascher, 1914 (e.g. *Chrysosphaera*)

Family 4. Apoikiidae Cavalier-Smith fam. n. *Apoikia* (heterotrophic phagotroph)

Order 4. Synurales Andersen 1987 (2 parallel cilia; phototrophs; no chlorophyll c2; silicified plate scales; non rhizopodial flagellates)

Family 1. Synuraceae Lemmerman, 1899 (e.g. *Synura*, *Tessellaria*)

Family 2. Mallomonadaceae Diesing, 1866 (*Mallomonas*)

Order 5. Hibberdiales Andersen, 1989 (phototrophs; no scales)

Family 1. Hibberdiaceae Andersen, 1989 (*Hibberdia*, *Chromophyton*)

Family 2. Chrysocapsaceae Pascher, 1912 (*Chrysocapsa*, *Chrysochaete*)

Order 6. Ochromonadales Pascher, 1910 em. (phagotrophs; phototrophs or heterotrophs; often loricate and/or colonial)

Family 1. Dinobryaceae Ehrenberg, 1834 (*Dinobryon, Kephyrion, Uroglena*)

Family 2. Chrysolepidomonadaceae** Peters and Andersen, 1993 (*Chrysolepidomonas, Epipyxis, Chrysonephele*) (some have non-silicified plate scales).

Includes 4 distinct *Spumella* clades

Chrysomonadia incertae sedis: Polylepidomonadaceae* Cavalier-Smith fam. n. Diagnosis: photosynthetic unicellular chrysomonads with single type of scales =plate scales with elongate perforations; centrioles almost orthogonal. Type genus *Polylepidomonas* Preisig and Hibberd 1983.

Subclass Hydrurophycidae Cavalier-Smith subcl. n. (epilithic multicellular thalli; phototrophs; no scales; phagotrophy not demonstrated)

Order Hydrurales Pascher, 1931 em.

Family 1. Chrysothallaceae Bourelly, 1968 (e.g. *Chrysothallus*, *Phaeoplaca*)

Family 2. Hydruraceae Rostafinsky, 1881 (*Hydrurus*)

The above classification includes all genera now on the rDNA tree and all with silicified scales, but may require revision when others are sequenced and the phenotype of major environmental DNA clades is known.

*We cannot assign this family to an order until sequences are available.

** Incorrectly placed in Chromulinales in algaebase (http://www.algaebase.org); Figure 4 places it robustly in Ochromonadales in a deep-branching clade with *Epipyxis* and *Chrysonephele*.

Table 2. *Paraphysomonas* and *Clathromonas* species and strain light microscope and TEM data

Species name (strain name)	GenBank 18S	Cell Length (CL)	Long Cilium (xCL)	Short Cilium (xCL)	Spine Scale		S/P ratio	Base Plate		Spine tip shape
					Spine length (µm)	Base width (µm)		Inflexion at edge?	Mid-point Annulus?	
P.* (*B.*) *ovalis (ARB)	JQ967331	5.2	**1.5-2**	**0.75**	1.5	0.8	1.9	y	n	rounded
P.*(*B.*) *segmenta (Ku3b2)	JQ967330	6.0	**2**	**0.5**	0.65	0.44	1.4	y	n	rounded
***P.* (*A.*) *imperforata*, Lucas**	/	4.5	**3-4**	**1**	1.0	0.77	1.3	n	y	unknown
***P.* (*A.*) aff. *imperforata* CCAP 935/13**	Identical to EF4232518 (C1) pos. 70-737	4.5	**3-3.5**	**1**	1.1	0.8	1.3	n	y/n	Rounded
***P.* (*A.*) aff. *imperforata* EP1**		4.7	**2.5**	**0.75-1**	0.79	0.71	1.1	n	y/n	Rounded
***P.* (*A.*) *lucasi* NC10-16**	JQ967323	3.6	**2.5-3**	**0.75**	0.92	0.7	1.3	n	n	Rounded
***P.* (*A.*) *mikadiforma* JBM02**	JQ967325	7.0	**3.5-5.0**	**1**	5.2	2.1	2.5	n	y/n	Acutely pointed
P.* (*A.*) *cuspidata PR26KB)	JQ967326	9.2	**2.5-3**	**0.75**	4.7	1.6	3.0	n	y	Acutely pointed
P.* (*A.*) *acuminata (PML6A)	JQ967329	9.0	**3.5-4**	**0.75-1**	5.2	1.9	2.8	n	y	Acutely pointed
P.* (*A.*) aff. *acuminata (CCL3C)	JQ967328	8.2	**3.5**	**0.75**	5.4	2.1	2.6	n	y/n	Acutely pointed
P.* (*A.*) aff. *acuminata (WA20KP)	JQ967327	9.5	**3.5**	**0.75**	5.3	2	2.6	n	y/n	Acutely pointed

P.* (*H.*) *hebes (Ind1)	JQ967320	4.7	**2.0**	**0.75**	1.4	0.6	2.4	n/y	n	Truncate
P.* (*H.*) *hebetispina (NC10-20)	JQ967321	5.3	**2**	**0.5-0.75**	1.2	0.5	2.5	n/y	y/n	Truncate slightly rounded
P.* (*H.*) *limna *(PML2A-e2)*	JQ967322	6.6	**2**	**0.75**	/	/	/	/	/	/
P.* (*P.*) *hemiradia (AU30KV)	JQ967318	9.9	**2**	**0.5-0.75**	4.6	1.8	2.6	y	n	Small oblique blunt
P.* (*P.*) aff. *uniformis (WA32KAG)	JQ967319	8.1	/	/	/	/	/	/	/	/
P.* (*P.*) *uniformis (WA28KT)	JQ967317	11.6	**1.5-2**	**0.5**	4.5	1.8	2.6	y	n	Small oblique blunt
P.* (*P.*) *cambrispina (WI34KN)	JQ967316	9.0	**2.0**	**0.5-0.75**	2.7	**1.2**	2.3	y	n	Small oblique blunt
P.* (*P.*) *vulgatana (W03)	JQ967313	11.3	**2**	**0.5**	/	/	/	/	/	/
P.* (*P.*) *vulgaris (PML2B)	JQ967314	9.0	**1.5-2**	**0.5**	3.9	2.1	2.1	y	n	Small oblique blunt
P.* (*P.*) aff. *vulgaris (SW02)	JQ967315	/	**/**	**/**	3.3	1.8	1.8	y	n	Small oblique blunt
P.* (*P.*) *vulgata vulgata (PML4B)	JQ967311	10.4	**1.5-2**	**0.5**	2.4	1.9	1.2	y	n	Small oblique blunt
P.* (*P.*) *vulgata elongata (PML8)		8.8	**1.5-2**	**0.5**	3.6	1.9	1.9	y	n	Small oblique blunt
P.* (*P.*) *caroni (CH2)	JQ967292	7.1	?	?	1.9	1.0	1.9	y	n	Small oblique blunt
P.* (*P.*) *petronia (J1)	GU220392	/	/	/	2.9	1.8	1.6	y	n	Small oblique blunt
P.* (*P.*) *variosa (Ind5)	JQ967296	8.3	**1.5**	**0.5**	2.9	1.2	2.5	y	n	Tapered - blunt
P.* (*P.*) *mantoni (BZ5a)	JQ967295	9.7	**1-4**	**0.5-1.0**	3.5	1.3	2.5	y	n	Tapered and oblique

P.* (*P.*) *balonovi (Bassen)	JQ967294	10.1	**1.5-2**	**0.5-0.75**	/	/	/	/	/	/
P.* (*P.*) *crocotilla (UPL1B)	JQ967308	8.0	**1.5**	**0.5**	3.7	1.2	3.1	y	n	Tapered - blunt
P.* (*P.*) *solis (GMCCL6)	JQ967309	8.3	**1.5-2**	**0.75**	2.6	1.1	2.6	y	n	Tapered - blunt
P.* (*P.*) *dimorpha (CA01)	JQ967310	6.7	**1.5-2**	**0.5-0.75**	3.2	1.1	3.0	y	n	Tapered - blunt
P.* (*P.*) *longispina (MEX3)	JQ967305	8.8	**2-2.5**	**0.5**	5.7	1.4	4	y	n	Tapered - blunt
P.* (*P.*) *stylaris (PML5D)	JQ967306	9.4	**2-2.5**	**0.75**	5.4	1.3	4.0	Y	n	Tapered to fine tip?
P.* (*P.*) *stylata (W02)	JQ967307	6.9	**2.5**	**0.75-1**	3.6	1.3	2.8	y	n	Tapered - pinched
P.* (*P.*) *tropica (BZ1)	JQ967304	7.6	**2.5**	**0.75**	/	/	/	/	/	/
P.* (*P.*) *sinensis (CH9)	JQ967303	9.8	**2.0**	**0.5-0.75**	2.6	1.3	2.1	y	n	Tapered with dull tip
P.* (*P.*) *edaphica (CH6)	JQ967302	9.1	**1.5**	**0.5-0.75**	/	/	/	/	/	/
P.* (*P.*) *indica (IND4)	JQ967299	/	/	/	/	/	/	/	/	/
P.* (*P.*) *terricola (GMBGL1)	JQ967301	9.7	**1.5-2**	**0.5**	3.2	1.0	3.1	y	n	Rounded
P.* (*P.*) *spiculosa (BZ8)	JQ967298	8.4	**2.0**	**0.5**	3.2	1.3	2.8	y	n	Rounded
C. butcheri (MD03)	JQ967291	3.3	**1.5-2**	**0.5-0.75**	n/a	n/a	n/a	n/a	n/a	n/a

The data are presented in clades as seen in the molecular analyses. All are the averages for each strain. Light microscope measurements are for live cells. As many measurements as possible were made for each criterion and averaged. These data show definite patterns corresponding to particular clades, especially, Long Cilium, Annulus and Inflexion. *Paraphysomonas* subgenera are indicated by the letter in brackets in species name.

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Table 3. All known species *Paraphysomonas* sensu stricto, in alphabetical order: nine previously established species (in bold), one raised to species level) and the 34 new species

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1 *P. acuminata*
2 ***P. antarctica* Takahashi (1987)**
3 *P. balonovi*
4 ***P. bandaiensis* Takahashi (1976)**
5 *P. cambrispina*
6 *P. caroni*
7 ***P. circumforaminifera* Wujek (1983)**
8 *P. crocotilla*
9 *P. cuspidata*
10 ***P. cylicophora* Leadbeater (1972)**
11 *P. dimorpha*
12 *P. edaphica*
13 ***P. foraminifera* Lucas (1967)**
14 *P. hebes*
15 *P. hebetispina*
16 *P. hemiradia*
17 ***P. imperforata* Lucas (1967)**
18 *P. indica*
19 *P. limna*
20 *P. longispina*
21 *P. lucasi*
22 *P. mantoni*
23 *P. mikadiforma*
24 ***P. oligocycla* Takahashi (1987)**
25 *P. ovalis*
26 *P. parahebes*
27 *P. perforata*
28 *P. petronia*
29 ***P. porosa* Dürrschmidt and Cronberg (1989)**
30 *P. segmenta*
31 *P. sinensis*
32 *P. solis*
33 *P. spiculosa*
34 *P. stylaris*
35 *P. stylata*
36 *P. terricola*
37 *P. tropica*
38 ***P. truncata* Preisig and Hibberd (1982a) stat. n.**
39 *P. uniformis*
40 *P. variosa*
41 ***P. vestita* (Stokes 1885) De Saedeleer (1929)**
42 *P. vulgaris*
43 *P. vulgata vulgata* and *P. vulgata elongata*
44 *P. vulgatana*

Figure legends

Figure 1 Bayesian tree for 329 ochrophyte 18S rDNA sequences (1672 nucleotide positions). To emphasize the tree's main features and fit it onto one page internal branches of all major clades are collapsed, with the number of species included in each shown on the right (their internal topology is shown in other figures). Support values are MrBayes posterior probabilities (left) and RAxML bootstrap percentages for 1000 pseudoreplicates to the right. Black dots mean maximal support for both, i.e. 1/100. Arrows suggest possible timings of the origins of scales and stomatocysts within the Chrysophyceae.

Figure 2 Bayesian tree for 329 ochrophyte 18S rDNA sequences showing only the branching order of *Paraphysomonas* sensu stricto in detail (1672 nucleotide positions). Support values are MrBayes posterior probabilities (left) and RAxML bootstrap percentages for 1000 pseudoreplicates to the right. Black dots mean maximal support for both, i.e. 1/100. All new sequences are in bold type (starting with 'JQ'). The number of identical sequences obtained in this study from different isolates is shown in parentheses; the 'vulgar' clade had the most commonly found 18S rDNA sequence. The schematic sketches indicate typical scale structure for each of the four subgenera, each corresponding to a single reproducible clade; note how isolates with thickened/inflexed rim to the base-plate of the spine scale group separately from those lacking a prominent rim; scale sizes are arbitrary. The ranges of cell length measurements (from this study only) are indicated beside the red lines. Sequences from freshwater strains are green, from marine strains blue, and soil strains brown.

Figure 3 Bayesian tree for 329 ochrophyte 18S rDNA sequences showing the branching order of Chrysophyceae other than *Paraphysomonas* sensu stricto and the immediate outgroup Picophagea only in detail. Support values are MrBayes posterior probabilities (left) and RAxML bootstrap percentages for 1000 pseudoreplicates to the right. Black dots mean maximal support for both, i.e. 1/100. The only new sequence (for *Clathromonas butcheri*) is in bold type.

Figure 4 Bayesian tree for 239 chrysophyte 18S rDNA sequences (1681 nucleotide positions). To emphasize the tree's main features and fit it onto one page internal branches of all major clades are collapsed, with the number of species included in each shown on the right. Support values are MrBayes posterior probabilities (left) and RAxML bootstrap percentages for 1000 pseudoreplicates to the right. Black dots mean maximal support for both, i.e. 1/100. Rooted on Picophagea (Synchromales and *Picophagus*).

Figure 5 Micrographs of two new species of subgenus *Acrospina*: ***Paraphysomonas ovalis, P. segmenta***. **A-F**, *P. ovalis,* **G-I***, P. segmenta*. **A**. DIC light micrographs of live *P. ovalis* cells. **B-F.** *P. ovalis* TEMs. **B**. Mastigonemes (double arrowheads) visible on whole cell. **C**. Flattened side of cell at base of cilia (arrowhead). **D**. Side view of spine scales near cell surface **E**. Aberrant base-plate forms (*). **F**. Single scales showing variation in density of base plate rim (inflection). **G**. DIC of live *P. segmenta* cells. **H**. Scales, showing the 'shoulder' of thickened shaft as it meets thinner end of spine (arrow). Thickness of the inflexion is variable but prominent. **I**. Scale on surface of cell. Scale bars: A and G, 5 μm. B and C, 1 μm. H, 0.2 μm. D, E, F and I, 0.5 μm.

Figure 6 Micrographs of three new species of subgenus *Acrospina*: ***Paraphysomonas acuminata, P. cuspidata, P. mikadiforma***. **A-B**, *P. acuminata*. **C-D**, *P.* aff. *acuminata* (WA20KP). **E**, *P.* aff. *acuminata.* (CCL3C). **F-G**, *P. cuspidata*. **H-I**, *P. mikadiforma*. **A**. DIC live cells of *P. acuminata,* spines visible (arrowhead). **B**. TEM of spine scales with oblique pointed tip and mid margin to base plate (arrow). **C**. DIC live cell *P.* aff. *acuminata* (WA20KP), spines visible (arrowhead) and typical long posterior cilium. **D**. TEM of spine scales with no real mid margin visible. **E**. *P.* aff. *acuminata* (CCL3C) TEM of scales, showing mid margin (arrowhead). **F**. DIC of *P. cuspidata*, long AF (double arrowhead) and spines visible (arrowhead). **G**. TEM of spine scales with oblique pointed tip and mid-margin on base plate (arrow). **H**. TEM of three spine scales of *P. mikadiforma* and with no dense base-plate rim and one contaminant scale (Cont.) with dense margin and different spine tip. Annular ridge clearly visible on two scales (arrow). **I**, DIC of live *P. mikadiforma* with visible spines (arrowhead). Scale bars; A, C, F and I, 5 μm. B, D, E, G and H, 1 μm.

Figure 7 Further micrographs of subgenus *Acrospina*: ***Paraphysomonas lucasi, P.* aff. *imperforata*** (EP1) and ***P. imperforata*** (CCAP 935/13). **A-B**, *P. lucasi*. **C-D**, *P.* aff. *imperforata* (EP1). **E-F**, *P. imperforata* (CCAP 935/13). **A**. TEM of detached scales showing the point at which the spine thins to a slight shoulder (arrow). **B**. DIC of live *P. lucasi* cells. **C**. DIC of live EP1 and larger dividing cell, shows four cilia (divide symbol). **D**. TEM EP1 scales showing mid-margin (arrowhead) and one uncommon scale with evidence of an inflexion at the rim on the base plate (double arrowhead). **E**. DIC of CCAP 935/13. **F**. scale showing mid-margin on base plate (arrowhead), broken spines (**). Scale bars: A-F, 0.5 μm.

Figure 8 Micrographs of all new species of subgenus *Hebetomonas*: ***Paraphysomonas hebes, P. limna, P. hebetispina***. **A-E**, *P. hebes.* **F**, *P. limna.* **G-M**, *P. hebetispina.* **A**. DIC of live *P. hebes* cells. **B-E**. *P. hebes* TEMs. **B**. Whole cell with both cilia and scales. **C.** Blunt tips of scales (arrowhead). **D** & **E**. Typical *P. hebes* scales. **F**. DIC of live *P. limna* cells, one with obvious beating envelope (*). **G**. DIC of live *P. hebetispina* cells, one with visible stalk (double arrowhead) and another with beating envelope (*). **H-M**. *P. hebetispina* TEMs. **H**. whole cell showing mastigonemes (small arrow), both cilia and scales. **I**. Loose scales showing range of size, gentle taper of spine and base plate with no obvious inflexion. **J**. Close-up of blunt tip of spine. **K**. A selection of scales showing obvious mid-margin on base plate (large arrow). **L**. another example of blunt tip of spine. **M**. Single scale with mid-margin on base plate (large arrow). Scale bars: A, F and G, 5 μm. B and H, 1 μm. C, 0.2 μm. D, E, I, J, K, L, and M, 0.5 μm.

Figure 9 Micrographs of new species of the most divergent subclade of subgenus *Hebetomonas*: ***Paraphysomonas hemiradia, P. uniformis.*** **A-E**, *P. hemiradia*. **F-G**, *P. uniformis*. **A**. DIC of live *P. hemiradia*. Scale cuticle visible (double arrowhead) on dividing cell (%). Unknown protrusion coming from one cell, possibly extrusome (arrow). **B**-**E**. *P. hemiradia* TEMs. **B.** Collection closely grouped scales with radial ribs. **C**. Two scales with radial ribs (arrowhead). **D**. Close up of base-plate with radial ribs. **E**. A selection of aberrant scale types without radial ribs (*) and one with radial ribs and a very wide spine (**). **F**. DIC of live *P. uniformis*, pyriform cell with visible scale cuticle (double arrowhead), large cell phagocytosed possibly smaller *Paraphysomonas* cell (*). **G**. TEM of *P. uniformis.* Detached scales. Scale bars: A and F, 5 μm. B, C, D, E and G, 1 μm.

Figure 10 Micrographs of three new species of the freshwater 'vulgar' subclade of subgenus *Paraphysomonas*: ***Paraphysomonas vulgaris, P. vulgatana, P. cambrispina.*** **A-B**, *P.* aff. *vulgaris*, (SW02). **C-D**, *P. vulgaris*. **E-F**, *P. vulgatana*. **G-H**, *P. cambrispina*. **A-B**. *P.* aff. *vulgaris* TEMs. **A**. A group of detached scales with a close-up of the spine tips. **B**. A selection of *P.* aff. *vulgaris* scales with varying lengths of spine as well as inflated spine bases (arrow). **C**. *P. vulgaris* TEM. Detached scales with inflated spine base and crease (arrow). **D**. DIC of live *P. vulgaris* cells of varying sizes. **E-F**. DIC of live *P. vulgatana* cells, one dividing (%). **F**. Enlarged image of boxed cell to show obvious scale cuticle (double arrowhead) and possible extrusome (arrowhead). **G**. DIC of live *P. cambrispina* cells. **H**. TEM of detached *P. cambrispina* scales, N.B. the scales are almost twice as small as the other species in this figure plate. Scale bars: C, D, E, F and G, 5 μm. A and B, 1 μm. H, 0.5 μm.

Figure 11 Micrographs of the two subspecies of ***Paraphysomonas vulgata*: *P. vulgata vulgata, P. vulgata elongata.*** **A-B**, *P. vulgata vulgata*. **C-D**, *P. vulgata elongata*. **A**. DIC of live *P. vulgata vulgata* cells with stalk (double arrowhead) and visible scale cuticle (arrowhead) and beginning to phagocytose (Phag.) **B**. *P. vulgata vulgata* TEMs showing detached scales with prominent inflation at base of spine (arrow) and base plate crease (*). **C**. *P. vulgata elongata* TEMs showing detached scales with base plate crease (*) and some with inflated base of spine (arrow). **D**. DIC of live *P. vulgata elongata* cells showing scale cuticle (arrowhead) and dividing cell (%). Scale bar: A and D, 5μm. B and C, 1μm.

Figure 12 Micrographs of new species in the second freshwater clade of subgenus *Paraphysomonas*: ***Paraphysomonas variosa, P. caroni, P. mantoni, P. balonovi.*** **A-F**, *P. variosa*. **G-K**, *P. caroni*. **M-L**, *P. mantoni*. **O**, *P. balonovi*. **A**. DIC of live *P. variosa* cells, dividing (division symbol) and with stalk (arrow). **B-F**. *P. variosa* TEMs. **B**. Close-up of tapering scale spine tip. **C**. Whole cell with attached scales, possible mal-formed spines (double arrowhead). **D**. Three detached scales showing diversity of size. **E**. Close-up of tapering spine tips. **F**. Attached scales showing possible mal-formed spines (double arrowhead) and example of shorter scale spine with wide inflated base (arrowhead). **G-J**. *P. caroni* TEMs. **G**. Whole cell with few cells attached (**) and possible exocysts (*). **H**. Scales at surface of cell. **I**. Scales at surface of cell showing slight oblique point at tapered spine tip. **J**. Detached scale. **K**. DIC of live *P. caroni* cells and contaminant (cont.). Contaminant cells as food (arrow). **L**. DIC of live *P. mantoni* cells, cuticle of scales visible (arrow). **M-N**. *P. mantoni* TEMs. Showing tapering scale tips and close-up of tapering spine tip (boxes). **O**. DIC of live *P. balonovi* cells and possible contaminant (cont.). Scale bar: A, K, L and O, 5 μm. B and E, 0.5 μm. D, F, H, I, J, M and N, 1 μm. G, 2 μm.

Figure 13 Micrographs of two new species of subgenus *Paraphysomonas* from soil: ***Paraphysomonas crocotilla, P. dimorpha.*** **A-G**, *P. crocotilla*. **H-M**, *P. dimorpha*. **A**. DIC of live *P. crocotilla* cells. **B-G**. *P. crocotilla* TEMs. **B**. Scales attached to cell, possible non spine scales (arrows). **C**. Possible extruded extrusomes (arrowheads). **D**. Close-up of possible extrusomes and tapering tip of spine scales (double arrowhead) and aberrant scale form with bulbous tip. **E**. group of detached scales and visible mastigonemes (*). **F**. Scales showing swollen spine bases; arrow marks shadowy marks similar to scale base plates possibly caused by scales washed off the grid during preparation. **G**. Detached scale. **H**. DIC of live *P. dimorpha* cells and one possible contaminant (cont.)

or dormant cell (see description), cuticle of scales visible (arrowhead). **I-M**. *P. dimorpha* TEMs. **I**. Whole cell with apparently just spine scales. **J**. Whole cells with a mix of spined and plate scales. Mastigonemes visible (*). **K**. Detached plate scales (arrow) from whole cell. **L**. Detached spine scales. **M**. Whole cell with only plate scales. Scale bar: A and H, 5um. B, 2 μm. C and D, 0.5 μm. E, F, G, I, J, K, L and M, 1 μm.

Figure 14 Micrographs of two new species of subgenus *Paraphysomonas* from the subclade with mixed habitat preferences: ***Paraphysomonas solis*** from soil*,* marine ***P. stylata***. **A-B**, *P. solis*. **C-G**, *P. stylata*. **A**. DIC of live *P. solis* cells one with visible scale cuticle (arrowhead). **B**. *P. solis* TEMs of detached scales showing tapering spine and tip (enlarged box) and aberrant small scale (*). **C**. DIC of live *P. stylata* cells showing stalk (double arrowhead) and scale cuticle (arrowhead). **D-G**. *P. stylata* TEMs. **D** & **G**. Scales. **E**. Close-up of pinched spine tips (arrow). **F**. Whole cell with scales. Scale bars: A and C, 5 μm. B and G, 1 μm. D and E, 0.5 μm. F, 2 μm.

Figure 15 Micrographs of two further new species of subgenus *Paraphysomonas* from the subclade with mixed habitat preferences**: *Paraphysomonas longispina, P. stylaris***. **A-B**, *P. longispina* (Mex3 – freshwater). **C-D**, *P. longispina,* (Mex1 - marine). **E**-**F**, *P. stylaris*. **A**. DIC of *P. longispina* (Mex3) live cells. **B**. *P. longispina* (Mex3) TEMs of detached scales, some with inflated spine bases (arrowhead). Aberrant scales (*) with lost spine or malformed spine. **C**. DIC of live *P. longispina* (Mex1) cells one having ingested another smaller of its own kind (**), other cell attached to floating detritus via stalk. **D**. *P. longispina* (Mex1) TEM of a single scale. **E**. *P. stylaris* TEMs of detached spine scales. **F**. DIC of live *P. stylaris* cells. Scale cuticle and spines just visible (arrow). Exocytosis observed (exo.) and late stage division (division symbol). Scale bar: A, C and F, 5 μm. B, D and E, 1 μm.

Figure 16 Micrographs of two closely related new species of subgenus *Paraphysomonas* from small soil subclade: ***Paraphysomonas sinensis, P. tropica***. **A-E**, *P. sinensis*. **F**, *P. tropica.* **A**. DIC of *P. sinensis* culture showing granule-like cells. **B-E**. *P. sinensis* TEMs. **B**. Scales with short spines or short protrusions (arrowheads). Large plate discs (*) with or possibly without central marking. **C**. Spined scales and non-spined plate scale (*). **D**. Tips of spines. **E**. Whole cell with boxed area indicating from where Figure 16B is taken. **F**. DIC of live *P. tropica* cells. Scale bar: A and F, 5 μm. B and C, 0.5 μm. D, 0.2 μm. E, 2 μm.

Figure 17 Micrographs of three closely related new species of subgenus *Paraphysomonas* from soil, showing stomatocysts of differing morphology: ***Paraphysomonas terricola, P. spiculosa, P. edaphica***. **A-B**, *P. terricola*. **C-E**, *P. spiculosa*. **F**, *P. edaphica*. **A**. DIC of live *P. terricola* cells with just visible scales (arrowhead) and plugged stomatocyst (*). **B**. *P. terricola* TEMs of detached scales. **C**. DIC of live *P. spiculosa* cells, one dividing (division symbol) and plugged stomatocyst (*). **D-E**. *P. spiculosa* TEMs. **D**. Scales attached to cell surface. **E**. Side view of scales and cilium hairs (**) and single scale including close-up of spine tip (box). **F**. DIC of live *P. edaphica* motile cell with cilia (on right), smaller more shrunken cell at top left (perhaps starved, apparently non-ciliate) and stomatocyst (*). Scale bar: A, C and F, 5 μm. B, 0.5 μm. D and E, 1 μm.

Figure 18 Micrographs of ***Clathromonas butcheri*** strain MD03. **A-G**, *C. butcheri*. **A**, DIC of live *C. butcheri* cells, beat envelope visible (arrowhead). **B-G**. *C. butcheri* TEMs. **B**.

Scales, mostly crown scales but one plate scale (arrow). **C**. Cell with only plate scales. **D**. Cell with mostly plate scales but one crown scale seen from the side (double arrowhead). **E**. Cell with mostly crown scales and a plate scale (arrow), some mastigonemes visible (**). **F**-**G**. Plate scales. Scale bar: A, 5 μm. B-G, 0.5 μm.

Figure 19 Comparison of the cells and scales of 15 of our 35 new species of *Paraphysomonas*. For each there are DIC light micrographs of live cells and an example of a spine scale all at the same relative magnifications. The diversity of size and proportions of the scales is just as notable as the size of the cells; only one species has an obviously oval base plate. The close-ups of spine tips are not all at the same scale, each being magnified sufficiently to show its characteristic shape; the differences in tips of the spines are quite subtle but stable within a strain and rather diverse; closely related species on Figure 2 generally have very similar spine tips. Scale bar: scales 1 μm. DIC live cells, 5 μm.

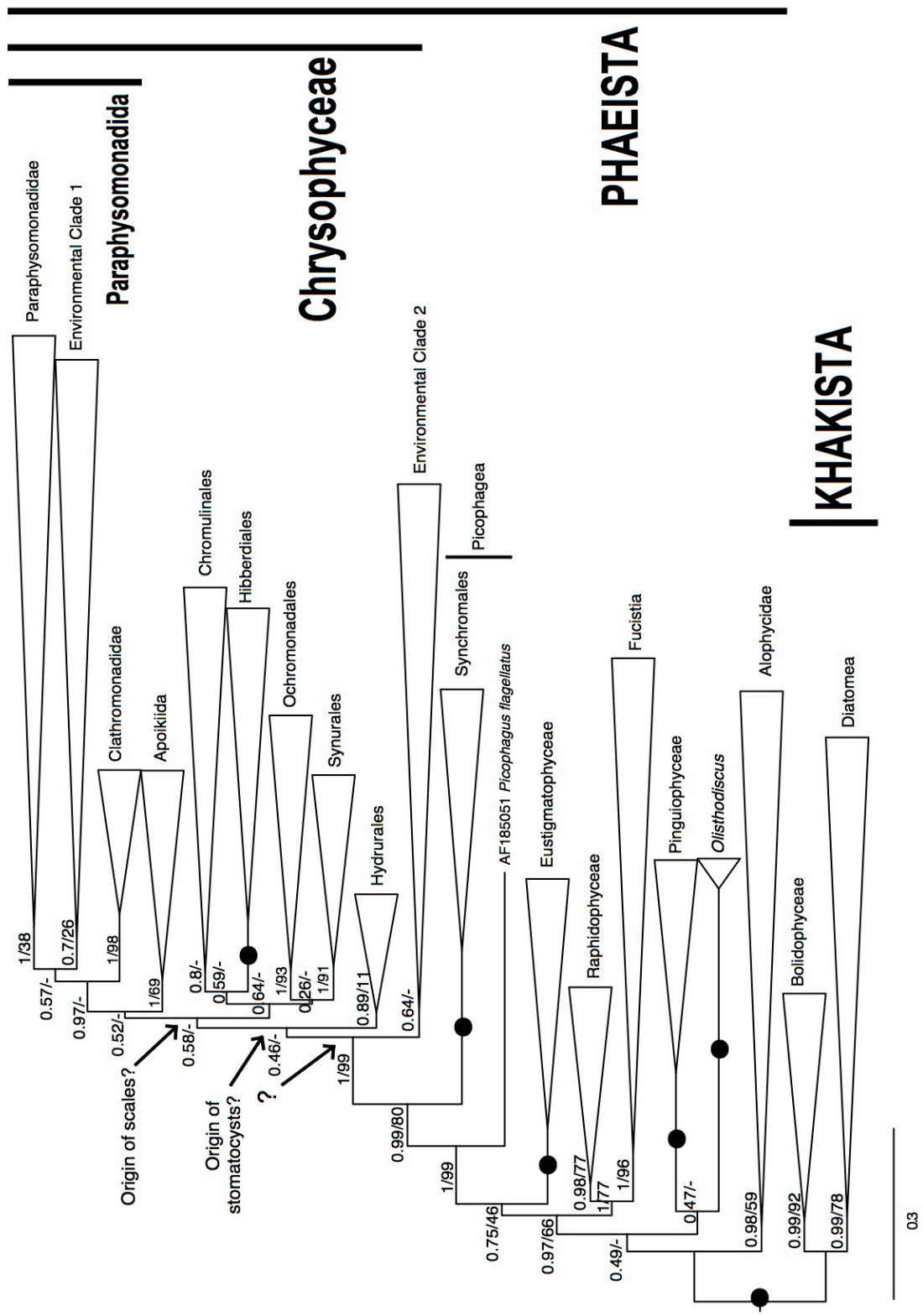
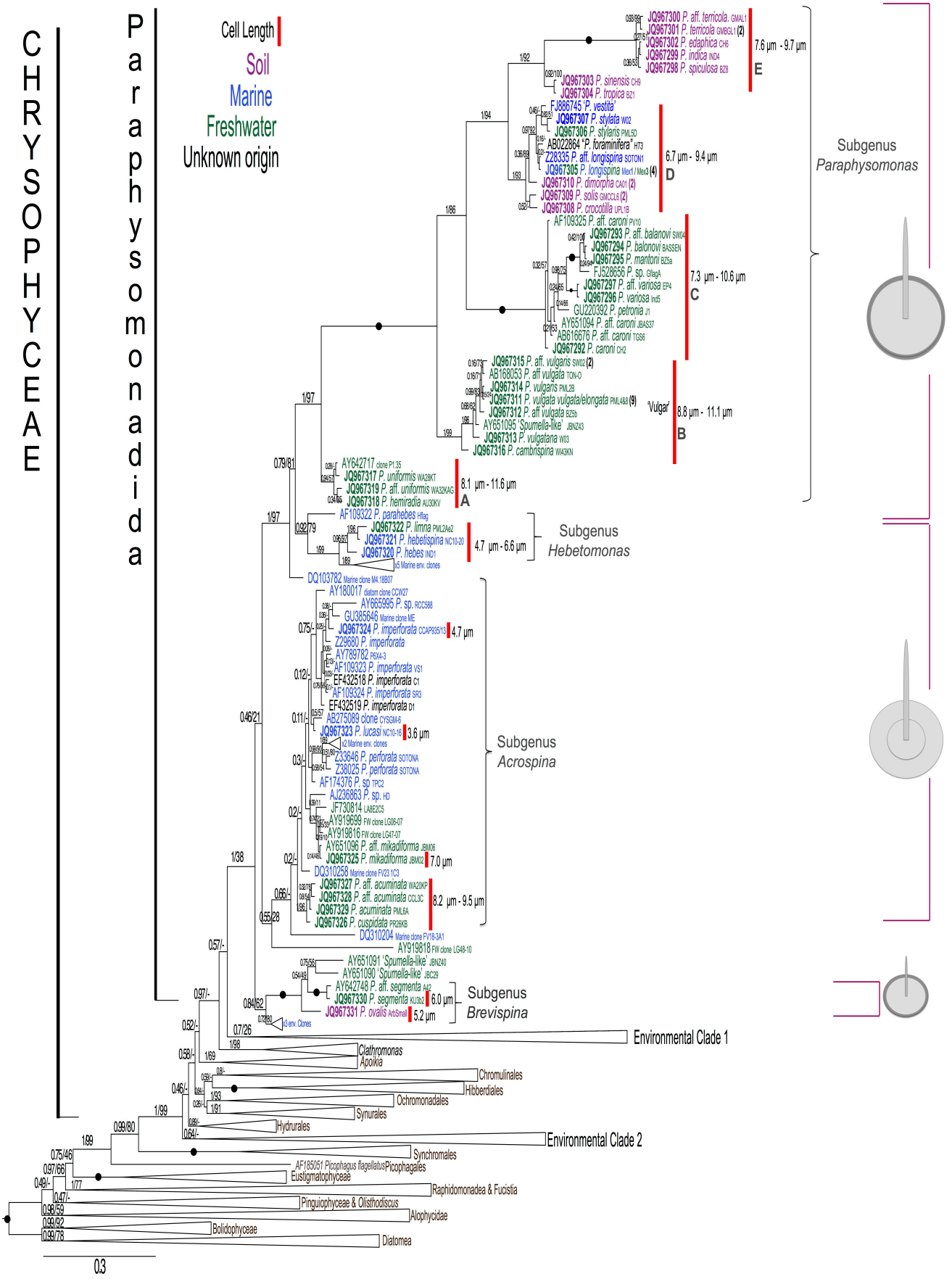

PHAEISTA
KHAKISTA
Chrysophyceae
Paraphysomonadida
Paraphysomonadidae
Environmental Clade 1
Clathromonadidae
Apoikiida
Chromulinales
Hibberdiales
Ochromonadales
Synurales
Hydrurales
Environmental Clade 2
Picophagea
Synchromales
AF185051 Picophagus flagellatus
Eustigmatophyceae
Raphidophyceae
Fucistia
Pinguiophyceae
Olisthodiscus
Alophycidae
Bolidophyceae
Diatomea
Origin of scales?
Origin of stomatocysts?
?
1/38
0.57/-
0.7/26
0.97/-
1/98
1/69
0.52/-
0.58/-
0.8/-
0.59/-
0.64/-
1/93
0.26/-
1/91
0.89/11
0.64/-
0.46/-
1/99
0.99/80
1/99
0.75/46
0.97/66
0.98/77
1/77
1/96
0.49/-
0.47/-
0.98/59
0.99/92
0.99/78
0.3

Figure 01


CHRYSOPHYCEAE
Paraphysomonadida
Cell Length
Soil
Marine
Freshwater
Unknown origin
JQ967300 P. aff. terricola GMAL1
JQ967301 P. terricola GMBGL1 (2)
JQ967302 P. edaphica CH6
JQ967299 P. indica IND4
JQ967298 P. spiculosa BZ8
7.6 µm - 9.7 µm
E
JQ967303 P. sinensis CH9
JQ967304 P. tropica BZ1
FJ886745 'P. vestita'
JQ967307 P. stylata W02
JQ967306 P. stylaris PML5D
AB022864 "P. foraminifera" HT3
Z28335 P. aff. longispina SOTON1
JQ967305 P. longispina Mex1 / Mex3 (4)
JQ967310 P. dimorpha CA01 (2)
JQ967309 P. solis GMCCL6 (2)
JQ967308 P. crocatilla UPL1B
6.7 µm - 9.4 µm
D
AF109325 P. aff. caroni PV10
JQ967293 P. aff. balanovi SW04
JQ967294 P. balonovi BASSEN
JQ967295 P. mantoni BZ5a
FJ528656 P. sp. GRagA
JQ967297 P. aff. variosa EP4
JQ967296 P. variosa Ind5
GU220392 P. petronia J1
AY651094 P. aff. caroni JBAS37
AB616676 P. aff. caroni TGS6
JQ967292 P. caroni CH2
7.3 µm - 10.6 µm
C
JQ967315 P. aff. vulgaris SW02 (2)
AB168053 P. aff vulgata TON-O
JQ967314 P. vulgaris PML2B
JQ967311 P. vulgata vulgata/elongata PML48B (9)
JQ967312 P. aff vulgata BZ5b
AY651095 'Spumella-like' JBNZ43
JQ967313 P. vulgatana W03
JQ967316 P. cambrispina WH3KN
'Vulgar'
8.8 µm - 11.1 µm
B
Subgenus
Paraphysomonas
AY642717 clone P1.35
JQ967317 P. uniformis WA28KT
JQ967319 P. aff. uniformis WA32KAG
JQ967318 P. hemiradia AU30KV
8.1 µm - 11.6 µm
A
AF109322 P. parahebes Hfag
JQ967322 P. limna PML2Ae2
JQ967321 P. hebetispina NC10-20
JQ967320 P. hebes IND1
4.7 µm - 6.6 µm
Subgenus
Hebetomonas
DQ103782 Marine clone M4.19B07
AY180017 diatom clone CCW27
AY665995 P. sp. RCC588
GU385646 Marine clone ME
JQ967324 P. imperforata CCAP935/13
4.7 µm
Z29680 P. imperforata
AY789782 PEK4-3
AF109323 P. imperforata VS1
EF432518 P. imperforata C1
AF109324 P. imperforata SR3
EF432519 P. imperforata D1
AB275089 clone CYSGM-6
JQ967323 P. lucasi NC10-16
3.6 µm
Z33646 P. perforata SOTONA
Z38025 P. perforata SOTONA
AF174376 P. sp TPC2
AJ236863 P. sp. HD
JF730814 LABE2C5
AY919699 FW clone LG06-07
AY919816 FW clone LG47-07
AY651096 P. aff. mikadiforma JBM06
JQ967325 P. mikadiforma JBM02
7.0 µm
DQ310258 Marine clone FV23 1C3
JQ967327 P. aff. acuminata WA20KP
JQ967328 P. aff. acuminata CCL3C
JQ967329 P. acuminata PML6A
JQ967326 P. cuspidata PR26KB
8.2 µm - 9.5 µm
Subgenus
Acrospina
DQ310204 Marine clone FV16-3A1
AY919818 FW clone LG48-10
AY651091 'Spumella-like' JBNZ40
AY651090 'Spumella-like' JBC29
AY642748 P. aff. segmenta Ad2
JQ967330 P. segmenta KU3b2
6.0 µm
JQ967331 P. ovalis AnSmall
5.2 µm
Subgenus
Brevispina
Environmental Clade 1
Clathromonas
Apoikia
Chromulinales
Hibberdiales
Ochromonadales
Synurales
Hydrurales
Environmental Clade 2
Synchromales
AF185051 Picophagus flagellatus
Picophagales
Eustigmatophyceae
Raphidomonadea & Fucistia
Pinguiophyceae & Olisthodiscus
Alophycidae
Bolidophyceae
Diatomea
0.3

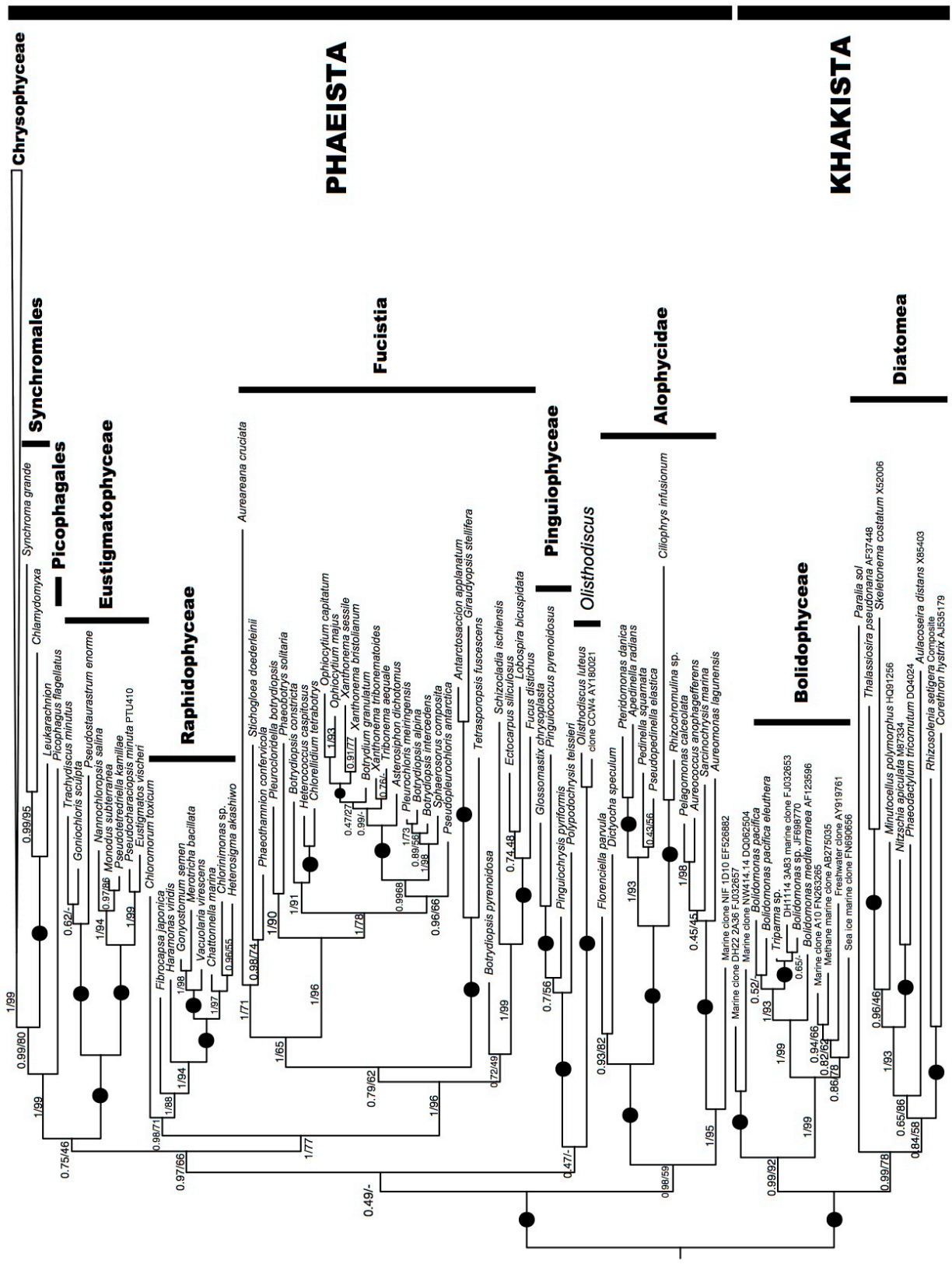

Chrysophyceae
Synchromales
Picophagales
Eustigmatophyceae
Raphidophyceae
PHAEISTA
Fucistia
Pinguiophyceae
Olisthodiscus
Alophycidae
Bolidophyceae
KHAKISTA
Diatomea
Synchroma grande
Chlamydomyxa
Leukarachnion
Picophagus flagellatus
Trachydiscus sculpta
Goniochloris sculpta
Pseudostaurastrum enorme
Nannochloropsis salina
Monodus subterraneus
Pseudotetraedriella kamillae
Pseudocharaciopsis minuta PTU410
Eustigmatos vischeri
Chloromorum toxicum
Fibrocapsa japonica
Haramonas viridis
Gonyostomum semen
Merotricha bacillata
Vacuolaria virescens
Chattonnella marina
Chlorinimonas sp.
Heterosigma akashiwo
Aureareana cruciata
Stichogloea doederleinii
Phaeothamnion confervicola
Pleurocloridella botrydiopsis
Botrydiopsis constricta
Heterococcus caespitosus
Chlorellidium tetrabotrys
Ophiocytium capitatum
Ophiocytium majus
Xanthonema sessile
Xanthonema bristolianum
Botrydium granulatum
Xanthonema tribonematoides
Tribonema aequale
Asterosiphon dichotomus
Pleurochloris meiringensis
Botrydiopsis alpina
Botrydiopsis intercedens
Sphaerosorus composita
Pseudopleurochloris antarctica
Antarctosaccion applanatum
Giraudyopsis stellifera
Tetrasporopsis fuscescens
Botrydiopsis pyrenoidosa
Schizocladia ischiensis
Ectocarpus siliculosus
Lobospira bicuspidata
Fucus distichus
Glossomastix chrysoplasta
Pinguiococcus pyrenoidosus
Pinguiochrysis pyriformis
Polypodochrysis teissieri
Olisthodiscus luteus
clone CCW4 AY180021
Florenciella parvula
Dictyocha speculum
Pteridomonas danica
Apedinella radians
Pedinella squamata
Pseudopedinella elastica
Ciliophrys infusionum
Rhizochromulina sp.
Pelagomonas calceolata
Aureococcus anophagefferens
Sarcinochrysis marina
Aureomonas lagunensis
Marine clone NIF 1D10 EF526882
Marine clone DH22 2A36 FJ032657
Marine clone NW414.14 DQ062504
Bolidomonas pacifica
Bolidomonas pacifica eleuthera
Triparma sp.
DH114 3A83 marine clone FJ032653
Bolidomonas sp. JF698770
Bolidomonas mediterranea AF123596
Marine clone A10 FN263263
Methane marine clone AB275035
Fragilariopsis cylindrus clone AY919761
Sea ice marine clone FN690656
Paralia sol
Thalassiosira pseudonana AF37448
Skeletonema costatum X52006
Minutocellus polymorphus HQ91256
Nitzschia apiculata M87334
Phaeodactylum tricornutum DQ4024
Aulacoseira distans X85403
Rhizosolenia setigera Composite
Corethron hystrix AJ535179
03

Figure 03

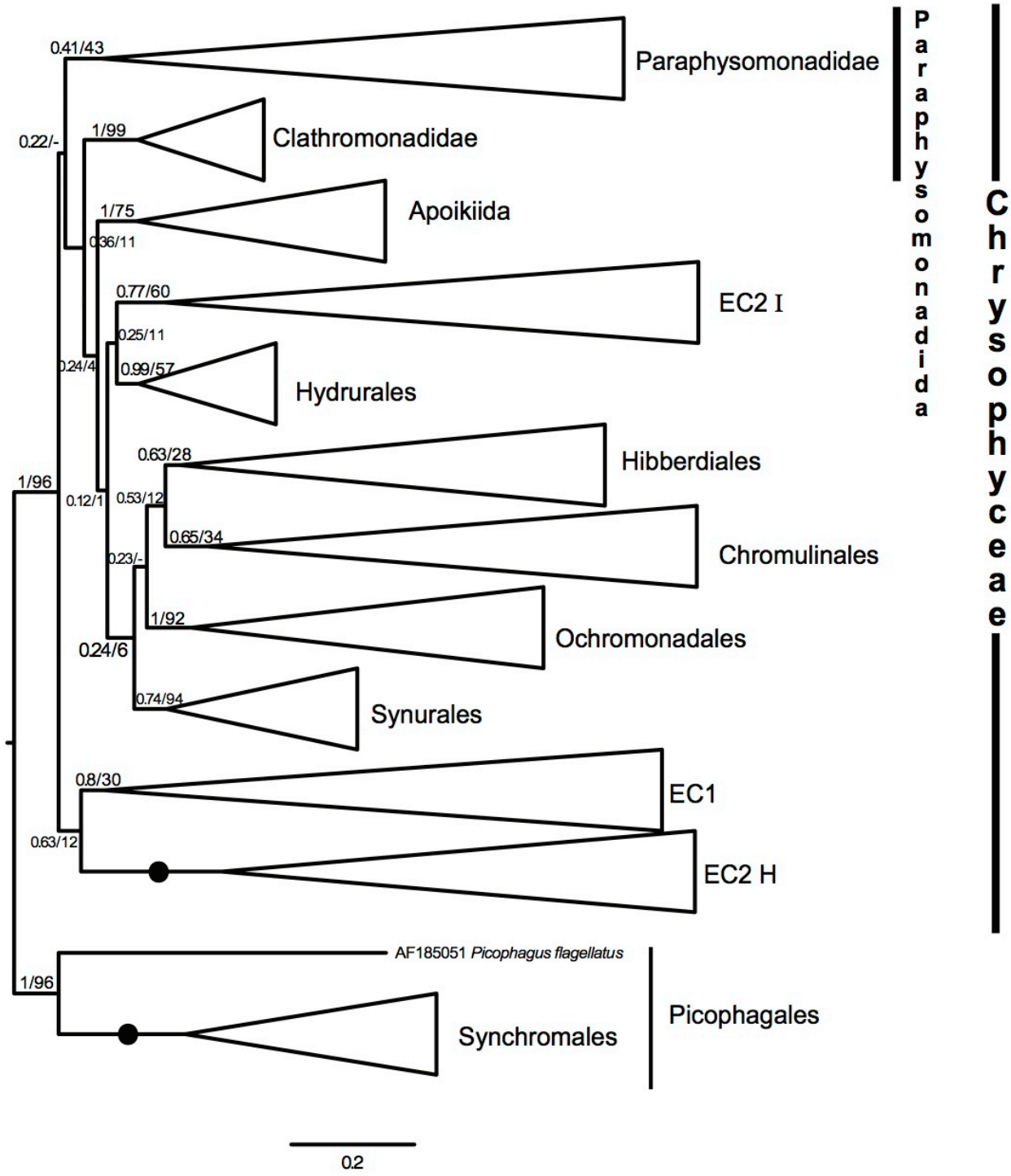

0.41/43
Paraphysomonadidae
1/99
0.22/-
Clathromonadidae
1/75
Apoikiida
0.36/11
0.77/60
EC2 I
0.25/11
0.24/4
0.99/57
Hydrurales
0.63/28
Hibberdiales
1/96
0.12/1
0.53/12
0.65/34
Chromulinales
0.23/-
1/92
Ochromonadales
0.24/6
0.74/94
Synurales
0.8/30
EC1
0.63/12
EC2 H
AF185051 Picophagus flagellatus
1/96
Synchromales
Picophagales
0.2
Paraphysomonadida
Chrysophyceae

Figure 04

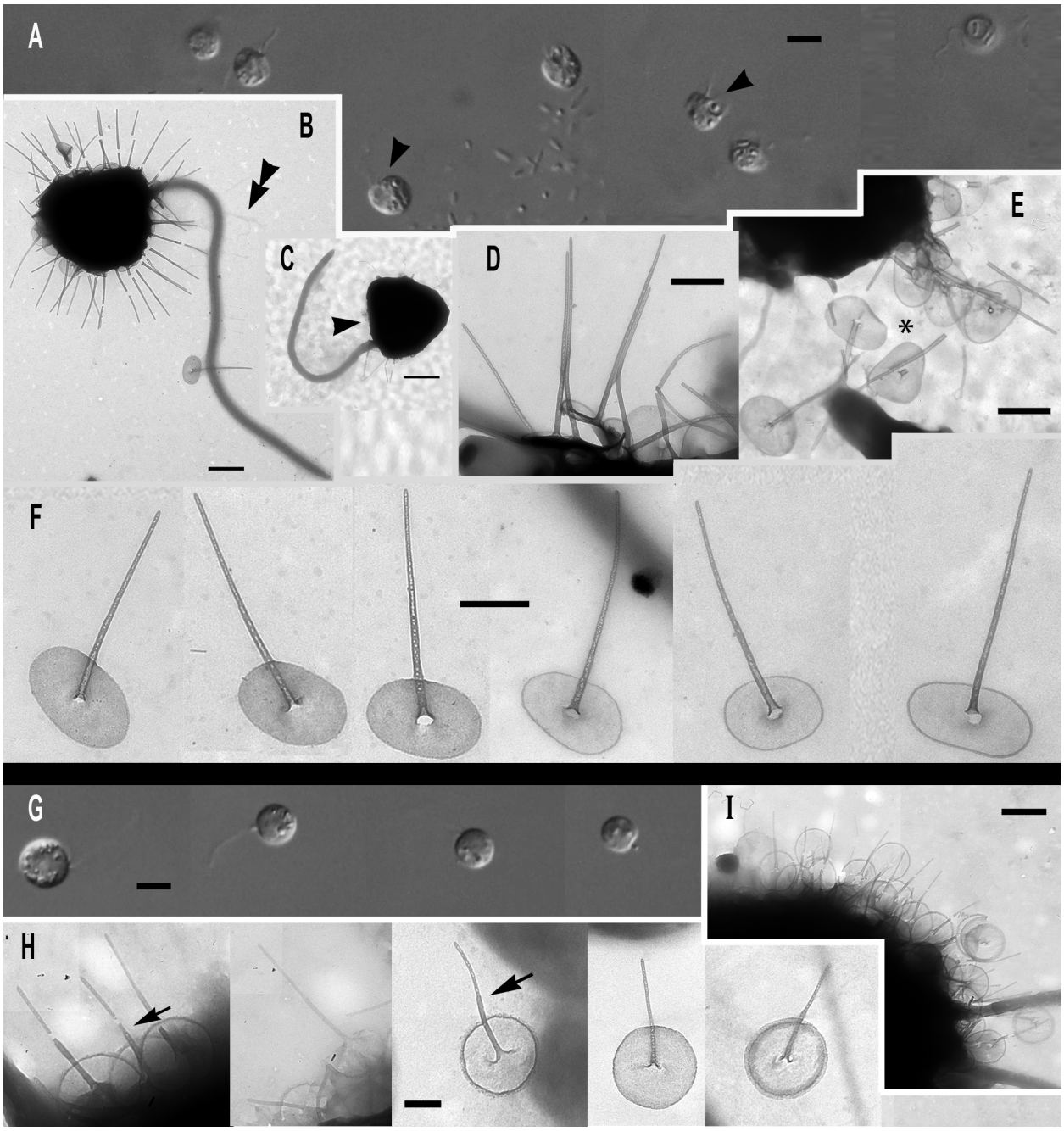


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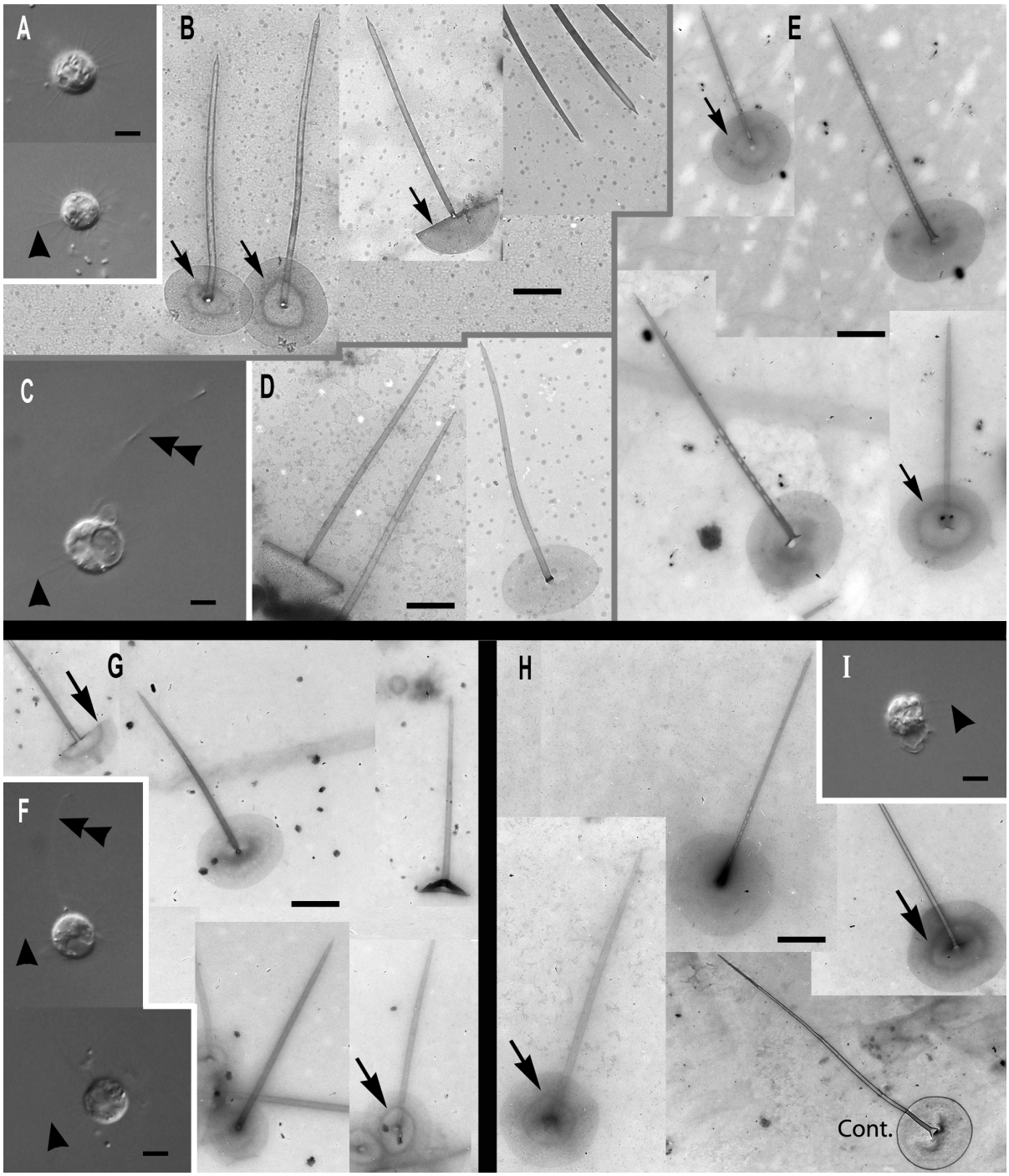


Figure 06

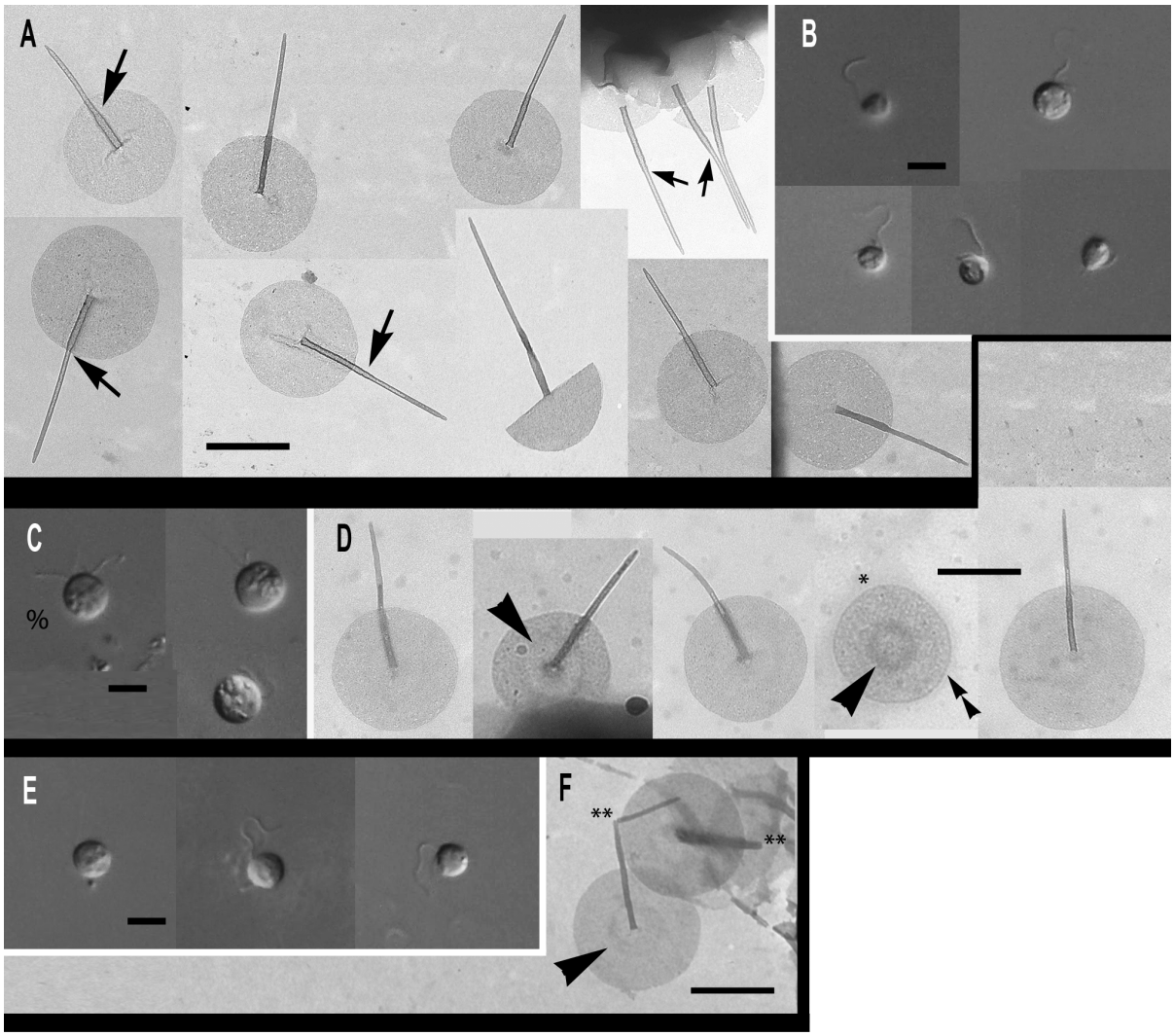


Figure 07

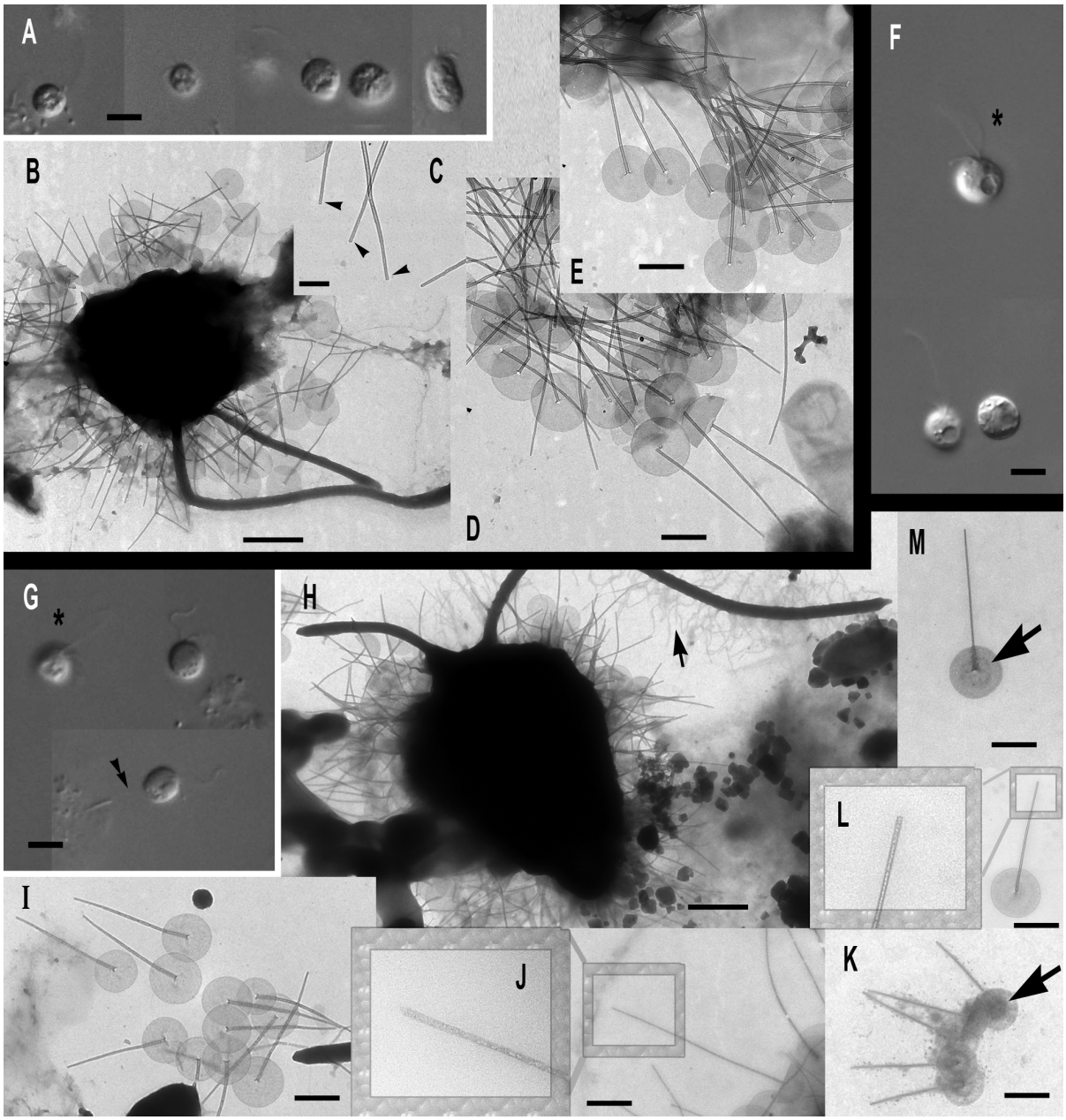


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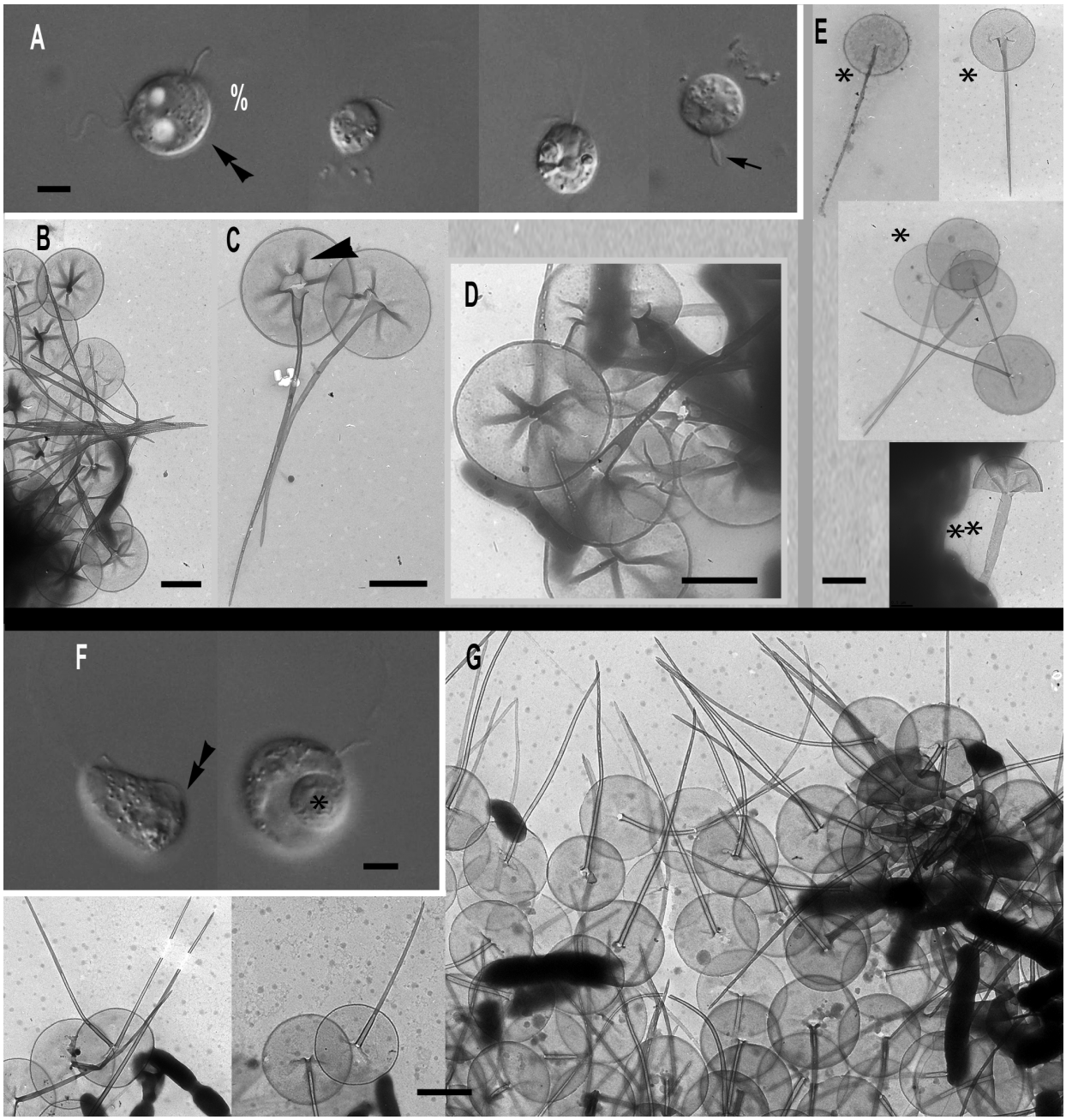


Figure 09

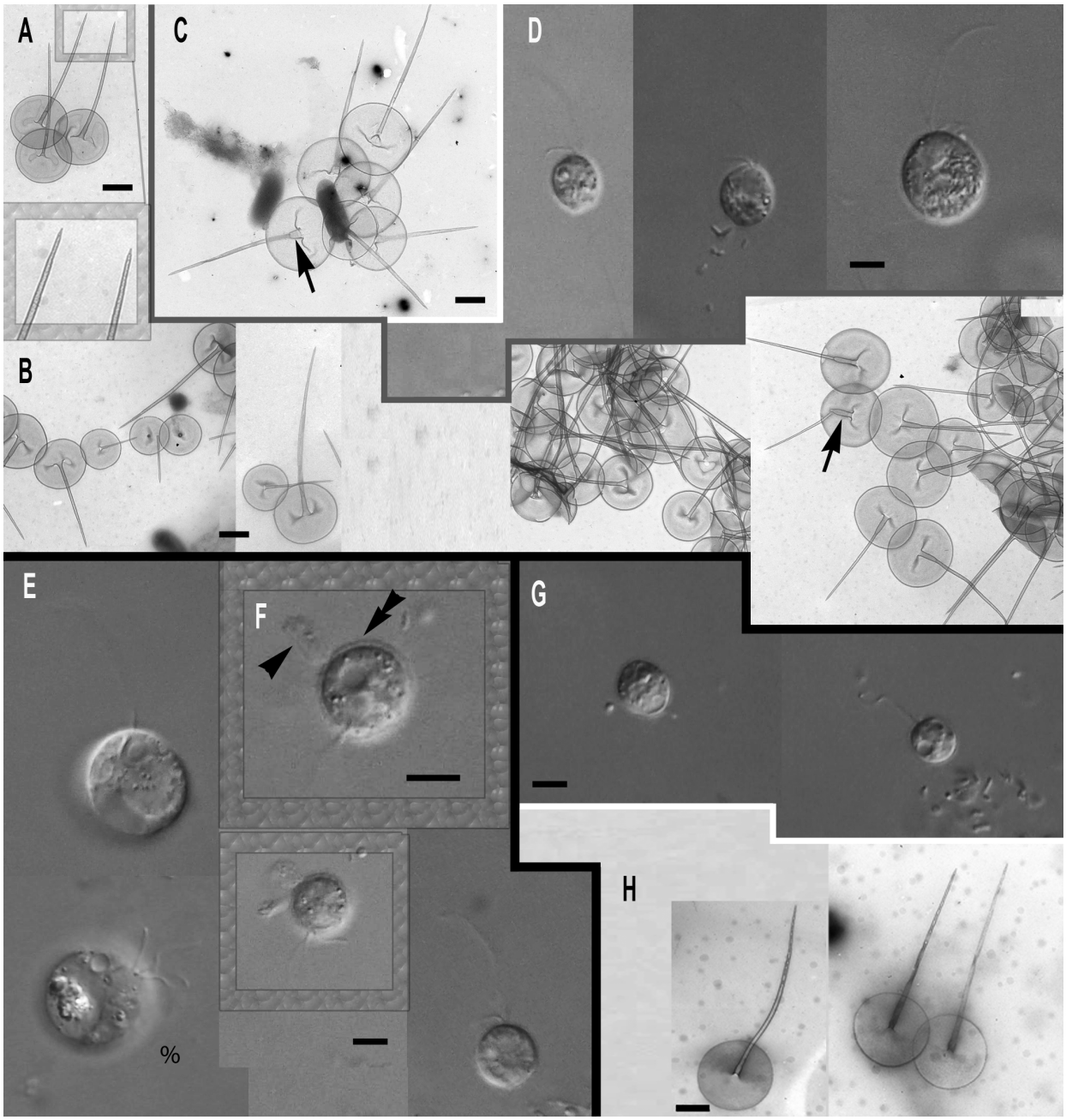


Figure 10

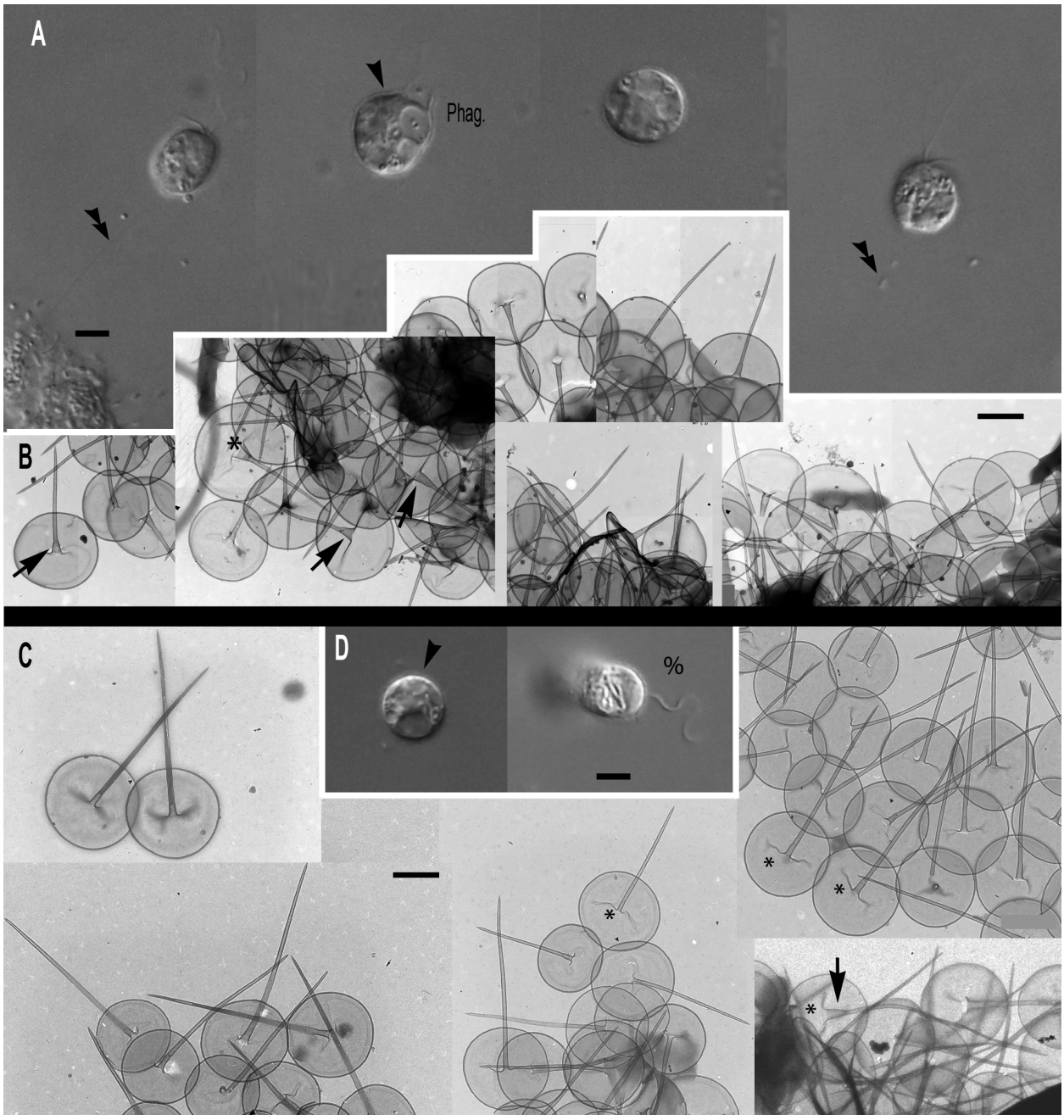


Figure 11

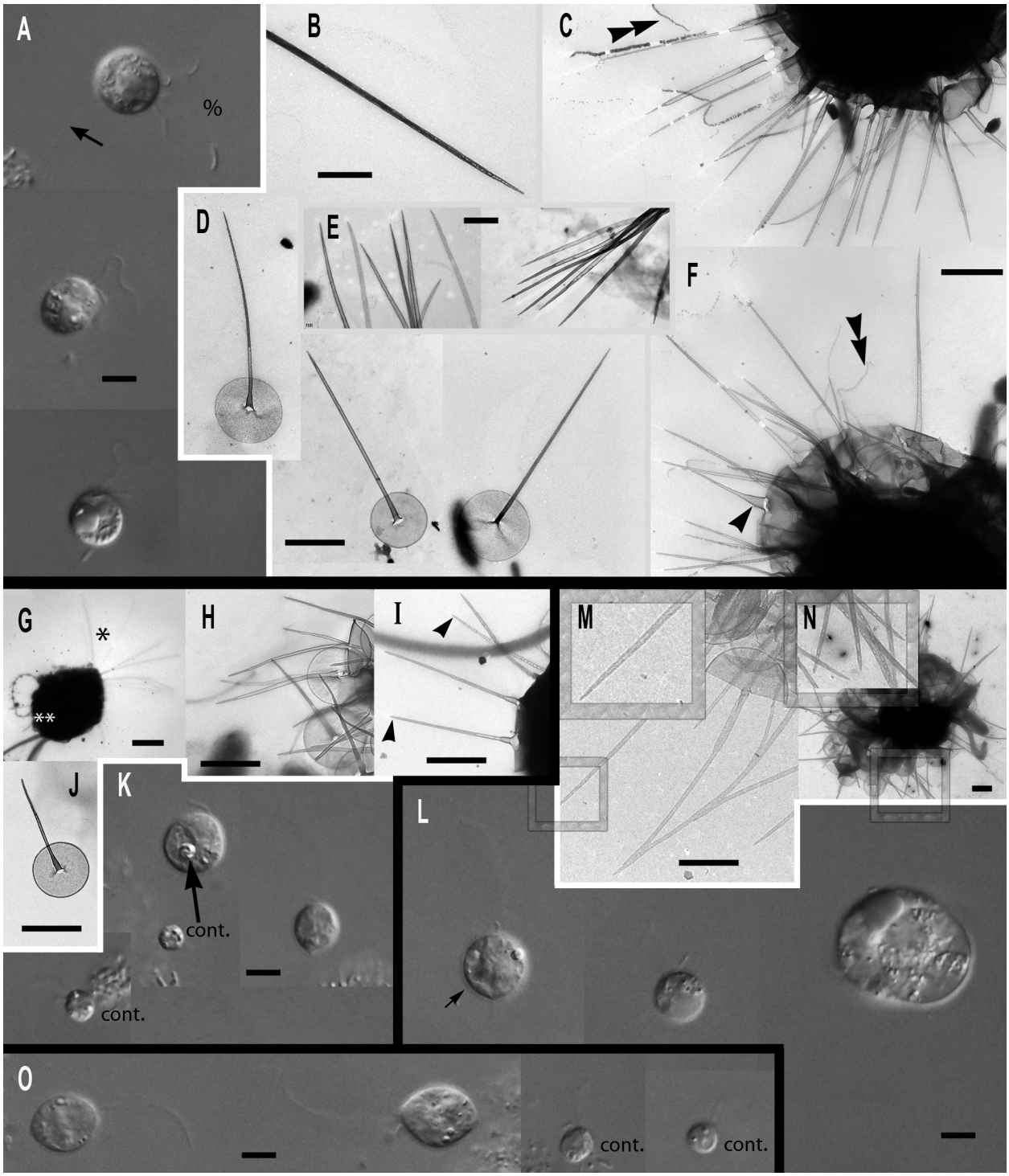


Figure 12

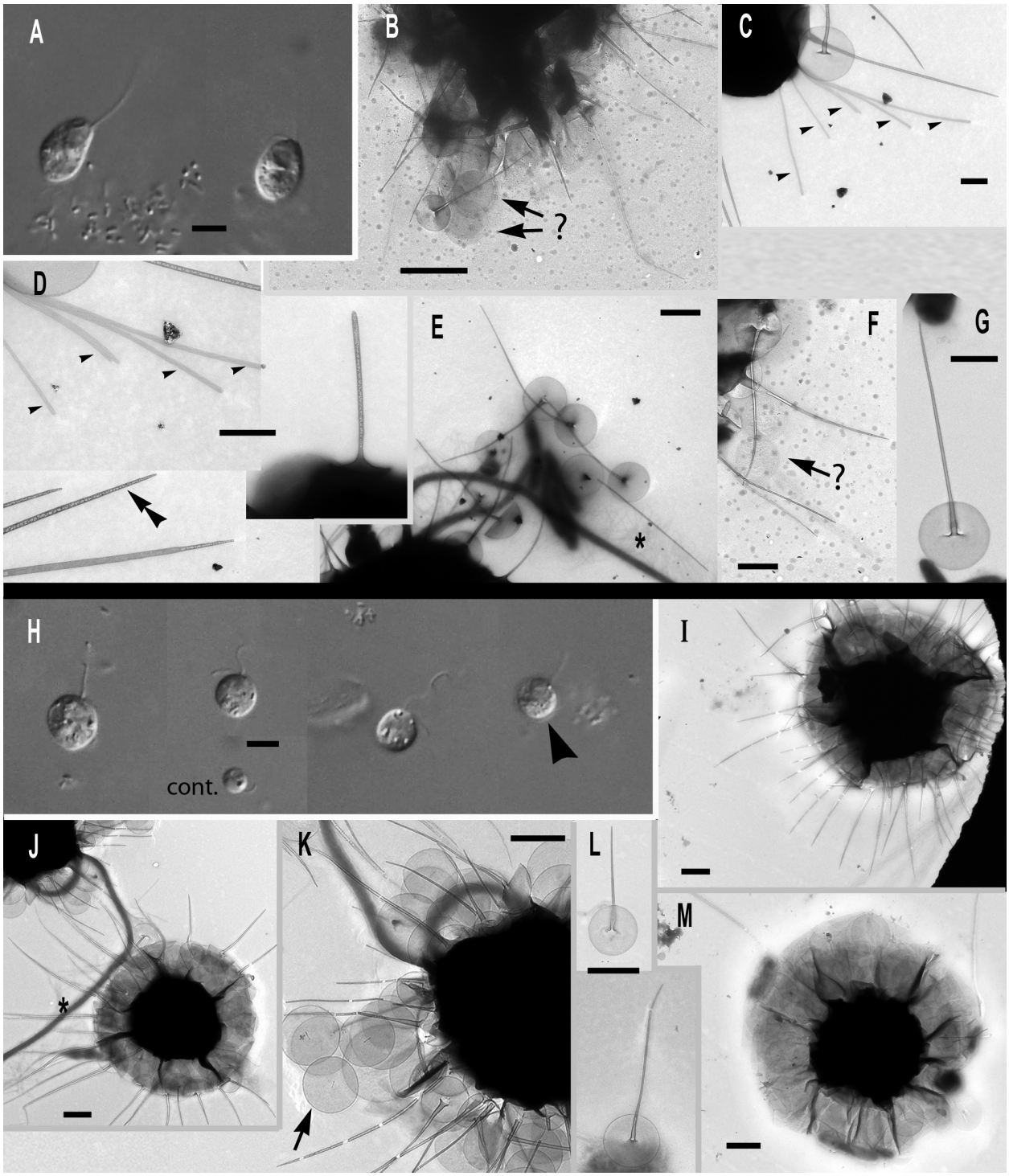


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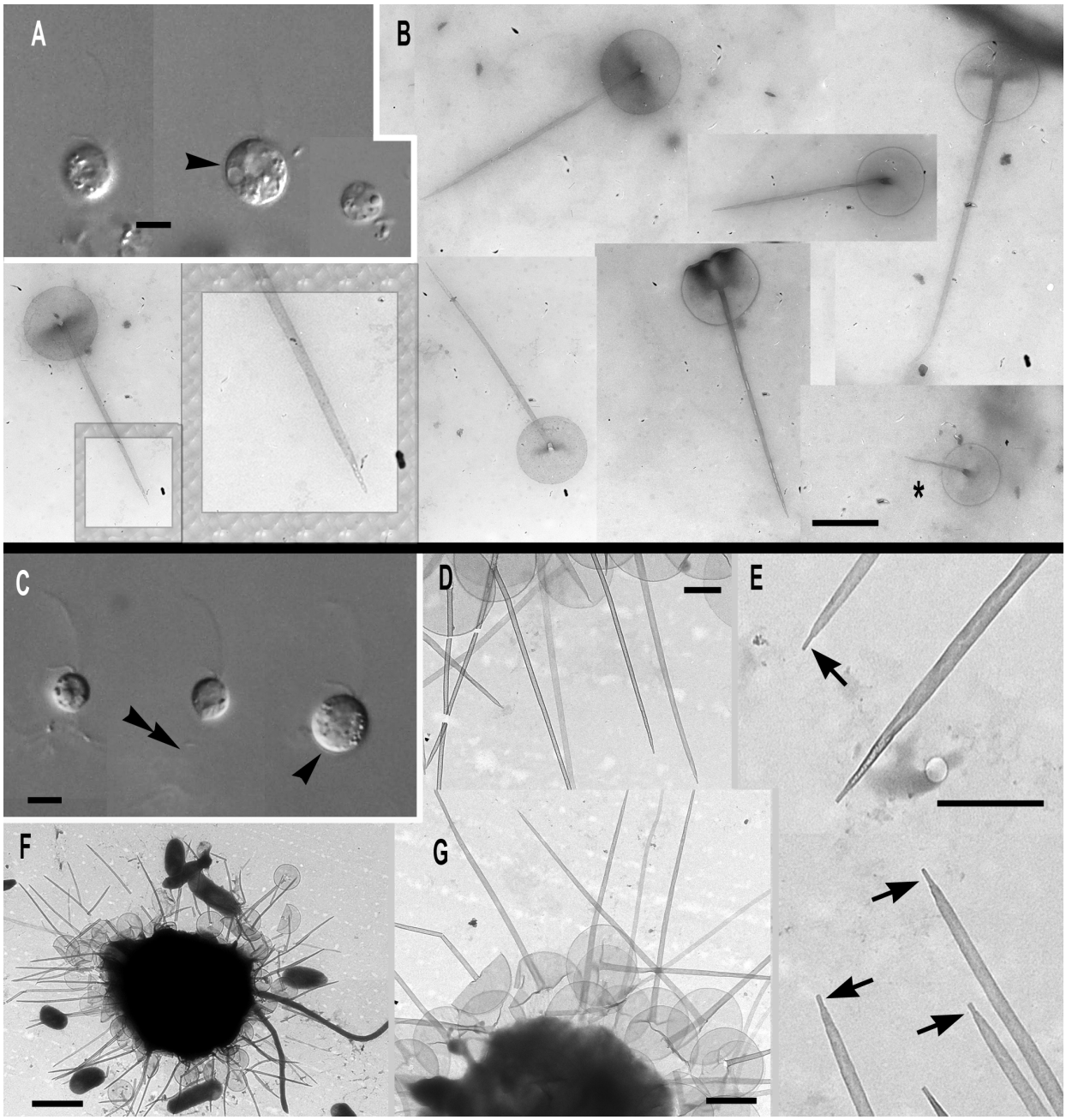


Figure 14

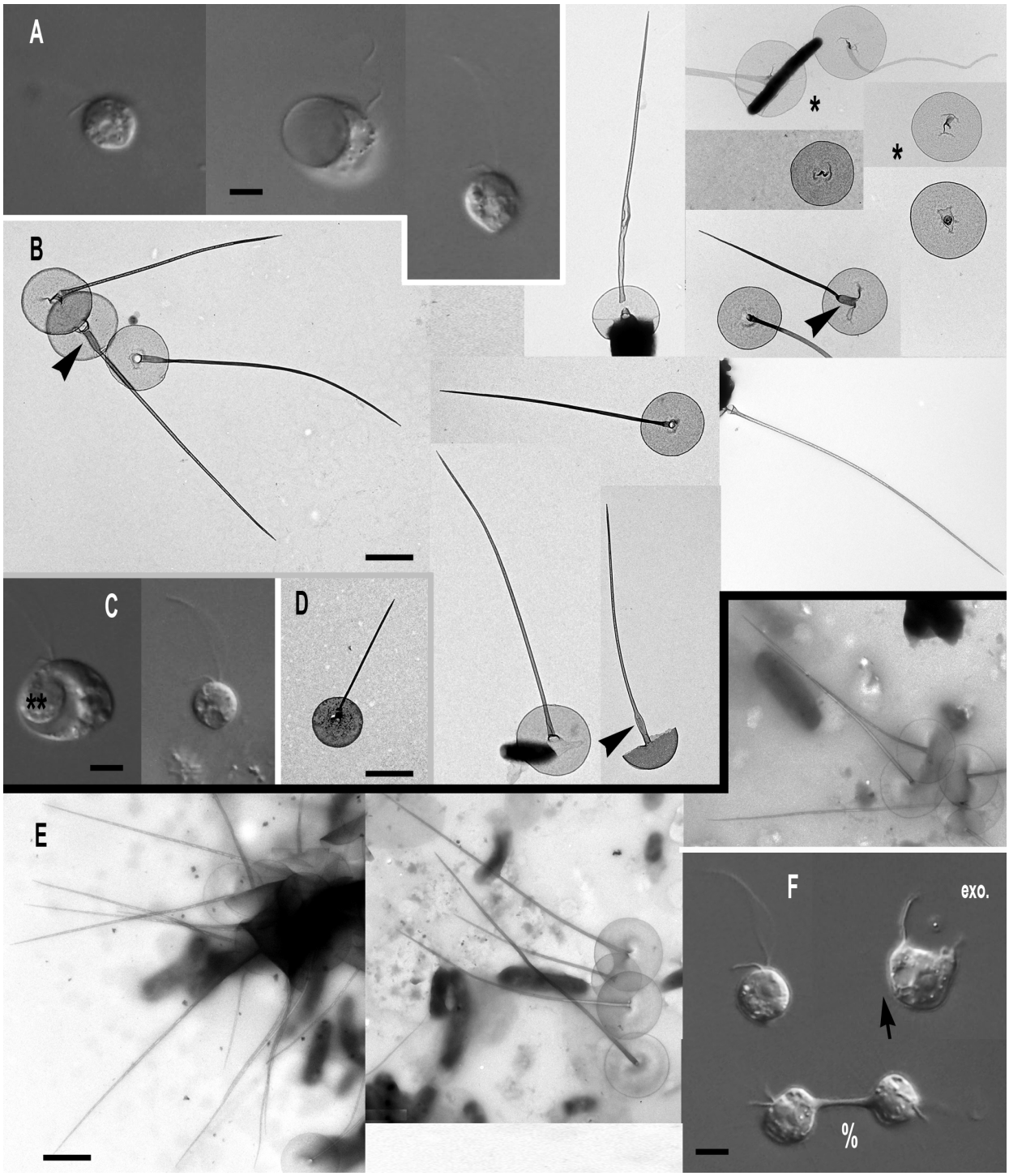


Figure 15

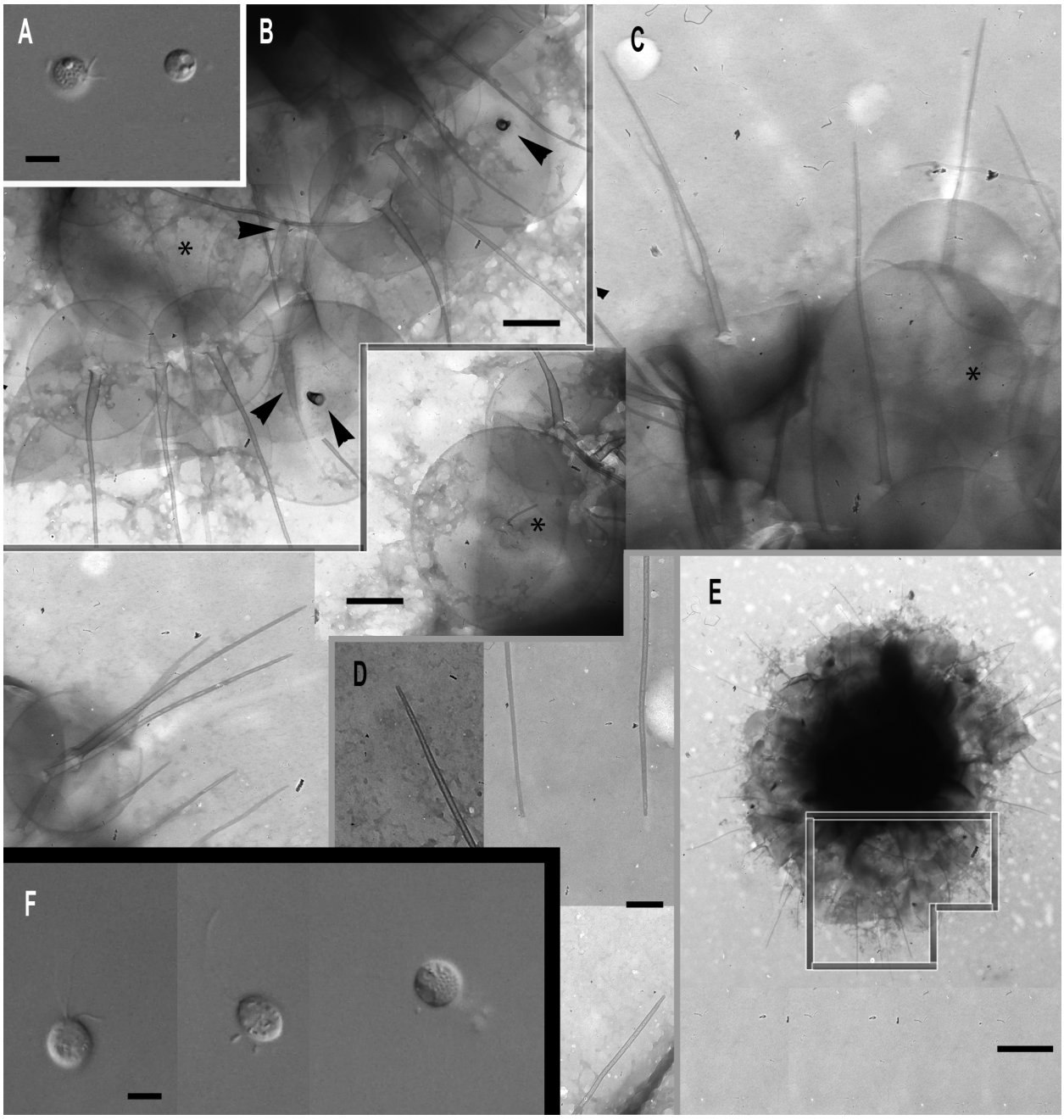


Figure 16

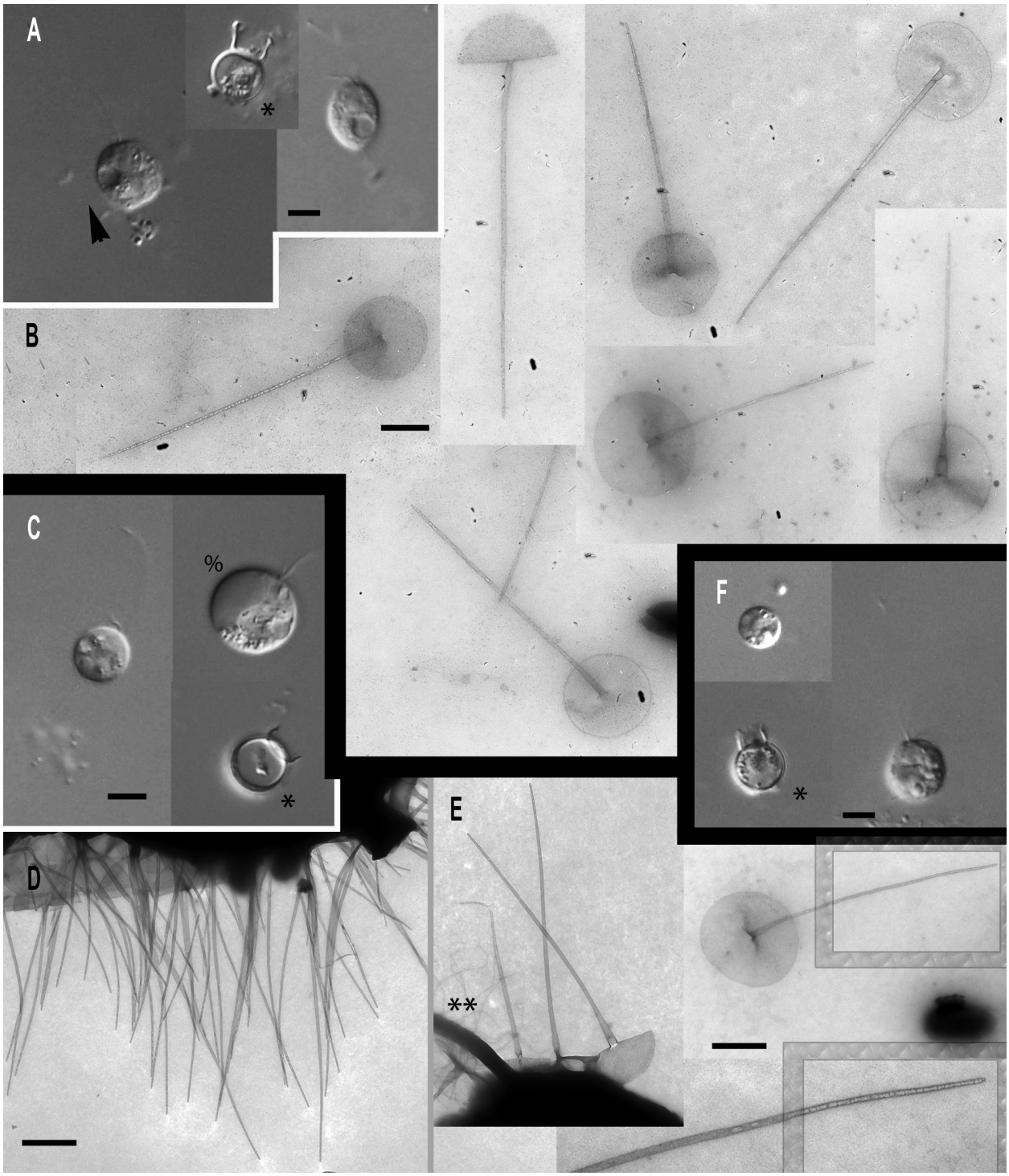


Figure 17

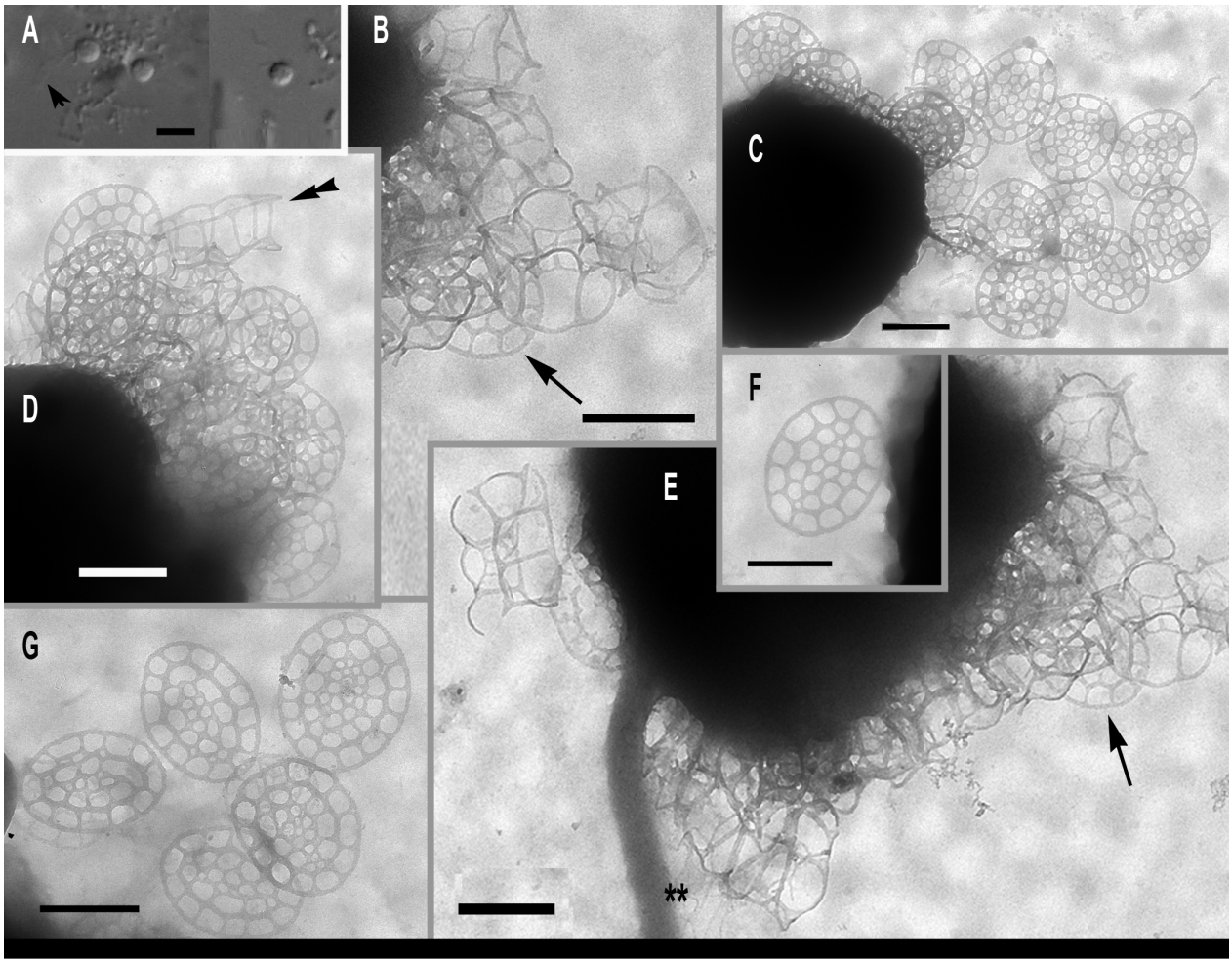


Figure 18

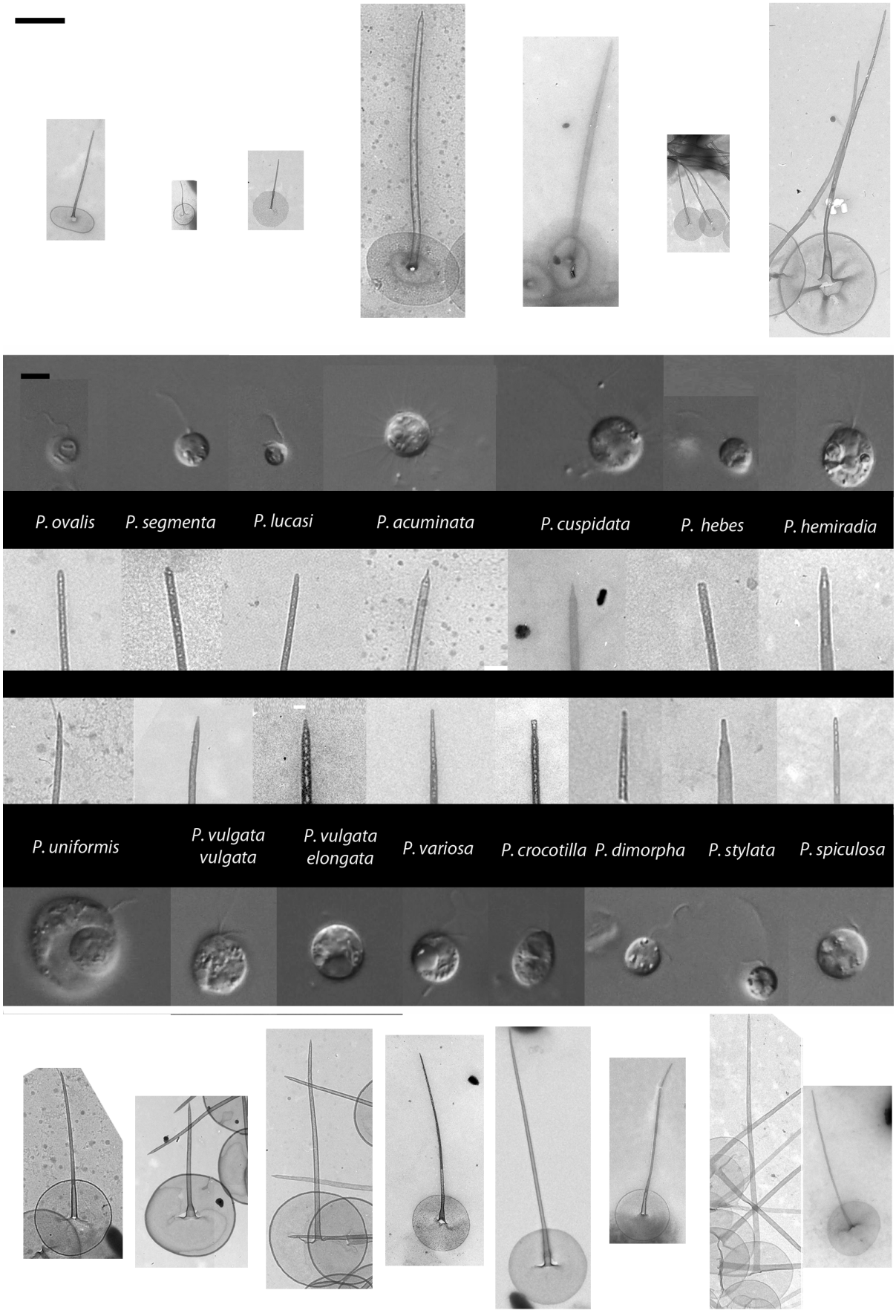

P. ovalis
P. segmenta
P. lucasi
P. acuminata
P. cuspidata
P. hebes
P. hemiradia
P. uniformis
P. vulgata vulgata
P. vulgata elongata
P. variosa
P. crocotilla
P. dimorpha
P. stylata
P. spiculosa

Figure 19

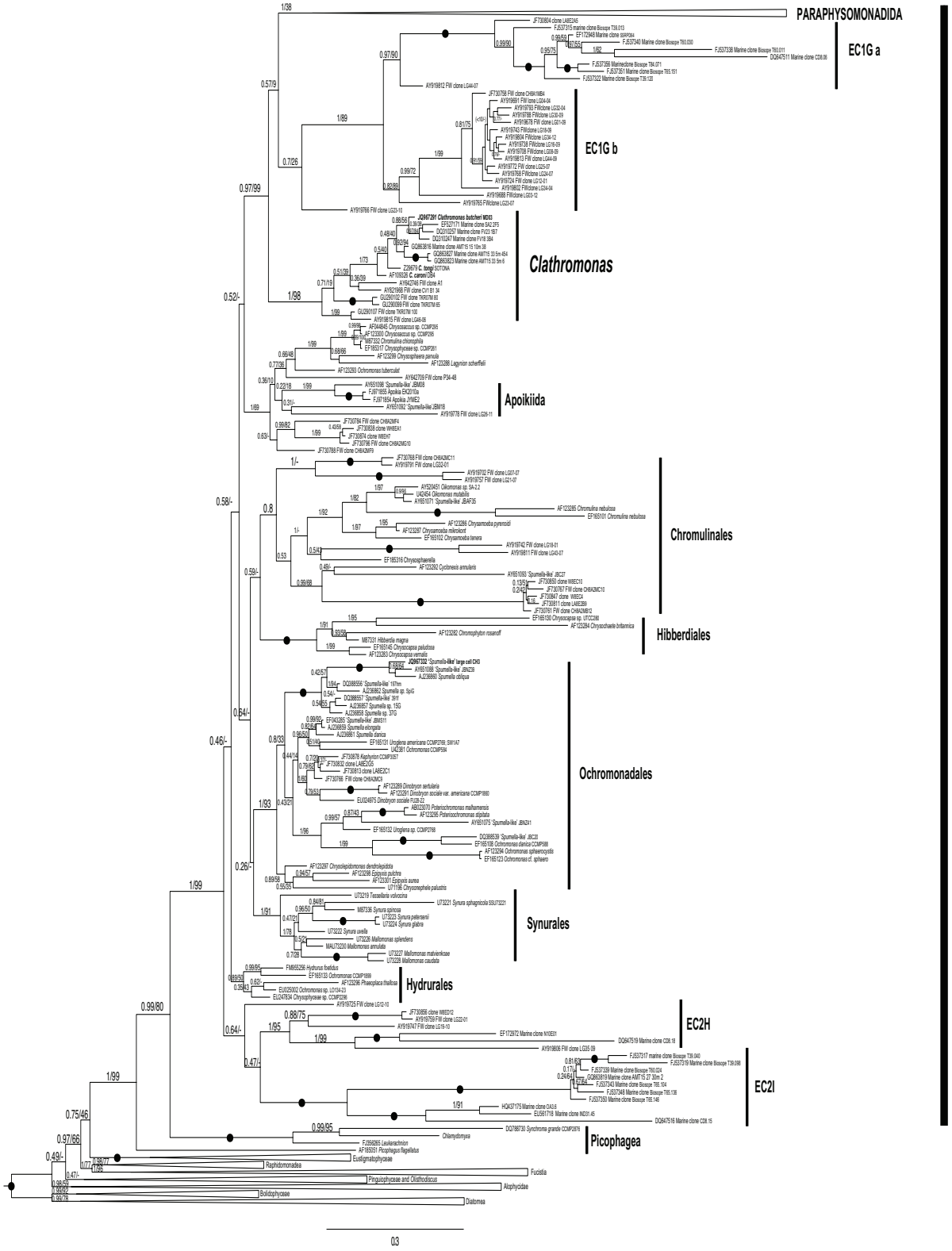
Supplementary material.

Figure S1 Ochrophyte tree showing rest of Chrysophyceae.

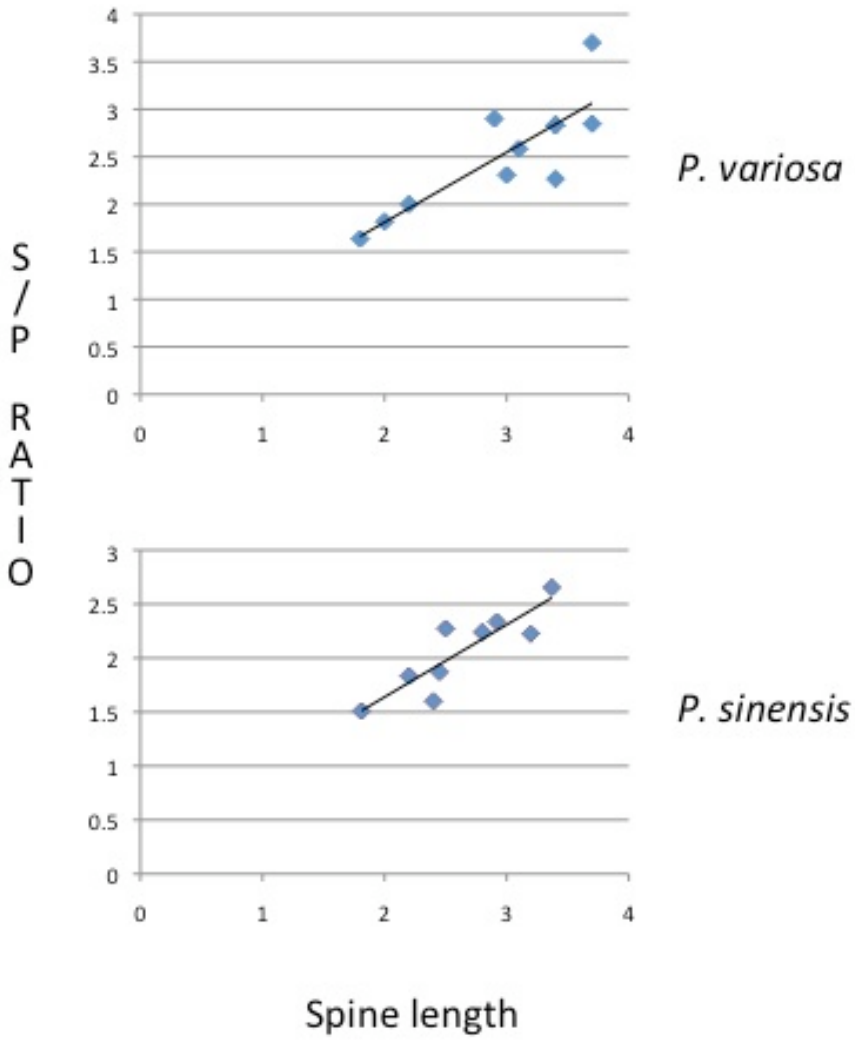
Uncollapsed clades of named Chrysophyeae and Environmental Clades, 1 and 2. MrBayes and RaXML values shown, respectively, at each node.

Figure S2 Scatter graph of *P. variosa* and *P. sinensis* scale data; spine length and S/P ratio.

A correlation is seen between the S/P ratio; the higher the value the longer the spine is to base-plate with, against spine length. The increase shows us how, for these species, as the spine length increases the base plate width decreases.


PARAPHYSOMONADIDA
EC1G a
EC1G b
Clathromonas
Apoikiida
Chromulinales
Hibberdiales
Ochromonadales
Synurales
Hydrurales
EC2H
EC2I
Picophagea
Chrysophyceae
Rhaphidomonadea
Eustigmatophyceae
Pinguiophyceae and Olisthodiscus
Fucidia
Alophrydae
Bolidophyceae
Diatomea
0.3

S1



Regression lines
P. variosa Y = 0.74 X + 0.34
P. sinensis Y = 0.67 X + 0.30

S2

CHAPTER FIVE

Paper for publication: thaumatomonads

This chapter of work is the collection of all the data accrued for thaumatomonad isolates that JMS cultured, sequenced and analyzed in the electron microscope. All of the data for the thaumatomonad work is contained within this chapter for which JMS did 100% of the laboratory work. The molecular analysis and production of trees was overseen by TCS for which JMS and TCS collaborated on inclusion of certain groups of taxa and for which types of phylogenetic tree analysis to perform. For this part of the work I attribute **90**% JMS, 10% TCS. The written manuscript was initiated and the first draft written by JMS after which TCS added to each section. The written work was **80**% JMS, 20% TCS.

This chapter is presented in the final state as it was submitted to European Journal of Protistology.

Scale evolution, sequence phylogeny, and taxonomy of thaumatomonad Cercozoa: 11 new species and new genera *Scutellomonas, Cowlomonas, Thaumatospina* and *Ovaloplaca*

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Abstract

We describe 11 new species of Thaumatomonadida using light and electron microscopy and rDNA gene sequences (18S, ITS1, 5.8S, ITS2). We found clear distinctions between major clades in molecular and morphological traits that support now splitting *Thaumatomastix* into three genera: new marine genera *Ovaloplaca* (oval plate-scales) and *Thaumatospina* (triangular plate-scales), both with distinctive radially-symmetric bobbin-based spine-scales, restricting *Thaumatomastix* to freshwater species with putatively non-homologous eccentric-spine scales and thicker triangular plate-scales. New genus *Scutellomonas* lacks spine-scales, having oval plate-scales with deeply-dished upper tier as in *Ovaloplaca*, with which it forms a clade having short/absent anterior cilium. *Cowlomonas* gen. n. is possibly naked. We describe two new *Allas* species, two new *Thaumatomonas*, and one new *Reckertia* species, and transfer *R. hindoni* to *Thaumatomonas*. Triangular-scaled *Reckertia* has varied plate-scales and ciliary scales. *Thaumatomonas* rDNA trees reveal two clades: *zhukovi*/*seravini* (predominantly triangular scales); *coloniensis*/*oxoniensis*/*lauterborni*/*constricta*/*solis* (scales mostly oval). We

hypothesize that the ancestor of Thaumatomonadidae had radially-symmetric bobbin-based spine-scales and triangular plate-scales, bobbin-based spine-scales being lost in one lineage and eccentric-spine scales evolving in *Thaumatomastix*. Bobbin-based spine-scales arguably evolved from triangular plate-scales and single-tier ciliary scales (O*valoplaca* and *Reckertia* only) from plate-scale rudiments. We present a unified scheme for scale evolution and development in Imbricatea.

Keywords: Imbricatea; Thaumatomonadida; Silica scale evolution; Spongomonadida, *Thaumatomastix*; Zoelucasida

Introduction

For over 700 million years mineralised cell surface scales have featured in protist evolution, the oldest known being phosphatic (Cohen and Knoll 2011). Thaumatomonads belonging to phylum Cercozoa are colourless gliding biciliates characterised by two-tier silicified scales made in silica-deposition vesicles partially embedded in giant mitochondria (Ota et al. 2012). Thaumatomonadida often feed by extending filose pseudopodia from a ventral groove and are grouped with the filose, purely amoeboid Euglyphida as class Imbricatea as both have often imbricate silica scales (Cavalier-Smith and Chao 2003), although the single-tier euglyphid scales are made in the Golgi region. Ribosomal DNA sequence evidence broadened Imbricatea to include also several scaleless taxa, e.g. Spongomonadida and Marimonadida (Howe et al. 2011a).

Recently Thaumatomonadida were grouped with Rotosphaerida, filose silica-scaled non-flagellates (families Pinaciophoridae, Clathrellidae, Rabdiophryidae, Rabdiocystidae, Luffisphaeridae), in a new superorder Placofila characterised by two-tier plate scales (Cavalier-Smith and Chao 2012), but no rotosphaerid sequence data are available to verify that postulated relationship. The biciliate Spongomonadida, with some ultrastructural similarities to Thaumatomonadida (Cavalier-Smith and Karpov 2012) and their likely closest relatives amongst sequenced taxa (Howe et al. 2011a), are grouped with Perlofilida (unsequenced filose non-flagellates *Pompholyxophrys*, *Acanthoperla*) as superorder Perlatia, which in contrast to Thaumatomonadida and Euglyphida secrete silicified perles instead of scales (Cavalier-Smith and Chao 2012). Placofila and Perlatia jointly constitute the imbricate subclass Placoperla (Cavalier-

Smith and Chao 2012); except for the secondarily scale-less thaumatomonad, *Esquamula lacrimiformis* (related to the scale-bearing *Peregrinia clavideferens*: Shiratori et al. 2012), all currently known Placoperla secrete either two-tiered silica scales or globular silica perles.

The other imbricate subclass Placonuda also has two superorders: Euglyphia with conspicuous imbricated single-tier plate scales and Nudisarca (Marimonadida, Nudifilidae: the only known imbricates other than *Esquamula* without silicified surface structures) (Cavalier-Smith and Chao 2012). At present Euglyphia include only the non-flagellate filose testate Euglyphida, but a non-filose biciliate, *Zoelucasa*, was recently discovered with scales so closely similar to those of the euglyphid *Sphenodenia* (Nicholls 2012a) that it might possibly belong in Euglyphia as sister to euglyphids. *Zoelucasa*, with ciliary pit and cilia arranged as in thaumatomonads, swims rather than glides and is the only scaly imbricate flagellate known outside Thaumatomonadida, and is here placed in a new imbricate order, Zoelucasida, seemingly phenotypically intermediate between thaumatomonads and euglyphids. The closest relatives to the putatively ancestrally scaly Imbricatea are Thecofilosea, which never have scales and are grouped with Imbricatea as superclass Ventrifilosa (Cavalier-Smith and Karpov 2012).

Most thaumatomonad species have been described solely from whole-mount electron micrographs of scale morphology in environmental samples. Thus, for most, the appearances of living cells or sequences and their trophic requirements are completely unknown. Recently, clonal cultures, sequencing, and electron microscopy were used to establish new species of *Thaumatomonas*, a new genus *Peregrinia,* and

to segregate *Reckertia* from *Thaumatomastix* (Howe et al. 2011a). That revision restricted *Thaumatomastix* to species having both spine-scales and plate-scales. Even then it was clear that the revised *Thaumatomastix* still included marine species with very different plate-scales: either triangular or oval; recently Nicholls (2012b) found that the spine-scales of two freshwater *Thaumatomastix* species are so different from those of marine species that we here consider them separate genera. We have now studied 34 new clonal cultures by electron and light microscopy and sequencing in order to clarify further thaumatomonad taxonomy, phylogeny, and scale evolution. We show that marine *Thaumatomastix* comprise two morphologically and genetically distinct clades with triangular versus oval plate-scales, and argue that their spine-scales are not homologous with those of freshwater *Thaumatomastix*, which include the not yet sequenced type species *Thaumatomastix setifera* (Lauterborn 1899).

We here establish two new genera for these marine '*Thaumatomastix*' clades and another new genus *Scutellomonas* for thaumatomonads with oval plate-scales but no spine-scales, which form a new clade. We have described two new species of *Allas*, two new species of *Thaumatomonas*, one new species of *Reckertia*, and transfer *Reckertia hindoni* Nicholls (2012b) to *Thaumatomonas.* We also resequenced three old *Thaumatomonas* cultures, the labelling of which had confused thaumatomonad taxonomy (Howe et al. 2011a) and now definitively dispel those confusions. Because *Thaumatomonas* and *Allas* 18S rDNA sequences are so similar, we sequenced ITS regions of 31 ribosomal (r) DNAs also to give more phylogenetic resolution and clearer species demarcation. Only four thaumatomonad ITS rDNA region sequences were previously known, though we discovered five more in GenBank misidentified as fungal. We found that ITS2 rDNA secondary structure helps define *Thaumatomonas* subclades

and species, and with ITS/5.8S rDNA region trees confirm the previously questionable phylogenetic distinctness of *Thaumatomonas* and *Allas*.

Materials and Methods

Culture isolation

Samples (1 - 5 g) of soil, marine littoral sand, seawater, or freshwater were placed in sterile 90 mm Petri dishes and drowned in media with a sterile (boiled) barley grain. Volvic® (Danone) was used for freshwater and soil samples. Artificial Seawater for Protists (ASWP) was made (Culture Collection of Algae and Protozoa (CCAP) recipe – www.ccap.ac.uk) and used for marine samples. Uniprotist clonal cultures were obtained from these crude cultures by single-cell picking and/or serial dilution. Strains BKARH9, GSPB9, HANTSF8 and MLTB12, were provided by K. Vickerman. Surviving strains representing new species have been placed in CCAP (accession numbers cited with diagnoses). Strain YPF609 (Nies Culture collection Japan: NIES-2378) was studied by A. Yabuki who kindly gave us his sequence and micrographs.

Microscopy

Phase contrast and differential interference contrast (DIC) light microscopy. Cultures were viewed and manipulated using phase contrast X20 and X40 objectives (Olympus IX70: Olympus CK40). Live high definition video recordings were made using a Sony HDV 1080i Handycam® mounted on a Nikon Eclipse 80i microscope with a water immersion x60 DIC objective lens (NA 1.0). Videos were uploaded to computer and cell

measurements taken from still frames. Stills were extracted using Final Cut Express HD 3.5.1 and Adobe Photoshop CS4 used to make the figures. Sometimes it was difficult to measure dimensions if cells did not lie completely flat (tilting causes foreshortening), so cells were measured at several stages of movement for accuracy, and results for each cell averaged. **Electron microscopy.** Whole-mount formvar-coated 200 mesh copper metal grids were viewed in an FEI Technai 12 electron microscope. Cells were fixed in glutaraldehyde diluted to 2.5% in freshwater or salt water media, dried on the grid, then washed with deionised water. YPF609 was fixed and photographed via SEM by A. Yabuki (Yabuki and Ishida 2011).

DNA extraction and sequencing

DNA was extracted as soon as possible after initial isolation, when cultures were in their first 'bloom' at the height of the growth curve. Cells were filtered through sterile Whatman™ GF/F glass fibre filters and DNA extracted using UltraClean® soil isolation kits (Mo Bio Laboratories, Camb Bio, UK). Eukaryote-wide* and cercozoan-specific** PCR primers (Bass and Cavalier-Smith 2004; Karpov et al. 2006) (from Invitrogen™ UK) were used for amplifying rDNA: for 18S we used either 25F* - 1801R* or 25F* - 1256R**; for ITS1+ITS2 we used 1259F** - 28SR1*, in 25 µl volumes. PCR programs were: 95°C for 5 min (initial denaturation) followed by 35 cycles: 95°C for 32 s, annealing 70°C (**) or 68°C (*) (depending on primers, shown by asterisks), extension 72°C for 2.5 min; final extension at 72°C was for 7 min. PCR products were electrophoresed on 1% agarose gels and visualised in UV by ethidium bromide. Polyethylene glycol was used to clean single bands (Howe et al. 2011a), but if multiple bands were present the correct size band was cut out and cleaned for sequencing

using GFX™ PCR DNA Band Purification Kit (QIAGEN®, GE healthcare UK). Internal sequencing primers were: 18S: 3NDF (5' GGC AAG TCT GGT GCC AG 3'), and Pre3NDF (5' CAG CAG GCG CGC AAA TTA CC 3') (Bass and Cavalier-Smith 2004). ITS1 and ITS2: PreBF (5' GTA GGT GAA CCT GCA GAA GGA TC 3'), and 384F (5' YTB GAT GGT AGT GTA TTG GA 3') (Dopheide et al. 2009). Sequencing was by Applied Biosystems BigDye® Terminator v. 3.1 and v. 1.1 Cycle Sequencing Kits and Applied Biosystems 3730/3730xl DNA Analyzer (Life Technologies) with Capillary-system.

Sequence editing, alignment, and phylogenetic analyses

Chromatogram reads were viewed and edited using Sequence Scanner v. 1.0 of Applied Biosystems. BioEdit was used for contig assembly. Additional 18S rDNA cercozoan sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/). To find closely related 18S and ITS rDNA environmental sequences in NCBI, we used our new sequences as well as published named thaumatomonad sequences as BLAST queries. Sequences were initially aligned using default settings of MAFFT v.6 Online version using secondary structure 'extremely slow Q-INS-I' setting (Katoh et al. 2009). MacGDE v. 2.4 (http://macgde.bio.cmich.edu/) was used to improve alignments manually.

We made two rDNA alignments. First, a large 18S rDNA alignment containing 273 taxa representing the three classes of infraphylum Monadofilosa closest to thaumatomonads: Imbricatea, Thecofilosea, and Sarcomonadea. Because the demarcation between Imbricatea and Thecofilosea is poorly resolved, we could not simply assume Thecofilosea to be the correct outgroup for Imbricatea, so it was essential to include a broadly representative selection of all major ventrifilosan

lineages (Imbricatea, Thecofilosea) and use sarcomonads as an unambiguous outgroup for them. This alignment contained many environmental sequences, some mislabeled 'thaumatomonad' and others genuinely thaumatomonad. As preliminary analysis suggested that some environmental sequences labeled thaumatomonad were actually sarcomonads, we included 73 sarcomonad sequences to establish their position. Unlike Howe et al. (2011a) we excluded the most distant monadofilosan class (Metromonadea), restricting our analysis to Ventrifilosa plus their immediate outgroup Sarcomonadea. By excluding more distantly related Cercozoa, we could align virtually the whole of 18S rDNA with confidence. Our second alignment was of the ITS1, 5.8S and ITS2 rDNA regions representing five thaumatomonad genera. This alignment embraced the last part of the 18S rDNA sequence (~400 nucleotides) and the beginning of 28S (~300 nucleotides) and yielded a more robust branching order for the *Thaumatomonas*/*Allas* part of the tree, which is poorly resolved by 18S rDNA alone. This ITS rDNA alignment also includes mislabeled 'fungal' environmental clones (Bowden et al. 2004; Hawkes et al. 2011; Waldrop et al. 2006) recognised here as thaumatomonad.

Maximum likelihood analyses used the GTRMIX model of RaXML-7.0.4 (Stamatakis 2006) with 1000 rapid bootstrap resamplings. Two different Bayesian methods were used. MrBayes v. 3.1.2 consensus trees were generated from two chains run for five million generations under a GTR adgamma evolutionary model with four gamma categories and covarion setting. One out of every 1000 trees were kept for analysis, and the first 450 trees excluded as burnin for both 18S and ITS. PhyloBayes v. 3.2 trees used the heterogeneous GTR-CAT model with default gamma settings (Lartillot et al. 2004) and two chains. Though both chains plateaued, even

after >75,140 cycles maxdiff was 0.50609, so the statistical criterion for convergence in a 'good' run was not met; therefore chains were summed separately (as well as together, excluding the first 1288 trees as burnin). As the topology was identical within the Thaumatomonadida/Spongomonadida clade and within all other major clades for each chain separately and the joint tree and support values closely similar, we arbitrarily used chain 1 only based on 73,850 trees for Fig. 1. The key difference between the two chains that accounts for their non-convergence was in the position of Discomonadida: weakly sister to Imbricatea in chain 1 (Fig. 1) and weakly sister to the imbricate subclass Placoperla only (i.e. within imbricates) in chain 2. As the PhyloBayes CAT-GTR heterogeneous model is evolutionarily more realistic than the homogeneous ML and MrBayes models (Lartillot et al. 2004), Figure 1 shows the PhyloBayes tree with support values for all three models mapped onto it.

ITS2 fold analysis

ITS2 rDNA region was located using an ITS2 annotation program (Koetschan et al. 2009) http://its2.bioapps.biozentrum.uni-wuerzburg.de/. The clipped 'annotated' sequences were folded using the online MFold program (Zuker 2003) http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form set at 37^{o}C. Each fold was checked by eye within the helix III region for compensatory base changes (CBCs) and hemi-CBCs (Coleman 2009) compared with closely related strains.

GenBank accession numbers: 18S rDNA. KC243105 - KC243105; ITS1, 5.8S rDNA and ITS2 submitted as contiguous sequences, KF577807-KF577836.

Results

We studied 23 newly isolated cultures and nine uncharacterised strains from K. Vickerman by sequencing 18S rDNA and transmission electron microscopy (TEM), though sequencing was unsuccessful for three and TEM for several. In order to clarify decisively the *Thaumatomonas* species-name confusions discovered by Howe et al. (2011a) we restudied similarly three key older cultures (one from Vickerman and two type cultures from Mylnikov). We also sequenced 18S rDNA from two dead unidentified clonal cultures from D. Bass. Fourteen sequences were novel. Thirty-one cultures were also sequenced across the internal transcribed spacer regions (ITS1, 5.8S, ITS2) of rDNA. Though our primary goal was to clarify the diversity and phylogeny of named thaumatomonads, we wished also to test whether environmental sequences annotated as 'thaumatomonads' really are from this group and to clarify the unresolved basal branching of Imbricatea, the class containing thaumatomonads. Because of its large size our monadofilosan 18S rDNA tree is split for clarity into Ventrifilosa (Figure 1.1) and the sarcomonad outgroup (Figure 1.2), which were consistently separated by all three methods. Support is weak for the tree's backbone (as previously: Howe et al. 2011a) except for the robust separation of cercomonads from other groups (1, 99%, 1) and the monophyly of Ventrifilosa by ML and MB.

Overall phylogeny within Ventrifilosa

Within Imbricatea, Placonuda and Placoperla are each reproducibly holophyletic with all three methods (Fig. 1.1). Within Placoperla, Spongomonadida are sister to all Thaumatomonadida in ML and MB (BS 58%, PP 0.86), but in PB spongomonads branch within Thaumatomonadida as sister to Esquamulidae and Peregriniidae (PP 0.61 Fig. 1.1). In ML and MB Thaumatomonadida were consistently

holophyletic with Peregriniidae and Thaumatomonadidae sisters, albeit with weak support (BS 53%, PP 0.77). Within Placonuda superorder Nudisarca is a clade with PB, but paraphyletic with ML and MB. Imbricatea are holophyletic using PB and ML, but with MB Placonuda branched more deeply (PP 0.56) as sister to all other Ventrifilosa. The MB and ML analyses never grouped *Discomonas* within Imbricatea, as did MB in Howe et al. (2011a), or even as sister to imbricates. However, PB showed the *Discomonas* clade either as sister to all other Imbricatea (Fig. 1 chain 1) or (chain 2) as sister to Placoperla only as in the MrBayes tree of Howe et al. (2011a). Only with MB is Ventricleftida sister to Thecofilosea (PP 0.77) in accordance with their classification and the MB tree in Howe et al. (2011a); with PB and ML Ventricleftida are extremely weakly sister to Imbricatea (PP 0.34, BS 5%).

Mataza hastifera (Yabuki and Ishida 2011) is always within Thecofilosea and in both Bayesian trees is sister to Ebriida plus environmental clades eEbriida and AB27503, with moderate support; in ML Matazida are weakly sisters to Cryomonadida (BS 18%). These trees consistently disagree strongly with both previous contradictory placements of *Mataza*: within Cryomonadida or as sister to all other Thecofilosea (Yabuki and Ichida 2011).

In separate 18S rDNA analyses (ML only, not shown as their presence or absence did not alter branching within the Thaumatomonadida/Spongomonadida clade) we examined the position of the long-branch Phaeodaria (Howe et al. 2011a) and of the even longer-branch Sainouroidea (Cavalier-Smith et al. 2009). When Phaeodaria sequences were added, they were sister to *Pseudodifflugia* cf. *gracilis*; *Pseudodifflugia* cf. *gracilis* was within Thecofilosea in every tree. When both

Phaeodaria and Sainouroidea were added they were mutually sisters, this clade being sister to *Pseudodifflugia*. However, when Phaeodaria were removed, Sainouroidea moved from Thecofilosea to Imbricatea, weakly sister to Euglyphida.

Thaumatomonadida 18S rDNA phylogeny

Eight known genera of Thaumatomonadida are in the tree (Fig. 1.1); at least four more are implied by the environmental sequences. The deepest branching of Thaumatomonadida is between Peregriniidae/Esquamulidae (a robust clade) and Thaumatomonadidae (always a clade but with weaker support). *Peregrinia* (freshwater) is sister to the soil sequence, Amb_18_1066 (EF023480) (Lesaulnier et al. 2008), this non-marine clade being robustly sister to marine *Esquamula lacrimiformis* (PP1, BS 100%, PP 1). Thaumatomonadidae consist of a single maximally supported freshwater clade (FW in Fig. 1.1) nested within four deeper-branching marine lineages, which are distinct on both ML and Bayesian trees. The large freshwater clade comprises *Thaumatomonas*, *Allas*, soil sequence Amb_18S_1164 (EF023728 and others not shown, EF023966, EF025032, EF023867: Lesaulnier et al. 2008), and another from a paddy field (AB534345: Takada and Morimoto 2010) and is sister to the marine *Reckertia*-containing clade. These sister clades both lack spine scales (in strains studied by both sequencing and EM). Three of the four deeper branching lineages of Thaumatomonadidae include species with spine scales that would previously formerly all have been lumped as '*Thaumatomastix*' (Howe et al. 2011a), but the fourth (uncultured sequence A15) is of unknown morphology. *Thaumatomonas* appears paraphyletic, with *Allas* sister to the *Ts seravini*/*zhukovi* subclade (abbreviations:

Thaumatomonas – Ts; *Thaumatomastix – Tx*; *Thaumatospina – Ta*). No environmental sequences were identified as *Thaumatomonas* or *Allas*.

Spine-scaled '*Thaumatomastix*' are paraphyletic, having two strongly supported clades: *Thaumatospina* gen. n. with triangular plate-scales (PP 0.99, BS 90%, PP 1), and *Ovaloplaca* gen. n. with oval plate-scales and spine-scales (PP 1, BS 100%, PP 1). *Ovaloplaca* is sister to a newly described genus, *Scutellomonas* gen. n. with oval plate-scales only (PP 1, BS 100%, PP 1) and an environmental clone 9-2.6 (AY620310) (Bass and Cavalier-Smith 2004) likely to have oval scales. Thus, all oval-scale thaumatomonads are molecularly more related than any are to the deepest triangular-scale *Thaumatospina* clade. Marine environmental sequence 'A15' (AY620317: Bass et al. 2005) is sister to all Thaumatomonadidae other that the deepest branching *Thaumatospina*. The two *Reckertia* species are weakly sister and group robustly with five marine sequences, possibly but not necessarily all *Reckertia*; three of these unidentified sequences form a robust clade R1 (Fig. 1.1) of sea ice sequences, FN690394, FN690393, FN690392 (Majaneva et al. 2011); EF100294 is from oxygen-depleted intertidal marine sediment (Stoeck et al. 2007), and GU385595 from seaweed/bait (Haska et al. 2012). This *Reckertia*-containing clade is reproducibly sister to the freshwater clade, not to either marine '*Thaumatomastix*' clade, reinforcing their generic distinction. Thus there are three major distinct thaumatomonad marine clades including known species, all lumped with the freshwater *Thaumatomastix* prior to Howe et al. (2011a).

Identifying environmental DNA clades

Two environmental sequences were misannotated as thaumatomonads, from the same soil study (Lesaulnier et al. 2008) from which many previous papers found numerous misannotations affecting other groups (e.g. Cavalier-Smith and Chao 2012; Cavalier-Smith and Scoble 2013; Howe et al. 2011a): Amb_18S_1199 (EF023758) is maximally supported as sister to *Nudifila*, placed below in the new family Nudifilidae: environmental sequence Elev_18S_823 (EF024516) is not even an imbricate or ventrifilosan sequence, but belongs to the deep-branching probable glissomonad clade Te that also includes a sequence (EF023937) even more bizarrely annotated as an apicomplexan eimeriid (Lesaulnier et al. 2008). Figure 1.1 does however reveal three distinct environmental clades lacking cultured representatives that are genuinely within Thaumatomonadidae, two from soil (AB534345, EF023728), one from marine sand (AY620317).

The three core NC1 sequences (Bass and Cavalier-Smith 2004) are robustly sisters to Marimonadida as Howe et al. (2011a) found, and probably belong in that order. However, Figure 1.1 sequence 7-6.6 (AY620336), which originally grouped weakly with NC1 (Bass and Cavalier-Smith 2004) or Euglyphida (Bass et al. 2005) or NC3 (Howe et al. 2011a), now consistently groups as sister to a robust clade comprising Nudifilidae, Clautriaviidae and NC2 (robustly sister to Clautriaviidae); below we make this entire clade a new placonudan order Variglissida. Figure 1.1 shows NC3 within Nudisarca (Placonuda) as sister to Marimonadida, NC1 and sequence 9-2.2. However, in both ML and MB, NC3 is sister to euglyphids and Nudisarca are paraphyletic.

Our trees confirm that two core NC4 sequences (AY620348 7-6.2; AY620314 9-1.4: Bass and Cavalier-Smith 2004) belong in Thecofilosea, whereas the marine sediment sequence 9-2.2 (AY620308), originally weakly grouping with NC4 (Bass and Cavalier-Smith 2004) belongs elsewhere. Sequence 9-2.2 is weakly (PP 0.22) sister to Marimonadida within Nudisarca in the heterogenous PB tree, but weakly sister to a possibly artefactual clade comprising Euglyphida and NC3 (NC3 is within Marimonadida by PB) on the two homogeneous model trees (ML 24%, MB 0.88). No tree placed 9-2.2 within glissomonads as it was with no support in Howe et al. (2011a); though its precise position is weakly supported, all three methods strongly exclude it from glissomonads.

Though our sampling of monadofilosan environmental sequences is more broadly representative than other studies, it is not exhaustive. For example, Fig. 1.1 includes eight environmental sequences related to *Cryothecomonas aestivalis*, mainly from the Arctic Ocean and sea ice, except two sister sequences (AB275093, AB275046) obtained from the Japanese east coast from a 'methane cold seep sediment' (Takishita et al. 2007); yet these all differ from the 13 *Cryothecomonas* Arctic environmental DNA sequences in the tree of Thaler and Lovejoy (2012), emphasising their high diversity in the Arctic. Within Ventricleftida we found a novel clade of marine environmental sequences (eVentri) strongly sister to *Ventrifissura* in PB (but the deepest branch of this clade is one node deeper in ML and MB).

Two sarcomonad environmental sequences (AB534506 Takada and Morimoto 2010, and EF024287 Lesaulnier et al. 2008) form a consistent clade that is sister to both Pansomonadida and Glissomonadida in PB and ML; oddly, with MB two NC7

environmental sequences, (AY620287 and AY620288: Bass et al. 2005) group as their sister instead of within Pansomonadida as with PB, ML and previously (Bass et al. 2005). We name this clade eSarcomonad (Fig 1.1); like clade Te (Howe et al. 2009) it is likely to be morphologically more glissomonad-like than cercomonad-like.

ITS1, 5.8S, and ITS2 rDNA phylogeny

To resolve the relationship between *Allas* and *Thaumatomonas* and other thaumatomonads, which was contradictory on PB, ML and MB 18S rRNA trees, we analysed a 1561-nucleotide alignment of 35 Thaumatomonadidae alone for their ITS1, 5.8S, and ITS2 rDNA sequences, which include ~700 nucleotides from adjacent 18S and 28S rRNA (Fig. 2). Five genera were represented, the tree being rooted between *Thaumatospina* and all other Thaumatomonadidae in accordance with the 18S rDNA tree (Fig. 1.1). In contrast to the 18S rRNA trees *Allas* and *Thaumatomonas* were both holophyletic mutually sisters on the MrBayes tree (Fig. 2), which supports their retention as separate genera (Howe et al. 2011a). The positions of named *Thaumatomonas* sequences within the two *Thaumatomonas* clades were exactly the same in ML and MB, though three of four misannotated 'fungal' clones behaved differently: in MB these three (DQ420715, DQ420716 and JQ666760: Waldrop et al. 2006) were sister to *Allas* plus *Thaumatomonas,* whereas in ML this long branch joined the positionally stable short-branch sequence HM240097 (Hawkes et al. 2011) within *Thaumatomonas*, as sister to the reproducible *Ts coloniensis*/*oxoniensis*/*lauterborni*/*constricta*/*solis* clade; (abbreviated below as C/L/S referring to the first species of its main sub-branches). Possibly the MB tree placed the long branch too deeply and all four 'fungal' clones are undescribed *Thaumatomonas*

related to the C/L/S clade; they must be undescribed (three species only as two are almost identical) since Figures 1.1 and 2 together now include all known *Thaumatomonas* species, assuming that Mylnikov's T-1 culture (which grouped closely and robustly with *Ts constricta* on Figure 1.1, but ITS rDNA is not sequenced) was correctly identified as *Ts lauterborni*. *Allas* was strongly holophyletic in both 18S (1/100%/1) and ITS (0.95/66%) rDNA trees. Relationships amongst *Ts* '*vancouveri', Ts coloniensis* and *Ts oxoniensis* are clearer than with 18S rDNA alone. *Scutellomonas* was consistently sister to *Reckertia,* which contradicts the 18S rRNA tree where it branched one node more deeply; however, the ITS tree is less taxon-rich near the base, so in this respect might be misleading as it does not include *Ovaloplaca*, the much closer true sister of *Scutellomonas*.

Phylogeny of the sarcomonad outgroup

Glissomonadida and Pansomonadida are consistently sister in MB and ML as in most previous studies. However with PB Pansomonadida branch well within Glissomonadida, albeit with weak support (Fig. 1.2), suggesting that their previous frequent (not invariable) exclusion by some other methods may have been a long-branch artefact, thus questioning their status as a separate order. Novel clade 7 (Bass and Cavalier-Smith 2004) forms three distinct clades, two within pansomonads and one (AY620284) their sister. The pansomonad/AY620284 clade is sister to a robust clade X/Y, previously assumed to be glissomonads (Howe et al. 2011b; Hess and Melkonian 2013), but since our analysis subclade X was identified as a more amoeboid new family Viridiraptoridae (Hess and Melkonian 2013) but probably belongs to the previously phylogenetically unplaced order Pseudosporida (Cavalier-Smith 1993); see

Discussion. With all methods the basal branching order within Glissomonadida differs in a few respects from Howe et al. (2011a,b), possibly owing to our improved alignment including more nucleotides and more extensive outgroups.

Even for the most distant outgroup Cercomonadida our trees are better resolved and more consistent than earlier. *Cercomonas* is a distinct clade in ML, but paraphyletic with PB and MB. Within Cercomonadidae, *Neocercomonas* plus *Filomonas* form a robustly supported clade in all three analyses (PP 0.99, BS 82%, PP 1.0), invalidating the assertion (Brabender et al. 2012) that bootstrap values are too low to support the monophyly of *Neocercomonas* and its distinction from *Cercomonas* (Cavalier-Smith and Karpov 2012).

ITS2 rDNA secondary structures of Thaumatomonadida

Thaumatomonas exhibited multiple isolates with the same 18S rDNA sequence, some with different or additional scale morphologies. Though the ITS rDNA region tree gave better resolution (Fig. 2), especially within the *Ts zhukovi*/*seravini* (Z/S) clade, and C/L/S clade, their internal branches are still so close together and in some cases poorly resolved, and scale morphologies so variable and not mapping simply onto the tree, that it was hard to use either trees or morphology to make clear boundaries between species. We therefore also used ITS2 rDNA secondary structure to help such demarcation, as Coleman (2009) suggested that organisms differing by at least one compensatory base change (CBC) in helix III would be unable to cross, and therefore are probably separate biological species. Figure 3 shows that ITS2 rDNA secondary structure is generally well conserved across Thaumatomonadidae and has the canonical structure of helices I-III. Structure was even more consistent within

Thaumatomonas enabling CBCs and hemiCBCs (hCBC) to be readily detected by comparing helical regions in closely related strains; these are summarised in supplementary Figures 1 and 2 together with the nucleotide differences in 18S rDNA and both ITS1 and ITS2. We found significant evolutionary change between some isolates resulting in CBCs, which could signify an altered function in the processing of the ribosomal RNA precursor. In the case of the Z/S clade, even though NC02 and NC03 differ from *Ts zhukovi* and each other by one CBC we decided to rank them only as different subspecies of *Ts zhukovi* because all three have the same 18S rDNA sequence and effectively indistinguishable scales. Another *Ts zhukovi* isolate HANTSF8 was also made a subspecies because of two hCBCs and distinct scale morphology. Interestingly, the type strain of *Ts seravini*, with four 18S rDNA differences from the *Ts zhukovi* type strain, had just one CBC compared with the *Ts zhukovi* type strain, whereas *Ts seravini* differs from the new *Ts zhukovi* subspecies by at least 2 CBCs; the number of hCBCs was consistently higher, making them useful additional species delimitation markers within the Z/S clade. The hCBC is a single-sided change, which is thermodynamically stable in RNA molecules and retains base-pairing; though implied to be half a CBC, hCBC frequency and distribution on the molecule of both CBCs and hCBCs can be independent (Caisová et al. 2011).

The ITS2 rDNA secondary structure fold for strain 4e (*Ts seravini varisquama*) was slightly different for helix III compared to the other isolates in its clade; part of the stem had an enlarged loop in the same place where CBCs were found in other closely related isolates (Fig.3). Therefore, the enlarged loop could account for the lower than expected CBC difference between 4e and two *Ts zhukovi* subspecies (*carolinensis* and *paracarolinensis*). Checking the folds by eye helped find this difference.

Setting boundaries between closely related *Thaumatomonas* species

Figure 2 shows that the *zhukovi*/*seravini* subclade has marked intrastrain variation in scale structure. We did not confirm that the type strain (T-3) of *Ts zhukovi* had all the scale types, triangular-like and oval, observed in the original description by Mylnikov and Mylnikov (2003); when examined in the TEM we saw only two cells, both with triangular scales only (Sup. Fig. 6). Seven strains in the Z/S clade were polymorphic for oval and triangular or quasi-triangular scales, whereas only two have just oval and one just triangular scales. Therefore in the Z/S clade one cannot use scale shape alone to identify species; morphology must be combined with sequences. Yet 18S rDNA sequences showed low divergence amongst these closely related *Thaumatomonas*; *Ts seravini* and *Ts zhukovi* differ by just four 18S rDNA nucleotides. In the *Ts coloniensis*/*oxoniensis* subclade, we found only one strain polymorphic for oval/triangular scales. Though scale structure is more uniform in this subclade low 18S rDNA divergence makes species discrimination difficult. It is unwise to make separate species just because strains differ in 18S rDNA by one or two nucleotides, unless one thoroughly excludes sequencing error and shows that ITS rDNA sequences are even more different. In addition to comparing ITS2 rDNA secondary structures among closely related strains to use CBCs and hCBCs as an aid to deciding species boundaries, we examined our larger 18S rDNA alignment with many more closely related *Thaumatomonas* isolates to seek likely sequencing errors (in new and published sequences) to reduce such errors in judgment. Overall we decided that our numerous new strains for these two *Thaumatomonas* subclades are all best included in four

existing species and a fifth 'species' (*Ts vancouveri*) should now be included in *Ts coloniensis* as a subjective junior synonym:

The *Ts zhukovi/seravini* clade (predominantly triangular scales). We sequenced 18S rDNA sequences from eight new strains of *Ts zhukovi* (T-3, UPL1Be2, UPL1Bf2, CCL4B, GMKL4, NC02, NC03, HANTSF8), of which three were made subspecies determined by ITS2 rDNA secondary structure: HANTSF8, NC02 and NC03. A ninth isolate, GMBGL1, with no 18S rDNA sequence, is also within this clade. *Ts zhukovi* type, T-3, had at least 12 nucleotide differences in ITS1 rDNA from other *Ts zhukovi* strains, and at least seven in ITS2 rDNA. However, ITS2 rDNA secondary structure yields no CBCs or hCBCs between *Ts zhukovi* strain T-3 and five other isolates: UPL1be2, GMBGL1, UPL1Bf2, CCL4B and GMKL4. Compared with T-3, HANTSF8 had no CBCs but 2 hCBCs, whereas NC02 had three CBCs and four hCBCs, and NC03 had two CBCs and 3 hCBCs, which made NC02 most divergent of the nine strains for ITS2 rDNA folding. ITS2 rDNA sequences for all nine isolates differed by at least one nucleotide, except for GMBGL1 and CCL4B, which have identical ITS2 (supplementary Figure 1). There were three 18S rDNA sequences for new *Ts seravini* strains: T-2, 4e, and CH8; ITS rDNA regions were sequenced only for T-2 and 4, maximally supported as sisters (Fig. 2).

The *Ts coloniensis/oxoniensis* subclade (predominantly oval scales) and suppression of *Ts vancouveri*. We isolated two strains effectively indistinguishable from *Ts coloniensis* (PML3A, Sup. Fig. 3GH, and MLTB12, Sup. Fig. 4D), and two other cultures (GMPL1, Sup. Fig. 3A – C, and EP3, Sup. Fig. 3D - F) similarly indistinguishable from the type strain of *Ts oxoniensis*. These were not included in the 18S rDNA tree

because the 18S rDNA sequences were identical to type strains. ITS2 shows at least 1 CBC and six hCBCs between these two groups (Sup. Fig. 2). The many new *Thaumatomonas* sequences revealed likely errors in published sequences, including *Ts vancouveri* (AF411264) (Howe et al. 2011a), which appears to have missing nucleotides and sequencing errors. Given its likely sequence errors, *Ts vancouveri* probably really has the same sequence as *Ts coloniensis* (both strains HFCC59 type, and HFCC93, which have identical 18S, and, apart from 3 single nucleotide indels that might be sequencing errors, identical 28S rDNA) except for one inserted C in *Ts coloniensis*, which could also be a sequencing error. ITS2 rDNA sequences are unavailable for the type strain of *Ts coloniensis* (HFC59 or for other original *Ts coloniensis* strain HFCC93), but ITS1 of HFCC93 was included in Figure 2 to represent the species as its branch was shorter on an ITS1 tree than was HFC59 whose ITS1 differs by two substitutions and three 1-2 nucleotide indels. The ITS1 rDNA sequence of *Ts 'vancouveri'* is almost identical to our PML3A *Ts coloniensis* strain and both branch so closely to HFCC93 *Ts coloniensis* (closer than does our second *coloniensis* strain, MLTB12) that we cannot treat them as separate species. The above-mentioned 'inserted C', in the 18S rDNA sequence, is the only definite difference between *Ts coloniensis* and *Ts oxoniensis* (HQ121429) but *Ts oxoniensis* has 77 undetermined nucleotides (n) in its sequence (Howe et al. 2011a) (Sup. Fig. 2). *Ts vancouveri* and *Ts oxoniensis* were made separate species by Howe et al. (2011a) using ITS2 folding analyses; two CBCs were found, making them likely different species (Coleman 2009, Wolf et al. 2013). Despite *Ts coloniensis* not having ITS2 rDNA sequence available for secondary structure analysis, in our new ITS2 rDNA CBC analysis the cluster including *Ts vancouveri* and our two new *Ts coloniensis* strains on the one hand and all three strains of *Ts oxoniensis* on the other consistently shows

the ITS2 rDNA difference previously used by Howe et al. (2011a) to separate *Ts vancouveri* and *oxoniensis,* which is congruent with molecular phylogenies (Fig. 2). However, as our new *Ts coloniensis* strains reveal that there is no clear ITS2 distinction between *Ts vancouveri* and *coloniensis*, we now make *Ts vancouveri* a synonym for *Ts coloniensis* but keep *Ts oxoniensis* as a distinct species because the original CBC distinction of Howe et al. (2011a) is confirmed in three independently isolated strains for each species (in disagreement with Karpov (2011), who made *Ts oxoniensis* a synonym of *coloniensis* and considered *Ts vancouveri* invalid because of incomplete scale description).

Sorting out the *Thaumatomonas* sequence labelling confusion

Howe et al. (2011a) concluded from contradictory labelling of essentially the same *Thaumatomonas* sequences in GenBank that labelling of either the *Thaumatomonas* genes sequenced by Ekelund et al. (2004) or the three source cultures originating from Mylnikov via Karpov and then Vickerman had been mixed up, as one of them was ostensibly the same T-2 type strain *Ts seravini* culture sequenced by Cavalier-Smith and Chao (2003) that Mylnikov gave them directly in 1994 in St Petersburg, but the sequences were contradictory. Ekelund (pers. comm.) now tells us that his strain labels were independent of those of Mylnikov and Karpov (1993); he coincidentally used virtually the same strain codes T1-T3 for different species: SCCAP T1' *Ts* sp., 'SCCAP T2' *Ts lauterborni*, and 'SCCAP T3' *Ts seravini*. By contrast Mylnikov's T-1 is *Ts lauterborni,* his T-2 is the *Ts seravini* species type strain*,* and T-3 is the *Ts zhukovi* species type strain. Howe et al. (2011a) reasonably assumed that the Ekelund labels without hyphens were derived from those of Mylnikov with hyphens; yet such

independent, contradictory use of essentially identical numbering by two labs does not explain all contradictions discovered by Howe et al. (2011a). We have now resequenced and reexamined scale ultrastructure of a fresh sample of the original *Ts seravini* (T-2) strain from Mylnikov's laboratory directly given in 2010 by Denis Tikhonenkov to TC-S at a conference. Its sequence is identical to the *Ts seravini* AF411259 of Cavalier-Smith and Chao (2003) and also that of Ekelund et al. (2004) labelled instead '*Ts lauterborni* SCCAP T 2 AY496045', but is very different from AY496044, of Ekelund et al. (2004) labelled 'SCCAP T 3 *Ts seravini*' (clearly mistakenly). Furthermore Figure 4G shows that this fresh T-2 *Ts seravini* culture from Mylnikov has uniformly triangular scales indistinguishable from those originally described for this type strain by Mylnikov and Karpov (1993) and with additional micrographs by Mylnikov and Mylnikov (2003). Therefore neither Mylnikov's nor our own laboratory has mixed up this culture with another; by contrast AY496044 and AY496045 certainly have the wrong names in Ekelund et al. (2004) and in GenBank, exactly as indirectly deduced by Howe et al. (2011a).

Complementary further clarification comes from two strains of our new species *Thaumatomonas constricta* that have very similar scales to *Ts lauterborni* of Mylnikov and Karpov (1993), which are quite distinct from those seen in the other three species: oval scale with no upper tier perforations (Fig. 5B & F). Our sequences for these are closest to AY496044 (*Ts seravini* SCCAP T3) of Ekelund et al. (2004), giving independent evidence that their sequence labelled '*Ts seravini* SCCAP T3' could not possibly be from *Ts seravini* but almost certainly came from *Ts lauterborni* (T-1 of Mylnikov). Therefore, at some stage there was almost certainly a straight swap of the names *Ts seravini* and *Ts lauterborni* for these two cultures during their travels

through four laboratories to Ekelund or else of their DNAs sequenced by Ekelund et al. (2004), whose names ought to be corrected in GenBank to cease the confusion.

This assignment for the first time of the correct species name (*lauterborni*) for AY496044, together with the Ekelund's information about his labels, invalidates the assumption (Howe et al. 2011a) that Ekelund's T1 was Mylnikov's T-1. Because no reply was received from an email query to Ekelund about that, Howe et al. (2011a) cautiously labelled sequence AY496046 ?*lauterborni*, which we now know was wrong. From Ekelund's new information, that sequence came from a soil strain isolated by Vickerman (originally labelled *Allas* sp.) and donated to Ekelund; Ekelund (pers. comm.) changed the name to *Thaumatomonas* sp. 'because they were then synonyms' (a mistake, for if he believed that, he should have used the older name *Allas* for all his *Thaumatomonas* sequences: see Howe et al. 2011a). Ekelund's *Ts* sp. sequence (AY496046) closely matches sequence U42446 *Thaumatomonas* sp., one of Vickerman's cultures published by Cavalier-Smith and Chao (1995). With additional *Thaumatomonas* sequences, it is now apparent that U42446 has sequencing errors, because of its manual sequencing using autoradiography, without which AY496046 matches it completely. Evidently Ekelund's *Ts* sp. from Vickerman was exactly the same strain as Vickerman's *Ts* sp. earlier sequenced by Cavalier-Smith and Chao (1995). Vickerman recently kindly gave us a culture from his collection labelled '*Thaumatomonas lauterborni* (Karpov)', but states that "On revival I am not sure what it is, as it does not seem to behave like the original description… So Karpov's isolate may have got mixed up with someone else's isolate (e.g. mine)." We sequenced its 18S rDNA and found its sequence the same as '*Ts* sp.' AY490646; we examined its scales and saw they were most similar to *Ts coloniensis* (Sup. 5). Probably therefore

Vickerman incorrectly relabelled that *Ts* sp. strain '*Ts lauterborni* Karpov' after giving the same correctly labelled *Allas*/*Th* sp. culture to Cavalier-Smith and Chao (1995), yielding sequence U42446, and independently to Ekelund et al. (2004), yielding AY490646. The later automated sequence AY490646 is more reliable than U42446 for Vickerman's *Ts* sp. strain, which should be accepted now as a *Ts coloniensis*. Confusingly, U42446 was said to have scales 'similar to *Ts lauterborni*' (Howe et al. 2011a), based on Vickerman's early observations when the only other species known was *Ts seravini* with triangular scales and *Ts zhukovi* and *coloniensis* had not yet been described; that statement can safely be taken to mean only that *Ts* sp. scales were oval with perforations in the upper tier rather than triangular, not that it was closer in detail to *lauterborni* than subsequently described species with broadly similar perforated oval scales. It therefore does not contradict our present observations or conclusions that decisively prove one swap of names and one wrong relabeling (so altogether three past name misassignments). Our revelation of past sequencing errors means that some fine detail of the *Ts coloniensis* part of the tree in Figure 1.1 must be dominated by sequence errors, not real differences; thus Figure 2 much more reliably represents diversity and relationships in that half of the *Thaumatomonas* clade.

An authentic sample of the *Ts zhukovi* type strain (T-3) (Mylnikov and Mylnikov 2003) was given directly by Denis Tikhonenkov to TC-S in 2010 at the same time as T-2. The 18S rDNA of *Ts zhukovi* (T-3) sequence differed from all previously published but differed from *Ts seravini* (T-2) by only four distinct nucleotides (top matrix Sup. Fig. 1). That proves that no previously published sequences under other names could mistakenly have come from *Ts zhukovi* (T-3), further corroborating our assignment of names to them. Only triangular scales were observed in our single TEM preparation

(two cells viewed) for *Ts zhukovi* (T-3) (Sup. Fig. 6D); no oval scales like those also pictured in the type description were seen.

Thus, the three Russian *Thaumatomonas* strains isolated by Mylnikov (two type) all now have reliably identified published 18S rDNA sequences as follows: *Ts lauterborni* (T-1) dead (AY496044); *Ts seravini* (T-2) (available as ATCC 50636) (AF41125, EF455776, AY496045); and from this study *Ts zhukovi* (T-3; available as CCAP 1974/4; KC243119). It is now clear that all three *Thaumatomonas* strains sequenced by Ekelund et al. 2004 (two wrongly named) were the same as the three sequenced earlier by Cavalier-Smith and Chao (1995, 2003), one (*Ts seravini*) sequenced a third time by Yoon et al. (2008). Tikhonenkov donated for our use his unpublished partial 18S rDNA sequence of *Ts zhukovi* T-3 with the culture; where it overlapped with ours his sequence was identical; KC243119 is a longer contig including his and ours. Figures 1 and 2 have the corrected species names for the Ekelund et al. (2004) sequences.

Taxonomic revisions, new species, and microscopic observations

As both phylogenetic trees indicate (Fig. 1 and Fig. 2), scale structure maps coherently onto phylogenetic trees and so is given special taxonomic emphasis.

Splitting *Thaumatomastix* into three genera. All *Thaumatomastix* as redefined by Howe et al. (2011a) are marine or freshwater thaumatomonads with spine scales as well as triangular or oval plate-scales. Their long spines are of two radically different kinds, with radially symmetric bases in marine species, but asymmetric ones in freshwater species (Nicholls 2012b), giving three separate combinations of distinctly

different scale types. Our trees showed that each of the two marine clade has either triangular or plate scales, never both, but both have similar radially symmetric spine scales; as these clades are not sisters, morphology and plate scale ultrastructure congruently show that they should be made separate genera: *Thaumatospina* for the deepest branching clade with triangular scales and long scale-free anterior cilium; *Ovaloplaca* with oval scales and, unlike all other thaumatomonads and all other Cercozoa, a very short anterior cilium clothed in tiny scales. *Ovaloplaca* is sister to another new marine genus (*Scutellomonas*) that like it has oval body plate-scales, but lacks spine scales, so cannot be included in *Thaumatomastix*.

Unfortunately, ultrastructure is unknown for the type species *Thaumatomastix setifera* (Lauterborn 1899). However, comparison of the original drawings of Lauterborn with the light micrographs of Nicholls (2012b) of the only other freshwater species, *Tx triangulata* and *Tx nigeriensis,* reveals a previously overlooked feature of cell structure shared by all three freshwater species and absent from all marine ones studied by light microscopy. This makes us reasonably confident that all three freshwater species should be kept in the same genus, so we restrict *Thaumatomastix* to them alone. These three freshwater species uniformly differ from all marine ones for which rDNA sequences are available in having a conspicuous 1 µm thick cell boundary layer visible in the light microscope as a 'boxed' look to the edge of the cell. This feature is evident from Nicholls' (2012b) ultrastructural studies of *Tx triangulata* and *Tx nigeriensis*; the boxed appearance is created by the plate scales' two-tier structure, in which the scales are about 1 µm thick and the two tiers are well separated by broad columns. This wide separation of the two tiers makes the ordered boundary layer of the cell more conspicuous than in marine species; on Lauterborn's

(1899) drawing it is 1 µm thick. All marine species so far described except *Tx tripus* (Takahashi and Hara 1984), which as discussed below might be in yet another genus, have thinner scales only about 0.5 µm thick (Nicholls 2012b; Thomsen et al. 1993); though most have not been seen as living cells, in two species of *Thaumatospina* studied by us and one by Chantangsi et al. (2010) and in our new *Ovaloplaca* the scale-containing boundary layer is significantly less thick (~0.6 µm) and thus distinctly less conspicuous. We therefore conclude that the plate scales of freshwater *Tx setifera* are about 1 µm thick, making it unlikely to belong in the same genus as any of the sequenced marine species, given the deep phylogenetic separation on our trees of thaumatomonads with substantially different scale types.

Accordingly, we here restrict *Thaumatomastix* to freshwater species with 1 µm thick plate scales, now excluding the marine species, which have scales twice as thin. As explained in more detail below, spine scales of the freshwater *Tx triangulata* and *Tx nigeriensis* are radically different from, and probably not homologous with, those of marine former *Thaumatomastix*; those of *Tx setifera* are more likely than not to be similar to these two freshwater species. The marine '*Thaumatomastix*' of Larsen and Patterson (1990) from Wailupe Beach, Hawaii, was not in our view reliably identified as the same species as freshwater *Tx setifera*. It was the same length and the illustrated cell a bit wider and otherwise indistinguishable from the *Thaumatomastix* sp. micrograph of Chantangsi et al. (2010) that our trees show is an undescribed *Thaumatospina* (if its scale layer was drawn to scale, it was only about 0.5 µm thick). Thus we are unaware of any convincing marine records of *Tx setifera*. We predict that genuinely freshwater *Tx setifera* will turn out to have triangular plate scales and non-

bobbin-based radially asymmetric spine scales like both other freshwater species, and frame the revised diagnosis on that assumption:

Thaumatomastix Lauterborn, 1899. **Revised diagnosis:** Freshwater non-plasmodial thaumatomonads with prominent nucleus, spine scales, and triangular 1 µm thick plate scales visible in light microscope as a peripheral double layer with 'boxed' substructure. Plate scales two-tiered with upper and lower tier of the same size. Spine scales like somewhat smaller plate scales with long eccentrically placed spine, without basal flange, stemming at a slant from beside central hole in two-tiered base. Plate scales and spine-scale triangular bases with gently rounded vertices. Posterior cilium longer than cell; non-scaly anterior cilium half to 2/3 cell length. Ventral pseudopodia. Type species: *Thaumatomastix setifera* Lauterborn, 1899. Other species: *Tx triangulata* (Balonov 1980), *nigeriensis* (Wujek 2008).

Two types of spine scale

Both marine former *Thaumatomastix* clades have spine scales that are very different from freshwater *Thaumatomastix*, but closely similar to each other, with a long spine stemming from a base that is several times smaller than a plate scale and bearing a circular flange a bit higher up that is narrower than the base plate. The base plate, flange, and the connector joining them are radially symmetric, but the connector flares out towards the base plate (Nicholls 2012b; Thomsen et al. 1993), this flared region becoming three physically distinct splayed out struts in *Ovaloplaca* alone. The flange is narrower than the base plate; apart from that, the overall appearance of spine scale bases of marine '*Thaumatomastix*' is like a radially symmetric bobbin. Therefore we refer to these two structurally related but distinct marine spine scales as

bobbin-based, to contrast them with the radically different spine scales of two freshwater species: *Tx triangulata* and *Tx nigeriensis* (Nicholls 2012b). Though not sufficiently emphasising their extremely different structure from marine spines, Nicholls (2012b) showed that both freshwater species have eccentric spine scales that consist of two closely associated structures: the spine itself (with no sign whatever of a radially symmetric bobbin-like base or flange) is eccentrically placed on a very broad base that closely resembles the plate-scales of the same species in being composed of two tiers of equal diameter. This two-tier spine scale-base has a somewhat smaller diameter than the standard spineless plate-scales in the same species, but with similar appearance and separation (~1 um) between the two tiers. The spine shaft is plain with a tapered tip, its extreme base having a subtle flare, and joined to a plate-scale-like base, not centrally and vertically as in bobbin-based scales, but to one side of its central hole and slanting at an angle.

We consider the spine scales of both marine clades as mutually homologous, so deduce that they originated in the last common ancestor of Thaumatomonadidae and were lost independently by *Scutellomonas* and the *Reckertia*/*Thaumatomonas*/*Allas* clade. We cannot regard the radically different spine scales of the freshwater species (*Thaumatomastix* sensu stricto) as directly homologous with the bobbin-type scales of the marine species (see discussion for detailed arguments about their homology and evolution).

Thaumatospina gen. n**. Diagnosis:** Marine thaumatomonads producing plasmodia. Bobbin-like two-tiered triangular plate-scales up to 0.5 µm thick, seen as a 0.6 µm thick boxed outer edge to cell by light microscopy; plate-scale tiers of equal

size, vertices rounded, differ in internal structure. Upper tier dishlike with broad margin and steep-sided smaller central triangular depression, whose sides appear in surface view as thin dense triangular line about 2 µm inside scale edge (in side view the dish floor and side junction is sharp right angle), unlike most *Reckertia* usually without corner holes (apparent exceptions *Ta patelliformis* and *dybsoeana*); upper tier connected to flatter lower tier by a strut just inside each corner of central depression, seen in surface view as elongate dense spot just inside vertex of inner triangular dense line. Long spines (visible in LM) extend centrally from radially-symmetric bobbin-like spine-scale bases with a flange some distance above base-plate; flange markedly narrower than basal disc; connector between flange and base plate distally and basally flared, often with triple-strutted substructure within basal flare; basal disc somewhat triangular with rounded vertices, small enough to fit snugly into central depression of plate scale; shaft between basal disc and flanged ridge and flared at base. Anterior cilium obvious, third to half cell length; cilium scales not reported. Type species *Thaumatospina vancouveri* sp. n. **Etym:** *Thaumato* Gk wondrous *spina* L. spine.

Type strains, illustrations, and sequences (or any two) in all species diagnoses below are to be regarded as part of a 'syntype', which comprises all or some of the following: living culture, illustrations, and sequence(s) as explained in Cavalier-Smith and Chao (2010). Cell measurements were from gliding single cells only (not plasmodia) for cell length (CL) and width (CW) at widest point; their ratio (ovalness ratio OR) objectively indicates approximate shape numerically (1.0 round; > 1.0 elongate; <1 wide). Posterior cilium (PC) length is an average; it is also shown as X CL ratio for ready comparison with average CL. Scale dimensions are averages with ranges in parentheses: scale length (SL) and scale width (SW). All scales are two-tiered and

often have a smaller top tier in *Thaumatomonas* and *Allas*. The bottom tier (bt) is the primary measurement given in diagnoses; oval scale averages lengths (oSL), width (oSW) and OR are given, with ranges in parentheses. Where possible, top tier measurements are given (also in Table 1). The *Thaumatomonas* and *Allas* scale's middle upper portion is joined to the perimeter of the upper tier by delicate perpendicular struts associated with holes or perforations on sides, which are not always distinct and often broken. The number of these lateral irregular-shaped holes given as a range refers to one side only.

We describe three new species with over ten 18S rDNA nucleotide differences and contrasting cell shape/growth forms, but very similar scales, most like those of *Ta patelliformis* except for spine scales having three unflared terminal teeth, not two flattened flared-out ones as in *patelliformis* (Takahashi and Hara 1984):

Thaumatospina vancouveri sp. n. Type Figure 6A-B. **Diagnosis**: CL 11.9 µm (8.6 - 18.6; N = 39); CW 6.7 µm (4.6 - 9.1); OR 1.8 (1.1 - 3); PC 17.7 µm (15 - 20.9); 1.6 X CL (1.1 - 2.4). Cells strongly elongate, bulbous; ventral groove up to 2/3 CL. AC, spines, and thin (0.6 µm) 'boxed' plate scale layer visible in LM (Fig. 6). PC tip sometimes has flaring bifurcation. Ventral finger-like branching pseudopodia, tips pointed. Larger polynucleated masses or aggregates common. Strongly curved tapering hollow spine-scale; tip slightly flared, open with three points. Spines highly curved, length 4.0 µm (3.3 – 5.3; N = 17), tips narrowly trifurcate, unflared; base plate more obviously triangular than in *Ta mexica* and *arabica*, width 0.64 µm (0.5 – 0.79); flange about half base plate width. 0.28 µm (0.19 – 0.40) from base plate. Two-tier rounded triangular plate-scales with inner electron-dense line of same shape; each side 1.2 µm (0.76 – 1.50; N = 20); electron-dense strut somewhat curved, thicker at inner edges of inner electron-dense line; central region varies, some scales (especially smaller ones) with lighter inner triangle with vertices opposite centre of flat sides (Fig. 6B arrows), some large ones with

similar central light triangular area with vertices opposite scale vertices, some with additional smaller central paler rough and indented/lobed circular of various widths; upper tier has punctate markings at outer margin (barely visible in Figure 6B), apparently small tooth-like projections from its underside. Type strain: **VWB1C** (From marine surf, Wreck Beach, Vancouver, Canada. JMS). Type 18S rDNA sequences **GenBank** KC243114. **Etym:** after Vancouver city**. Comment**: As cell size was so variable several clonal cultures were made from single picked cells, but size variability remained. The offset light triangle on some plate scales might be a print left by a spine scale basal disc attached to it in vivo, which could explain why only some scales show it; however, the fact that *Ta arabica* has a similar offset pale triangle but circular base plates argues against this.

Thaumatospina mexica sp. n. Type Figure 6C-F. **Diagnosis**: Round to oval, bulbous, AC in constant motion when gliding. Finger-like pseudopodia. Cell size 6.5 - 8.4 µm long in TEM, shrunken (no live cells measured). Multiciliate and polynucleate gliding cells common. Spine-scales visible in LM. Spine-scales and triangular body-scales as for *Ta vancouveri* except body-scales consistently have roughly lobed central depression with larger, lighter rounded area overlain on the central inner electron-dense rounded triangle (vertices never opposite flat side as in *vancouveri*). Spine-scales curved to almost straight, length 4.1 µm (3.1 – 5.6; N = 18); base plate much less triangular than *Ta vancouveri*, slightly less circular than *Ta mexica*, width 0.58 µm (0.5 – 0.64); flange height from base plate 0.28 µm (0.21 – 0.40). Triangular scales 1.2 µm (0.93 – 1.43; N = 28). Type strain: **TxMex** (Marine, Mexico. JMS). 18S/ITS1&2 rDNA sequences **GenBank** KC243115/KF577833. **Etym:** *Mexica* from Mexico**. Comment**: 18S rDNA has a large 400 bp insert (position 1158 - 1558) (not repeated elsewhere) that does not BLAST to any GenBank sequence. Repeated PCR with different primers yielded the same insert.

Thaumatospina arabica sp. n. Type Figure 7B - C. **Diagnosis**: larger than *Ta vancouveri*, CL 14.9 µm (12.7 - 18.6; N = 13); CW 10 µm (9.1 - 11.8); OR 1.5 (1.2 - 1.9); PC 20.8 µm (20 -

22.7); 1.4 X CL (1.1 - 1.6). Oval to round and bulbous, long AC. Glides slowly and jiggly. Spine-scales obvious in LM. Refractile granules common in cell posterior. Lamellar and finger-like pseudopodia, possibly reticulose, sometimes produced ventrally when sessile. Ventral groove up to 2/3 length of cell. Spine-scales as in *Ta mexica* but flange edge more rounded than in *Ta mexica* and *vancouveri* (no measurements as scale bar absent from micrographs); spine hollow, flares out below flange as three struts onto near-circular base plate, tapers incompletely to subtle, short sharp trifurcate tip, two outer points more prominent than third. Two-tiered triangular plate-scales with rounded vertices, distinct inner margin, obvious evenly spaced punctate underside margin; prominent inner line with inner three-pronged mark inside each corner; central deep triangular depression shows light central triangular area with 60° offset as in some *vancouveri* scales. Type strain: **ThxDubai**. (Marine surf, Dubai. JMS). Type 18S/ITS1&2 rDNA sequences **GenBank** KC243113/KF577834. **Etym:** *arabica* L. from Arabia**. Comment**: The cell in Fig. 12 of Takahashi and Hara (1984) had spine tips more like *Ta arabica* than the type *Ta patelliformis* (their fig. 9), so might be one of the three species described here not *Ta patelliformis*. Thomsen et al. (1993) found another example of similar spines. Takahashi and Hara (1984) said there was no spine variation on the same cell but that on others spines are straighter with slightly flattened bifurcate tips. Possibly *Ta patelliformis* in these earlier papers included more than one species. '*Thaumatomastix'* sp. of Chantangsi et al. (2010), robustly sister to but genetically distant from our three species, is a fourth (undescribed) *Thaumatospina* with slightly longer spines.

New combinations. We transfer six former marine *Thaumatomastix* with similar scales to *Thaumatospina*. They fall into two contrasting morphological groups; plate scales of (b) are so different from those studied here that they might eventually merit recognition as a distinct subgenus (or genus if sequencing supported that)**:**

(a) Three species with spines and plate scales similar to *Ta vancouveri*

Thaumatospina patelliformis comb. n. Basionym *Chrysosphaerella patelliformis* Takahashi and Hara (1984 p. 106). Synonym *Thaumatomastix patelliformis* (Takahashi & Hara) Beech and Moestrup, 1986

Thaumatospina curvata comb. n. Basionym *Thaumatomastix curvata* Nicholls (2012b)

Thaumatospina sablensis comb. n. Basionym *Thaumatomastix sablensis* Nicholls (2012b) (The figure show that its diagnosis statement that spine-scales are 40 to 52 μm long is obviously a typo for 4.0-5.2.)

(b) Three species with substantially larger plate scales (struts nearer centre than edge) than *Ta vancouveri* and shorter, thicker less curved spines

Thaumatospina tauryanini comb. n. Basionym *Thaumatomastix tauryanini* Mikrjukov (2002)

Thaumatospina inornata comb. n. Basionym *Thaumatomastix inornata* Nicholls (2012b)

Thaumatospina gwaii comb. n. Basionym *Thaumatomastix gwaii* Nicholls (2012b)

Thaumatomastix tripus (Takahashi & Hara) Beech and Moestrup, 1986 differs from *Thaumatospina* as here defined in having its plate scales with 1 μm separation between the tiers; it differs from freshwater *Thaumatomastix* as here redefined by having radially symmetric spine scales with bobbin-like bases, and much longer spines than either *Thaumatospina* or *Thaumatomastix*. It therefore does not fit into any genus as here defined; until we have sequences for *T. tripus* and/or *Tx setifera* we leave it in *Tx*, even though it may not belong there; like Beech and Moestrup (1986) and Nicholls (2012b) we now consider *T. tripus* a thaumatomonad (not *Chrysosphaerella*) and that the original mention of a chloroplast, and perhaps also contractile vacuole (unusual in marine species), were errors. It might belong to the uncultured marine lineage branching between *Thaumatospina* and *Ovaloplaca*/*Scutellomonas*; its plate-scale central region is obviously different from the three

Thaumatospina we sequenced and from the other six species by having three radiating lines from the centre as in *Tx triangulata* (Nicholls 2012b).

Ovaloplaca gen. n**. Diagnosis:** Marine non-plasmodial thaumatomonads; light microscopy shows inconspicuous 0.6 µm plate-scale layer and conspicuous spines. Two-tiered oval plate-scales up to 0.5 µm thick, both tiers of similar size, upper rim thicker, or upper tier somewhat (up to 15%) smaller; often perforated in species-specific patterns. Long spines extend centrally from radially symmetric bobbin-like spine-scale bases less than half the width of a plate-scale, with flange above base-plate; flange markedly narrower than base plate; connector between flange and base plate distally and basally flared, with triple-strutted substructure resembling base of Eiffel tower within basal flare. Anterior cilium small and inconspicuous, sometimes bearing much smaller round to oval single-tier scales. Ventral branching, long, slender pseudopodia. Type species: *Ovaloplaca salina* (Birch-Andersen) comb. n. **Etym:** *Ovalis* L. oval, *placa* Galician plate, referring to oval plate-scales. We describe one new species and make four new combinations:

Ovaloplaca yabukii sp. n. Type Figure 8E-G. **Diagnosis**: CL 11.1 µm (N = 1); CW 10.5 µm (x - x); OR 1.0; PC 11 µm; 1X CL; AC not noticed. Cells glide seldom, usually gyrate in one place. Spine scale 5.18 µm (4.5 - 6.2; N = 9) with longitudinally triple ribbed spine with shortly trifurcate tip, slightly contorted but not completely twisted; straight or strongly curved near tip; prominent basal flange splays out as three short, broad struts onto rounded-triangular or round base (base ~0.5 µm diameter). Oval plate scales; tiers same size; prominent two-level central transverse band separates two D-shaped bowls; bowls regularly perforated all round edges, rarely (as *O. salina* typically is extensively) with very few in centre. oSL x oSW; 1.29 x 0.73 µm (1.2 - 1.4 x 0.6 - 0.9; N = 6); OR 1.8 (1.4 - 2.2). Type strain: **YPF609** (TKB-345 deposited

as **NIES-2378**. Marine. Tokyo Bay, Japan. A. Yabuki). Type 18S rDNA sequence differs by one nucleotide (C at position 678 instead of T at 715) from *O. salina* Norwegian strain UIO286 (FR846196) (Ota et al. 2012) in a variable region (also a few undetermined nucleotides): **GenBank** KC243118. **Etym:** after Akinori Yabuki (isolator and provider of the type sequence and micrographs). **Comment**: Very similar to *O. salina* (Beech and Moestrup 1986), which is more elongate and whose spine scales vary more in length. *O. salina* has only one definite nt difference. Because the type strain of *O. salina* no longer exists we accept UIO286 as representing it, as it is extremely well characterised and like the type was from Scandinavia, and treat Yabuki's Japanese strain as a separate extremely closely related species distinguishable by 18S rDNA sequence and plate scale perforation pattern (*O. yabukii* more often has perforations in the central region of the D-shaped depressions); *O. salina* has oval AC scales. Takahashi and Hara (1984) noted that Japanese *O. salina* was more variable in plate-scale perforation patterns and spine tip tooth length than the type or other Danish samples. Yabuki's culture is more uniform and most similar to their Fig. 18, and we did not see either the extremes of plate scales with no perforations or perforated all over the D-shaped regions reported previously. Several similar, subtly distinct cryptic species may coexist in Japanese seas. Plate scales of *O. salina* and *yabukii* have an unperforated broad central transverse band where upper and lower tiers are not in contact, unlike the uniformly perforated *O. multipora* or even more different unperforated *O. asymmetrica* scales.

Four new combinations: *Ovaloplaca salina* comb. n. Basionym *Chrysosphaerella salina* Birch-Andersen, 1973. Synonym *Thaumatomastix salina* (Birch-Andersen) Beech and Moestrup, 1986.

Ovaloplaca bipartita comb. n. Basionym *Thaumatomastix bipartita* Beech and Moestrup, (1986)

Ovaloplaca multipora comb. n. Basionym *Thaumatomastix multipora* Nicholls (2012b)

Ovaloplaca asymmetrica comb. n. Basionym *Thaumatomastix asymmetrica* Nicholls (2012b)

Scutellomonas gen n. **Diagnosis**: Colourless, heterotrophic dorso-ventrally flattened marine thaumatomonads, sometimes floating; round to oval; trailing posterior cilium emerges from subapical pit at bulbous anterior end; anterior cilium not visible in light microscope, possibly sometimes present as very short stub. Lobose, branching, finger-like pseudopodia produced ventrally from central furrow. Cell covered in two-tiered oval, unperforated plate-scales often with thick central longitudinal electron-dense line - or two lines closely parallel; upper tier appears similar size as lower tier, joined to upper tier conspicuously by inner electron-dense oval with struts at each end and at centre of sides where it is somewhat pinched in laterally and less conspicuously by these four struts at intermediate positions. Spine scales absent. Type species *Scutellomonas patella* sp. n. **Etym:** *Scutella* L. saucer or flat bowl, describing scale shape; *monas* Gk unit.

Scutellomonas patella sp. n. Type Figure 8A-D. **Diagnosis**: CL 10.6 µm (7.7 - 12.7; N = 15); CW 8.5 µm (6.4 - 10); OR 1.3 (1.1 - 1.5); PC 15.2 µm (12.3 - 19.31) 1.4 x CL (1.2 - 1.8). Round to oval, dorso-ventrally flattened cell, wobbles slowly when gliding; bulbous anterior. Ventral groove along nearly entire cell. Fast moving pseudopodia extend just over 1X CL. Refractile granules. Round cysts. Acronematic posterior cilium end often stretches when sticks to substrate during gliding. Oval two-tier plate-scale commonly (not always) has central thick line, sometimes as two close thin lines; electron-dense oval inner margin appearing ‘pinched-in’ where mid and end struts join lower and upper tiers. Prominent thickened margin on both tiers. oSL x oSW; 0.77 x 0.53 µm (0.63 - 0.91 x 0.45 - 0.64; N = 30); OR 1.5 (1.2 - 1.7). Type strain: **CA05**. (Marine. Monterey Bay, California, USA. JMS). Type 18S/ITS1&2 rDNA sequences **GenBank** KC243116/KF577835. **Etym:** *patella* L. dish. **Video**:

http://dx.doi.org/10.6084/m9.figshare.100962. **Comment**: PC length was difficult to measure on gliding cells because acronematic tips varied in elasticity and stuck to the substrate during gliding, extending/stretching the cilium. No AC observed in light microscope (a white spot sometimes seen at the head of the groove might be an end-on view of a ciliary stub), but very short scale-less cilium observed in TEM, perhaps pre-divisional because not in all specimens. An 18S rDNA sequence, AY180026 from an oxygen-depleted marine environment (Stoeck and Epstein 2003) is probably another *Scutellomonas* as it shares very distinct nucleotide signatures throughout with ours. *Scutellomonas* is most similar to *Thaumatomastix thomseni* (Tong 1997), not mentioned by Howe et al. (2011a) but transferred here to *Scutellomonas*. Tong described her cell as different from a similar one in (Vørs 1992b) ('unkendt flagellat' - unknown flagellate fig 6.48.); our scales were slightly smaller than Tong's (a wider range including smaller ones). Vørs and we did not observe cilium scales, but Tong wrote that even though she did not see a second shorter cilium its presence was implied by small scales like those typically on the AC of other *Thaumatomastix*, which in hers had inner perforations around the margin. However, as Tong studied mixed protist cultures these tiny scales might have come from another cell. We do not consider our isolate conspecific with '*Tx' thomseni* because Tong's specimen had a mucilage sheath unlike ours and its central furrow reached only half way down the cell not all along it.

New combination *Scutellomonas thomseni* comb. n. Basionym *Thaumatomastix thomseni* Tong (1997); synonym *Reckertia thomseni* Karpov (2011) - its oval scales mean that it was wrongly put in *Reckertia*, where they are always triangular.

New *Reckertia* species: *Reckertia gemma* Type Figure 8H-J. **Diagnosis**: Smooth fast-gliding oval to round dorso-ventrally flattened cell with distinct ciliary pocket apically off centre. CL 11.6 µm (10 - 13.2; N = 14); CW 9.7 µm (7.7 - 11.8); OR 1.2 (1.1 - 1.3); PC 19.1 µm (18.2 - 20.5) 1.6 x CL (1.5 - 1.8). Posterior portion of cell body raised slightly into water column when gliding. Ventrally branching lamellipodia produced centrally, possibly reticulose. Large

highly refractile irregular ball-like cell masses frequent, with multiple cilia. Clumps of irregular-shaped cells often glide/jiggle. Body scales with plain triangular base (~ 0.45 µm each side) crowned with extremely delicate, very open upper tier connected to corners of lower tier by delicate struts that branch dichotomously distally and proximally; upper tier has three-fold symmetry, consisting of a thicker triangular raised marginal band, each side bearing three more slender forked (Y-shaped) connectors joined at the centre by the fork stems; distal to this latticework is a denser triangular domed 'jewel' with curved edges connected at its corners by three extremely slender dichotomous branching strands on either side of the centre of each marginal band inside the junctions with the six forked ends of the Y-shaped connectors, forming a 'crown' that is a third layer distal to the upper tier. Jewel triangle vertex to vertex 0.17 µm (0.15 - 0.2). Oval scales with thickened margin and central thick line thickly coat AC (Fig. 8I); oSL 0.31 µm x 0.17 µm (0.25 - 0.36 x 0.15 - 0.19). Type strain **VWB1Thas** CCAP 1950/1. (Marine surf, Wreck Beach. Vancouver, Canada. JMS). Type 18S/ITS1&2 rDNA sequences **GenBank** KC243117/KF577831. **Etym:** *gemma* L. jewel. **Comment**: Scale struts and connectors often broken (thus hard to measure) but the jewel remains intact. Although pseudopodia are generally extruded from the centre of the ventral side, they sometimes seemed to come from lateral surfaces as in the type *Reckertia sagittifera* (Conrad 1920) that had an extremely long AC, very different from *R. gemma*'s which is short (Fig. 8J). Even so, a cell with similar dimensions to Conrad's observed in EM (Thomsen et al. 1993, figure 3) had ciliary scales similar to *R. gemma*, but a different type of triangular crowned scale with no apparent jewel. *Reckertia dybsoeana* (Thomsen et al. 1993, figure 15) has similar scales with a jewel in the crown of the triangular scale like *R. gemma*, but it is not the same species since its cilium scales are circular or nearly so with a non-elongated central density, markedly unlike the strongly elongated ovals in *R. gemma*, and the triangular jewel of *R. dybsoeana* has straighter sides (Thomsen et al. 1993 figures 16, 17 shows body-scales of an unnamed Danish species which could have been *R. gemma*; their Finnish scale (Fig. 14) is probably another undescribed

species). Figure 3A of Gooday et al. (2006) called *Tx dybsoeana* was certainly misidentified (the central three-fold part symmetric of its upper tier has strongly inward curving edges as in Fig. 14 of Thomsen et al. 1993 (but is proportionately much larger), not straight or slightly outward curving edges as in *R. dybsoeana*), and is probably an undescribed *Reckertia* species scale with apparently two distinct superimposed upper-tier structures of three-fold symmetry analogous to but very different in detail from *R. gemma* or *dybsoeana* scales, suggestive of an intermediate morphology between the highly dissected upper tier of *R. gemma* and *dybsoeana* and more sheet-like upper tiers of *R. filosa* (Howe et al. 2011a) and *R. formosa* that both lack the third crown layer (see discussion for further comparisons). Their Fig. 3B *Thaumatomastix* sp. is a different undescribed *Reckertia* with even narrower three-fold central upper tier region without a gem. We suspect that Fig. 3C of Gooday is also misidentified as *R. formosa* as its upper tier seems smaller than the lower, with supporting struts nearer its corners than in *R. formosa* where both tiers are equal in size. *R. dybsoeana* was misspelt in the transfer of *Thaumatomastix dybsoeana* to *Reckertia* by Howe et al. (2011a) because the legend of the type illustration (Figure 14-17) misspelt it as dybsoena, which contradicted that in the original diagnosis (Thomsen et al. 1993); the correct name is *Reckertia dybsoeana* (Thomsen et al.1993).

Two new *Allas* species from soil. Both have quasi-oval, but bilaterally asymmetric, two-tier scales with a median lateral strut on one side only connecting the upper and lower tiers, as in *Allas* aff. *diplophysa* (Howe et al. 2011a) but unlike *Thaumatomonas* which has end struts only. Both species are smaller than *A. diplophysa* and their scale upper tiers have many more and smaller lateral holes (Fig. 9) and are obviously mutually different. 18S rDNAs have 19 nucleotide differences between the three species; we noted two distinct *Allas* 18S rDNA sequence signatures: TCCAT and GACT (at positions 1395 and 1717 of AF411626).

Allas multipora sp. n. Type Figure 9A-B. **Diagnosis**: Gliding cells often rounded, smaller on average than *A. diplophysa*. Prominent AC and ventral groove from apical end. CL 12.2 µm (10 - 13.6; N = 16); CW 9.6 µm (7.3 - 13.2); OR 1.3 (1 - 1.7); PC 13.8 µm (12.3 - 15.9) 1.1 x CL (1 - 1.4). Branching pseudopodia not readily observed, quickly retracted. Two-tier scales with three struts supporting upper tier; end struts predominantly single and broad (two closely positioned narrow ones in *A.* aff. *diplophysa*), narrow at base broadening considerably at upper end, appearing in surface view as dense U shape with terminal granules at each end of trampoline-like upper tier; lateral strut splayed at each end, giving slightly forked appearance, narrower distally than end struts, in surface view resembling a small dense 'c' (rarely missing). Some struts misshapen, with two struts at same end. Up to nine small lateral holes on strut free side; fewer on strutted side as hole beside lateral strut is larger. Oval scales; oSL x oSW 0.61 x 0.4 µm (0.44 - 0.84 x 0.28 - 0.49; N = 28; OR 1.7 (1.2-2.2). Type strain: **GSPB9** CCAP 1903/3. (molehill soil, Galicia, Spain. K. Vickerman. 2001). Type 18S/ITS1&2 rDNA sequences **GenBank** KC243107/KF577829. **Etym:** *Multi L.* many *pora* L. pores. **Comment**: Figure 9B multiple oblique views make it clear that though the lower tier of *Allas* scales is essentially elliptical, the upper tier is a laterally squashed triangle with a structurally homologous strut supporting each vertex; degree of triangularity varied, some being more oval than others; the most oval scales were measured. Being even smaller than *A.* aff. *diplophysa*, it is even less like the type species *A. diplophysa* (average size 18 µm), and merits a new name.

Allas media sp. n. Type Figure 9C-E. **Diagnosis**: Large polynucleate and smaller gliding cells. Smooth gliding with obvious AC; smaller cells with proportionally longer PC than *A. diplophysa*. Filose pseudopodia retracted quickly and often raised in water-column. CL 11.4 µm (9.6 - 13.2; N = 20); CW 8.9 µm (7.3 - 10); OR 1.3 (1.2 - 1.6); PC 17.5 µm (14.1 - 19.1) 1.5 x CL (1.3 - 1.6). Scale lower tier oval; upper tier rarely oval with only end struts (e.g. Fig. 9D near bottom right), usually a non-equilateral triangle with rounded vertices each with one U or C shaped strut; up to 10 very small upper-tier holes/perforations on longest side; two end holes

much smaller than in *A. diplophysa* or *A. multipora*, sometimes not much larger than hole beside lateral strut. End struts as in *A. multipora* but U more sharply curved; end holes large, circular or somewhat D-shaped. Scales; oSL x oSW; 0.58 x 0.47 µm (0.48 - 0.68 x 0.38 - 0.62; N = 41); OR 1.2 (1-1.4). Type strain: **BKARH9**. (Beaver Creek Arizona, USA. K. Vickerman. 2001). Dried riverbed sediment. Type 18S/ITS1&2 rDNA sequences **GenBank** KC243108/KF577830. **Etym:** *medius* L. middle, because number of perforations and shape of scale intermediate between other species. **Comment**: *A. media* 18S rDNA sequence differs from *A. multipora* by more than 15 nucleotide differences, and from *A.* aff. *diplophysa* by 6; the MrBayes ITS tree that makes *A.* aff. *diplophysa* and *media* sisters better fits this than ML trees that do not. If that topology is correct *Allas scales* probably had numerous lateral holes ancestrally, reduced in *A.* aff. *diplophysa*, and also only three struts consistent with their ancestral scale having been triangular as argued in the discussion. Two other strains from K. Vickerman had the same 18S rDNA sequence: BKARH11 (dried Arizona riverbed); SH4_B4 (Sourhope, Lancaster, UK), so *A. media* is widely distributed and common.

Two new *Thaumatomonas* species from soil

Thaumatomonas solis. sp. n. Type Figure 5C-D. **Diagnosis**: Cells broad, often irregular with flared posterior. Branching ventral pseudopodia. CL 14.2 µm (10.9 - 15.9; N = 5); CW 11 µm (8.6 - 12.7); OR 1.3 (1.2 - 1.5); PC 15.9 µm (N = 2); 1.2 x CL (1 - 1.5 N = 2). Two-tiered oval scales with plain base and margin. Top tier with three or five struts at each end and a central oblong plate with 4 - 8 lateral holes on each side; end holes roughly D-shaped; oSL x oSW; 0.57 x 0.31 µm (0.5 - 0.7 x 0.27 - 0.36; N = 26); OR 1.9 (1.6 - 2.3). Type strain: **BZ8** CCAP 1974/3 (soil, Brazil. JMS). Type 18S rDNA sequence **GenBank** KC243111. **Etym:** *solum* L. soil. **Comment**: Most cells are dorso-ventrally flattened, but some are so irregularly shaped to be unstable when gliding. Scales most resemble *Ts coloniensis* and *Ts oxoniensis*, but *Ts solis* on average has more lateral holes and often only three end struts; when there are five the two end ones are typically closer

together suggesting accidental doubling. The closest sequence from strain ATCC50250 (AF411261) has one nucleotide different at the end, possibly an error in which case it should be the same species. Strain CH9 (China, soil) *Ts* aff. *solis* with six clear 18S rDNA nucleotide differences (KC243112) died before cellular comparisons could be made so is not made a new species as it probably deserved; ITS trees suggest that the *Ts solis*/ *Ts* aff. *solis* lineage is sister to the *Ts coloniensis*/*lauterborni*/*constricta* clade (Fig. 2); PhyloBayes for 18S rDNA shows the same (Fig. 1), so its placement as sister to all other *Thaumatomonas* plus *Allas* in the ML and MrBayes 18S rDNA trees is probably a long-branch artefact. This lineage has three distinct nucleotides absent elsewhere in the Monadofilosa alignment (positions 993, 995, 1082).

Thaumatomonas constricta sp. n. Type Figure 5E-G. **Diagnosis**: Smoothly gliding elongate, oval cells; posterior sometimes acutely pointed. Long branching pseudopodia readily produced, sometimes broad in larger cell aggregates. CL 12.2 µm (6.8 - 15.9; N = 11); CW 7.4 µm (5 - 9.6); OR 1.7 (1.2 - 2.2); PC 15.5 µm (11.4 - 18.2) 1.3 x CL (0.9 - 2.5). Elongate oval body scales; bottom tier plain oval with thickened margin; Top tier narrower centrally than at curved ends, each supported by three (occasionally four) narrow struts beside large hole; slightly domed centrally; lateral holes absent; oSL x oSW; 0.57 x 0.28 µm (0.44 - 0.72 x 0.22 - 0.35; N = 25); OR 2.1 (1.7 - 2.4). Type Strain **CH3** (soil, China. JMS. 2009). Type 18S/ITS1&2 rDNA sequences **GenBank** KC243110/KF577826. **Etym:** *constrictus* L. compressed - to emphasize that the trampoline-like upper tier central element is narrower than its denser curved supporting ends. **Comment**: Another *Ts constricta* strain from Brazil (BZ1, CCAP 1974/2) with identical (but terminally partially missing) 18S rDNA sequence (KC243109) had the same ITS1 sequence, and three differences in ITS2 rDNA (KF577825) but no CBCs and similar scales (Fig. 5B) except that the upper tier seemed to sag downwards rather than bow upwards. BZ1 has a flared acronematic PC tip (figure 5A), not noted in live type strain CH3, though TEM shows a subtly tapered tip with a lighter swollen membranous region (Fig. 5G), similar to *Ts zhukovi* strain NC02 (Fig. 10H). The dead Panama strain P106 (sequence KF577827) has >20

differences in ITS2 rDNA from *Ts constricta*, with two CBCs and seven hCBCs making them likely different species, but not describable without micrographs. The scales look most similar to *Ts lauterborni* (Mylnikov and Karpov 1993) but dimensions are different: *Ts contricta* is thinner.

Transfer of *Reckertia hindoni* to *Thaumatomonas*: We regard freshwater *'Reckertia' hindoni* (Nicholls 2012b) with triangular two-tier scales as a *Thaumatomonas* for four reasons: (1) no scales were observed on the anterior cilium (anterior ciliary scales occur in both phylogenetically well characterised *Reckertia* plus the type species *R. sagittifera*, but in no *Thaumatomonas*); (2) *R. hindoni* scales are completely different from the triangular crowned open lattice type seen in *Reckertia gemma* and *dybsoeana*; (3) their struts are more like those of *Thaumatomonas* triangular scales than the curious tripartite type seen in those *Reckertia* with sheet-like upper tiers (e.g. *R. filosa*: Howe et al. 2011a, or *R. formosa*) and the conspicuous lateral holes along each side, closely resemble those of all triangular scales of *Thaumatomonas*, notably *Ts seravini*, whereas no phylogenetically characterised *Reckertia* have such an arrangement of lateral holes; (4) All *Reckertia* are marine. *R. hindoni* scales are most similar to the perforated triangular scales *Ts seravini*, but *R. hindoni* does not have extra-big corner holes, and the other perforations of *Ts seravini* are larger and the scale vertices are less gently curved in *R. hindoni*. Considering the variation in perforation size amongst closely related species (*Allas*, Fig. 9), such differences do not argue against *R. hindoni* being a *Thaumatomonas* so we remove it from *Reckertia* (see discussion). *Thaumatomonas hindoni* comb. n. Basionym *Reckertia hindoni* Nicholls (2012b p. 12)

Four new *Thaumatomonas* subspecies: We isolated eight strains of *Thaumatomonas* strains with very similar rDNA sequences to *Ts zhukovi* (Fig. 2). Two of these (NC02/3) form a distinct subclade and differ sufficiently in ITS2 rDNA secondary structure (sup. Fig. 1) that it could be argued that they may be separate species. However Fig. 2 places them as sister to a clade of five strains that do not differ from each other or from *Ts zhukovi* in ITS2 CBCs or hCBCs, and whose scales do not distinguish them unambiguously from *Ts zhukovi*. As we would regard these five clones as *Ts zhukovi*, we decided not to treat to NC02/3 as separate species, but make them new subspecies of *zhukovi* to recognise their ITS distinctiveness. Their scales are very similar: predominantly oval with two inter-tier end struts but some are more irregular with a third strut making them non-equilateral triangles (Figs 10AB, GI). *Thaumatomonas zhukovi* originally had a mixture of oval scales, equilateral and irregular triangles and even ones with four struts, but when we re-examined it we saw only equilateral triangles (Supplementary Fig. 6D). Strikingly the five new strains with *zhukovi*-like ITS2 had different ranges of scale morphology: two (GMKL4, GMBGL1; Fig. 10C,D) had only oval scales with 4 or 5 struts at each end and 5-8 lateral holes on each side, and the large terminal holes were shaped like a curved sausage (as in *Ts seravini varisquama*), not circular as in all other *Ts zhukovi*; the other three (UPL1Bf2 CCAP 1974/1, UPL1Be2, CCL4B; supplementary Fig. 6) had a mixture of oval and triangular scales, usually not exactly equilateral; their oval scales differ from those of GMKL4, GMBGL1 in end strut structure, having a single broad end strut with denser edges, which in UPL1Bf2 only may be partially or completely subdivided into two probably separate struts; lateral holes of UPLBf2 are very similar to those of GMKL4/GMBGL1, but UPL1Be2 and CCL4B have fewer larger lateral holes (4-5 per side). The eighth new

zhukovi-like strain (HANTSF8) also had a mixture of ovals and non-equilateral triangles but groups on the tree weakly as sister to two *Ts seravini* strains, which branch from within the *Ts zhukovi*-like strains. Furthermore the new *Ts seravini* strain (4e) has a mixture of oval and equilateral triangular scales in contrast to the original *Ts seravini* (T-2, whose scales were all equilateral triangles (Mylnikov and Mylnikov 2003)). These observations indicate that scale shape is developmentally and evolutionarily quite labile in the *zhukovi*/*seravini* clade, but that developmental variability may vary from strain to strain (and may even have changed during long-term culture of T-3), and is not a sound basis for making separate species. Thus scale variation in our nine new strains and apparent absence now of oval scales from T-3 have almost broken down the original morphological distinctions between *Ts zhukovi* and *seravini*; it would be not unreasonable to make *Ts seravini* just a subspecies of *zhukovi*. However, for now we conservatively retain it as a separate species because T-2 differs from all 10 other strains studied here in one ITS2 CBC. We treat HANTSF8 as a subspecies (*saxoni*) of *zhukovi* and 4e as new subspecies *varisquama* of *Ts seravini*; morphologically we can say that in this present usage *Ts seravini* has either equilateral triangle or oval scales whereas *Ts zhukovi* has oval and or typically non-equilateral triangular scales. For clarity we first revise the nominal subspecies diagnosis of *Ts zhukovi* to contrast it with the others:

Thaumatomonas zhukovi zhukovi Mylnikov in Mylnikov and Mylnikov (2003). **Revised diagnosis:** CL 6.6 – 13.1 µm; CW 4.5 – 8.2 µm, PC 15 – 19.4 µm, 1.1 – 2.3 x CL. Scales variable: triangular or a mixture of triangular, oval and irregular: triangular scales equilateral or often irregular with elongated upper-tier apertures and broadly fused struts sometimes with two fusion points distinctly visible. Oval scales have either distinct struts and small upper-tier

perforations with between 3 - 8 upper-tier perforations on one side, or broadly fused struts and darkened round or elongated upper-tier apertures with 2 - 8 perforations along each side; the terminal hole typically circular, rarely elongated curved sausage-shape. Type strain clone T-3 (Mylnikov and Mylnikov 2003). **Comment:** Five new *Ts zhukovi* strains form a distinct ITS rDNA clade because ITS1 of the type T-3 differs by 24 – 25 nucleotides, but we treat them as the same species because ITS2 rDNA is so similar and UPL1B strains have virtually the same scale morphology as T-3, though the two GM strains, one from Oxford and the other from Keele University, England, clearly differ (Table 3 gives provenance of *Thaumatomonas* strains).

Thaumatomonas zhukovi saxoni sp. n. Type figure 4, A-B. **Diagnosis**: CL 10.3 µm (7.3 - 12.7; N = 23); CW 7.8 µm (5.9 - 10.9); OR 1.3 (1 - 1.9); PC 11.1 µm (6.8 - 12.7) 1.1 x CL (0.9 - 1.4). Round to elongate cell with ventral groove up to 2/3 length of cell. AC short. Sometimes asymmetric apical notch at ciliary pocket. Finger-like pseudopodia produced ventrally from centre of stationary cell, sometimes extensive, up to 3x CL. Scales two-tier: oval and irregular-shaped triangular, 2 – 10 lateral upper-tier holes with circular aperture at either end; end holes circular. Bottom tier; oSL x oSW; 0.63 x 0.3 µm (0.45 - 0.85 x 0.28 - 0.38; N = 36); OR 1.9 (1.4 - 2.3). Type Strain **HANTSF8** (Molehill, Longstock, Hampshire. UK. KV). ITS1 & 2 rDNA sequence **GenBank** KF577808. Differs by two hCBCs in ITS2 rDNA secondary structure from the *Ts zhukovi zhukovi* type T-3 strain. **Etymol**: *saxoni* L. as collected from a former Saxon region of the UK. **Comment**: Scales are very regular but some show a propensity to become triangular: rather like *Allas* but very different from T-3. The top tier bows upward, not always visible in TEM. The upper-tier lateral perforations can be up to two holes more than any seen in *Ts zhukovi zhukovi*. This subspecies is the most rounded (OR 1.3) of all *Ts zhukovi zhukovi* strains studied.

Thaumatomonas zhukovi carolinensis subsp. n. Type Figure 10G - I. **Diagnosis**: CL 12.2 µm (8.6 - 15.6; N = 20); CW 6.8 µm (4.6 - 9.1); OR 1.8 (x - x); PC 14.4 µm (11.4 - 16.4) 1.2 x CL (1

- 1.6). Smooth gliding with short AC constantly beating. PC tip noticeably plastic, often momentarily sticking to substrate. Oval and triangular two-tiered scale often with two broadly fused struts at either end with 3 - 5 lateral upper-tier holes. Circular to oval upper tier end apertures. Oval scales more common than triangular; end holes somewhat oval. oSL x oSW; 0.55 x 0.33 µm (0.50 - 0.59 x 0.29 - 0.35; N = 10 x); OR 1.7 (1.5 - 2). Type Strain: **NC02**. (Soil, near stream, North Carolina, USA. JMS. 2010). ITS1 & 2 rDNA sequence **GenBank KF577814**. Differs by three ITS2 rDNA CBCs from T-3 *Ts z. zhukovi* (Mylnikov and Mylnikov 2003), and four hCBCs. **Etym:** From Carolina state.

Thaumatomonas zhukovi paracarolinensis subsp. n. Type Figure 10A - B. **Diagnosis**: CL 10.7 µm (7.3 – 13.6; N = 16); CW 6.8 µm (4.1 – 9.1); OR 1.6 (1.1 – 2.5); PC 14 µm (11.4 – 15.9) 1.3 x CL (1.0 – 1.7). Smooth gliding with AC constantly beating. PC end noticeably plastic, often momentarily sticking to substrate. Mainly oval, sometimes irregular triangular scales; ovals with two broadly fused struts at each end; end holes usually circular, rarely slightly laterally oval; 2 - 5 upper-tier lateral holes. oSL x oSW; 0.54 x 0.32 µm (0.45 - 0.73 x 0.28 - 0.38; N = 18); OR 1.7 (1.3 - 1.9). Type strain **NC03** from moss and lichen, North Carolina, USA. JMS. 2010. 18S rDNA sequence same as *Ts z. zhukovi* T-3. Type ITS1 & 2 rDNA sequence **GenBank** KF577815. ITS2 rDNA has two 2 CBCs and 3 hCBC differences from T-3 *Ts z. zhukovi* and one CBC compared with *Ts z. carolinensis*. **Etym:** *para* Gk. beside, as it is sister to *T. z. carolinensis*.

Thaumatomonas seravini varisquama subsp. n. Type Figure 4C,D. **Diagnosis**: Differs from *Ts seravini seravini* T-2 (with identical 18S rDNA) by having oval scales as well as triangular ones. CL 11.9 µm (6.8 - 14.6; N = 18); CW 9.5 µm (4.6 - 13.2); OR 1.3 (0.9 - 1.9); PC 14.7 µm (8.2 - 16.8) 1.2 x CL (1 - 1.4). Cell posterior often wide and irregular. Smooth gliding with small AC. Stationary cells make branching finger-like and lamellar pseudopodia. Cells sometimes attached together by pseudopodia and glide or wobble. Oval scales have 4-5 struts at each end; about 5 lateral holes per side; large end holes not circular as in *Ts z. zhukovi* and

saxoni, but a curved sausage-shape as in *Ts z. carolinensis*/*paracarolinensis* oSL x oSW; 0.56 x 0.31 µm (0.48 - 0.61 x 0.28 - 0.34; N = 6); OR 1.9 (1.6 - 2.1). Triangular scale (roughly equilateral) 0.48 µm (0.46 - 0.52; N = 3). Type strain **4e** (Woodland soil, moss and lichen, Oxford, UK. JMS. 2009). Type ITS1 & 2 rDNA sequences **GenBank** KF577824. ITS2 differs from nominal subspecies by an enlarged loop in helix III where CBCs are usually present. **Etym:** *vari* L. various s*quama* L. scale. **Comment**: TEM yielded only few, often broken scales; large polynucleate cells with multiple cilia exhibited few scales.

***Thaumatomonas* aff. *seravini* strain IVY18a** (from an ivy leaf, Oxford, U.K. A. Howe, 2007). This strain groups with *Ts seravini* on trees and could be that species. However its 18S rDNA sequence (KC243106) has one significant difference (pos. 1659) from all the other 23 *Thaumatomonas* sequences in a conserved region, so it might a separate species, but without scale or ITS sequence data we cannot verify that. The original *T. seravini* sequence AF411259 (ATCC 50636) had a missing nucleotide, probably an error as a nucleotide is present in this position in all other monadofilosan sequences including our resequenced T-2. We briefly describe this strain: CL 10.7 µm (7.7 - 12.3; N = 14); CW 7.38 µm (4.6 - 8.6); OR 1.5 (1.2 - 1.7); PC 13.1 µm (11.4 - 15.9) 1.2 x CL (1.1-1.5). Smooth gliding oval to round also egg-shaped; cell shape and size vary, sometimes quite broad with irregular asymmetric indent in posterior half. Stationary cells include aggregates; branching pseudopodia readily formed, varying in thickness, sometimes with eruptive broad ends.

New Genus *Cowlomonas* Diagnosis: Very fast gliding, sometimes floating, dorso-ventrally flattened and rounded colourless heterotrophic biciliate with two cilia of unequal length emerging from subapical pit continuous with a ventral groove. Prominent anterior hood extending as a ridge ventrally towards the posterior on the right side. Pseudopodia emerge ventrally from posterior end. Like *Esquamula* centrioles divergent, not parallel as in most thaumatomonads; unknown whether

scales present or absent. **Type species *Cowlomonas planata*** sp. n. Type Figure 7A.

Diagnosis: CL 11.8 µm (9.1 - 13.6 N=4); CW 10 µm (6.8 - 11.8 N=4); CL x CW ratio 1.2 (1-1.34 N=4); AC 8.3 µm (6.8-10 N=3); PC 20.6 µm (20.5 - 20.9 N=3); PC = 1.84 x CL (1.5 - 2.25 N=3)**.** Broadly elliptical, dorso-ventrally flattened gliding cell with distinct anterior hood, two conspicuous subapical cilia of different lengths; longer PC reflexed backwards during gliding can point forwards during pseudopodial feeding. Cell glides smoothly, very fast, with trailing PC; AC constantly beats stiffly, not undulatorily. In floating/swimming cell PC in constant sporadic motion, jerking the cell, PC repeatedly relaxes then stiffens, slightly bent at midpoint. Stationary/feeding cell extends thick finger-like, weakly branching, mainly blunt pseudopodia ventrally from sometimes visible posterior opening; cilia then inactive. Two or three highly refractile bodies up to 3 µm. Cysts not observed. Unlike *Protaspa*, but like *Esquamula*, nucleus not readily seen by light microscopy. Strain VWB1B, now dead, from marine surf, Wreck Beach, Vancouver, Canada. **Etym:** *Cowl* E. a monk's hood, L. *planus* flat. **Comments**: Cultured using 50% artificial seawater, there were never enough *Cowlomonas* cells to sustain a stable culture and allow DNA extraction. A pico-sized colourless chrysomonad contaminant, possibly a food source, was often attached to the gliding cells via a long stalk and trailing behind higher up in the water column; if *Cowlomonas* required eukaryotic food, that could explain why single-cell picking attempts to obtain a mono-protist culture were fruitless. It is most similar to the only known scale-free thaumatomonad *Esquamula lacrimiformis* (Shiratori et al. 2012), also marine. *Esquamula lacrimiformis* differs in shape (teardrop rigid cell, commonly with granulated surface) and is smaller (4.5-11.3 X 3.9-8.8), though size overlaps, with shorter cilia (anterior half that of *Cowlomonas*, posterior three-quarters. Their behaviour is similar: smoothly gliding cells with thick ventral pseudopodia, but the anterior cilium of *Esquamula* is almost motionless. We do not place *Cowlomonas* in *Esquamula* because cell shapes differ, *E. lacrimiformis* pseudopodia were not commonly emitted from the posterior as in *Cowlomonas*, and especially because we failed to prove the absence of scales (three attempts at TEM whole mount

preparation failed even to show cells). *Cowlomona*s and *E. lacrimiformis* both have a deep ciliary pocket that continues as a ridge along the ventral side of the cell (Shiratori et al. say 'groove' although the continuation of the flagella pocket seems more a ridge than groove), but *Cowlomonas* is more flattened and has an obvious hood at the apical end, absent in *Esquamula*. The lack of an obvious large nucleus as in the thecofilosean *Protaspa*, which also combines an anterior ciliary pit and ventral pseudopod-emitting groove, makes it less likely to belong in Cryomonadida than in Thaumatomonadida. The other major flagellate group combining posterior ciliary gliding and pseudopodia is Sulcozoa (Cavalier-Smith 2013), but we saw no evidence of a distinct dorsal pellicle, and the ciliary pit and its surrounding cowl continued on the right side by a ventral ridge is much more like *Esquamula* than any known sulcozoan. No sign of scales as are evident in *Thaumatospina arabica* (Fig. 10b) was seen by light microscopy so we place *Cowlomonas* in Esquamulidae; even if scales were present that position need not be wrong as sequence trees show that *Esquamula* lost them secondarily.

Two superficially somewhat similar marine flagellates were considered as potential relatives because of their cowl-like anterior end, ciliary pits, and posterior gliding cilium: *Abollifer prolabens* (Vørs 1992a) and *Heterochromonas opaca* (Lee and Patterson 2000). However, neither has pseudopodia and *Cowlomonas* is thus distinct from both. *Abollifer* glides on its anterior cowl-like swelling, with its rear end is raised high above the substratum. It has only one cilium (a second sometimes present is presumably predivisional). *H. opaca* has similar cell shape and two similarly proportioned cilia to *Cowlomonas* but is larger. Neither *Abollifer* nor *Heterochromonas* has been placed in a phylum; we suspect that they are mutually related, probably both Cercozoa, but not close to *Cowlomonas*; we place them incertae sedis within Ventrifilosa.

New ventrifilosan orders and family

Several undoubted ventrifilosan genera for which rDNA sequences are available have not been placed in orders. To rectify that deficiency we establish:

Variglissida Cavalier-Smith ord. n. **Diagnosis:** scale-free cercozoan zooflagellates with only one long posterior cilium used for gliding on surfaces; either dorsally rigid cells with ventral groove and no pseudopodia and reduced or absent anterior cilium (Clautriaviidae Cavalier-Smith in Cavalier-Smith and Scoble, 2013) or soft-bodied cells with filose branching and reticulose pseudopodia and prominent anterior cilium (Nudifilidae Cavalier-Smith in Howe et al., 2011a)**.** Phylogenetically most closely related to Marimonadida and Euglyphida; grouped with them in imbricate subclass Placonuda.

Discomonadida Cavalier-Smith ord. n. **Diagnosis:** as for sole included family: **Discomonadidae** Cavalier-Smith fam. n. **Diagnosis:** rigid, round disc-like dorso-ventrally flattened gliding zooflagellates with two conspicuous cilia, with divergent centrioles sub-apically inserted behind apical notch; large nucleus conspicuous in light microscope as in *Protaspa*, but unlike them with no pseudopodia; glide on posterior shorter cilium. Type genus *Discomonas* (Chantangsi and Leander 2010). Placed incertae sedis within cercozoan superclass Ventrifilosa.

Though no sequences are available for *Zoelucasa*, its imbricate scales suggest that it is related to euglyphid filose amoebae in Imbricatea, but Nicholls (2012a) considered that it cannot be placed in an existing order. Its parallel ciliary bases, reflexed posterior cilium and large prominent nucleus are all features shared with thaumatomonads, but as its scales differ from any so far known in thaumatomonads, strongly overlap like those of euglyphids and are not known to be made in association with mitochondria, and it lacks a ventral groove and posterior ciliary gliding, we do not place it in Thaumatomonadida. The absence of pseudopodia and presence of cilia equally strongly differentiate it from Euglyphida, so we establish a new order and family for it within superorder Euglyphia:

Zoelucasida Cavalier-Smith ord. n. **Diagnosis:** as for sole included family: **Zoelucasidae** Cavalier-Smith fam. n. **Diagnosis:** non-gliding biciliates cells with reflexed posterior cilium with parallel centrioles, large nucleus, and rigid lorica of large imbricate, probably silicified oval scales; scales probably double-layered, with meshwork connecting the smooth unperforated layers. Type genus *Zoelucasa* Nicholls, 2012.

Discussion

Our results strengthen the view that scale structure and rRNA phylogeny are highly congruent in thaumatomonads (Howe et al. 2011a): the seven silica-scaled thaumatomonad genera now sequenced group discretely and logically according to scale morphology on our trees. Our most striking findings are of two phylogenetically distinct clades of marine former *Thaumatomastix*, for which we established two new genera, *Ovaloplaca* and *Thaumatospina*, and the discovery of *Scutellomonas*, which has oval plate scales like its sister *Ovaloplaca*, but no spine scales. Discovery of another *Reckertia* species confirms that *Reckertia* form a distinct clade. As previously emphasized (Howe et al. 2011a), molecular divergence in 18S rDNA between *Thaumatomonas* species is low, but by sequencing also ribosomal DNA ITS regions for numerous new strains, studying ITS2 secondary structure, and resequencing several strains we were able to sort out definitively a long-standing historical muddle about which sequences belong to which species (see Howe et al. 2011a), establish two new species and four new subspecies, put one old 'species' in synonymy with *Thaumatomonas coloniensis*, and show that *Allas* (with two new species) and *Thaumatomonas* are phylogenetically and morphologically distinct.

Splitting *Thaumatomastix* into three genera

Howe et al. (2011a) removed species without spine scales from *Thaumatomastix* and put them in *Reckertia* (Conrad 1920), of which there are now 10 species not 11, since the freshwater *R. nigeriensis* was shown to have eccentric spine scales and moved back to *Thaumatomastix* (Nicholls 2012b), so *Reckertia* is at present exclusively marine. Our trees confirm that neither clade with spine scales groups with *Reckertia*, and thus fully support that narrowing of the genus. Until recently all spine-bearing *Thaumatomastix* studied ultrastructurally were marine and it had been assumed that their spine scales were homologous with those of the freshwater type species *Tx setifera* Lauterborn and that they therefore belong to the same genus. However, Nicholls (2012b) has shown that two freshwater *Thaumatomastix* species (*triangulata*, *nigeriensis*) have spine scales that cannot be regarded as homologous with those of marine *Thaumatomastix*. Beech and Moestrup (1986), who first moved *C. triangulata* to *Thaumatomastix*, gave too little weight to the marked differences in spine-scale bases between *C. triangulata* and marine *Thaumatomastix* that we emphasised above. Howe et al. (2011a) moved *Thaumatomastix triangulata* (originally *Chrysosphaerella triangulata* (Balonov 1980)) back to *Chrysosphaerella* because it contradicted the original description of *Thaumatomastix*. But Nicholls (2012b) returned *C. triangulata* to *Thaumatomastix* because he found a cell with scales very similar to Balonov's first description of *C. triangulata*, but which lacked chloroplasts in contradiction to Balonov. Though we cannot exclude the possibility that Balanov really described a *Chrysosphaerella*, we provisionally accept Nicholl's (2012b) assumption that Balanov made several mistakes when describing it and interpretation of this species as a freshwater *Thaumatomastix* with scales very similar to *Tx nigeriensis*.

The type species *Thaumatomastix setifera* of Lauterborn (1896, 1899) had spine scales and a prominent 1 μm thick layer at the surface of the cell resembling small boxes (Lauterborn 1896, 1899), which we interpret as the plate-scale layer in side view. The LM images and drawings of *Tx nigeriensis* (Nicholls 2012b) resemble the type description of *Thaumatomastix setifera*, for which ultrastructural detail of the scales can never certainly be known. All three freshwater species, *Tx nigeriensis*, *Tx triangulata*, and *setifera*, exhibit a distinct eccentric nucleus, spine scales, boxed cell boundary layer, and pseudopodia. All other former *Thaumatomastix* species are marine, and all except *Thaumatospina tripus* have plate scales with the two tiers separated by ~0.5 μm, not 1 μm as in freshwater species.

Howe et al.'s (2011a) argument that *C. triangulata* should not be in the same genus as marine *Thaumatomastix* with spines remains completely sound. Balonov's pictures of *Tx triangulata* show eccentric spines coming from a bobbin base nearer the size of the plate scales, which is entirely different from the marine '*Thaumatomastix*' species (now *Thaumatospina* and *Ovaloplaca*), which have radially symmetric spine bases. Our restriction of *Thaumatomastix* to the three freshwater species and establishing those new genera now completely reconciles that argument of Howe et al. (2011a) with Nicholl's (2012b) reinterpretation of *Tx triangulata*. Before Nicholls (2012b), only the plate scales of *Tx triangulata* had been reported (never spine scales) since its first description (Thomsen et al. 1993; Vørs 1992a) so our present reinterpretation of spine-scale base evolution was impossible before Nicholls discovered its spine scales and showed they were morphologically like those of *Tx nigeriensis*, and very unlike those of any marine '*Thaumatomastix*'. We therefore disagree strongly with the assumption that *Thaumatomastix* sensu stricto spine bases

are homologous with those of marine species, as implied schematically in Thomsen et al. (1993).

We explain below how we think these two grossly dissimilar spine-scale morphologies evolved independently from plate-scale ancestors by modifying their biogenesis in radically contrasting ways. Though no sequences are known for any freshwater *Thaumatomastix*, we predict that they will not branch with either marine clade, but somewhere within the more derived freshwater clade comprising *Thaumatomonas*, *Allas*, and two deep-branching environmental DNA lineages, either (or both) of which could turn out to be genuine freshwater *Thaumatomastix*. In both freshwater species plate-scales are triangular with rounded corners, somewhat like those of *Thaumatospina* or *Reckertia* (Nicholls 2012b).

Scale evolution within Thaumatomonadida

Each major thaumatomonad clade has a distinct broadly uniform scale morphology, which maps logically onto the trees (Fig. 2). The main limitation for reconstructing the history of their scale morphology is that we do not yet know where *Gyromitus* fits on the tree. This is important because its scales are oval latticed hollow cylinders with strongly outrolled upper and lower rims and a sieve-like sheet across its lower tier, markedly different from both the two-tier plate scales and the flanged spine scales of Thaumatomonadidae. As all three of them and the morphologically distinct but somewhat similar scales of *Peregrinia*, are finally made in mitochondria-associated vesicles (Mylnikov and Mylnikov 2012; Ota et al. 2012; Swale and Belcher 1974, 1975), we suggest that all four diverged from a common ancestor in ancestral thaumatomonads that first evolved this mitochondrial association, which is found only

in thaumatomonads. *Gyromitus* is currently classified in Peregriniidae (Howe et al. 2011a) and it is possible that environmental sequence Amb_18S_1066 that is sister to *Peregrinia* is a *Gyromitus* rather than another *Peregrinia*. The structure and symmetry of the upper and lower tier connections are so substantially different (Fig. 11) that we do not accept the inclusion of *Peregrinia* within *Gyromitus* by Karpov (2011). All trees indicate that *Esquamula* is a peregrinid that has lost scales. Our PhyloBayes tree (Fig. 1.1) suggests that Spongomonadida may also be derived from thaumatomonads by scale loss and is consistent with the idea that silica scales evolved in the common ancestor of Thaumatomonadida and Euglyphida – the ancestral imbricate (Cavalier-Smith and Chao 2003) and were lost one more time in the common ancestor of Nudisarca, which are apparently sisters of Euglyphida.

The simplest interpretation of silica scale evolution in imbricates is that scales were ancestrally single-tier plate-scales made in the Golgi complex, as in Euglyphida. We suggest that the lower tier of plate-scales in Thaumatomonadidae and the proximal sheet in Peregriniidae are homologous to each other and to the single-tier euglyphid scale. We postulate that even today in Thaumatomonadida lower-tier scale rudiments first assemble in Golgi vesicles and that these are then moved to the mitochondria for completion and adding the upper tier. We regard association with mitochondria as a shaping device for the upper tier only, and unnecessary for the ancestral first steps of lower tier assembly. Consistently with this hypothesis, developing two-tier plate scales are always oriented with the upper tier facing and projecting into the invaginated mitochondrial envelope (Mylnikov and Mylnikova 2012; Ota et al. 2012; Swale and Belcher 1974, 1975). Figure 11 suggests how the major kinds of imbricate scales may have diverged from a common ancestor.

Gyromitus plate scales do not have a definite upper tier and the upper flange is connected to the lower tier by a continuous lattice, not by discrete widely separated struts or groups of struts as in all other genera; both features are plausibly the ancestral condition for thaumatomonad plate-scales as they could simply have evolved by extending the ring into the shaping mitochondrion. In *Peregrinia,* the upper tier lacks a central sheet and consists of laterally inrolled sides supported by terminal struts. The spine-scales with central spine and narrow flange found in *Thaumatospina* and *Ovaloplaca* only clearly evolved in the last common ancestor of Thaumatomonadidae after it diverged from the Peregriniidae/Esquamulidae clade that lacks spine-scales, and were lost in the common ancestor of the *Reckertia/Allas/Thaumatomonas* clade and independently by *Scutellomonas.* We consider the flange as homologous with the rim of the continuous upper tier in body plate-scales and that it became narrower than the lower tier when the central region of the organic precursor material was pushed out to form the hollow spine. The fact that the flange is connected to the base plate by 3-fold symmetric struts in these radially symmetric spines strongly suggests that plate-scales at the time of this differentiation were themselves triangular with three symmetrically placed corner struts which evolved directly into the spine-scale struts. In *Ovaloplaca salina* (Ota et al. 2012) the three struts are all clearly basally dichotomous, as they also appear to be in *Thaumatospina vancouveri* (Fig. 6B), *mexica* (Fig. 6C), and *arabica* (Fig. 7C); the base of each spine-scale strut is also clearly flared outwards in *Ovaloplaca yabukii* (Fig. 8F), but moderately so in *O. asymmetrica* and scarcely so in *O. multipora* (Nicholls 2012b). The single corner struts of all three *Thaumatospina* described here can also be interpreted as basally dichotomous. We therefore suggest that each subflange strut in

Thaumatospina/*Ovaloplaca* is homologous with and evolved from a single basally dichotomous corner strut of triangular spine scales present in the common ancestor of Thaumatomonadidae.

In *Reckertia gemma* the single corner struts of the triangular scales are also basally dichotomous (Fig. 8H), as they also are in *R. filosa* (Howe et al. 2011 fig. 7B4), though interpretation is complicated by the presence of a third density that may not be basal which makes them look tripartite in surface view; their fig. 4B5). The micrographs for *R. dybsoeana* have insufficient resolution to show whether the strut base is merely flared or truly dichotomous (Thomsen et al. 1993); those of *R. formosa* are somewhat different with an upper part resembling an inverted hollow cone that underlies the upper tier corner perforation and a narrow proximal portion that is only slightly flared at its base. We suggest that *Reckertia* retained triangularity of its plate-scales from the ancestor of Thaumatomonadidae with the same type of basally dichotomous strut, and that its triangular scale architecture is homologous with that of *Thaumatospina* plate-scales. On that interpretation the oval plate-scales of the *Ovaloplaca*/*Scutellomonas* clade (mutually similar in both genera, but markedly different from the oval scales of *Thaumatomonas* and *Peregrinia*) evolved secondarily from such triangular scales by addition of a fourth basally dichotomous/flared strut: in *Scutellomonas* the structure is clearest – one basally flared strut at each end of the oval and one at each side at its pinched-in waist.

Reckertia species have triangular scales (curved vertices) of two seemingly distinct morphologies. Most similar to the triangular plate scales of *Thaumatospina* (*Ta triangulata*, *Ta tripus*, *Ta patelliformis*) are scales with a partially sheet-like upper tier

joined to the bottom tier by large hollow 'wine glass' struts (*R. formosa*) or by short rods at the corners (*R. filosa*). These differ from *Thaumatospina* plate scales primarily by having three prominent corner holes located just centrally to the corner struts (*R. filosa*) or over their wine-glass cavity (*formosa*); some species also have less conspicuous elongated lateral holes (elongated in *Ta filosa*; possibly not present in *Ta formosa*). Superficially quite different are scales with a very open, latticed upper-tier, often with a dense crown-like central structure raised on longer varyingly slender supports that meet the sides of the triangle, not the vertices as do inter-tier struts (e.g. *R. gemma, R. sagittifera*, *R. spinosa*, *R. dybsoeana, R. splendida*). As the crown-scale *R. gemma* is closely related to *R. filosa* with sheet-like upper tier (Fig. 1.1) there is no need to place them in separate genera, and the differences must be able to evolve relatively simply.

The *R. filosa* type with sheet-like upper tier is more like that of outgroups, suggesting that the open crown type may have evolved from it simply by greatly expanding pre-existing holes in the upper tier, leaving only slender supports after such expansion. Enlarging corner holes only by a more moderate amount than in most species would produce an intermediate structure with a central triangular sheet very like that of Fig. 3A of Gooday et al. (2006), misidentified as *Tx dybsoeana*; as well as its central sheet being broader than in *R. dybsoeana*, Fig. 3A shows two additional curved loops interconnecting the vertices across the corner holes unlike in *R. dybsoeana* or any *Thaumatospina*: though distinct from all described *Reckertia* its multiple perforations suggest membership of that genus - as this was a single isolated scale there is no evidence that it donor organism had separate spine-scales like *Thaumatospina*. Still further enlargement of corner holes only would yield an even

more open structure like that of Gooday et al. Fig. 3B with a broad residual central 3-armed structure, or with slightly less than that ones like Fig. 14 of Thomsen et al. (1993). Enlarging lateral holes only and pushing the central region out distally would yield structures like species 1 and 2 of Thomsen and Ikävalko (1997), whereas substantially enlarging both corner and lateral holes might yield *R. fusiformis* and *fragilis* (though interpreting their often broken structure is hard). Enlarging both types of holes would yield a suitable precursor of *R. gemma* scales but a third tier would need to be added to make the jewel. Interpreting Fig. 3A,B of Gooday et al. 1993) is difficult from a single scale seen in aerial view, as one or both might have lower tier perforations also contributing to the observed structure, unlike all described thaumatomonads.

R. splendida from Antarctic and Arctic sea-ice (Thomsen et al. 1995; Thomsen and Ikävalko 1997) was removed from *Thaumatomastix* (Howe et al. 2011a) because unlike *Thaumatospina* it has only one scale type with a large two-tiered triangular base and central 'spine' with three very long dichotomously-based struts but no flange and which connect to the side of the rail-like marginal band of the upper tier of a plate scale; it is thus not structurally homologous with spine-scales of *Thaumatospina*/*Ovaloplaca*. Its superficially spine-like central projection, extends from the junction point of the three lateral supports, which are probably homologous with the crown-like tripartite central structures of '*Tx*' species 1 and 2 in Thomsen and Ikävalko (1997), both probably undescribed *Reckertia*; the *R. splendida* central projection is terminally bulbous and internally tripartite, and probably homologous with the terminal point of such tripartite crowns of other *Reckertia* species; it almost certainly elongated entirely independently of the differently tipped spines of

Thaumatospina/*Ovaloplaca* and *Thaumatomastix*; unlike them it is not a second differentiated scale type but a simple modification of a multiperforated plate scale. The triangular hole that makes the base of each lateral support dichotomous in *R. splendida* is probably homologous with the lateral holes of *R. filosa*; the three lateral supports could be the residues left after a vast expansion of corner holes. *R. gemma* arguably duplicated this dichotomous lateral structure when evolving the jewel layer, so unlike most latticed *Reckertia* it has 6 forked lateral supports not just three.

The unusual scale diversity of *Reckertia* is consistent with its being a relatively deep-branching clade (compared with *Thaumatomonas*/*Allas*) likely to be over a hundred million years old. We suggest that the entire *Reckertia*-containing clade is probably *Reckertia*; it will be interesting to determine how its varied scale types map across this clade. *R. splendida* and most other Arctic species (Thomsen and Ikävalko 1997) might belong in the sea-ice environmental subclade R1 (Fig. 1.1). Figure 11 assumes that upper-tier corner holes evolved just once in the common ancestor of *Reckertia* and *Allas*/*Thaumatomonas* and were secondarily lost in *Reckertia igloolika*, which can be tested by establishing its position on the sequence tree. It is noteworthy that both sequenced non-polar *Reckertia* (*filosa* and *gemma*), which are closely related on Fig. 1.1, have oval ciliary scales; conceivably therefore *Reckertia* with round ciliary scales (e.g. *R. formosa*, *R. dybsoeana*: Thomsen et al. 1993) are more distant and might branch in sister clade R1. However, though the micrographs are less clear, it appears that *R. dybsoeana*'s jewel may constitute a third tier as in *R. gemma*, in which case it is perhaps more likely to be phylogenetically closer to it than to the other Arctic species; possibly these other two three-tier open lattice scale types evolved from a sheet-like ancestor independently of the others that lack a third-tier domed gem. The presence

of oval ciliary scales in both sequenced *Reckertia* and in *Ovaloplaca* suggests that oval ciliary scales may have been ancestral for the whole clade, which might have become secondarily circular and smaller in Arctic species.

In contrast to *Thaumatospina*, when *Thaumatomastix* eccentric spines evolved later, the spine grew not from the central area but from the rim to one side of a central hole (which if present when it originated would have prevented central spine formation); in this spine type the plate-scale upper tier remained the same diameter as the lower tier and was not narrowed into a flange. These key differences strongly suggest that the two types of spines evolved independently from two-tier plate-scales with originally equal-sized upper and lower tiers by separate morphogenetic processes. It is hard to see how the eccentric *Thaumatomastix* spine could have evolved directly from the *Thaumatospina*/*Ovaloplaca* type.

The much smaller single-tier oval scales on the anterior cilium of *Ovaloplaca* and *Reckertia* only could have evolved in their common ancestor after it diverged from *Thaumatospina* by interrupting the developmental pathway of body scales before they left the Golgi for adding the upper tier in the mitochondria, while the rudiment was still very small; we predict that these ciliary scales will be found to pass directly from the Golgi to the ciliary base without a mitochondria-associated phase. Ciliary scales were probably lost independently by *Scutellomonas* that drastically shortened its anterior cilium and by the ancestor of *Thaumatomonas*/*Allas*/*Thaumatomastix* that retained a relatively long one.

In the common ancestor of the *Allas*/*Thaumatomonas* clade lateral and morphologically distinct corner or end perforations evolved in the strongly upwardly

bowed upper tier which is at least somewhat smaller than the lower tier. As its outgroup, *Reckertia*, has triangular scales it is likely that the *Allas*/*Thaumatomonas* ancestor also had triangular scales. *Allas* itself uniformly has scales with an oval base but rounded triangular upper tier supported by three dichotomously based struts, which could have evolved simply by squashing the upper tier laterally during development. The ancestor of *Thaumatomonas* however arguably evolved oval scales differently by suppressing altogether one of the three ancestral struts and keeping one only at each end. The *Ts seravini*/*zhukovi* clade's ancestor clearly kept both such two-strut oval scales and three-strut triangular scales, but some descendant lineages lost the triangular version and others lost the oval ones. Within *Ts zhukovi*, some strains retained the putatively ancestral single broad terminal struts as in triangular scales, but some subdivided these into a larger number of narrower struts (as detailed in Results). Interestingly, scale development in the *Ts zhukovi* type strain T-3 still seems developmentally or evolutionarily labile in culture, as when it was first isolated it had both triangular and oval scales (and irregular ones, some even with four vertices), but a decade later we saw only triangular ones. An analogous example of rapid ultrastructural changes (eyespot degeneration) during prolonged laboratory cultivation was recently noted in a dinoflagellate (Moldrup et al. 2013).

By contrast the ancestor of the *Ts lauterborni*/*solis* clade evolved multiple terminal struts and at the same time lost the triangular version of the scale, which seems to have re-evolved in just one lineage; one explanation for that is that the pattern formation process for *Thaumatomonas* plate scales can in principle produce either three struts or two struts depending on tweaking some quantitative parameter (e.g. diffusion rates or binding interactions of morphogens or inhibitors), which can be

relatively easily changed or reversed in evolution. The evolution of multiple narrow end struts instead of just one broad one might also be a consequence of increasing its dimension and thus easily evolve in parallel. The broad single struts of *Thaumatomonas* and *Allas* appear as a less dense 'curtain' connecting their more electron-dense curved edges. The curtain is clearly seen in *A. multipora* and *A. media* (Fig. 9B and D, respectively) and *Ts zhukovi* strain UPL1Be2 (Sup. Fig. 6G). Multiple end struts are clearly separated structures that appear as black dots from above, as seen in *Ts zhukovi* strain GMKL4 (Fig. 10D), *Ts oxoniensis* strain EP3 (Sup. Fig. 3E) and *Ts solis* (Fig. 5D).

Our interpretation of *Thaumatomonas* scale evolution makes it likely that the PhyloBayes tree showing the *Ts lauterborni*/*solis* clade, as holophyletic is correct. It is simpler to suppose that end struts multiplied only once in the ancestor of this clade than separately in *Ts solis* as would be the case if the trees by other methods that place *Ts solis* more deeply before the divergence from the *Ts zhukovi*/*seravini* clade were right. The correct identification here of all sequenced *Thaumatomonas* and much improved sampling has enabled a much simpler and more comprehensive interpretation of *Thaumatomonas* scale evolution than was previously possible. Isolation of two new *Allas* species with the same general kind of scale as *A. diplophysa*, with non-equilateral triangular upper tier and oval lower tier, both distinct in upper tier perforation detail, confirms our earlier view that *Allas* and *Thaumatomonas* can be reasonably treated as distinct genera (Howe et al. 2011a); our PhyloBayes trees, which uses the most evolutionarily realistic heterogeneous model, suggest that they are sisters and each is holophyletic.

Thaumatomonas coloniensis MLTB12 from a chalk cliff in Malta is the only strain in the *lauterborni*/*solis* clade that apparently has triangular scales and oval scales with broad struts, more like those seen in the *zhukovi* clade, as well as what could be the usual oval scales (Sup. Fig. 4D). We do not revise the description of *Ts coloniensis* to include the greater variety of scales found in our single TEM preparation of strain MLTB12 because it was technically poor and no repeat preparation could be made to verify the scale types. As we did not isolate the culture ourselves we also cannot be sure it was clonal or say whether scale morphology changed since isolation. The numerous *Thaumatomonas* strains studied show that cells with the same 18S rDNA can produce different scale types, indicating that in this genus scale morphology can evolve faster than 18S rDNA genes and be extremely discriminating taxonomically.

Morphological differences between thaumatomonad genera

All thaumatomonad genera, as now defined, group distinctly on trees (Fig. 1.1 & Fig. 2), and show clearly correlated morphological differences. *Reckertia* and *Ovaloplaca* are the only thaumatomonads with scales on their anterior cilium. *Reckertia* cells are oval to round and more dorso-ventrally flattened than *Thaumatomonas* cells, which appear more convex. *Thaumatospina* cells are inflated, not flattened, and a long AC (up to half cell length) and spine scales are visible in the light microscope, and readily produce pseudopodia. *Scutellomonas* has a very distinct bulbous anterior end, no visible AC and is the fastest glider, along with *Cowlomonas,* which is distinctive in producing ventral pseudopodia from a posterior opening. In *Cowlomonas* the long AC flicks to one side of the cell. The AC for *Thaumatomastix* Lauterborn (1896, 1899) was almost the same length as the posterior. The AC in

Ovaloplaca yabukii and *O. salina* (Ota et al. 2012) is very short (barely visible), further support for their being a different genus from *Thaumatomastix* and *Thaumatospina*. The AC of *Thaumatomonas* and *Allas* are always a lot shorter than *Thaumatospina* and some published *Reckertia*; however the *Reckertia* in this study also have a short AC and glide faster than *Thaumatomonas*, and have an obvious flagella pocket (notch) off-centre. Therefore AC length varies considerably in *Reckertia*.

Despite the above differences, not all thaumatomonad genera can be distinguished unambiguously in the light microscope, the *Allas*/*Thaumatomonas* distinction especially, though all genera and most species can be recognised by electron microscopy. There is inconclusive evidence that *Allas* may not produce pseudopodia as readily as *Thaumatomonas*, but they cannot safely be distinguished in the light microscope. Within *Thaumatomonas* DNA sequences also are necessary for clearly distinguishing the closely related species in which scale variation is high. All three species of *Allas* now described have uniform two-tier scales with oval lower tier and a non-equilateral triangular upper tier supported by three broad struts that flare dichotomously at their base: one at each end and one on the side. Those of *Thaumatomonas* are much more variable, the majority being oval with oval upper tier and only end struts, but in some strains both tiers are triangular with a single broad basally dichotomous strut at each vertex. Most strains in the *Ts coloniensis*/*lauterborni*/*solis* clade have only oval scales, but one *Ts coloniensis* strain (ML2B12) has non-equilateral triangular scales as well. *Ts seravini* always has equilateral triangular scales, either alone (type) or mixed with oval two-strut scales. *Thaumatomonas zhukovi* always has 2-strut oval scales and seven of the nine *Ts zhukovi* also have at least a few non-equilateral triangular or irregular scales. None of

these *Thaumatomonas* scale types is the same as those of *Allas*, which unlike any *Thaumatomonas* scales always combine oval lower tier and triangular upper tier.

Some former *Thaumatomastix* spines are hollow and some are winged, but this is only really visible when using TEM not SEM, which can hide structural internal details. The triangular body-scaled *Thaumatomastix* now called *Thaumatospina* (*Ta tauryanini*, *tripus, patelliformis, vancouveri*, *arabica*, *mexica*) all have hollow-shaft spine-scales (Mikrjukov 2002; Takahashi and Hara 1984). However, oval scale former *Thaumatomastix*, now called *Ovaloplaca*, may have either winged spine-scales with a hollow proximal half (*O. salina*) or smooth hollow shaft spine-scales with bifurcate distal ends*, O. biparta* (Beech and Moestrup 1986; Thomsen et al. 1993).

A difficulty in distinguishing *Reckertia* scales from the triangular variety of *Thaumatomonas* and *Thaumatospina* was exemplified by Nicholls (2012b) who noted resemblance between *Ts hindoni* and *Thaumatomastix* sp. #5 (figure 40 - 43 of Thomsen and Ikävalko (1997), which we considered an unidentified *Reckertia* (Howe et al. 2011a). *Thaumatomastix* sp. #5 has two plates of nearly equal size connected at the corners by hollow, short tubes; one plate has submarginal densely spaced perforations. *Ts hindoni* had a base plate nearly the same size as the upper tier with no hollow tube struts, just joins at the corners. *Ts hindoni* has a bowed upper tier with straight edges (surface view) similar to our *Ts seravini* (T-2) strain: Figure 4F, comparable with that seen in Nicholls' (2012b) Figure 10C; far right scale side view bows upward making visible the corner strut. Nicholls (2012b) did not report cilium scales. All *Reckertia* have anterior cilium scales whereas *Thaumatomonas* do not. Thus all characters support the transfer of *R. hindoni* to *Thaumatomonas*. *Thaumatospina* also might potentially be

misidentified as *Reckertia* in the absence of spine scales or cilia, because we found that *Ta mexica* sp. n. sometimes produces cells with only triangular scales despite other cells from the same clonal culture having both triangular and spine scales (Fig. 8D and 8F). However, *Reckertia* all have anterior cilium scales, unlike *Thaumatospina*, a clear distinction between then genera.

Thaumatomonad posterior cilium acronematic tip

Four thaumatomonad genera, *Scutellomonas*, *Thaumatospina*, *Allas* and *Thaumatomonas*, displayed variable length acronematic posterior cilia. Acronemes have apparently not been described before for any thaumatomonad. *Scutellomonas* has a prominent thin acroneme that sticks to the substratum causing it to stretch before retracting back with the gliding cell. *Thaumatospina* (Fig. 7A), *Allas* and *Thaumatomonas* (Fig. 5A) do not have as long or prominent and thin acroneme as *Scutellomonas* (Fig. 8A); it instead appears more flattened and flared or bifurcate. In other protists permanent acronemes often arise when ciliary central pair microtubules extend further than the outer doublets, differences in the length of such protrusion accounting for different acroneme lengths. Temporary acronemes can arise in gliding flagellates if the membrane tip sticks to the substratum when the rest of the cilium is moved forwards by kinesin motors interacting with the microtubules during gliding, which would leave an elastic membranous tip empty of microtubules whose length would depend simply on the lag between membrane de-adhesion and forward gliding. Variable lags in de-adhesion could explain the variable acroneme length and sudden retraction seen here and in some other gliding flagellates, notably the sulcozoan *Mantamonas plastica* (Glücksman et al. 2011).

Improving monadofilosan phylogeny

Of key importance for better understanding imbricate scale evolution is to determine by sequencing whether Zoelucasida and Rotosphaerida really belong in Imbricatea. It is also important to study their scale development to see if they are made in association with mitochondria as in Thaumatomonadida, or not as in Euglyphida. Through our improved understanding of thaumatomonad plate-scale homologies and evolution summarised in Figure 11, we have become sceptical of the idea that the diverse scales of rotosphaerids are more closely related to thaumatomonad two-tier strutted scales than to those of other imbricates (Cavalier-Smith and Chao 2012). Whilst it remains reasonable to consider rotosphaerids as imbricates, a direct relationship between Rotosphaerida and Thaumatomonadida seems more questionable. Because of its parallel ciliary bases we place Zoelucasida in subclass Placoperla (within superorder Placofila because of its scales rather than perles) not with euglyphids in subclass Placonuda; none of the flagellate members of the superorder Nudisarca (Cavalier-Smith and Chao 2012), the apparent sister group to Euglyphida on the PB tree (Fig. 1.1), has the combination or two parallel ciliary bases with one cilium directed forwards and one backwards shared by Thaumatomonadida and *Zoelucasa*. Ciliary and nuclear features of *Zoelucasa* suggest an affinity with thaumatomonads, and it is possible that their apparently double-layered scales are related to those of the peregrinid *Gyromitus* despite the notable absence of the second layer in the central region and much thicker rim in *Gyromitus*. Though such a relationship is speculative, the already known phenotypic diversity in the putative *Peregrinia*/*Esquamula*/*Spongomonas* subclade is sufficiently great to leave open the possibility that *Zoelucasa* might belong in or near to unidentified marine flagellate

subclade MF1 (Fig. 1.1). Though another plausible candidate for a clade in that position is the aciliate filose Perlofilida (*Acanthoperla*, *Pompholyxophrys*), which like spongomonads (putative sister of clade MF1 on the PB tree) secrete a surface layer of silicified perles, and also require sequencing to verify their inclusion in Imbricatea and Placoperla, one member of MF1 (5B) is known to be a flagellate, which tends to argue against this (though not decisively as cercozoan clades can have mixtures of flagellates and filose amoebae).

No previously published large-scale monadofilosan tree used the evolutionarily more realistic heterogeneous CAT-GTR gamma models, as in our PhyloBayes CAT-GTR 18S rDNA tree, which is also by far the most comprehensive taxonomically to date for Monadofilosa other than metromonads. Our trees also gave apparently improved phylogenies in several respects, notably support for the holophyly of the imbricate subclasses Placonuda and Placoperla, superorder Nudisarca, and new order Variglissida as distinct holophyletic groups, as well as the previously controversial distinctness of *Neocercomonas* from *Cercomonas* and the affinities of several environmental DNA clades. For the first time our CAT-GTR tree suggests that spongomonads may have evolved from thaumatomonads by scale loss, and that pansomonads may have evolved from glissomonads. In view of the established ability of CAT-GTR models to reduce long-branch artefacts compared with homogeneous models (Lartillot et al. 2007, 2008; Philippe et al. 2009; Roure et al. 2013), these novel findings, which are important for cercozoan phylogeny though only weakly supported on single-gene trees, need testing by multigene analyses. The positions of Discomonadida and Ventricleftida within Ventrifilosa, and of Sainouroidea all remain

unclear, so multi-gene trees will be required to determine whether each is closer to Imbricatea or Thecofilosea.

Clades X and T 'glissomonads' which together form one of the two robust subclades (the other being clade Y) which are sister to pansomonads on our PhyloBayes tree have now been identified as a new family of algivorous amoeboflagellates, Viridiraptoridae (Hess and Melkonian 2013). Their amoeboid phenotype and position on our PhyloBayes tree as sister to pansomonads rather than to any established (less amoeboid) glissomonad group suggests that rather than assigning them to Glissomonadida (Hess and Melkonian 2013) they might more appropriately be considered early diverging pansomonads, if this alternative phylogeny proves to be correct. Alternatively, because viridiraptorids resemble some of the disparate flagellates assigned to *Pseudospora* and have been suggested to be fairly closely related to certain biciliate species of that possibly polyphyletic genus (Hess and Melkonian 2013), they could equally well – even preferably – be assigned to the still older alga-eating order Pseudosporida (Cavalier-Smith 1993), whose diagnosis they fit much better than the previous diagnosis of either Glissomonadida or Pansomonada. Accordingly we here assign clades T/X and Y all to Pseudosporida, and transfer that order from Endomyxa (where it previously was in the absence of sequence evidence for it being either an endomyxan or filosan merely because of its life-style resemblance to vampyrellids (Bass et al. 2009), which are Endomyxa (Hess et al. 2012); earlier it was in Filosa: Cavalier-Smith and Chao 2003). If the Fig. 1.2 phylogeny is confirmed by stronger evidence, it would probably be desirable to reduce Pansomonadida and Pseudosporida both to suborders within a broadened Glissomonadida. The putative pansomonad/pseudosporid subclade seems to have become secondarily more

amoeboid than in ancestral glissomonads; however until clade Te is also identified phenotypically we cannot be sure that the deepest branching glissomonads were not also more amoeboid in their trophic phase. When the e-sarcomonad clade is characterised it may prove appropriate to expand Glissomonadida further to include it also.

The discovery of this green-alga eating sarcomonad clade emphasises that our knowledge of cultured sarcomonads is currently strongly biased towards bacterivorous species. Some other novel clades might also require a eukaryote food supply before they can be cultured. The culture techniques of this paper were similarly biased towards bacterivorous thaumatomonads; future research should bear in mind that some uncultured thaumatomonad lineages may also feed on algae or other eukaryotes, as postulated above for *Cowlomonas* and *Thaumatomastix tripus* as least appears to eat diatoms (Thomsen et al. 1993), so more complex culture procedures deliberately supplying potential algal, fungal, or protozoan foods may be necessary to characterise them.

Brabender et al. (2012) claimed that bootstrap values are too low to support the monophyly of *Neocercomonas* (Cavalier-Smith and Karpov 2012). However, its distinction from *Cercomonas* with our well-aligned 1785-nucleotide positions is unambiguous. Brabender et al. (2012) found *Cercomonas* and *Neocercomonas* intermixed on their tree, presumably an artefact arising because their alignment of only 1069 nucleotides excluded too many informative positions; it was unwise to use so few for a cercomonad tree as far more can always be accurately aligned with sufficient effort. Their paracercomonad tree used 1539 positions; even so, our

alignment seems better as our tree had only two contradictions between Bayesian and ML trees (the two most basal branches where our ML tree differed in topology from theirs). Their tree had at least six such contradictions; probably many more as they omitted such details for seven insignificantly supported 'clades', most not even present on our much better supported trees. Unlike theirs, none of our trees put *Brevimastigomas* in a clade sister with *Metabolomonas*. Their Bayesian tree did not even recover monophyly of *Paracercomonas* or two of its subclades (B1a, B1b); all three were strongly supported (maximally for B1b) on all our trees. Even within these subclades the branching order on our more convincingly supported trees was different.

Habitat conservatism and monadofilosan environmental sequences

Our trees show that thaumatomonads very rarely shifted habitat between marine and fresh water. *Reckertia*, *Thaumatospina, Ovaloplaca*, and *Scutellomonas* have been found only in marine environments. The *Thaumatomonas/Allas* clade is wholly from fresh water or soil and groups robustly with two deeper branching environmental clades (Fig. 1.1), either of which could be (or be related to) the missing freshwater *Thaumatomastix* species. The rest of the thaumatomonad tree is marine except for *Peregrinia clavideferens* and its freshwater environmental sister sequence. This small freshwater clade is sister to the scaleless marine thaumatomonad, *Esquamula lacrimiformis* (Shiratori et al. 2012). The marine environmental clade sister to *Reckertia,* 'R1 sea ice clade' (Fig. 1.1), is a prime candidate to hold species like *R. splendida* from sea ice (Thomsen et al. 1995). It seems that thaumatomonads were ancestrally marine and freshwater/soil was colonised only twice - if Spongomonadida

really nests phylogenetically within Thaumatomonadida they are a third transition to freshwater.

We suggest that the imbricate subclass Placonuda was also ancestrally marine; its superorder Nudisarca is entirely marine whereas Euglyphida can be marine or (usually) freshwater. Combining the extensive euglyphid tree of Heger et al. (2010) with our more complete outgroup tree suggests that one major exclusively freshwater euglyphid clade of three families (Euglyphidae, Assulinidae, Trinematidae) colonised freshwater early on before it diversified. The other two clades, Paulinellidae and Cyphoderiidae, remained marine initially; within each the ancestor of perhaps only one subclade much later changed to freshwater habitats (*Paulinella* and a *Cyphoderia* subclade). Thus, on present evidence, Imbricatea were ancestrally marine but colonised freshwater at least six times. Their immediate outgroups, Discomonadida and Ventricleftida, are exclusively marine, strengthening the idea of a marine ancestry. A nudisarcan position for marine sequence 9-2.2 close to marimonads, as observed here, is more reasonable than the previous (unsupported) position within the virtually entirely soil/freshwater glissomonads (Howe et al. 2011a), which was probably a long-branch artefact.

The more distant Thecofilosea were probably also ancestrally marine, as most still are; *Rhogostoma* colonised soil and *Pseudodifflugia* is said to be marine, freshwater or soil. Thus, Ventrifilosea were almost certainly ancestrally marine. By contrast, virtually all Sarcomonadea are from soil or fresh water whereas Metromonadea are all marine.

We suggest that Monadofilosa were originally marine and that there may have been no more than about 10 transitions to soil or fresh water, and scarcely any switches back to a marine habitat. Our present estimate of seven marine to freshwater transitions if Sarcomonadea are holophyletic, and eight if they are paraphyletic as many 18S rDNA trees marine trees weakly suggest (Howe et al. 2011a), is probably somewhat too low as sampling is incomplete. Orders Perlofilida and Rotosphaerida (both probably imbricates: Cavalier-Smith and Chao 2012) have not yet been placed on the 18S rDNA tree. Within Sarcomonadea only one cercomonad has been isolated from a marine habitat (*Paracercomonas marina*; Karpov et al. 2006) but even that grows better in fresh water; it is unclear whether detection of some cercomonad DNA in littoral marine samples simply reflects the washing of cysts from land to sea or whether they can actually grow in seawater – most strains tested cannot, though tolerance of low levels of salt is widespread (Bass et al. 2007). Distribution on our tree of the numerous environmental sequences is consistent with marine to freshwater transitions being distinctly rare in monadofilosan evolution. Similar saline versus non-saline habitat conservatism over hundreds of millions of years is general in other protists (see citations in Bass et al. (2009), Cavalier-Smith and Chao (2012) and Heger et al. (2010)).

Acknowledgements

We thank Denis Tikhonenkov and Alexander Mylnikov for strains and an unpublished sequence; Keith Vickerman for strains; Flemming Ekelund for information; Pavel Škaloud for help with ITS2 analysis; Takashi Shiratori and Ken Ishida for pre-publication data for *Esquamula* and *Scutellomonas* strains; and Akinori Yabuki for

permission to include his unpublished sequence and micrographs of *Ovaloplaca yabukii*; BBSRC for a studentship (JMS); and NERC for a research grant.

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TABLE 1. *Thaumatomonas* and *Allas* species cell and scale measurements: type strains in bold; lines separate the main clades. Top left of table shows key.

/ no data
~ approximately
? unknown
PC posterior cilium
bt bottom tier
tt top tier

	Light microscopic live-cell measurements µm					Electron microscopic measurements µm					
						Oval scales			Observations		NOTES
	CL Cell length	**CW Cell width**	**OR**	**PC**	**PC x CL**	**oSL bt/tt** oval Scale Length	**oSW bt/tt** oval Scale Width	**OR** ovalness ratio	**triangle scales?**	**lateral holes?**	
Ts zhukovi zhukovi (T-3) (Mylinkov and Mylnikov 2003)	6.6 - 11.6	4.5 - 5.5	~ 1.8	15-18	~1.6 - 2.3	**bt** 0.6 - 0.75	**bt** 0.3 - 0.4	/	yes	3 - 5 holes	triangular, oval, and intermediate scales
UPL1Be2 *Ts z. zhukovi*	10.4	7	1.5	14.3	1.3	0.61/0.55	0.34/24	bt 1.8 tt 2.3	yes	3 - 8 holes	round circle apertures at either end of scale form broad struts
GMBGL1 *Ts z. zhukovi*	**12.5**	7.5	1.7	19.4	1.5	0.50/0.43	0.31/0.21	bt 1.6 tt 2.1	not observed	5 - 7 holes	3 - 5 solid struts on oval scales (no 18S rDNA sequence)
UPL1Bf2 *Ts z. zhukovi*	10.6	8.2	1.8	14.1	1.3	**0.66/0.56**	0.36/0.22	bt 1.8 tt 2.8	yes	3 - 8 holes	round circle apertures at either end of scale form broad struts
CCL4B *Ts z. zhukovi*	10.2	7.7	1.4	13.1	1.2	0.57/0.47	0.32/0.20	bt 1.8 tt 2.5	yes	2 - 4 holes	round circle apertures at either end of scale form broad struts
GMKL4 *Ts z. zhukovi*	**13.1**	8.2	1.6	18.1	1.4	0.51/0.44	0.30/0.20	bt 1.6 tt 2.2	not observed	3 - 8 holes	3 - 5 solid struts (scale)
HANTSF8 ***Ts z. saxoni***	10.3	7.8	1.3	11.1	1.1	**0.63/0.56**	0.30/0.22	bt 1.9 tt 2.6	~yes (triangle-like)	2 - 10 holes	round circle apertures at either end of scale form broad struts
NC02 ***Ts z. carolinensis***	12.2	6.8	1.8	14.4	1.2	0.54/0.44	0.32/0.18	bt 1.7 tt 2.4	yes	2 - 5 holes	round circle apertures at either end of scale form broad struts
NC03 ***Ts z. paracarolinensis***	10.7	6.8	1.6	14	1.3	0.54/0.44	0.32/0.18	bt 1.7 tt 2.4	yes	3 - 5 holes	round circle apertures at either end of scale form broad struts
Ts seravini seravini (T-2) (Mynikov and Karpov 1993)	9.9 - 16.5	6.6 - 11.6	~ 1.5	20 - 33	2	Triangle 0.7	none	none	yes	~ 5 holes	round circle apertures at either end of scale form broad struts

CH8 *Ts s. seravini*	10.3	7.5	1.4	14.1	1.4	Triangle 0.5	none	none	yes	3 - 5 holes	round circle apertures at either end of scale form broad struts
4e ***Ts s. varisquama***	11.9	9.5	1.3	14.7	1.2	Triangle 0.48 oval 0.56/0.49	oval 0.31/0.21	bt 1.9 tt 2.4	yes	3 - 4 holes	oval scales; 3 - 5 solid struts. Scales difficult to measure - broken
IVY18a *Ts* sp.	10.7	7.4	1.5	13.1	1.2	/	/	/	/	/	molecularly closet to *T. seravini* but with an 18S rDNA nucleotide difference
Ts lauterborni (T-1) (Mylnikov and Karpov, 1993)	8.3 - 14.9	5 - 8.3	~ 1.7	14.4	2	bt ~ 0.7	bt ~ 0.35	?	no	none	
Ts constricta CH3	12.2	7.4	1.7	15.5	1.3	0.57/0.44	0.28/0.08	bt 2.1 tt 5.3	no	none	scale top tier is very narrow with no lateral holes
BZ1 *Ts contricta*	10.8	7.5	1.5	14.9	1.4	0.57/0.47	0.27/0.08	bt 2.1 tt 6.4	no	none	scale top tier is very narrow with no lateral holes
Ts coloniensis (HFCC59) (Wlyezich et al 2007)	9.9 - 14.9	3 - 5.8	~ 2	16 - 18	~ 1.2	bt 0.50-0.65	bt 0.35-0.45	~bt 1.5	no	~ 5 - 7 holes	4 - 5 solid struts (scales)
Ts coloniensis-like ('lauterborni' from K. Vickerman)	9.1 - 14.6	8.2 - 11.8	1.3	13.3	1.1	/	/	/	/	/	
Ts coloniensis 'vancouveri' (3108W2)	8 (from fixed)	?	?	~ 9.6	1.2	/	/	/	no	"several"	4 solid struts (data from Howe et al, 2011a). Small measurement due to shrinkage of fixed cell?
PML3A *Ts coloniensis*	11.8	7	1.7	16.7	1.5	0.52/0.45	0.30/0.22	bt 1.8 tt 2.1	no	5 - 7 holes	3 - 5 solid struts (scales)
MLTB12 *Ts coloniensis*	13.4	7.4	1.8	17.6	1.3	0.53/0.46	0.29/0.21	bt 1.8 tt 2.2	yes	4 - 6 holes	Broad struts at either end of oval scale and other scales with smaller solid struts. Oval and triangular scales observed
Ts oxoniensis (Hinksey)	9.5	~6	1.6	18.5	2	/	/	/	no	4 holes	4 - 5 solid struts (scales)
GMPL1 *Ts oxoniensis*	13	8.1	1.6	17.5	1.4	0.50/0.40	0.27/0.20	bt 1.9 tt 2.2	no	4 - 6 holes	3 - 5 solid struts (scales)

EP3 *Ts oxoniensis*	12.9	7.4	1.7	15.5	1.3	0.51/0.44	0.28/0.21	bt 1.8 tt 2.2	no	5 - 7 holes	3 - 5 solid struts (scales)
Ts solis BZ8	14.2	11	1.3	15.9	1.2	0.57/0.49	0.31/0.23	bt 1.9 tt 2.7	no	4 - 8 holes	3 - 5 solid struts (scales)
Ts solis CH9	no data	/	/	/	~1.3	0.55/0.47	0.33/0.18	bt 1.7 tt 2.6	no	~ 5 - 7	3 - 4 solid struts (scales)
Allas aff. *diplophysa* (ATCC50635 Howe et al, 2011a)	13 - 20	?	?	13 - 20	~1.1	~ 0.5	bt ~ 0.35	?	lateral strut triangular	up to 4	distinct large lateral holes on scale upper tier
Allas multipora GSPB9	12.2	9.6	1.3	13.8	1.1	0.61/0.53	0.4/0.25	bt 1.7 tt 2.2	lateral strut triangular	up to 8	bottom tier of scale notably wider than top tier
Allas media BKARH9	11.4	8.9	1.3	17.5	1.5	0.58/-	bt 0.47	bt 1.2	lateral strut triangular	up to 10	scales appear in between oval to triangular in shape

TABLE 2. *Reckertia, Ovaloplaca, Scutellomonas and Thaumatospina* cell and scale measurements: Top left of table shows key.

/ no data ~ approximately oSW oval Scale Width oSL oval Scale Length OR ovalness ratio	Light microscope live-cell measurements µm					Electron microscopic measurements - µm				NOTES
						Spine scale		**Plate scale**		
	CL	**CW**	**OR**	**PC**	**PC x CL**	**Spine length**	**Base width**	**Oval**	**Triangular**	
Reckertia filosa (JJP-2003 Howe et al, 2011a)	11.4	?	?	17 - 20	1.8 - 2	/	/	/	~ 0.55	oval AC scales 0.33 - 0.37 x 0.2 - 0.22
Reckertia gemma VWB1 THAS	11.6	9.7	1.2	19.1	1.6	/	/	/	~ 0.45 each side	oval AC scales 0.25 - 0.36 x 0.15 - 0.19
Ovaloplaca salina OTA et al, 2012	10.7	7.5	1.4	~ 15	~ 1.5	~ 5.4	/	oSL ~ 1.3 oSW ~ 0.9	none	scales measured from Figure 2. Ota et al., 2012
Ovaloplaca yabukii YPF609	11.1	10.5	1.1	11	1	5.18	/	oSL 1.3 oSW 0.7	none	one cell measured
Scutellomonas patella CA05	10.6	8.5	1.3	15.2	1.4	no spine scale	no spine scale	0.77 x 0.53	none	
Thaumatospina vancouveri VWB1C	11.9	6.7	1.8	17.7	1.6	4	0.64	none	1.2	
Thaumatospina mexica TxMEX	/	/	/	/	/	4.1	0.58	none	1.2	observed in LM but not measured (large 18S rDNA insert)
Thaumatospina arabica TxDUBAI	14.9	10	1.5	20.8	1.4	/	/	none	yes	

TABLE 3. Provenance of *Thaumatomonas* isolates

Strain	**Species**	**Provenance**
T-1	*Thaumatomonas lauterborni*	Borok, Russia
BZ1	*Thaumatomonas constricta*	?, Brazil
CH3	*Thaumatomonas constricta*	Yangshuo, China
T-2	*Thaumatomonas s. seravini*	Borok, Russia
4E	*Thaumatomonas s. varisquama*	Oxford, United Kingdom
T-3	*Thaumatomonas z. zhukovi*	Borok, Russia
GMKL4	*Thaumatomonas z. zhukovi*	Staffordshire, United Kingdom
UPL1Be2	*Thaumatomonas z. zhukovi*	Oxford, United Kingdom
UPL1Bf2	*Thaumatomonas z. zhukovi*	Oxford, United Kingdom
CCL4B	*Thaumatomonas z. zhukovi*	Oxford, United Kingdom
GMBGL1	*Thaumatomonas z. zhukovi*	Oxford, United Kingdom
HANTSF8	*Thaumatomonas z. saxoni*	Hampshire, United Kingdom
NC02	*Thaumatomonas z. carolinensis*	North Carolina, USA
NC03	*Thaumatomonas z. paracarolinensis*	North Carolina, USA
Hinksey	*Thaumatomonas oxoniensis*	Oxford, United Kingdom
GMPL1	*Thaumatomonas oxoniensis*	Staffordshire, United Kingdom
EP3	*Thaumatomonas oxoniensis*	Exmoor, United Kingdom
HFCC59	*Thaumatomonas coloniensis*	Cologne, Germany
PML3A	*Thaumatomonas coloniensis*	Oxford, United Kingdom
MLTB12	*Thaumatomonas coloniensis*	?, Malta
3108W2	*Thaumatomonas coloniensis*	Vancouver, Canada
CH9	*Thaumatomonas solis*	Yunnan, China

Figure legends

Figure 1. PhyloBayes CAT-GTR gamma tree for 273 Monadofilosa 18S rDNA sequences (1785 nucleotide positions) with environmental sequences in bold. The tree is across two pages: Fig. 1.1, Ventrifilosa: Imbricatea and Thecofilosea; Fig. 1.2, Sarcomonadea. Arrows indicate new species from this study. Asterisks indicate new Thaumatomonadidae species type strains from this study. Environmental clades mentioned in text: R1 environmental marine sister to *Reckertia*; MF1 environmental Marine Flagellate clade 1; NC1, NC3, and NC4, from Bass and Cavalier-Smith (2004); eVentri Ventricleftida; eEbriida sister to Ebriida; eSarcomonad. Paracercomonads labeled according to Brabender et al. (2012). FW shows the base of the major freshwater clade within Thaumatomonadidae; deeper branches in this family are exclusively marine. Support values are PhyloBayes posterior probabilities (left), RAxML GTR gamma bootstrap percentages for 1000 pseudoreplicates (middle), MrBayes posterior probabilities (right). Black dots indicate maximal support for all, i.e. 1/100/1. The tree is rooted between cercomonads and glissomonad/pansomonads in accord with the cercozoan-wide tree of Howe et al. (2011a). The robust clade lying between Pansomonadida and Glissomonadida, which includes Viridiraptoridae (Hess and Melkonian 2013) and clade Y are here assigned to Pseudosporida rather than Glissomonadida for reasons explained in the discussion.

Figure 2. MrBayes GTR gamma covarion tree for 35 species of Thaumatomonadidae based on ITS1, 5.8S, ITS2 rDNA sequences (1561 nucleotide positions) including ~400 nucleotides of the end of 18S rDNA and the first ~300 nucleotides of 28S rDNA. Sketches of scales from each strain are shown; for *T. z. zhukovi* T-3 parentheses embrace additional scale types observed in the original publication (Mylnikov and Mylnikov 2003); for MLTB12 parentheses embrace scales observed from one bad preparation unable to confirm which type. Asterisks indicate species type strains, and arrows indicate new subspecies. Smaller anterior cilium scales for *Reckertia* are shown to the right of the body-scales. Some genera and species from Figure 1 have no ITS sequences available. Support values are MrBayes posterior probabilities (left) and RAxML bootstrap percentages for 1000 pseudoreplicates (right). Black dots indicate maximal support for both, i.e. 1/100.

Figure 3. Secondary structure of ITS2 rDNA folds. **A**. Examples of *Thaumatomonas* species: *Ts constricta*, *Ts seravini varisquama, Ts z. zhukovi, Ts oxoniensis*, *Ts s. seravini*. Enlarged loop of *Ts seravini varisquama* labeled on the third helix. **B**. *Reckertia gemma*. **C**. *Thaumatospina*: *Ta arabica* and *Ta mexica*. **D**. *Allas*: *A. media* and *A. multipora*. **E**. *Scutellomonas patella*. All

secondary structures have similar folds and show four clear helices (labeled on *Thaumatomonas seravini varisquama*).

Figure 4. A-B, *Thaumatomonas zhukovi saxoni* (HANTSF8). **C-D**, *Thaumatomonas seravini varisquama* (4e). **E**, *Thaumatomonas* sp. (IVY18a). **F-G**, *Thaumatomonas seravini seravini* (T-2).

A. Body-scales of *Ts zhukovi saxoni* (HANTSF8); less common triangular-like (*), bowed upper tier and end strut (arrow). **B**. DIC of live *Ts z*. *saxoni* (HANTSF8); i – ii, one gliding cell in two focal planes (two-way arrow), i, anterior cilium (AC) and, ii, posterior cilium (PC), iii – iv, one feeding cell shown in two focal planes (two-way arrow), iii, cilium pit (PIT) and, iv, pseudopodia. Cell with ventral groove (double arrowhead) and anterior cilium (AC). Tilted side view of cell (*) with anterior cilium (AC). **C**. DIC of live *Ts s*. *varisquama* (4e); i – ii, gliding partially divided cell (%) shown in two focal planes (two-way arrow), with posterior cilium (PC), iii, two feeding cells with pseudopodia, broad lamellipodia (*), iv – v, two gliding cells with broad posterior end and anterior cilium (AC), vi, three feeding cells joined via pseudopodia each with posterior cilium (PC). Gliding dividing cell (%) with anterior cilium (AC). Gliding cell with anterior labeled (ant.). **D**. Body-scales of *Ts s. varisquama* (4e) showing five narrow end struts on oval scales and single broad vertex struts on triangular scales. **E**. DIC of live *Ts* sp. (IVY18a; 18S groups closely with *Ts seravini*); i – ii, double feeding cell on two focal planes (two-way arrow) with branching pseudopodia (double arrowhead), iii, single cell with pointed posterior, anterior indicated (ant.). **F**. Side view of triangular *Ts s*. seravini (T-2) body-scale with corner strut (arrow). **G**. Body-scales of *Ts s*. *seravini* (T-2). Scale bars: B, C and E, 10 µm. A, D, F and G, 0.5 µm.

Figure 5. A-B, *Thaumatomonas constricta* (BZ1). **C-D**, *Thaumatomonas solis* (BZ8 type). **E-G**, *Thaumatomonas constricta* (CH3 type). **H,** *Thaumatomonas solis* (CH9).

A. DIC of live *Ts constricta* (BZ1); i – ii, gliding cells showing posterior cilium (PC), ii, with two posterior cilia (**), iii, two feeding cells joined via long thin pseudopodium (double arrowhead), iv, gliding cells with flared end of posterior cilium (*), v, two gliding cells with anterior cilium (AC) and one with ventral groove (arrow). **B**. *Ts constricta* (BZ1) body-scales, side view of end strut (double arrowhead). **C**. DIC of *Ts solis* (BZ8) cells; oblong cell with two sets of cilia (**), i, iii, and iv – v, gliding cells with broad posterior ends (Post.) and visible anterior cilium (AC), ii, feeding cell, iii, ventral groove (arrow). **D**. Body-scales of *Ts solis* (BZ8 type) aberrant scale (*); arrows indicate discrete narrow end struts. **E**. DIC of live *Ts constricta* (CH3); i – ii, two feeding cells with thick branching pseudopodia (double arrowheads), small cell (sm.), and round cyst, iii – iv, gliding cells, anterior end (ant.), ventral groove (arrow). **F**. TEM of *Ts constricta* (CH3)

body-scales, side view with end struts (double arrowhead). **G**. TEM of *Ts constricta* (CH3) cell with posterior and anterior cilia (PC, AC). **H**. Body-scales of *Ts solis* (CH9). Scale bars: A, C and E 10 µm. B, D, F and H 0.5 µm, G 2 µm.

Figure 6. A-B, *Thaumatospina vancouveri* (VWB1C). **C-F,** *Thaumatospina mexica* (TxMex).

A. DIC of live *Ta vancouveri* (VWB1C); i – ii, same cell (two-way arrow) in two focal planes showing a broad pseudopodium (double arrowhead), posterior and anterior cilia (PC, AC), prominent nuclei (*). Close-up of flared end of posterior cilium (arrow in bordered window). iii – iv, two gliding cells with prominent AC and ventral groove (arrow). Aggregate refractile mass of cells (**). **B**. TEM of *Ta vancouveri* (VWB1C) body-scales, some plate scales with light inner triangle shape (arrow). Three normal spine-scales and one aberrant with highly curved spine and elongate base (*). Close-up of trifurcate tip (window). **C-G,** TEM of *Ta mexica* (Mex), **C**. Body-scales, aberrant spine-scale with two spines (*); punctate markings on the margin of the upper tier – magnified in window with asterisks (**). **D**. Cell with no spine-scales. **E**. Spine-scale; flared and pointed tip (left), upper flange and base plate (right). **F**. Whole cell with spine and plate-scales. **G**. Triangular plate-scales, and two spine-scales. Scale bars: A 10 µm. B, C and G 1 µm, D and F 2 µm, E 0.5 µm.

Figure 7. A, *Cowlomonas planata* (VWB1B). **B-C**, *Thaumatospina arabica* (Dubai).

A. DIC of two live *C. planata* (VWB1B); i – x, movement series of same cell, vii – viii, ventral ridge continuation of the cell's anterior hood (double arrowhead), x, ventral pseudopodia at posterior end (arrowhead), xi – xiii, movement series of same cell, xi, posterior and anterior cilia (AC, PC) coming out of hood. **B**. DIC of live *Ta arabica* (Dubai); i – ii, spine-scales (double arrowhead), i, ii, and v – vi, anterior cilium (AC), iii, iv and viii, posterior cilium (PC), iii – iv, feeding cells with branching pseudopodia (arrowhead), vii, ventral groove (arrow). **C**. TEM of *Ta arabica* (Dubai) spine and two-tiered plate-scales. Punctate markings on upper tier underside margin of triangular plate scales (*) next to bobbin base of spine-scale. Scale bar: A and B 10 µm, C unknown scale.

Figure 8. A-D, *Scutellomonas patella* (CA05). **E-G,** *Ovaloplaca yabukii* (YPF609). **H-J**, *Reckertia gemma* (VWB1Thas).

A. DIC of live *S. patella* (CA05); i–v, series of a cell creeping with active pseudopodia; ii, posterior cilium and anterior orientation of the cell (ant.); vi, a cell with ventral groove (arrow); vii – ix, series of a third cell, gliding, vii shows a ridge at the anterior end (*); ix and x, thinning posterior cilium distal end, which appears acronematic (arrowhead), close-up (window); x - xi,

same cell in different focal plane, xi showing subapical ciliary pit. **B**. TEM of *S*. *patella* (CA05), showing body-scales and posterior cilium with thin tip. **C**. TEM of *S. patella* (CA05) cell with very short second cilium (*), possibly pre-divisional. **D**. Body-scales of *S. patella* (CA05) showing four main struts (**). **E**. Phase contrast micrograph of *O. yabukii* (YPF609) showing posterior cilium (PC) and spine-scales (double arrowhead). **F-G**. SEM of body-scales of *O. yabukii* (YPF609); dished oval scales with upper-tier inner perforations, and ribbed spine-scales with proximal flange above base-plate and tripartite basally flared struts linking them. **H**. Body-scales of *R. gemma* with triangular base-plate (arrowhead) and inner margin; seen on three body scales (double arrowhead); corner long strut has short bifurcation onto lower margin creating small aperture (arrow). Side view of central, domed upper tier 'jewel' (*). **I.** Cilium scales of *R. gemma* (VWB1Thas). **J**. DIC of *R. gemma* (VWB1Thas); i – ii, four gliding cells showing anterior cilium (AC) and angled apical dent at ciliary pocket; iii, anterior cilia of two cells; iv – v, (binucleate and tetraciliate) feeding cells on different focal planes (two-way arrow); v, branching pseudopodia, vi group of gliding cells showing posterior cilia (PC), vii – viii, gliding cells with anterior end indicated (ant.), with large nuclei with dense nucleolus visible (near ciliary bases). Scale bars: A, E and J 10 µm. B, and G 2 µm. C and F 1 µm, H and I 0.2 µm, D 0.5 µm.

Figure 9. A-B, *Allas multipora* (GSPB9). **C-E**, *Allas media* (BKARH9).

A. DIC of live gliding *A. multipora* (GSPB9); anterior and posterior cilia (AC, PC). i, thin ventral groove (arrow), ii and iv – v, cells with pointed and/or 'ragged' posterior ends in contrast to iii, with broad rounded posterior **B**. Body-scales of *A. multipora* (GSPB9); side view of broad lateral strut (double arrowhead). **C**. TEM of whole mount *A. media* (BKARH9) cell, anterior and posterior cilia (PC, AC), PC with thin tip. **D**. Body-scales of *A. media* (BKARH9). Side view of scale with broad strut (double arrowhead). **E**. DIC of live *A. media* (BKARH9), i – ii, same polynucleate round mass (two-way arrow) in different focal planes, ii, with cilium, iii, gliding cell mass with two posterior cilia (PC), iv, ventral groove (double arrowhead) on gliding cell, anterior and posterior cilia (AC, PC), v, complete length of posterior cilium on gliding cell (PC), vi, granulated ventral groove (arrow). Scale bars: A and E 10 µm. B and D, 0.5 µm, C 2 µm.

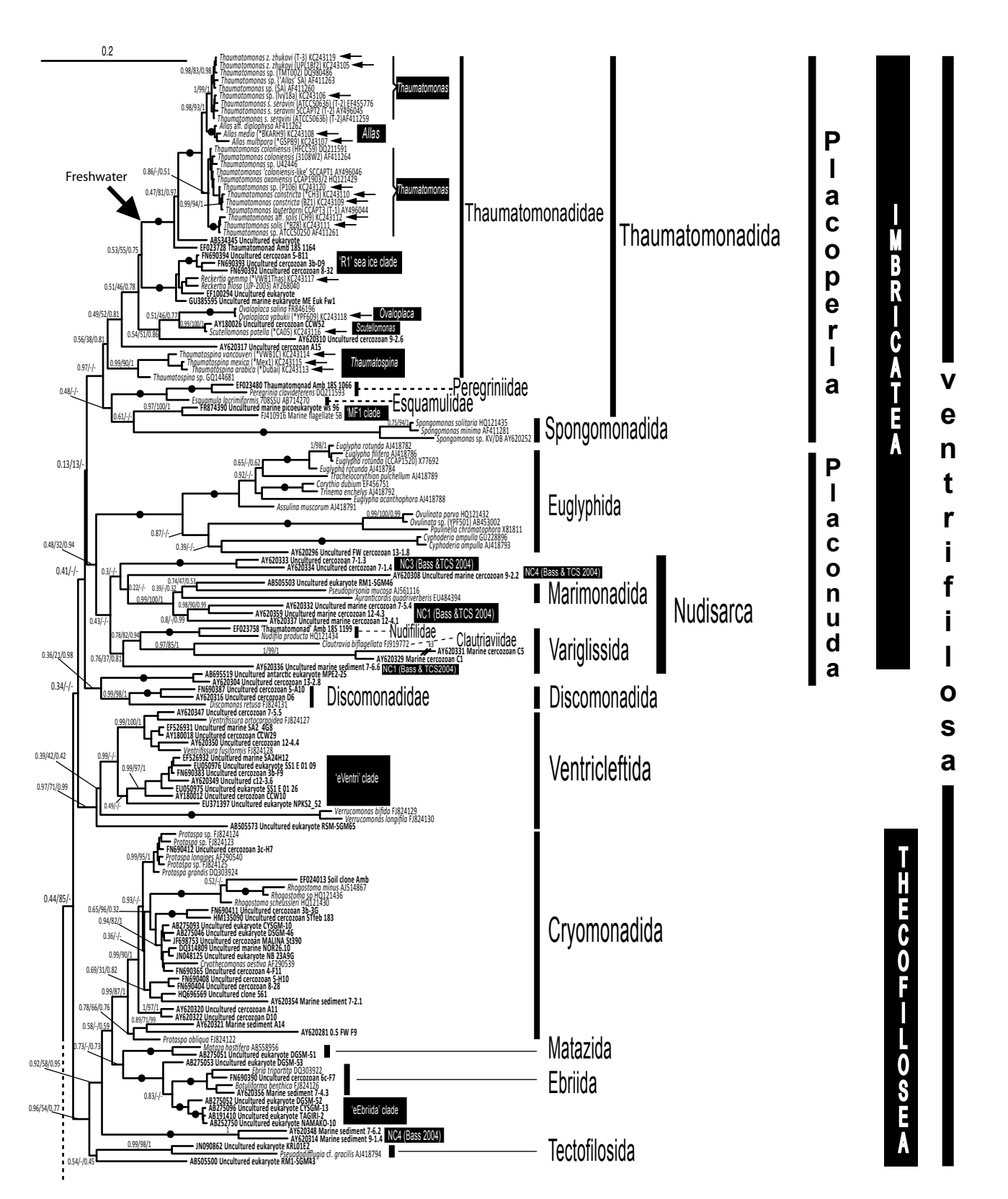
Figure 10. A-B, *Thaumatomonas zhukovi paracarolinensis* (NC03). **C-D**, *Thaumatomonas zhukovi zhukovi* (GMKL4). **E-F**, *Thaumatomonas zhukovi zhukovi* (GMBGL1). **G-I**, *Thaumatomonas zhukovi carolinensis* (NC02).

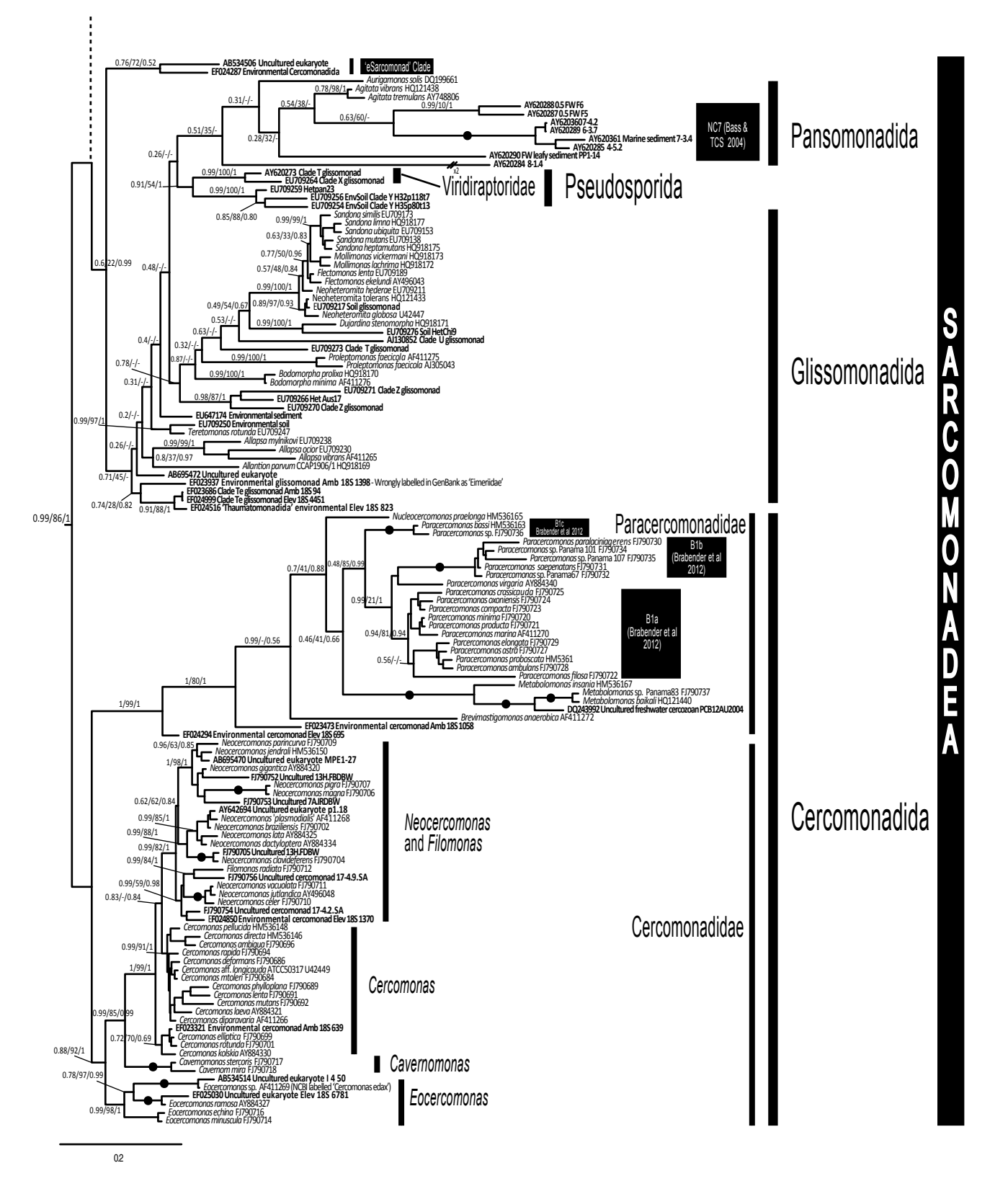
A. DIC of *Ts z*. *paracarolinensis* (NC03); i, elongate dividing gliding cell (%), ii, small round cell, iii – iv, same gliding cell shown on two focal planes (two-way arrow) posterior cilium (PC), v – vi,

same small round feeding cell on two focal planes (two-way arrow) with pseudopodia (double arrowhead), vi, large feeding cell with branched pseudopodia (double arrowheads). Refractile aggregate of cells of various sizes (*). **B**. Body-scales of *Ts z*. *paracarolinensis* (NC03). **C**. DIC of live *Ts z. zhukovi* (GMKL4); i, feeding cell with pseudopodia (double arrowheads), ii, gliding cell with pointed posterior and visible anterior cilium (AC), iii, gliding cell with two sets of anterior and posterior cilia (AC, PC). **D**. Body-scales of *Ts z. zhukovi* (GMKL4). **E**. Body-scales of *Ts z. zhukovi* (GMBGL1). **F**. DIC of live *Ts z. zhukovi* (GMBGL1); i, feeding cell with branching pseudopodia (double arrowhead), ii – iv, gliding cells with visible posterior and anterior cilia (PC, AC), ii – iii, tapered posterior. **G.** TEM of *Ts z. carolinensis* (NC02) body-scales **H**. TEM of whole *Ts z. carolinensis* (NC02) cell. **I**. DIC of live *Ts z. carolinensis* (NC02) cells; i, small round cell at the right of an oval gliding cell with anterior end indicated (ant.), ii, gliding cell with posterior obtusely angled (flick), anterior cilium indicated on gliding cells (AC), anterior of gliding cell (ant.), anterior cilium on gliding cells (AC), iii – iv, feeding cells show broad pseudopodia (double arrowhead) and posterior cilium (PC). Scale bars: A, C, F and I 10 µm, B, D, E and G 0.5 µm, H 2 µm.

Figure 11. Scale evolution in Imbricatea. Ancestrally, imbricates probably had unadorned oval single-tier scales made in Golgi-derived cisternae like those of Euglyphida. Thaumatomonadida alone evolved a novel shaping process by attachment of the Golgi cisternae to the mitochondrial envelope and its invagination to make a second intramembrane space for addition of the upper tier. Peregriniidae retained the simple oval shape; *Gyromitus* has a symmetric latticed wall connecting the lower and upper tiers, whereas *Peregrinia* modified this to make distinct struts positioned at the ends of the scale only. The ancestor of Thaumatomonadidae evolved three symmetric struts making the plate scales triangular and differentiated them into two types: plate scales, and more radically modified spine scales in which the upper tier was extended centrally as a long hollow spine and contracted laterally to make a basal flange connected to the lower tier by three struts; fused distally but splaying out dichotomously at their base. This bobbin-based spine scale was retained by *Thaumatospina* and *Ovaloplaca* but lost independently by *Scutellomonas* and the clade including *Thaumatomonas*, *Allas*, and *Reckertia* (and putatively *Thaumatomastix*). *Thaumatomastix* scales evolved non-homologous lateral spines without a flange. Within Thaumatomonadidae, oval scales evolved thrice independently: the ancestor of the *Scutellomonas*/*Ovaloplaca* clade evolved numerous symmetrically arranged almost invisible struts around the inner oval; *Allas* made the lower tier only oval and squashed the triangular upper tier laterally; *Thaumatomonas* made the lower tier oval and the upper tier oblong, but the *Ts*

zhukovi/*seravini* ancestor initially retained triangular scales also. The *Allas*/*Thaumatomonas* ancestor evolved lateral and end/vertex perforations in the bowed upper tier. End struts increased in number independently in the *Ts lauterborni*/*solis* ancestor and at least one sublineage of *Ts zhukovi*. Single tier anterior ciliary scales (*Ovaloplaca* and *Reckertia* only) evolved, probably from single tier scale rudiments prior to migration to the mitochondria in the common ancestor of all Thaumatomonadidae except *Thaumatospina*, and were lost by *Scutellomonas* and the *Thaumatomonas*/*Allas*/*Thaumatomastix* ancestor.


0.2
Thaumatomonas z. zhukovi (T-3) KC243119
Thaumatomonas z. zhukovi (UPLBF2) KC243105
Thaumatomonas sp. (TMT002) DQ980486
Thaumatomonas sp. ('Allas' SA) AF411263
Thaumatomonas sp. (SA) AF411260
Thaumatomonas sp. (Ivy18a) KC243106
Thaumatomonas s. seravini (ATCC50636) (T-2) EF455776
Thaumatomonas s. seravini SCCAPT2 (T-2) AY496045
Thaumatomonas s. seravini (ATCC50636) (T-2)AF411259
Allas aff. diplophysa AF411262
Allas media (*BKARH9) KC243108
Allas multipora (*GSP89) KC243107
Thaumatomonas coloniensis (HFCC59) DQ211591
Thaumatomonas coloniensis (3108W2) AF411264
Thaumatomonas sp. U42446
Thaumatomonas 'coloniensis-like' SCCAPT1 AY496046
Thaumatomonas axoniensis CCAP1903/2 HQ121429
Thaumatomonas sp. (P106) KC243120
Thaumatomonas constricta (*CH3) KC243110
Thaumatomonas constricta (BZ1) KC243109
Thaumatomonas lauterborna CCAPT3 (T-1) AY496044
Thaumatomonas aff. solis (CH9) KC243112
Thaumatomonas solis (*BZ8) KC243111
Thaumatomonas sp. ATCC50250 AF411261
Thaumatomonas
Allas
Thaumatomonas
Thaumatomonadidae
Thaumatomonadida
Placoperla
Freshwater
AB534345 Uncultured eukaryote
EF023728 Thaumatomonad Amb 18S 1164
FN690394 Uncultured cercozoan 5-B11
FN690393 Uncultured cercozoan 3b-D9
FN690392 Uncultured cercozoan 8-32
'R1' sea ice clade
Reckertia gemma (*VWB1Thas) KC243117
Reckertia fissa (JIP-2003) AY268040
EF100294 Uncultured eukaryote
GU385595 Uncultured marine eukaryote ME Euk Fw1
Ovaloplaca salina FR846196
Ovaloplaca yabukii (*YPF609) KC243118
AY180026 Uncultured cercozoan CCW52
Scutellomonas patella (*CA05) KC243116
Ovaloplaca
Scutellomonas
AY620310 Uncultured cercozoan 9-2.6
AY620317 Uncultured cercozoan A15
Thaumatospina vancouveri (*VWB1C) KC243114
Thaumatospina mexica (*Mex1) KC243115
Thaumatospina arabica (*Dubai) KC243113
Thaumatospina
Thaumatospina sp. GQ144681
EF023480 Thaumatomonad Amb 18S 1066
Peregrinia clavidefera DQ211593
Peregriniidae
Esquamula lacrimiformis T08SSU AB714270
FR874390 Uncultured marine picoeukaryote WS 96
Esquamulidae
MF1 clade
FJ410916 Marine flagellate 5B
Spongomonas solitaria HQ121435
Spongomonas minima AF411281
Spongomonas sp. KV/DB AY620252
Spongomonadida
Euglypha rotunda AJ418782
Euglypha filifera AJ418786
Euglypha rotunda (CCAP1520) X77692
Euglypha rotunda AJ418784
Trachelocorythion pulchellum AJ418789
Corythio dubium EF456751
Trinema enchelys AJ418792
Euglypha acanthophora AJ418788
Assulina muscorum AJ418791
Euglyphida
Ovulinata parva HQ121432
Ovulinata sp. (YPF501) AB453002
Paulinella chromatophora X81811
Cyphoderia ampulla GU228896
Cyphoderia ampulla AJ418793
Placonuda
AY620296 Uncultured FW cercozoan 13-1.8
AY620333 Uncultured cercozoan 7-1.3
AY620334 Uncultured cercozoan 7-1.4
NC3 (Bass &TCS 2004)
AY620308 Uncultured marine cercozoan 9-2.2
NC4 (Bass &TCS 2004)
AB505503 Uncultured eukaryote RM1-SGM46
Pseudopirsonia mucosa AJ561116
Aurantiamoeba quadriverberis EU484394
Marimonadida
Nudisarca
AY620332 Uncultured marine cercozoan 7-5.4
AY620359 Uncultured marine cercozoan 12-4.3
AY620337 Uncultured marine cercozoan 12-4.1
NC1 (Bass &TCS 2004)
EF023758 'Thaumatomonad' Amb 18S 1199
Nudifila producta HQ121434
Nudifilidae
Clautraviidae
Clautravia biflagellata FJ919772
AY620331 Marine cercozoan C5
AY620329 Marine cercozoan C1
Variglissida
AY620336 Uncultured marine sediment 7-6.6
NC1 (Bass &TCS2004)
AB695519 Uncultured antarctic eukaryote MPE2-25
AY620304 Uncultured cercozoan 13-2.8
FN690387 Uncultured cercozoan 5-A10
AY620316 Uncultured cercozoan D6
Discomonas retusa FJ824131
Discomonadidae
Discomonadida
AY620347 Uncultured cercozoan 7-5.5
Ventrifissura artocaroidea FJ824127
EF526931 Uncultured marine SA2_4G8
AY180018 Uncultured cercozoan CCW29
AY620350 Uncultured cercozoan 12-4.4
Ventrifissura fusiformis FJ824128
EF526932 Uncultured marine SA24H12
EU050976 Uncultured eukaryote SS1 E 01 09
FN690383 Uncultured cercozoan 3b-F9
AY620349 Uncultured c12-3.6
EU050975 Uncultured eukaryote SS1 E 01 26
AY180012 Uncultured cercozoan CCW10
EU371397 Uncultured eukaryote NPKS2_52
'eVentri' clade
Verrucomonas bifida FJ824129
Verrucomonas longifila FJ824130
Ventricleftida
AB505573 Uncultured eukaryote RSM-SGM65
Protaspa sp. FJ824124
Protaspa sp. FJ824123
FN690412 Uncultured cercozoan 3c-H7
Protaspa longipes AF290540
Protaspa sp. FJ824125
Protaspa grandis DQ303924
EF024013 Soil clone Amb
Rhagostoma minus AJ514867
Rhagostoma sp. HQ121436
Rhagostoma schelisseri HQ121430
FN690411 Uncultured cercozoan 3b-3G
HM135090 Uncultured cercozoan STeb 183
AB275093 Uncultured eukaryote CYSGM-10
AB275046 Uncultured eukaryote DSGM-46
JF698753 Uncultured cercozoan MALINA SI390
DQ314809 Uncultured marine NOR26-10
JN048125 Uncultured eukaryote NB 23A9G
Cryothecomonas aestiva AF290539
FN690365 Uncultured cercozoan 4-F11
FN690408 Uncultured cercozoan 5-H10
FN690404 Uncultured cercozoan 8-28
HQ696569 Uncultured clone S61
AY620354 Marine sediment 7-2.1
AY620320 Uncultured cercozoan A11
AY620322 Uncultured cercozoan D10
AY620321 Marine sediment A14
AY620281 0.5 FW F9
Cryomonadida
Protaspa obliqua FJ824122
Matazoa hastifera AB558956
Matazida
AB275051 Uncultured eukaryote DGSM-51
AB275053 Uncultured eukaryote DGSM-53
Ebria tripartita DQ303922
FN690390 Uncultured cercozoan 6c-F7
Batuliforma benthica FJ824126
AY620356 Marine sediment 7-4.3
Ebriida
AB275052 Uncultured eukaryote DGSM-52
AB275096 Uncultured eukaryote CYSGM-13
AB191410 Uncultured eukaryote TAGIRI-2
AB252750 Uncultured eukaryote NAMAKO-10
'eEbriida' clade
AY620348 Marine sediment 7-6.2
AY620314 Marine sediment 9-1.4
NC4 (Bass 2004)
JN090862 Uncultured eukaryote KRL01E2
Pseudodifflugia cf. gracilis AJ418794
AB505500 Uncultured eukaryote RM1-SGM43
Tectofilosida
IMBRICATEA
ventrifilosa
THECOFILOSEA


SARCOMONADEA
Pansomonadida
Glissomonadida
Pseudosporida
Viridiraptoridae
Paracercomonadidae
Cercomonadida
Cercomonadidae
'eSarcomonad' Clade
NC7 (Bass & TCS 2004)
B1c Brabender et al 2012
B1b (Brabender et al 2012)
B1a (Brabender et al 2012)
Neocercomonas and Filomonas
Cercomonas
Cavernomonas
Eocercomonas
AB534506 Uncultured eukaryote
EF024287 Environmental Cercomonadida
Aurigamonas solis DQ199661
Agitata vibrans HQ121438
Agitata tremulans AY748806
AY620288 0.5 FW F6
AY620287 0.5 FW F5
AY6203607-4.2
AY620289 6-3.7
AY620361 Marine sediment 7-3.4
AY620285 4-5.2
AY620290 FW leafy sediment PP1-14
AY620284 8-1.4
AY620273 Clade T glissomonad
EU709264 Clade X glissomonad
EU709259 Hetpan23
EU709256 EnvSoil Clade Y H32p118t7
EU709254 EnvSoil Clade Y H35p80t13
Sandona similis EU709173
Sandona limna HQ918177
Sandona ubiquita EU709153
Sandona mutans EU709138
Sandona heptamutans HQ918175
Mollimonas vickermani HQ918173
Mollimonas lachrima HQ918172
Flectomonas lenta EU709189
Flectomonas ekelundi AY496043
Neoheteromita hederae EU709211
Neoheteromita tolerans HQ121433
EU709217 Soil glissomonad
Neoheteromita globosa U42447
Dujardina stenomorpha HQ918171
EU709276 Soil HetChi9
AJ130852 Clade U glissomonad
EU709273 Clade T glissomonad
Proleptomonas faecicola AF411275
Proleptomonas faecicola AJ305043
Bodomorpha prolixa HQ918170
Bodomorpha minima AF411276
EU709271 Clade Z glissomonad
EU709266 Het Aus17
EU709270 Clade Z glissomonad
EU647174 Environmental sediment
EU709250 Environmental soil
Teretomonas rotunda EU709247
Allapsa mylnikovi EU709238
Allapsa ocior EU709230
Allapsa vibrans AF411265
Allantion parvum CCAP1906/1 HQ918169
AB695472 Uncultured eukaryote
EF023937 Environmental glissomonad Amb 18S 1398 - Wrongly labelled in GenBank as 'Eimeriidae'
EF023686 Clade Te glissomonad Amb 18S 94
EF024999 Clade Te glissomonad Elev 18S 4451
EF024516 'Thaumatomonadida' environmental Elev 18S 823
Nucleocercomonas praelonga HM536165
Paracercomonas bassi HM536163
Paracercomonas sp. FJ790736
Paracercomonas paralaciniagerens FJ790730
Paracercomonas sp. Panama 101 FJ790734
Parcercomonas sp. Panama 107 FJ790735
Paracercomonas saepenatans FJ790731
Paracercomonas sp. Panama67 FJ790732
Paracercomonas virgaria AY884340
Paracercomonas crassicauda FJ790725
Paracercomonas oxoniensis FJ790724
Paracercomonas compacta FJ790723
Parcercomonas minima FJ790720
Paracercomonas producta FJ790721
Paracercomonas marina AF411270
Parcercomonas elongata FJ790729
Paracercomonas astra FJ790727
Paracercomonas proboscata HM5361
Paracercomonas ambulans FJ790728
Paracercomonas filosa FJ790722
Metabolomonas insignia HM536167
Metabolomonas sp. Panama83 FJ790737
Metabolomonas baikali HQ121440
DQ243992 Uncultured freshwater cercozoan PCB12AU2004
Brevimastigomonas anaerobica AF411272
EF023473 Environmental cercomonad Amb 18S 1058
EF024294 Environmental cercomonad Elev 18S 695
Neocercomonas parincurvo FJ790709
Neocercomonas jendrali HM536150
AB695470 Uncultured eukaryote MPE1-27
Neocercomonas gigantica AY884320
FJ790752 Uncultured 13H.FBDBW
Neocercomonas pigra FJ790707
Neocercomonas magna FJ790706
FJ790753 Uncultured 7AJRDBW
AY642694 Uncultured eukaryote p1.18
Neocercomonas 'plasmodialis' AF411268
Neocercomonas brazilliensis FJ790702
Neocercomonas lata AY884325
Neocercomonas dactyloptera AY884334
FJ790705 Uncultured 13H.FDBW
Neocercomonas clavideferens FJ790704
Filomonas radiata FJ790712
EJ790756 Uncultured cercomonad 17-4.9.SA
Neocercomonas vacuolata FJ790711
Neocercomonas jutlandica AY496048
Neocercomonas céler FJ790710
FJ790754 Uncultured cercomonad 17-4.2.SA
EF024850 Environmental cercomonad Elev 18S 1370
Cercomonas pellucida HM536148
Cercomonas directa HM536146
Cercomonas ambiqua FJ790696
Cercomonas rapida FJ790694
Cercomonas deformans FJ790686
Cercomonas aff. longicauda ATCC50317 U42449
Cercomonas mtolert FJ790684
Cercomonas phylloplana FJ790689
Cercomonas lenta FJ790691
Cercomonas mutans FJ790692
Cercomonas laeva AY884321
Cercomonas diparavaria AF411266
EF023321 Environmental cercomonad Amb 18S 639
Cercomonas elliptica FJ790699
Cercomonas rotunda FJ790701
Cercomonas kolskia AY884330
Cavernomonas stercoris FJ790717
Cavernom mira FJ790718
AB534514 Uncultured eukaryote I 4 50
Eocercomonas sp. AF411269 (NCBI labelled 'Cercomonas edax')
EF025030 Uncultured eukaryote Elev 18S 6781
Eocercomonas ramosa AY884327
Eocercomonas echina FJ790716
Eocercomonas minuscula FJ790714
02

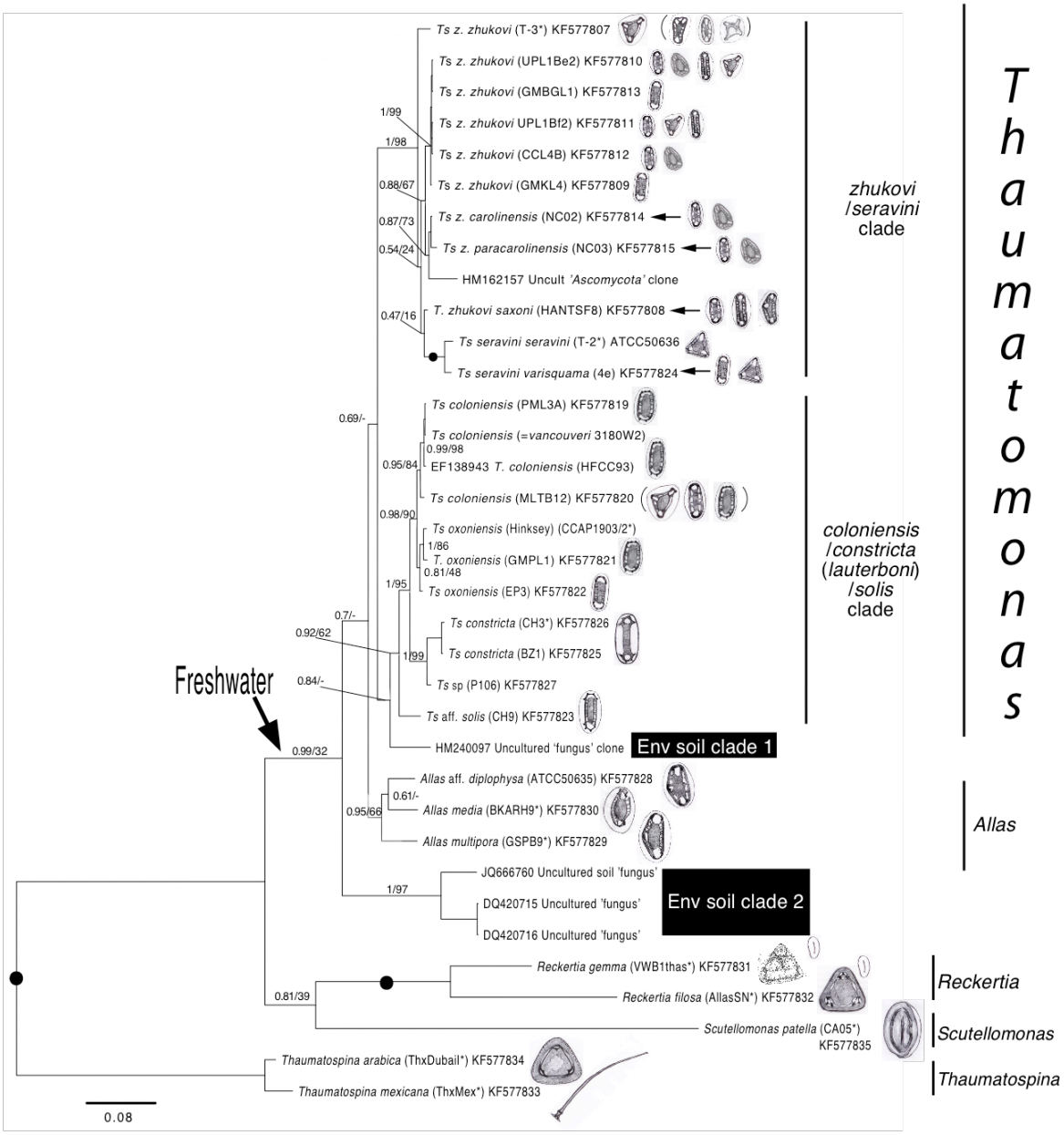

Ts z. zhukovi (T-3*) KF577807
Ts z. zhukovi (UPL1Be2) KF577810
Ts z. zhukovi (GMBGL1) KF577813
Ts z. zhukovi UPL1Bf2) KF577811
Ts z. zhukovi (CCL4B) KF577812
Ts z. zhukovi (GMKL4) KF577809
Ts z. carolinensis (NC02) KF577814
Ts z. paracarolinensis (NC03) KF577815
HM162157 Uncult 'Ascomycota' clone
T. zhukovi saxoni (HANTSF8) KF577808
Ts seravini seravini (T-2*) ATCC50636
Ts seravini varisquama (4e) KF577824
Ts coloniensis (PML3A) KF577819
Ts coloniensis (=vancouveri 3180W2)
EF138943 T. coloniensis (HFCC93)
Ts coloniensis (MLTB12) KF577820
Ts oxoniensis (Hinksey) (CCAP1903/2*)
T. oxoniensis (GMPL1) KF577821
Ts oxoniensis (EP3) KF577822
Ts constricta (CH3*) KF577826
Ts constricta (BZ1) KF577825
Ts sp (P106) KF577827
Ts aff. solis (CH9) KF577823
HM240097 Uncultured 'fungus' clone
Env soil clade 1
Allas aff. diplophysa (ATCC50635) KF577828
Allas media (BKARH9*) KF577830
Allas multipora (GSPB9*) KF577829
JQ666760 Uncultured soil 'fungus'
DQ420715 Uncultured 'fungus'
DQ420716 Uncultured 'fungus'
Env soil clade 2
Reckertia gemma (VWB1thas*) KF577831
Reckertia filosa (AllasSN*) KF577832
Scutellomonas patella (CA05*) KF577835
Thaumatospina arabica (ThxDubail*) KF577834
Thaumatospina mexicana (ThxMex*) KF577833
0.08
Freshwater
1/99
1/98
0.88/67
0.87/73
0.54/24
0.47/16
0.69/-
0.95/84
0.99/98
0.98/90
1/86
0.81/48
1/95
0.7/-
0.92/62
1/99
0.84/-
0.99/32
0.61/-
0.95/66
1/97
0.81/39
zhukovi /seravini clade
coloniensis /constricta (lauterboni) /solis clade
Thaumatomonas
Allas
Reckertia
Scutellomonas
Thaumatospina

Figure 02

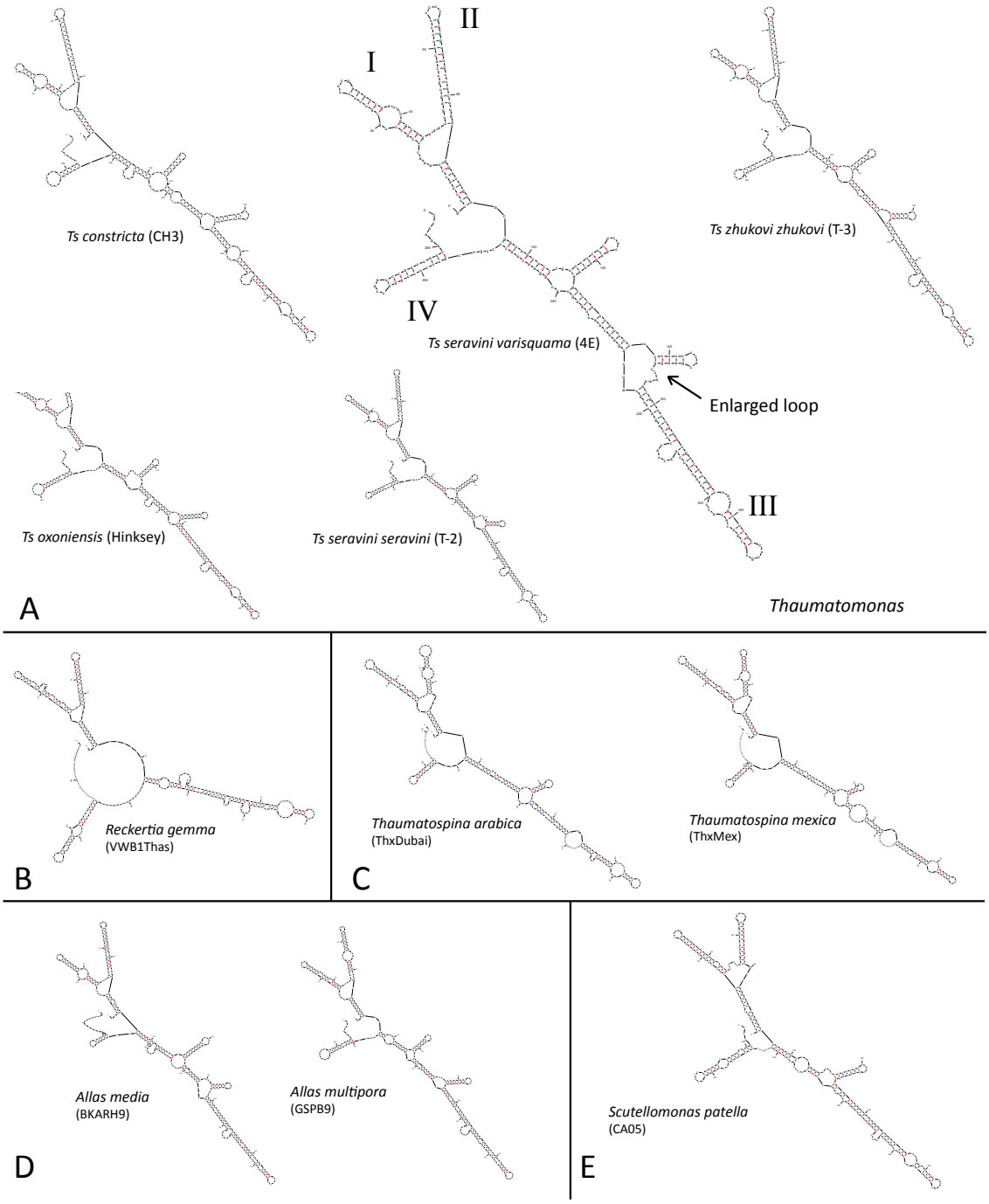

I
II
III
IV
Ts constricta (CH3)
Ts zhukovi zhukovi (T-3)
Ts seravini varisquama (4E)
Enlarged loop
Ts oxoniensis (Hinksey)
Ts seravini seravini (T-2)
A
Thaumatomonas
Reckertia gemma
(VWB1Thas)
B
Thaumatospina arabica
(ThxDubai)
Thaumatospina mexica
(ThxMex)
C
Allas media
(BKARH9)
Allas multipora
(GSPB9)
D
Scutellomonas patella
(CA05)
E

Figure 03

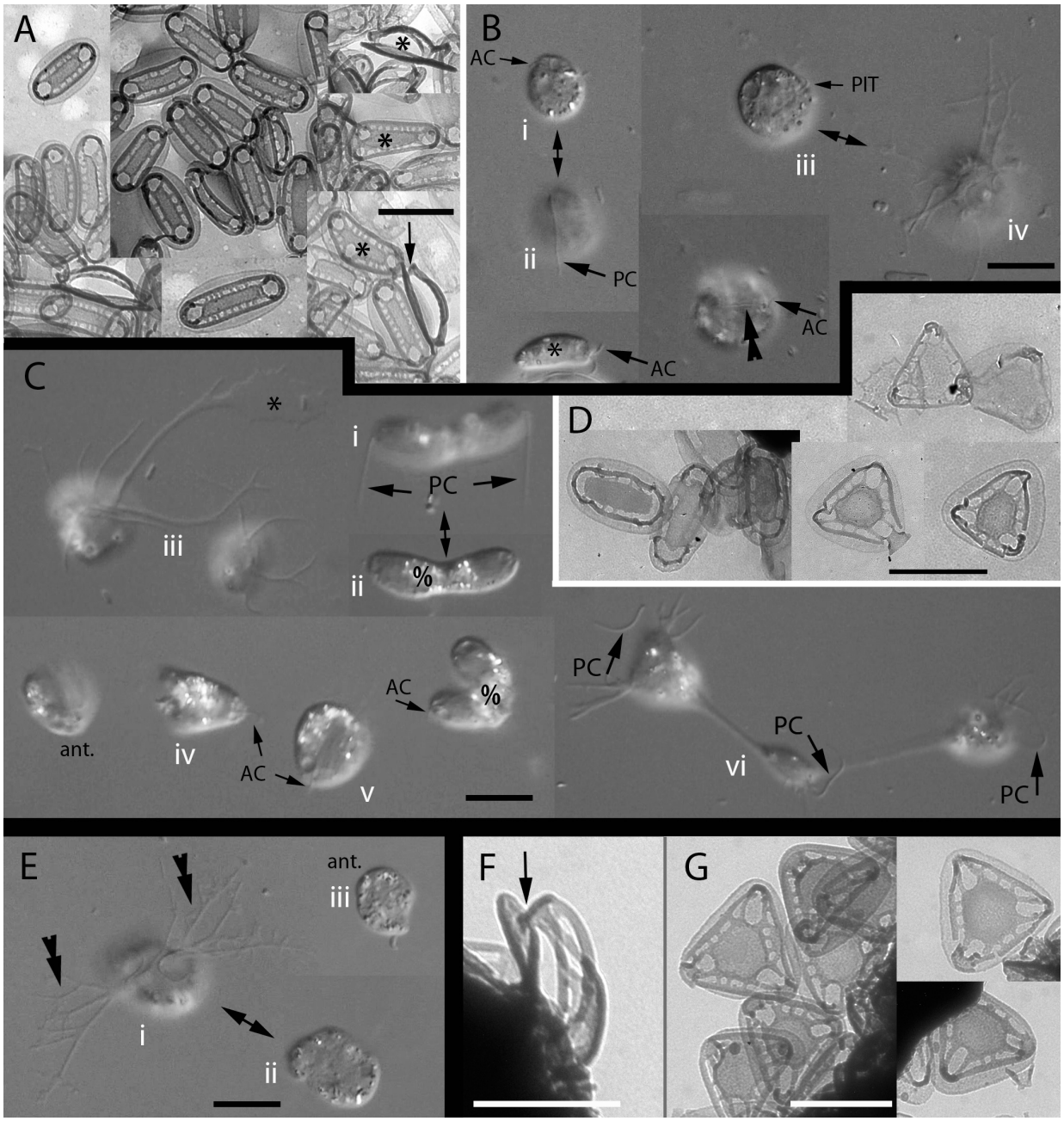

A
B
AC
i
ii
PC
iii
PIT
iv
AC
AC
C
i
PC
ii
iii
D
ant.
iv
AC
AC
v
PC
PC
vi
PC
E
ant.
iii
i
ii
F
G

Figure 04

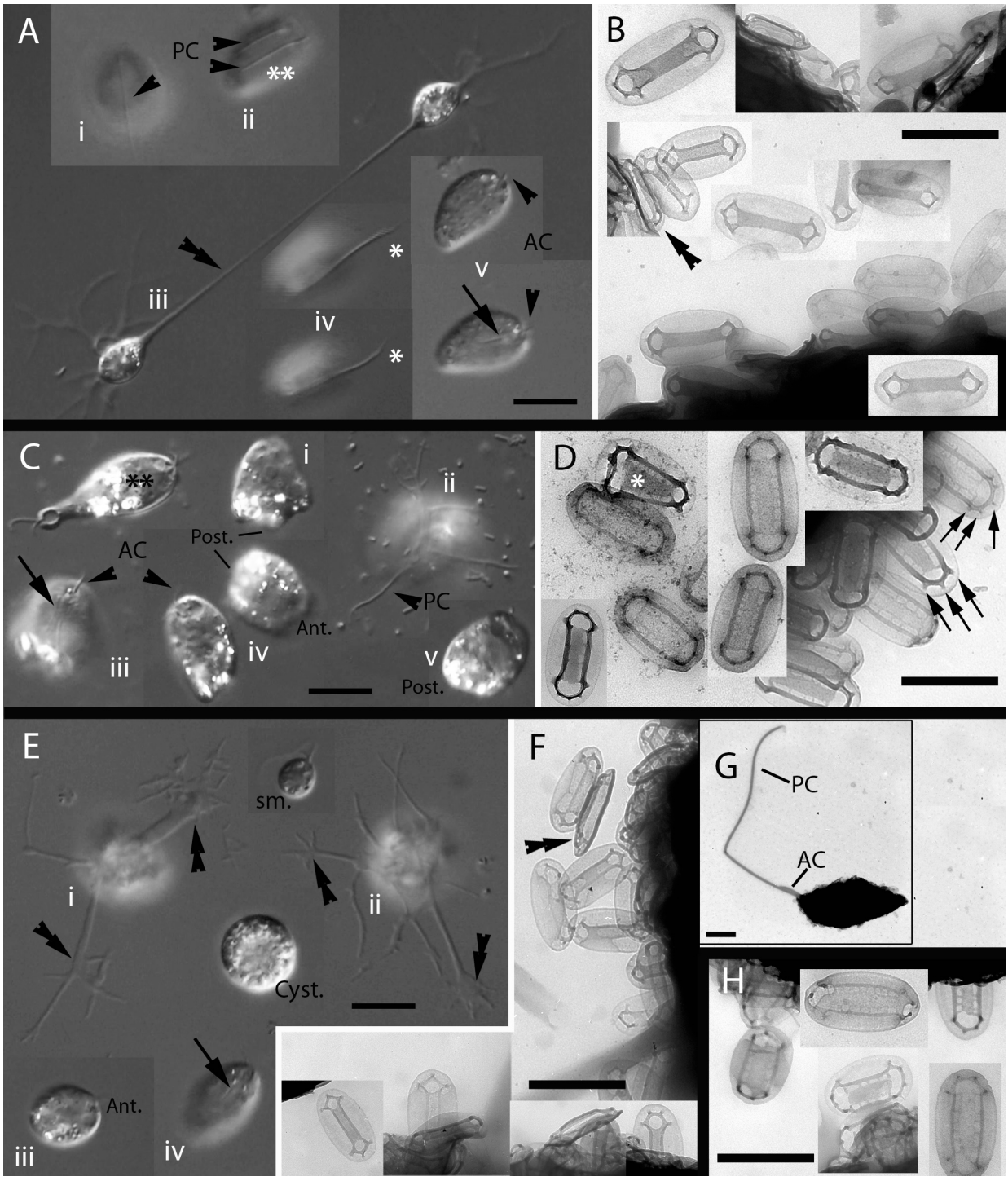

A
PC
**
i
ii
iii
iv
v
AC
*
*
B
C
**
i
ii
Post.
AC
PC
Ant.
iii
iv
v
Post.
D
*
E
sm.
i
ii
Cyst.
Ant.
iii
iv
F
G
PC
AC
H

Figure 05

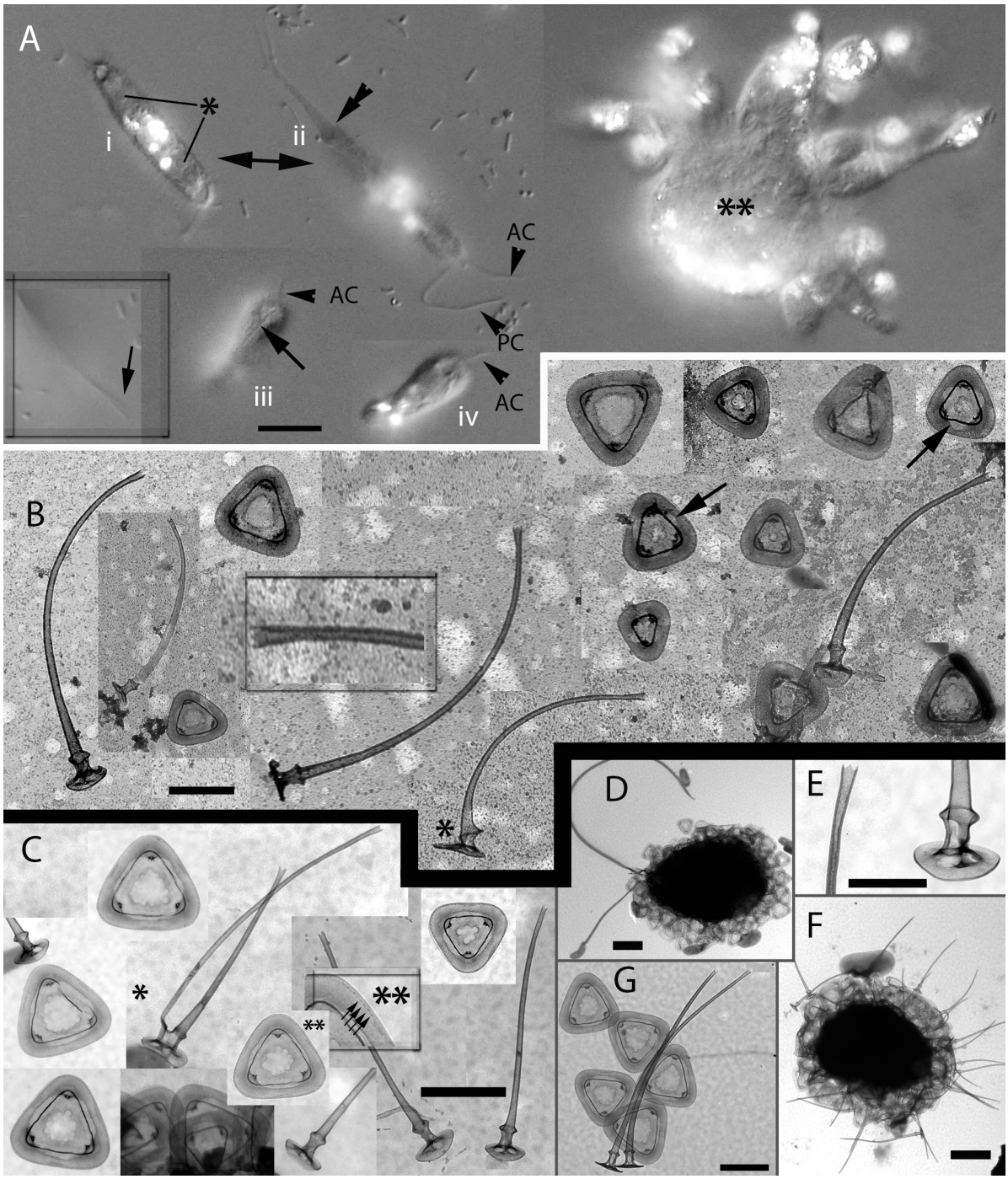


Figure 06

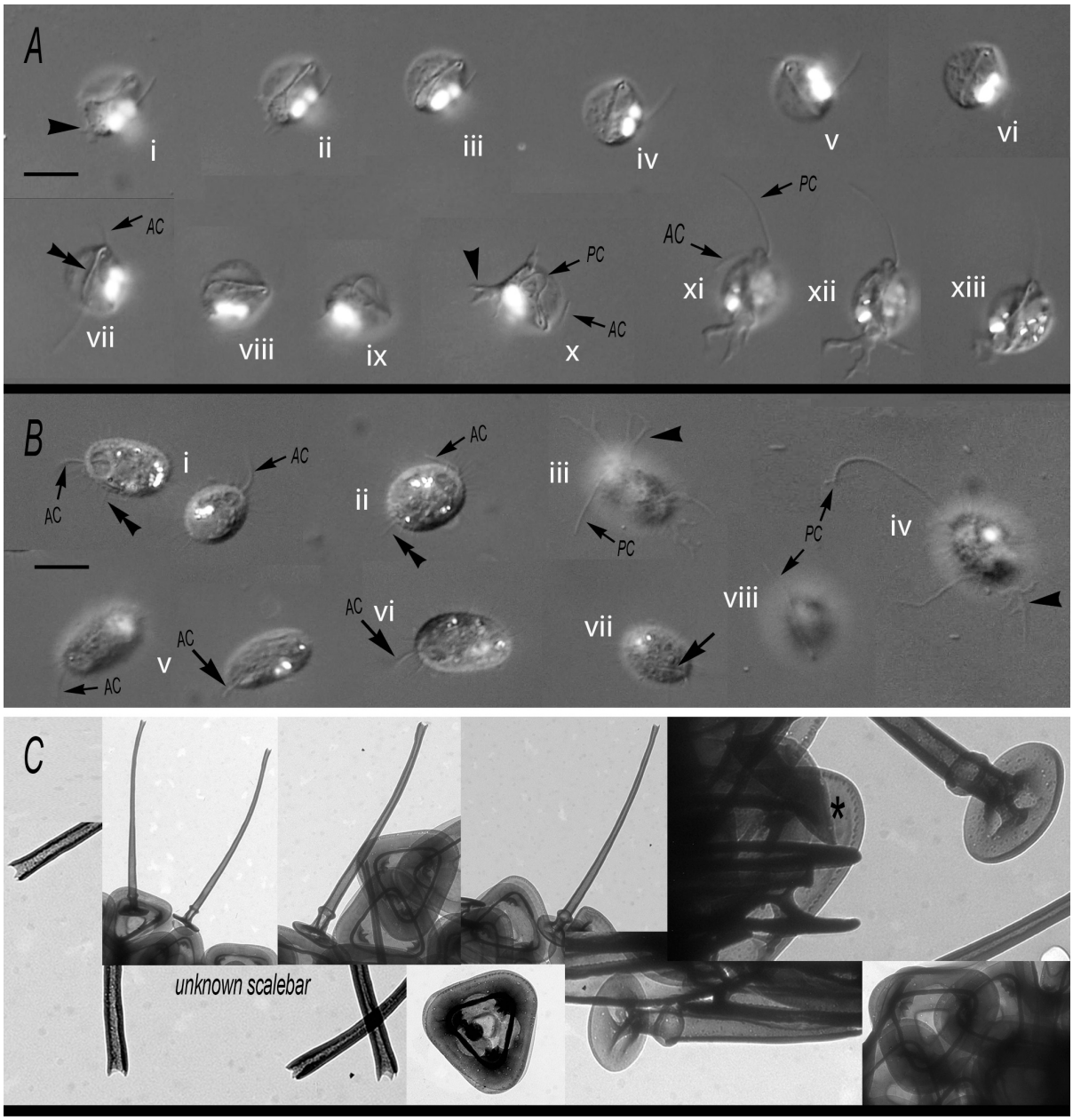

A
i
ii
iii
iv
v
vi
AC
PC
vii
viii
ix
x
xi
xii
xiii
B
i
ii
iii
iv
v
vi
vii
viii
C
*
unknown scalebar

Figure 07

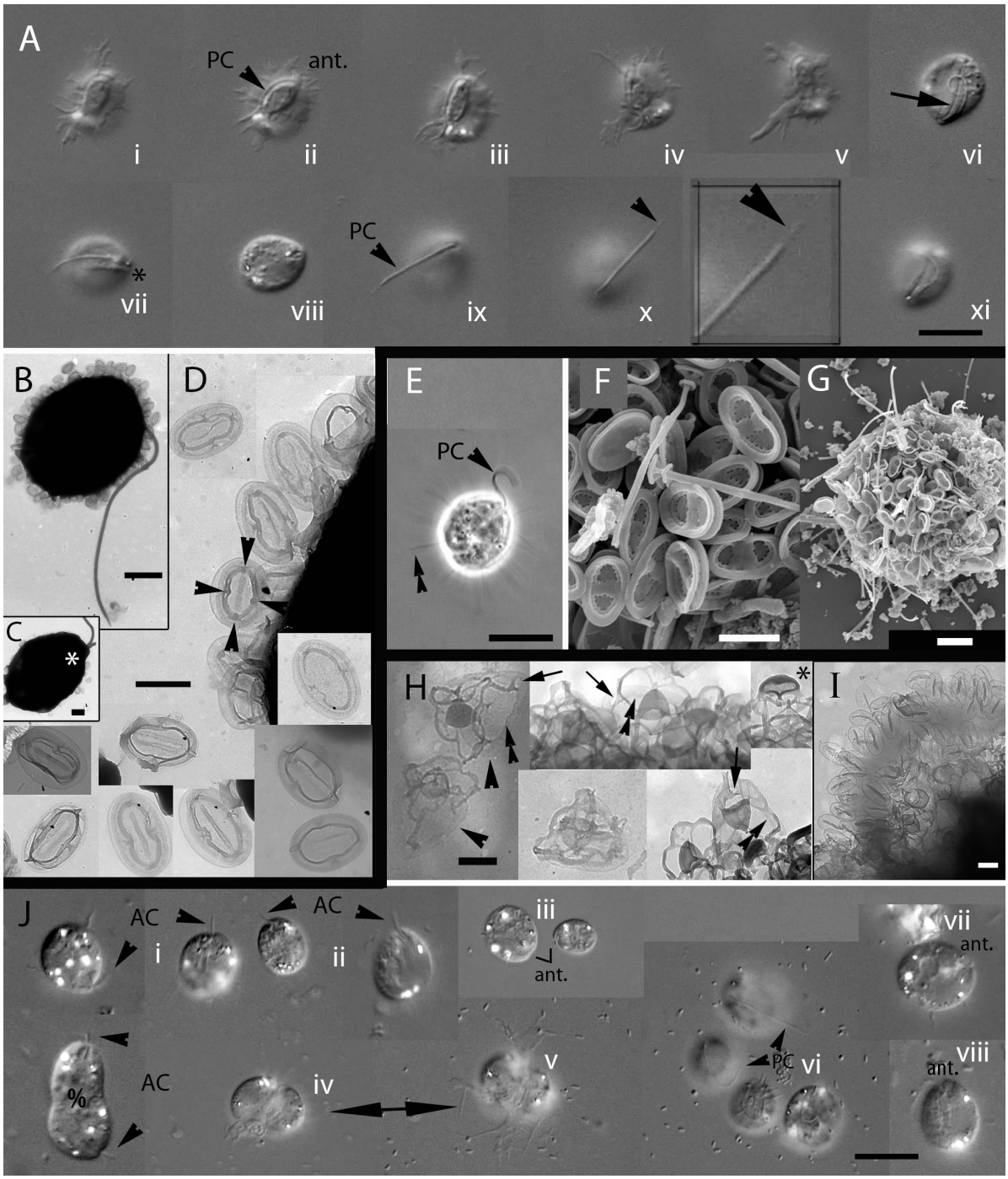

A
PC
ant.
i
ii
iii
iv
v
vi
vii
viii
PC
ix
x
xi
B
C
D
E
PC
F
G
H
I
J
AC
AC
i
ii
iii
ant.
vii
ant.
AC
%
iv
v
PC
vi
viii
ant.

Figure 08

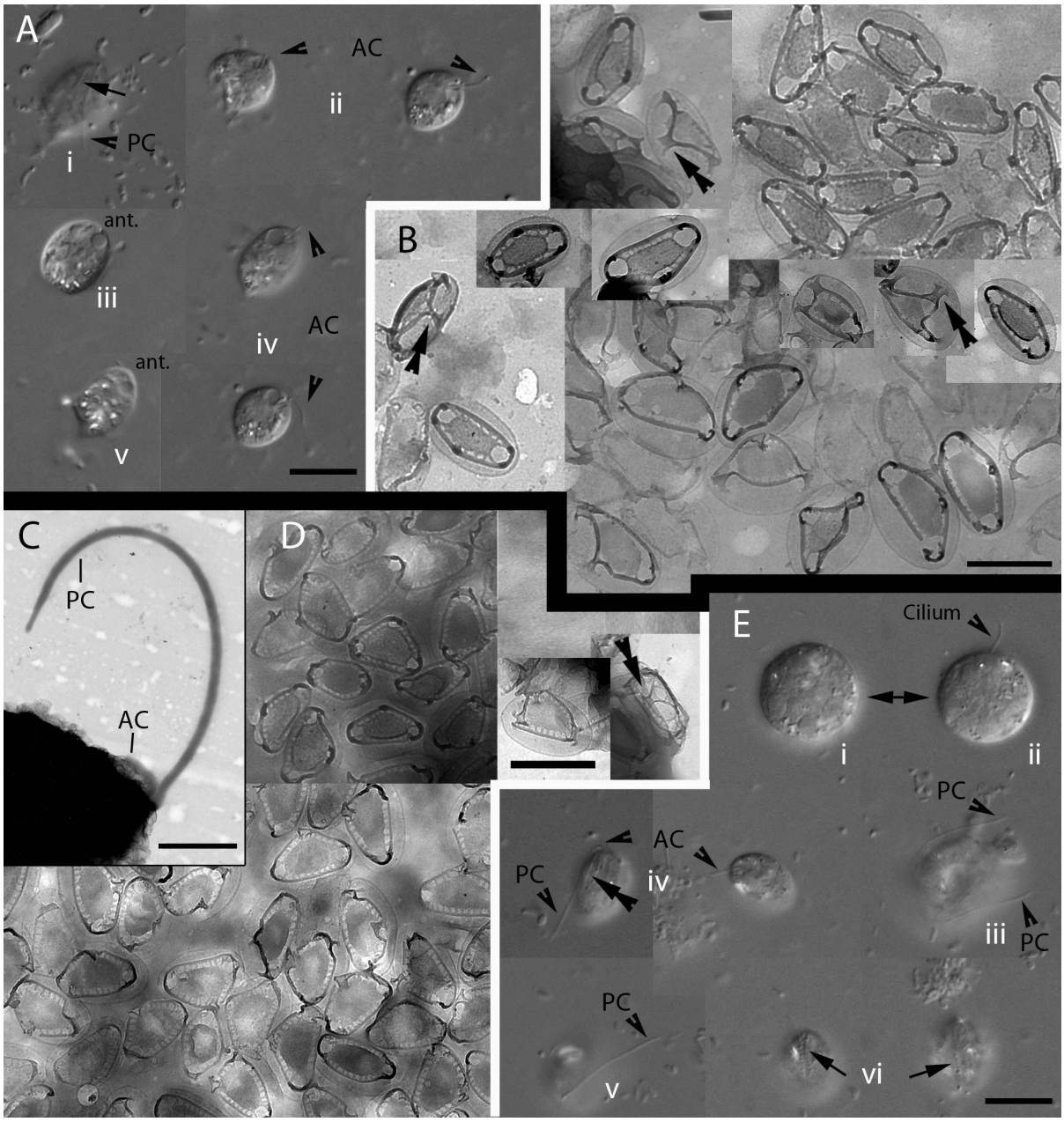

A
AC
ii
PC
i
ant.
iii
AC
iv
ant.
v
B
C
PC
AC
D
E
Cilium
i
ii
PC
AC
PC
iv
iii
PC
PC
v
vi

Figure 09

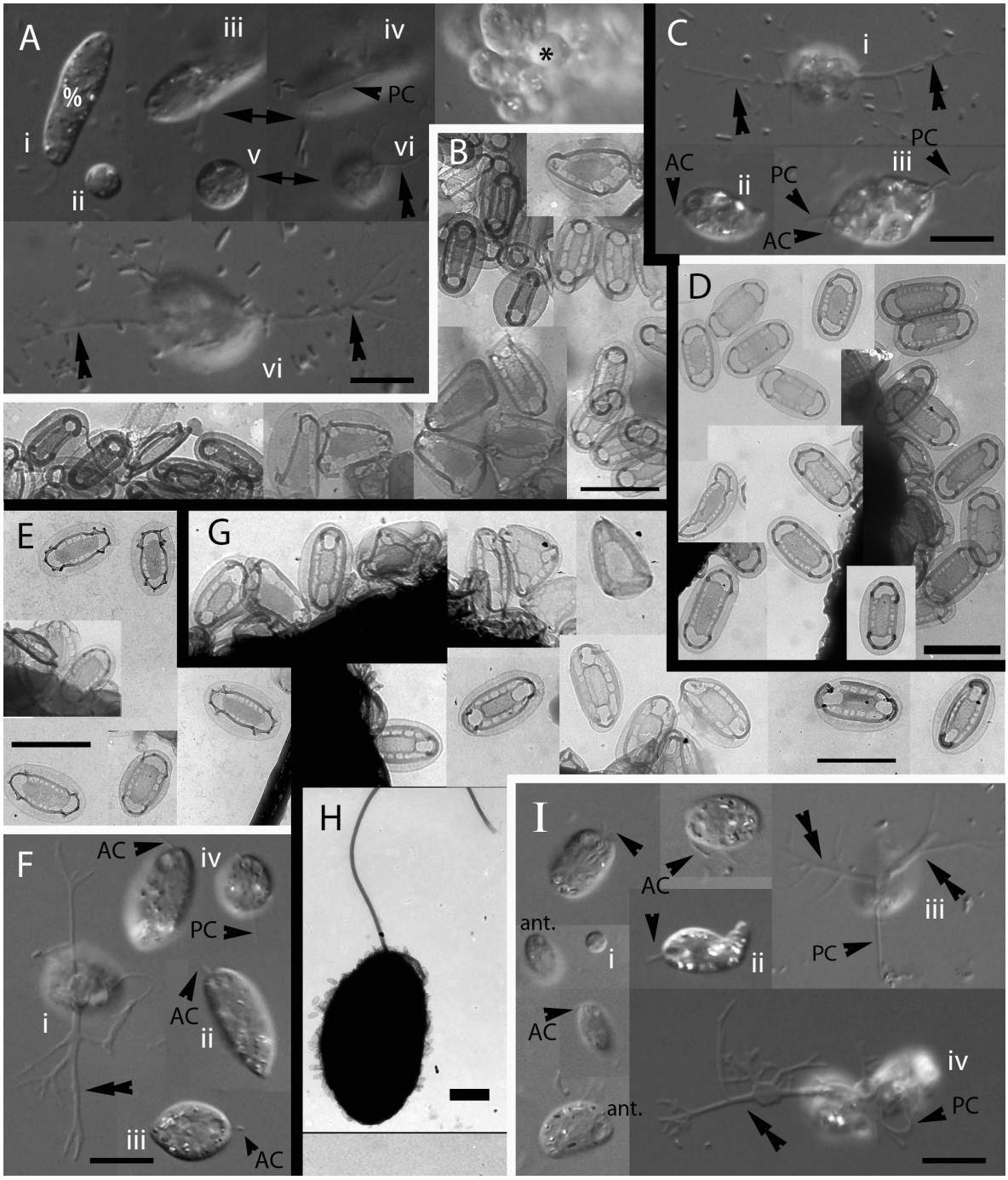

A
i
ii
iii
iv
PC
v
vi
vi
*
B
C
i
PC
AC
ii
PC
iii
AC
D
E
G
F
AC
iv
PC
i
AC
ii
iii
AC
H
I
AC
ant.
i
ii
iii
PC
AC
iv
ant.
PC

Figure 10

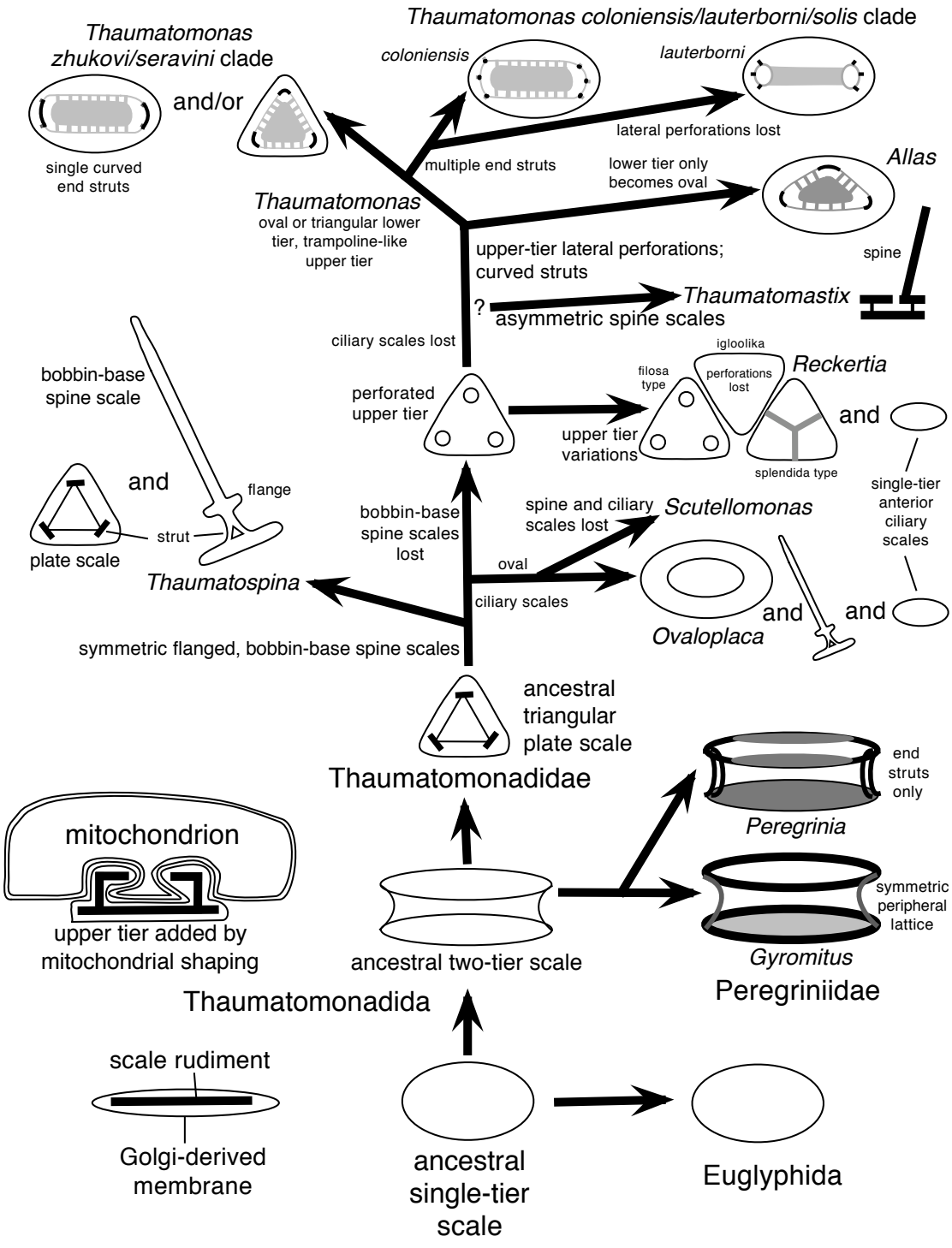

Thaumatomonas coloniensis/lauterborni/solis clade
Thaumatomonas zhukovi/seravini clade
coloniensis
lauterborni
and/or
single curved end struts
lateral perforations lost
multiple end struts
lower tier only becomes oval
Allas
Thaumatomonas
oval or triangular lower tier, trampoline-like upper tier
upper-tier lateral perforations; curved struts
spine
?
Thaumatomastix
asymmetric spine scales
ciliary scales lost
igloolika
filosa type
perforations lost
Reckertia
bobbin-base spine scale
perforated upper tier
upper tier variations
and
splendida type
single-tier anterior ciliary scales
and
flange
strut
plate scale
bobbin-base spine scales lost
spine and ciliary scales lost
Scutellomonas
oval
Thaumatospina
ciliary scales
and
and
Ovaloplaca
symmetric flanged, bobbin-base spine scales
ancestral triangular plate scale
Thaumatomonadidae
end struts only
Peregrinia
mitochondrion
upper tier added by mitochondrial shaping
ancestral two-tier scale
symmetric peripheral lattice
Gyromitus
Peregriniidae
Thaumatomonadida
scale rudiment
Golgi-derived membrane
ancestral single-tier scale
Euglyphida

FIGURE 11

Supplementary Figure 1. Clade *zhukovi*/*seravini*: molecular comparison. Top section of figure shows 18S rDNA nucleotide differences between *Ts z*. *zhukovi* isolates and two *Ts s*. *seravini* isolates. Nucleotide positions marked 'n' are undetermined and '/' is a deletion. The middle section shows a matrix of CBCs and hCBCs in ITS2 rDNA secondary structure folds between both clades, which identify three main groups of strains separated by thick lines, and show that two *Ts z.* subspecies (*carolinensis* and *parcarolinensis*) are markedly more different than the others. The bottom matrix details the number of nucleotide differences between the isolates in ITS1 rDNA and ITS2 rDNA, which again identify the three groups.

Supplementary Figure 2. Clade *coloniensis*/*oxoniensis*: molecular comparison of strains. Top section of the figure is a matrix of the 18S rDNA nucleotide differences emphasising very little difference at this molecular level; *Ts coloniensis* '*vancouveri*' has five likely sequencing errors shown here (in bold). Dashes '/' indicate deletion, 'n' indicate undetermined nucleotides. '~' Indicates missing data. *Ts oxoniensis* has more than 77 undetermined nucleotides in two sections of its sequence. Both *Ts coloniensis* sequences DQ211591 and DQ211592 have one notable different nucleotide (C). The middle section of the figure shows the number of CBC and hCBC differences between the isolates in ITS2 rDNA secondary structure; CBCs clearly differentiate *Ts coloniensis* and oxoniensis, but different isolates of the same species show no CBC between them. (There are no ITS2 rDNA data for the type strain HFCC93 of *Ts coloniensis*). The bottom left matrix gives the total number of nucleotide differences for ITS1 rDNA and ITS2 rDNA. The bottom right matrix gives the total number of ITS2 rDNA secondary structure CBCs and hCBCs between the isolates.

Supplementary Figure 3. A-C, *Thaumatomonas oxoniensis* (GMPL1). **D-F**, *Thaumatomonas oxoniensis* (EP3). **G-H**, *Thaumatomonas coloniensis* (PML3A).

A. DIC of live *Ts oxoniensis* (GMPL1); i, two feeding cells with branching pseudopodia (double arrowheads), ii, gliding cells with short anterior cilium (AC), iii – iv, one gliding cell in two focal planes (two-way arrow), iii, anterior cilium (AC) and ventral groove (arrow), iv, pointed posterior end. **B**. TEM whole cell of *Ts oxoniensis* (GMPL1). **C**. Body-scales of *Ts oxoniensis* (GMPL1). **D**. TEM of two *Ts oxoniensis* (EP3) cells bearing scales, attached via pseudopodium (see strain EP3 DIC comparison - F (*)). **E**. Body-scales of *Ts oxoniensis* (EP3), side view of scale showing upper tier bows upward and end strut (double arrowhead), aberrant scale (*). **F**. DIC of live *Ts oxoniensis* (EP3); i, two feeding masses joined via pseudopodium (*) and adjacent small round cell, ii, gliding cells with anterior end indicated (ant.), iii, feeding cell mass with multiple cilia (arrowheads) and branching pseudopodia (double arrowheads), iv, partially

divided gliding cell with two anterior cilia (AC). **G**. DIC of live *Ts coloniensis* (PML3A); i – ii, feeding cell with pointed posterior shown in two different focal planes (two-way arrow) with posterior cilium (PC), iii, gliding cell with pointed posterior and anterior cilium (AC), iv, gliding cell with pointed posterior, v, two small rounded cells with no cilia, vi, two smaller gliding cells. **H**. Body-scales of *Ts coloniensis* (PML3A). Scale bars: A, G and F 10 µm, B and D 2 µm, C, E and H 0.5 µm.

Supplementary Figure 4. A-C, *Thaumatomonas seravini seravini* (CH8). **D-E**, *Thaumatomonas coloniensis* (MLTB12).

A. DIC of live *Ts s. seravini* (CH8); i – ii in two focal planes (two-way arrow) with three trailing posterior cilia (PC), iii – iv and x – xi, two large round polynucleate feeding cells (* and **) shown on two focal planes (two-way arrow), iv and xi, branching pseudopodia, v, gliding cells with posterior cilium (PC) and ventral groove (arrow), vi, gliding cell with anterior cilium (AC), vii, gliding cell with ventral groove (arrow), viii, oval gliding cell with anterior end indicated (ant.), ix, two large cells attached via cilium (double arrowhead). Possibly misdivided, double gliding cell (%). **B**. Body-scales *Ts s. seravini* (CH8). **C**. TEM of fixed *Ts s. seravini* (CH8) cells showing cilia and attached body-scales. **D**. Body-scales of *Ts coloniensis* (MLTB12), arrows pointing to solid terminal struts characteristic of the clade containing this species. **E**. DIC of live *Ts coloniensis* (MLTB12) cells; i – ii, gliding cell in two focal planes (two-way arrow) with posterior cilium (PC), iii, gliding cell with posterior obtusely angled (flick) and anterior end indicated (ant.), iv, two gliding cells with pointed posterior and anterior cilium (AC), v, round refractile cyst. Scale bars: A and E 10 µm, B and D 0.5 µm, C 2 µm.

Supplementary Figure 5. TEM showing Keith Vickerman's culture 'lauterborni (?)' body scale compared to *T. lauterborni*-like scale (L) and *T. coloniensis*-like (C) scale. Vickerman's is more similar to C.

Supplementary Figure 6. A-C, *Thaumatomonas zhukovi zhukovi* (UPL1Bf2). **D**, *Thaumatomonas zhukovi* (T-3). **E-F**, *Thaumatomonas zhukovi zhukovi* (CCL4B). **G-H**, *Thaumatomonas zhukovi zhukovi* (UPL1Be2).

A-B. *Ts z. zhukovi* (UPL1Bf2) body-scales with struts (arrows). **C**. DIC of live *Ts z. zhukovi* (UPL1Bf2); i – iii, egg-shaped gliding cells with anterior cilium (AC), iv, feeding cell with broad pseudopodia (double arrowhead) and posterior cilium (PC), v, gliding cell with anterior cilium (AC). **D**. *Ts z. zhukovi* (T-3) body-scales. **E**. Body-scales of *Ts z. zhukovi* (CCL4B), side view of scale with strut (double arrowhead); right-hand picture shows single broad end struts. **F**. DIC of

live *Ts z. zhukovi* (CCL4B); i, double gliding cells (%) with anterior cilium (AC), ii, gliding cell with anterior cilium (AC) and contractile vacuole. **G**. Body-scales of *Ts z. zhukovi* (UPL1Be2); side-view of scale with bowed upper-tier and single broad end strut (double arrowhead) and slightly oblique view of broad end strut (arrow). **H**. DIC of live *Ts z. zhukovi* (UPL1Be2); i, gliding cell with anterior cilium (AC), ii, gliding cell with posterior cilium (PC), iii, feeding cell with pseudopodia and posterior cilium (PC), iv, partially divided (?) gliding cell (%). Scale bars: A, B, D, E and G, 0.5 µm. C, F and H, 5 µm.

Supplementary Figure 7. ITS1 and ITS2 rDNA molecular analysis for *Allas*: **A**. *A.* aff. *diplophysa;* **B**. *A. media* and **C**. *A. multipora.* Top of figure shows the ITS2 rDNA secondary structure folds, a matrix grid showing the number of nucleotide differences between each sequence for ITS1 and ITS2 rDNA sequence, A and B are the most different. The bottom half of the figure shows each ITS2 rDNA nucleotide different between the strains revealing a mosaic pattern. Blocks of similarity between the strains are shown in different parts of the molecule, e.g. A and C share a similarity block between nucleotides 33 – 35, where B and C are more similar in a larger section between nucleotides 76 – 82. White blocks indicate deletion.

Supplementary Figure 8. ITS1 & ITS2 rDNA results for *Thaumatomonas*: **A.** *Ts constricta* (BZ1); **B.** *Ts constricta* (type strain CH3) and **C**. *Ts* sp. (106). Top of the figure shows the nucleotides that are different between the three strains; A and B have no differences in ITS1 but 22 different from C. A and B have four differences between them in ITS2 rDNA but have 22 and 23 differences between them and C, respectively (shown in left matrix) confirming that C is the most divergent. CBC analysis of the ITS2 rDNA folds reveals no CBCs or hCBCs between A or B, but they both have 2 CBC and 7 hCBCs against C (right matrix). The three two-dimensional ITS2 rDNA folds reveal little apparent difference in structure bottom of figure.

18S rDNA seqeunce differences

	1	2	3	4	5	6	7	8	9	10	11	12	13
Ts zhukovi zhukovi KC243119 T-3	t	c	t	**n**	**n**	**n**	g	t	t	g	c	c	g
Ts zhukovi zhukovi KC243105 UPL1be2	t	c	t	g	g	g	g	t	t	g	c	c	g
Ts zhukovi zhukovi UPL1Bf2	t	c	t	g	g	g	**n**	t	t	g	c	c	g
Ts zhukovi zhukovi GMBGL1	?	?	?	?	?	?	?	?	?	?	?	?	?
Ts zhukovi zhukovi GMKL4	t	c	t	g	g	g	g	t	t	g	c	c	g
Ts zhukovi zhukovi CCL4B	**n**	**n**	**n**	g	g	g	g	t	t	g	c	c	g
Ts zhukovi saxoni HANTSF8	t	c	t	g	g	g	g	t	t	g	c	c	g
Ts zhukovi carolinensis NC02	t	c	t	g	g	g	g	t	t	g	c	c	g
Ts zhukovi paracarolinensis NC03	t	c	t	g	g	g	g	t	**n** x30	g	c	c	g
Ts seravini seravini AF411259 ATCC50636 T-2	t	c	t	g	g	g	g	/	t	**c**	**g**	**a**	**t**
Ts seravini varisquama 4e	t	c	t	g	g	g	g	t	t	**c**	**g**	**a**	**t**

Number of ITS2 rDNA hCBCs and CBCs

		Ts zhukovi zhukovi (T-3) A	*Ts zhukovi zhukovi* UPL1Be2 B	*Ts zhukovi zhukovi* UPL1Bf2 C	*Ts zhukovi zhukovi* GMBGL1 D	*Ts zhukovi zhukovi* GMKL4 E	*Ts zhukovi zhukovi* CCL4B F	*Ts zhukovi saxoni* HANTF8 G	*Ts zhukovi carolinensis* NC02 H	*Ts zhukovi paracarolinensis* NC03 I	*Ts seravini seravini* T-2 J	*Ts seravini varisquama* 4E K
***Ts zhukovi zhukovi* T-3**	A		**0**	**0**	**0**	**0**	**0**	2	4	3	5	6
***Ts zhukovi zhukovi* UPL1Be2**	B	**0**		**0**	**0**	**0**	**0**	2	4	3	5	6
***Ts zhukovi zhukovi* UPL1Bf2**	C	**0**	**0**		**0**	**0**	**0**	2	4	3	5	6
***Ts zhukovi zhukovi* GMBGL1**	D	**0**	**0**	**0**		**0**	**0**	2	4	3	5	6
***Ts zhukovi zhukovi* GMKL4**	E	**0**	**0**	**0**	**0**		**0**	2	4	3	5	6
***Ts zhukovi zhukovi* CCL4B**	F	**0**	**0**	**0**	**0**	**0**		2	4	3	5	6
***Ts zhukovi saxoni* HANTF8**	G	**0**	**0**	**0**	**0**	**0**	**0**		3	5	7	6
***Ts zhukovi carolinensis* NC02**	H	3	3	3	3	3	3	1		3	7	6
***Ts zhukovi paracarolinensis* NC03**	I	2	2	2	2	2	2	2	1		5	5
***Ts seravini seravini* T-2**	J	1	1	1	1	1	1	1	2	1		1
***Ts seravini varisquama* 4E**	K	1	1	1	1	1	1	**0**	1	**0**	**0**	

number of hCBCs

number of CBCs

number of nucleotide differences in ITS1 rDNA sequence

		T-3 A	UPL1Be2 B	UPL1Bf2 C	GMBGL1 D	GMKL4 E	CCL4B F	HANTF8 G	NC02 H	NC03 I	T-2 J	4E K
***Ts zhukovi zhukovi* T-3**	A		7	9	5	6	5	11	16	24	41	38
***Ts zhukovi zhukovi* UPL1Be2**	B	25		4	2	1	2	14	21	25	44	43
***Ts zhukovi zhukovi* UPL1Bf2**	C	25	**0**		4	3	4	15	22	27	46	45
***Ts zhukovi zhukovi* GMBGL1**	D	25	**0**	**0**		1	**0**	12	19	23	42	41
***Ts zhukovi zhukovi* GMKL4**	E	24	1	1	1		1	13	20	24	43	42
***Ts zhukovi zhukovi* CCL4B**	F	24	1	1	1	**0**		12	19	23	42	41
***Ts zhukovi saxoni* HANTF8**	G	17	17	17	17	16	16		23	25	42	40
***Ts zhukovi carolinensis* NC02**	H	24	13	13	13	12	12	15		18	45	48
***Ts zhukovi paracarolinensis* NC03**	I	25	13	13	13	12	12	16	2		44	50
***Ts seravini seravini* T-2**	J	31	36	36	36	35	35	21	36	37		31
***Ts seravini varisquama* 4E**	K	39	33	33	33	32	32	34	29	29	22	

number of nucleotide differences in ITS2 rDNA sequence

<10
>10
>20
>30
>40

S1

18S rDNA sequence differences

	1	2	3	4	5	6	7	8	9	10	11	More info
DQ211591-2 *Ts coloniensis*	/	t	c	t	/	a	g	a	**c**	/	**~**	
AY496046 *Ts coloniensis*-like	/	t	c	t	/	a	g	a	t	/	**~**	
Ts coloniensis PML3A	/	t	c	t	/	a	g	a	t	/	g	
AF411264 *Ts coloniensis* '*vancouveri*'	/	t	c	t	**t**	**/**	**t**	**/**	t	/	**a**	
Ts coloniensis MLTB12	/	t	c	t	/	a	g	a	t	/	g	
HQ121429 *Ts oxoniensis*	**n**	**n**	**n**	**n**	/	a	g	a	t	**n**	g	77 Ns
Ts oxoniensis GMPL1	/	t	c	t	/	a	g	a	t	/	g	
Ts oxoniensis EP3	/	t	c	t	/	a	g	a	t	/	g	

Number of ITS2 rDNA Compensatory Base Changes (CBCs) and hemiCBCs

		A	B	C	D	E	F	G
Ts coloniensis	**A:** ***Ts coloniensis*** HFCC93		/	/	/	/	/	/
	B: *Ts coloniensis* PML3A	/		1	2	4	2	6
	C: *Ts coloniensis* 3108W2 '*vancouveri*'	/	0		1	4	2	4
	D: *Ts coloniensis* MLTB12	/	0	0		3	1	4
Ts oxoniensis	**E:** ***Ts oxoniensis*** CCAP1903/2	/	2	2	2		2	1
	F: *Ts oxoniensis* GMPL1	/	2	2	2	0		4
	G: *Ts oxoniensis* EP3	/	2	2	2	0	0	

number of hCBCs

number of CBCs

number of nucleotide differences in ITS2 rDNA sequence

number of nucleotide differences in ITS1 rDNA sequence

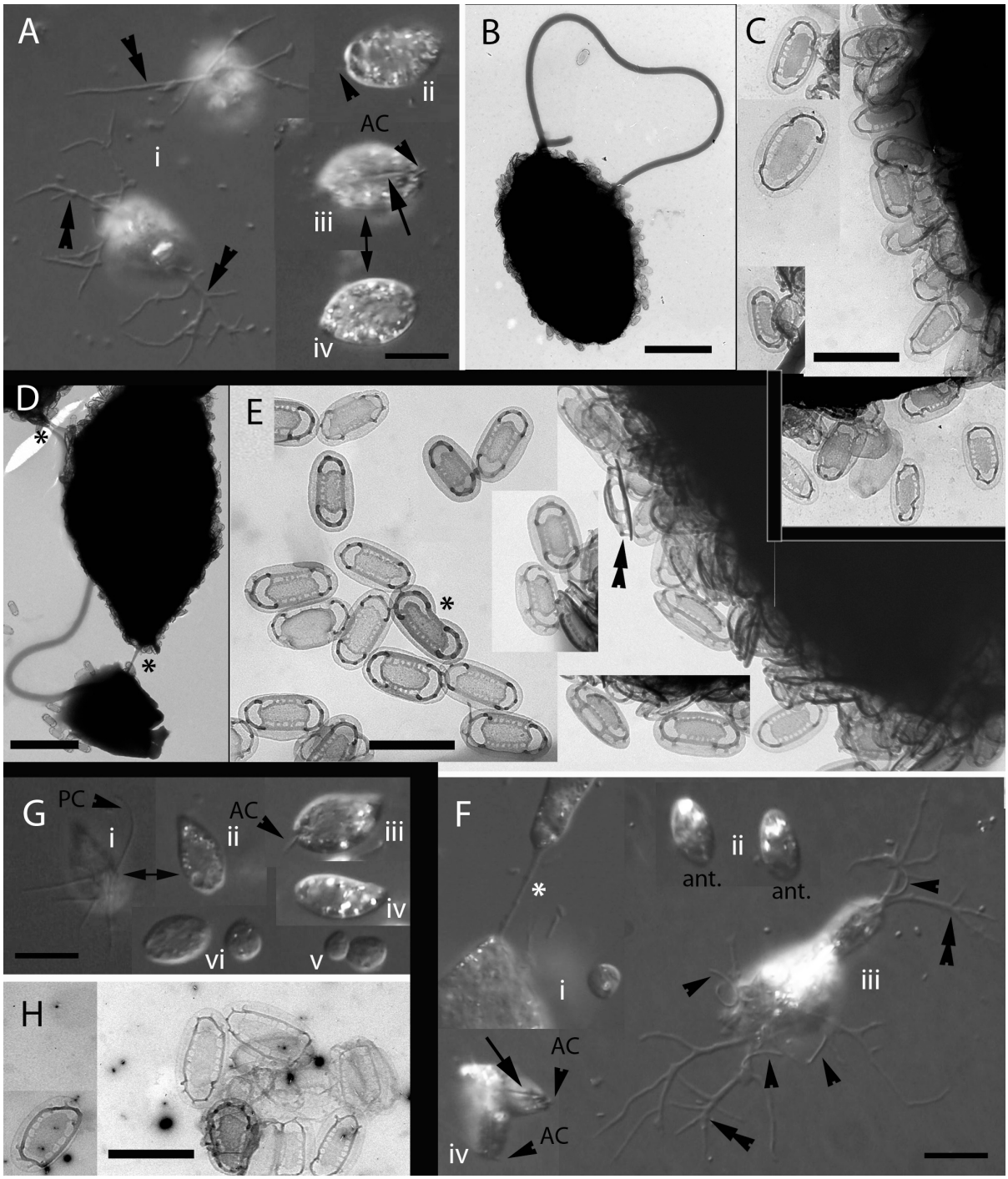
		A	B	C	D	E	F	G	H
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Ts coloniensis HFCC93	B	8		No data	No data	No data	No data	No data	No data
Ts coloniensis PML3A	C	8	**0**		5	8	25	25	21
Ts coloniensis = 3108W2 'vancouveri'	D	8	**0**	**0**		5	21	22	18
Ts coloniensis MLTB12	E	9	13	13	13		21	21	13
Ts oxoniensis CCAP1903/2	F	22	17	17	17	25		10	12
Ts oxoniensis GMPL1	G	10	13	13	13	10	14		18
Ts oxoniensis EP3	H	11	15	15	15	11	22	5	

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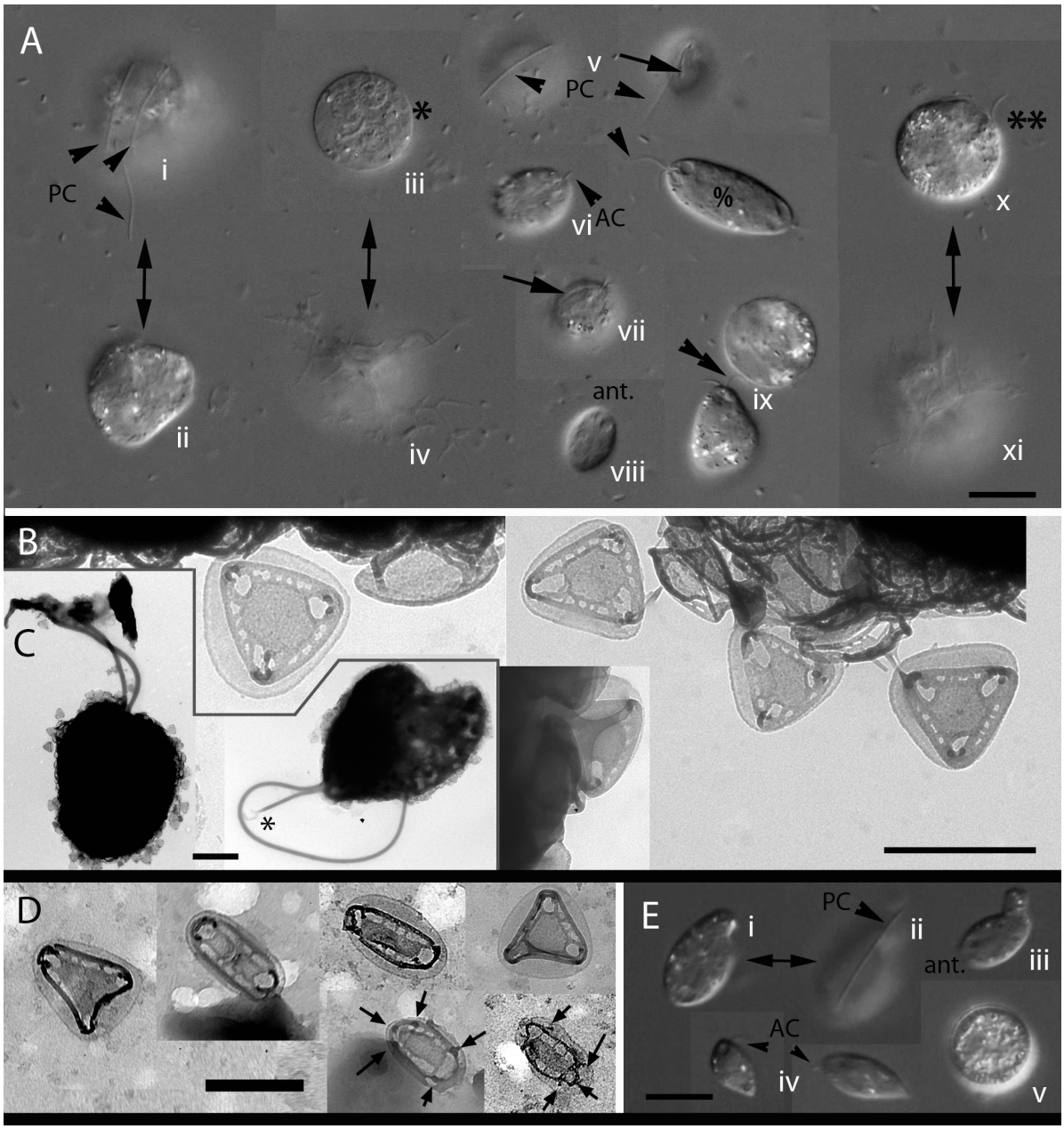
Total number of CBCs and hCBCs

		A	B	C	D	E	F	G
Ts coloniensis HFCC59, HFCC93	A		No data	No data	No data	No data	No data	No data
Ts coloniensis PML3A	B	No data		1	2	7	4	8
Ts coloniensis = 3108W2 'vancouveri'	C	No data	1		1	6	4	6
Ts coloniensis MLTB12	D	No data	2	1		5	3	6
Ts oxoniensis CCAP1903/2	E	No data	7	6	5		2	1
Ts oxoniensis GMPL1	F	No data	4	4	3	2		5
Ts oxoniensis EP3	G	No data	8	6	6	1	5	

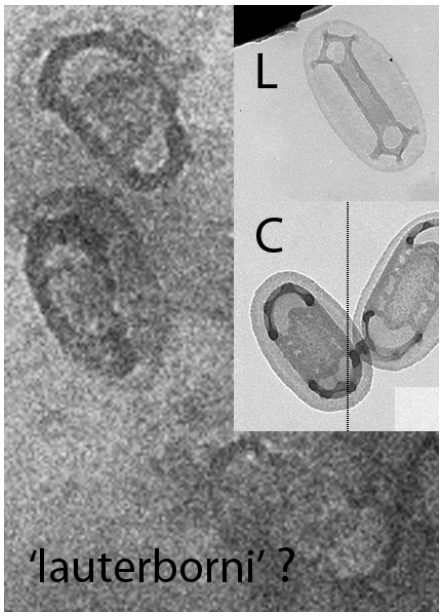
S2


A
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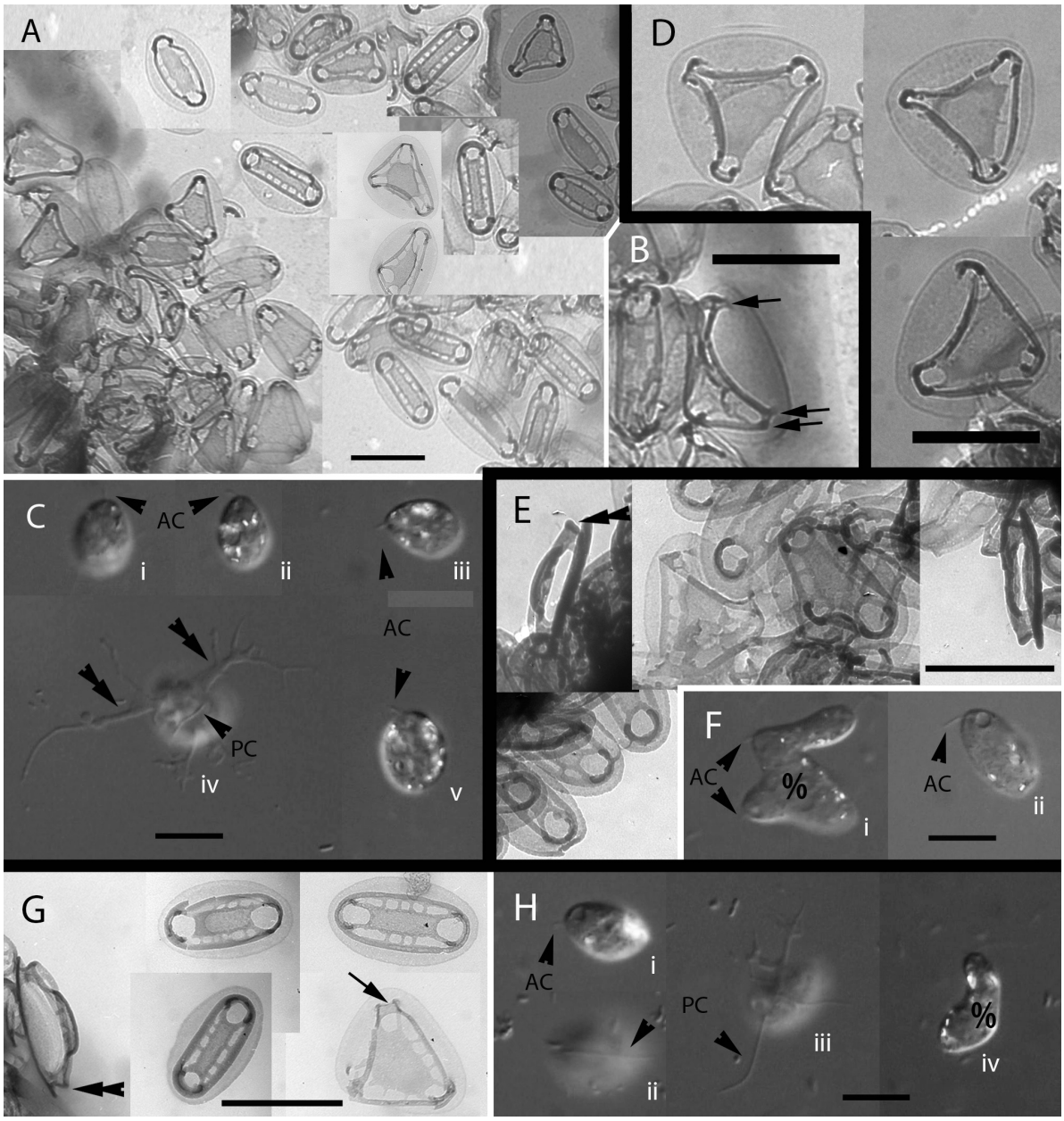
S3


A
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iii
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iv
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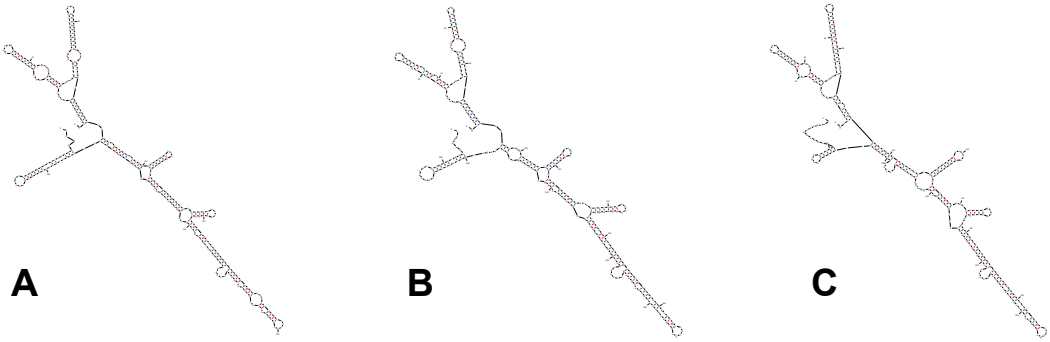
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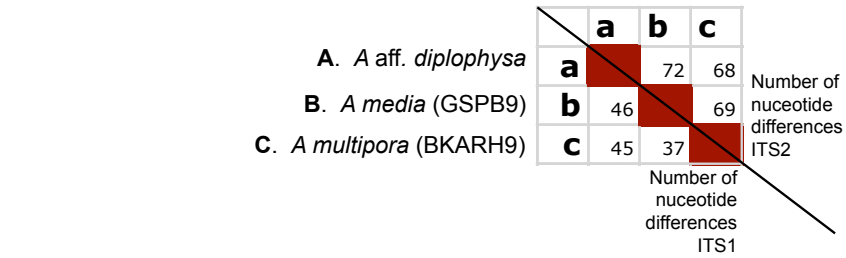


S5

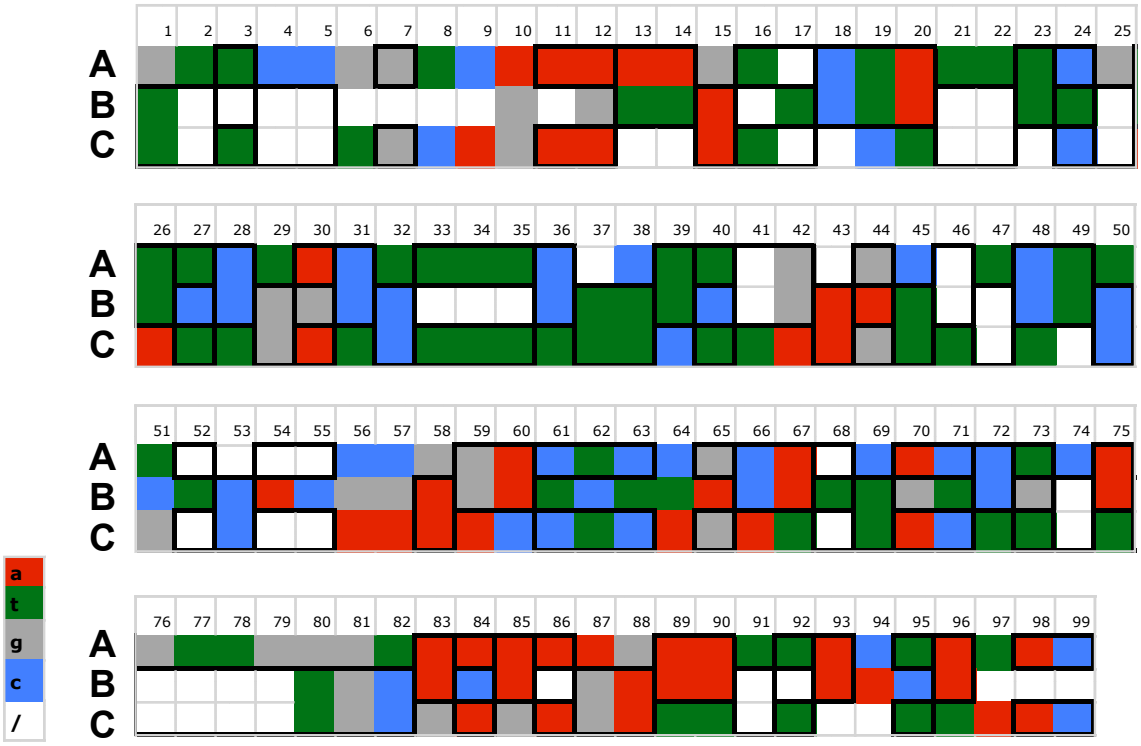


S6



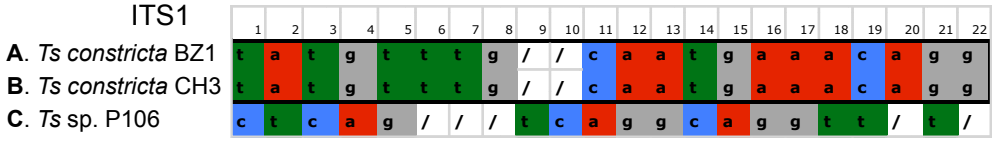


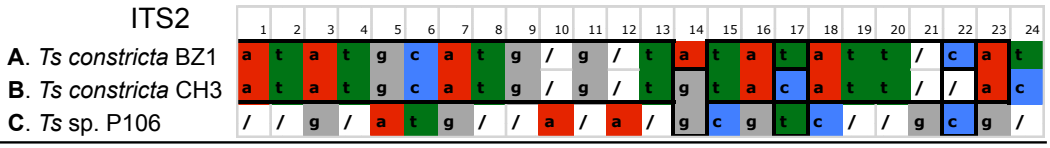
ITS2 rDNA nucleotide differences between the strains

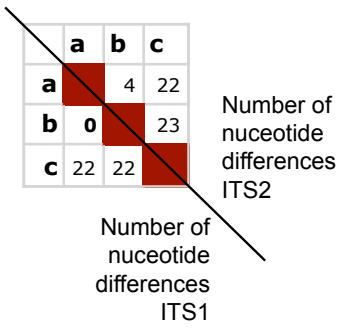


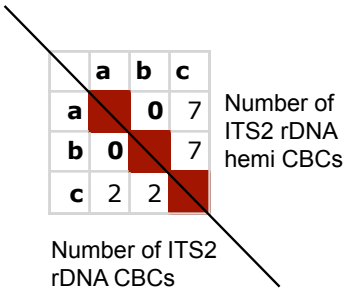
S7

Nucleotide differences between these closely related strains

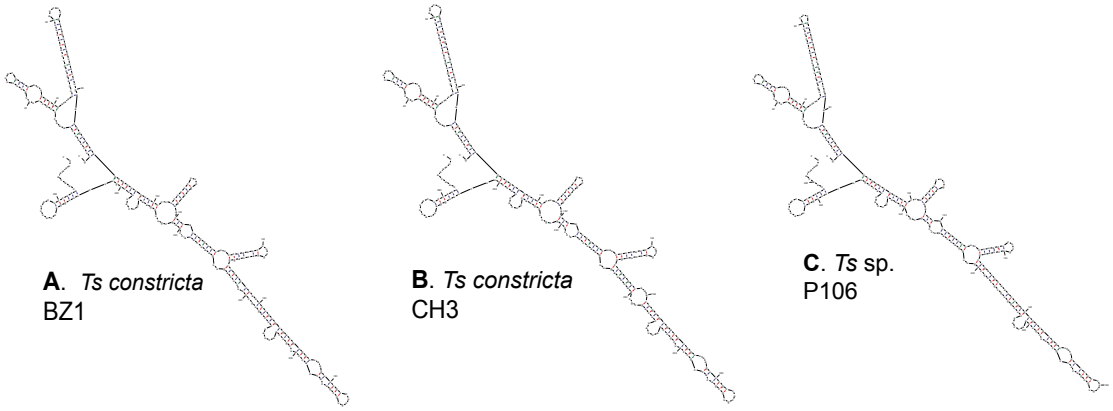








ITS2 rDNA folds



S8

CHAPTER SIX

General discussion and conclusions

This thesis achieved its aim to explore the diversity and improve the taxonomy of two silica-scaled protist groups using clonal culturing, microscopical, and molecular methods.

Nearly one hundred isolates were cultured, molecularly and morphologically investigated to improve the taxonomy of two well known and common protist groups, paraphysomonads and thaumatomonads. Forty-six new species of protist were described using crude and biased culturing methods (Chapter 2), which, if modified slightly, raise new isolates (Appendix B).

A review of the existing literature for one of the taxonomic groups, *Paraphysomonas*, (Chapter 3) showed that this speciose genus was morphologically heterogeneous (Preisig and Hibberd 1982a; Preisig and Hibberd 1982b; Preisig and Hibberd 1983) and molecular phylogenetic trees showed that sequences purportedly from the same species often appeared in very different places, which was often interpreted as misidentifications. New molecular and morphological scale data from this thesis quickly showed a great molecular (18S rDNA) and morphological divergence between the isolates, and that the majority of the 34 newly characterized paraphysomonad species would have previously been lumped as one of two known species, *P. imperforata* and *P. vestita* (Chapter 4). When thaumatomonads were examined in the same way as paraphysomonads, 18S rDNA molecular divergence was

not so great between *Thaumatomonas* isolates with notably different scale morphology so more divergent ribosomal molecules were sequenced and used in phylogenies as well as secondary structure analysis (Chapter 5).

It has been demonstrated in this thesis that molecular sequencing of ribosomal DNA genes has been an important tool in determining species within morphological groups by showing that morphology easily maps on to phylogenetic trees and clustering them into distinct groups (Chapter 4 and 5). ITS2 rDNA folding was useful to delineate species that were sometimes morphologically ambiguous and which had low 18S rDNA molecular divergence. For example, *Thaumatomonas seravini* make triangular scales and *Ts zhukovi* make oval and triangular scales and they had compensatory base changes (CBCs), which confirmed their distinct species status (Chapter 5).

1. Scale variation within *Paraphysomonas* and *Thaumatomonas*

Now that *Paraphysomonas* is a morphologically unified genus containing species that produce only spine scales, a more detailed inventory of spine scaled morphological characters could and has begun (Chapter 4). This thesis has identified discrete features of *Paraphysomonas* spine scales that have become part of the taxonomic identity of these species and specifically the subspecies groups. Groups that show tapered, acutely pointed, or flattened spine tips belong to distinct molecular clades. These scale features map well on to 18S rDNA phylogenetic trees and show great molecular divergence. From these data, one can now apply the same expectation of genetic variation to scaly and non-scaly relatives: *Spumella*, a scaleless counterpart, and *Clathromonas*, a scaly molecularly close relative. The environmental sequences of

these organisms can now reflect more of the expected morphological (scale) diversity between the scaly protists, but not the scaleless organisms. The divergence of sequences may also reflect an ecological specificity to environment yet to be discovered. The discrete mapping of morphology on phylogenetic trees drove a clearer path toward the identification of these distinct morphological characters as well as showing how this level of molecular diversity is reflected in scale morphology.

The *Thaumatomonas* genus is not as speciose as *Paraphysomonas*, however, related genera like *Thaumatomastix,* even though formerly revised to include only species with spine scales (Howe et al. 2012), still required further taxonomic refinement since published sequence trees and ones from this thesis showed that the genus was paraphyletic (Ota et al 2012). Additional sequencing and characterization of newly acquired isolates of other closely related thaumatomonads helped to morphologically refine other thaumatomonad species into morphologically homogeneous genera and created new genera: *Scutellomonas, Ovaloplaca* and *Thaumatospina* (former *Thaumatomastix*). Thaumatomonad scale morphology is now distinct for each scaly genus.

Scale variation within *Paraphysomonas* was previously excessively broad. Retention of just nine former species and an additional 35 newly described from this thesis reaffirms the diverse nature of the group but in a more homogeneous way from revealing numerous subtle differences in the spine scales. Scale size within the genus is wide-ranging; *P. segmenta* has spine scales from 0.52 – 0.73 µm long, when compared to *P. longispina* and *P. stylaris* whose spines are up to ten times as long; 2.2 – 7.3 µm. The broad spine scale size differences are also reflected in the subgenera; *P. brevispina*

0.52 – 1.9 µm; *P. acrospina* 0.8 – 6.7 µm; *P. hebetomonas* 0.9 – 5.6 µm; *P. paraphysomonas* 1.3 – 7.3 µm, however, each of these subgenera are morphologically homogeneous according to their spine tips. The scale size variation observed within these subgenera of *Paraphysomonas* reveals an overall broad natural variation. Spine scale size variation is also present within a single species but the range of variation is not consistent, some species have a wide range of spine scale lengths 2.2 – 7.3 µm (*P. longispina*) and others have a shorter range *P. uniformis* 4.0 – 5.3 µm. The broad or narrow range of variation of spine scale lengths for *Paraphysomonas* species are themselves indicative of the species and have been seen before in the literature (Manton and Leedale 1961). However, even though this thesis did not specifically test the absolute levels of scale variation within species, attempts were made to understand if the dimension of the spine scale differed via spine/plate (S/P) ratios; the length of the spine divided by the width of the base plate. The S/P ratios were useful to show how the dimensions of the scales were similar among the subgenera groups. The S/P ratios indicated that similar spine scale dimensions were present in subgenera and the ranges of these ratios were small in some subgenera, especially *P. brevispina* species, with an average S/P ratio between 1.1 and 1.3, indicating that the spine measurement of a scale is never much more than 1.3 x the width of its base plate. When compared to scales with longer spines as those seen in *P. acrospina* the S/P ratio ranges are between 2.5 and 3.0, thus indicating that the dimension of the scales between the subgenera do not vary greatly. This knowledge could be useful when looking at published *Paraphysomonas* literature, which shows spine scales, to see which kind of species it is mostly likely related. Manton and Leedale (1961) studied a strain with scales most similar-looking to species within the subgenus *Paraphysomonas*.

The range of spine length is broad (~2.2 – 8.3 µm) and the base plate range is less broad (1.2 – 2.5 µm). The S/P ratio is between 2.1 – 3.7, which means the spine length can get over three and half times the baseplate width. The averages of these scale measurements fit with those found in the subgenus *Paraphysomonas*; although it is more difficult to assign it specifically to any clade it is likely a novel species. Even so, given the large size of the cell and the spine scale measurement averages it is likely most related to *P. hemiradia* clade than any other in this thesis. The broad range of scale size for one species of *Paraphysomonas* is confirmed here but the spine tips have shown uniformity throughout the clades and contradict previous notions that they are not useful in identification (Thomsen 1975). Spine shape and tip were fundamental in observing the main morphological scale difference between *Paraphysomonas* subgenera.

Scale variation withtin the *Thaumatomonas* isolates was not as broad as the variation seen within the *Paraphysomonas* isolates; the *Thaumatomonas* scale sizes are of similar dimension, shape and structure, and the repertoire of scales produced by the isolates seemed to vary, some produced triangular scales more than oval. Molecular rDNA phylogenies grouped the species that mainly produced triangular scales (*T. zhukovi/seravini*) separately from those that mostly produce oval scales (*T. coloniensis/oxoniensis/lauterborni*). Comparing two-tiered strutted scale structures was more of a challenge than spine scales, which often lie flat and can easily be measured whereas the two-tier scales often do not lie flat and overlap with other scales making interpretation of structure difficult. The strutted scales of *Thaumatomonas* showed scale variation within the species with some typically having more or fewer struts than others (*T. coloniensis*-like scales), and the ovalness ratio

(length of scale divided by width) of the oval scales did not reveal any obvious pattern between the isolates or their clades. The repertoire of scale types and frequency of scale kinds (triangular and oval) produced by a *Thaumatomonas* isolate would appear to be a good indicator of which of the two main clades an isolate would belong if no sequence data were available.

2. Cell variation

Cell size of *Paraphysomonas* mapped well onto phylogenetic trees and the range of average cell length of the species in this study was between 3.6 – 11.6 µm. The length of the long cilium was distinct within one subgenus, *P. acrospina*; 2.5 – 5 x the cell length, much longer than those found in the other subgenera, 1.5 – 2 x cell length. Gliding thaumatomonad cells showed lower intra-species variation in size than *Paraphysomonas*, although small cells were present in some thaumatomonad cultures the shapes of the cell varied greatly. Many of the thaumatomonad cultures exhibited plasmodial stages which might suggest a stage after genetic exchange within their complex life cycle; flagellate, filopodial amoebae, plasmodia and cyst (Karpov 2010/11), *Paraphysomonas* exhibit stages of encystment, stalked and swimming. Many of the thaumatomonad plasmodial cell stages revealed various numbers of cilia and sometimes glided and showed pseudopodia, and plasmodial stages were observed in *Thaumatospina vancouveri* showing that plasmodia are seen among (former) *Thaumatomastix* species (Zolotarev et al. 2011). Thaumatomonadidae showed some anterior cilium length variation between the genera; *Thaumatospina* species showed distinctly long anterior cilium (AC) length, whilst other genera, *Allas* and *Thaumatomonas* had notably shorter AC. Moreover, I observed that perhaps *Allas*

does not produce pseudopodia as readily as other thaumatomonad cells. These observations whilst preliminary show how cellular function is diverse within the thaumatomonads.

3. Molecular variation

As already mentioned, the variation of 18S rDNA divergence is different between the two groups studied. Heterokont *Paraphysomonas* appears much more divergent than cercozoan *Thaumatomonas*, which could be due to a number of reasons, one of which could be from the way in which I isolated thaumatomonads may have been too crude to select for different genotypes or just that thaumatomonads evolved more recently. Thirdly, it could also mean, any genetic exchange the thaumatomonads are postulated to be performing during their life stages could reduce the divergence of genes through this process (Cohan 1994; Mayr 1982), but this genomic aspect would need to be explored further.

4. The consequences of splitting the lumped

Revising the taxonomy for paraphysomonads and thaumatomonads to make morphologically homogeneous genera meant that the original genera had to be split. These newly revised genera are morphologically more easily recognizable and 18S rDNA sequence trees corroborate these revisions. The revised *Paraphysomonas* genus contains 44 species of which 35 are newly described that would have been lumped into just nine known species (only nine species of the original taxonomy were kept in the genus). These results show that simple but careful investigations into scaly cryptic species complexes can reveal clear morphological and molecular diversity.

This thesis sought to reorganize the taxonomy of two well-known groups of protists so that more meaningful work and interpretation of ecology, phylogeny and biogeography could be done and in doing so has reinforced the use of scales and morphology as reliable characters to identify protists to the genus-level. In many cases they are also distinctive for species, but in some one needs sequences as well as (or instead of) scales for accurate species identification. With the knowledge gained about detail of spine scale morphology of *Paraphysomonas*, one can easily see that treating all protist genera the same in regard to morphospecies is not useful and considerations about morphospecies must be made case-by-case (Bass and Boenigk 2011; Boenigk et al. 2012). *Thaumatomonas* did not have as much 18S rDNA molecular diversity or apparent scale diversity as observed for *Paraphysomonas*, which had comparatively easily recognisable molecular divergence and morphological scale differences. *Thaumatomonas* species have very little 18S rDNA divergence between them but by increasing the molecular resolution and using different analysis, such as ITS2 rDNA folding, helps to resolve differences between more cryptic isolates (Chapter 5). It would be interesting to view *Paraphysomonas* at a higher molecular resolution to observe the folding of the ITS2 rDNA sequence to see if this method recognizes the same distinct species by CBCs, in this way a more comparative designation of species can be observed between two very different groups of protists.

The species unit is useful to understand the evolution of a group of protists and the diversity of a functional group of organisms within nature. Ecologists have recognized paraphysomonads and thaumatomonads as important groups of protists because they contribute considerably in biomass within common environmental niches (Arndt et al. 2000; Charvet et al. 2011; Patterson and Lee 2000). To unlock the

veritable functional groups or operational taxonomic units (OTUs) of a larger 'black box' of ecological function one must try to understand the diversity of these major groups. Here I have made important steps towards the understanding of scaly protist diversity and these data can be used to identify more subsets of both groups and towards designing group-specific primers. In this way, the identities of larger groups of protist can be broken down into functional groups.

In this short discussion, I have shown ways (in addition to the points made in Chapters 4 & 5) in which this research affects other aspects of protistology research that could affect views of taxonomy and how we judge species as units within protistology research. These species have shown a level of diversity never seen before, which in turn shows how relatively little we have to do to see it by using simple culturing, sequencing, and electron microscopical techniques. I have accrued a lot of data with simplified methods of culturing and in doing so I have shown a lot about morphology and molecular diversity of silica-scaled protists.

Conclusion

The combined use of morphology and sequence data has improved the taxonomy of two very common protist groups and has revealed how important the range of morphological diversity is to taxonomy and how this matches levels of genetic diversity.

This thesis has shown how different the results of morphology and genetic diversity have been for just two protist groups. This in turn shows how our interpretation of diversity for any group of protists might be challenged.

This kind of research is important for improving taxonomy of protists, which is ultimately the foundations on which ecology, biodiversity and biogeography is placed. The rate of clonal culturing efforts must be maintained if not increased in the light of ever-cheaper sequencing of environmental DNA revealing hidden diversity. Culturing clonal cultures are at least as important now as eDNA sequencing is for discovering new protists that could give us more insights in to the evolution of cellular functions, evolution, and the diversity of life. Cultured strains such as those I have placed in public culture collections can be used for all kinds of cell biological, ultrastructural and physiological research impossible with environmental 18S rDNA sequences alone.

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Appendices

A. Phylogeny of Heterokonta: *Incisomonas marina,* a uniciliate gliding opalozoan related to *Solenicola* (Nanomonadea), and evidence that Actinophyrida evolved from raphidophytes. T Cavalier-Smith and **Scoble JM**. (2013). European Journal of Protistology. 49, 328-353

B. Novel cultured protists identifying deep-branching environmental DNA clades of Cercozoa: New genera *Tremula, Micrometopion, Minimassisteria, Nudifila, Peregrinia.* Howe AT, Bass D, **Scoble JM**, Lewis R, Vickerman K, Arndt H, Cavalier-Smith T. (2011). Protist. 162, 332-372

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European Journal of Protistology 49 (2013) 328–353

European Journal of PROTISTOLOGY

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Phylogeny of Heterokonta: *Incisomonas marina*, a uniciliate gliding opalozoan related to *Solenicola* (Nanomonadea), and evidence that Actinophryida evolved from raphidophytes

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Received 6 July 2012; received in revised form 29 September 2012; accepted 30 September 2012
Available online 4 December 2012

Abstract

Environmental rDNA sequencing has revealed many novel heterokont clades of unknown morphology. We describe a new marine heterotrophic heterokont flagellate, *Incisomonas marina*, which most unusually lacks an anterior cilium. It glides and swims with its cilium trailing behind, but is predominantly sedentary on the substratum, with or without a cilium. 18S rDNA sequence phylogeny groups *Incisomonas* strongly within clade MAST-3; with others it forms a robust sister clade to *Solenicola*, here grouped with it as new order Uniciliatida, placed within new class Nanomonadea encompassing MAST-3. Our comprehensive maximum likelihood heterokont phylogeny shows Nanomonadea as sister to MAST-12 plus Opalinata within Opalozoa, and that Actinophryida are not Opalozoa (previously suggested by distance trees), but highly modified raphidomonads, arguably related to *Heliorapha* (formerly *Ciliophrys*) *azurina* gen., comb. n. We discuss evolution of Actinophryida from photosynthetic raphidophytes. Clades MAST-4,6-11 form one early-branching bigyran clade. *Olisthodiscus* weakly groups with Hypogyristea not Raphidomonadea. Phylogenetic analysis shows that MAST-13 is all *Bicosoeca*. Some gliding uniciliates similar to *Incisomonas marina* seem to have been misclassified: therefore we establish *Incisomonas devorata* comb. n. for *Rigidomastix devoratum*, revise the genus *Rigidomastix*, transfer *Clautriavia parva* to *Kiitoksia*. We make 17 new familes (13 heterokont (three algal), two cercozoan, two amoebozoan).
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Keywords: *Actinosphaerium*; *Incisomonas*; Aurophyceae; Uniciliatida; Opalozoa; *Heliorapha*

Introduction

Historically, the smallest protists have been the most understudied, being hardest to identify because their size makes it difficult to observe any conspicuous morphological features. It is important to know more about them because they are key grazers in food webs, playing an especially large role, with bacteria, in oligotrophic habitats where it is more difficult for most other organisms to sustain life (Caron et al. 1999). Environmental DNA amplification studies have looked at the biodiversity of these ecologically key small (pico- and nano-sized) protists, revealing a large number of novel 18S rDNA clades within the chromist infrakingdom Heterokonta (Kolodziej and Stoeck 2007; Lara et al. 2011; Massana et al. 2002, 2004). Many are marine and named MAST ('marine stramenopiles'; 'stramenopiles' is an unnecessary recent synonym for the longer established classical 'heterokonts'); these are abundant in marine plankton and must be ecologically more important than previously realised (Massana 2011), but others with relatively little overlap with marine

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0932-4739/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.ejop.2012.09.002

ones are from fresh water (Lara et al. 2011; Richards and Bass 2005; Richards et al. 2005). Heterokonta, the sister group to Alveolata (Burki et al. 2009; Cavalier-Smith 2010a), include a huge variety of nutritional and cellular forms ranging from algae (both unicells like chrysomonads and diatoms and multicellular brown algae), through to phagotrophic flagellates, heliozoan-like phagotrophs, gut-symbiotic anaerobes and the net-like Labyrinthulea, making it phenotypically one of the most evolutionary diverse branches of the eukaryotic tree (Cavalier-Smith and Chao 2006). This diversity of feeding modes and body forms is key to understanding their ecology and the evolutionary relationships amongst the major clades within the group, so characterising nutritional modes and body forms of the 15 or more environmental heterokont clades is of major significance for marine and freshwater biology (Lara et al. 2011; Massana et al. 2009). Fluorescence in situ hybridisation using specific rDNA probes suggests that several MAST clades consist of small uniciliate or biciliate phagotrophic heterotrophs (Kolodziej and Stoeck 2007; Massana et al. 2002, 2006), but little else is known about them.

These novel heterokont clades are mostly deep, but insecurely placed, branches within the basal or early diverging heterokont phylum Bigyra (Cavalier-Smith and Chao 2006), though a few seem to be more closely related to Pseudofungi. Only one of the 13 MAST clades described by Massana et al. (2002) and subsequently has so far had a described species assigned to it (the uniciliate *Solenicola* which belongs in clade MAST-3: Gómez et al. 2011), yet molecular survey work is still yielding even more sequences of unknown significance (Massana 2011). Discovering the morphology of MAST clades is important for understanding the previously underestimated biodiversity of phagotrophic bigyran heterokonts and for marine ecology. Making uniprotist cultures of these flagellates available is essential for physiological studies and to enable phylogenetic analyses using genes other than 18S rDNA. Because basal branching within Heterokonta is poorly resolved on single-gene rDNA trees (Cavalier-Smith and Chao 2006; Gómez et al. 2011; Massana et al. 2002; Park and Simpson 2010), a combination of several well-conserved genes will be essential for improving the tree and better understanding heterokont early evolutionary diversification (Riisberg et al. 2009).

This paper has four aims: (1) to describe a new marine phagotrophic heterokont of distinctive morphology, *Incisomonas marina*, that we recently brought into stable uniprotist culture in the presence of food bacteria; we show by 18S rDNA phylogeny that it belongs to the MAST-3 clade, and together with its relative *Solenicola* identifies a major MAST-3 subclade as being essentially posteriorly uniciliate, here treated as new order Uniciliatida. (2) Reform the classification of a few uniciliate phagotrophic protists of previously uncertain taxonomic position from which *Incisomonas* needs to be clearly distinguished. (3) To provide a taxonomically more comprehensive maximum likelihood (ML) analysis of Heterokonta than any previously, so as to place both MAST and freshwater heterokont clades more robustly within groups for which cultured representatives are available and reevaluate the deep-branching tree topology of heterokonts; our analyses show there are fewer distinct MAST clades than previously thought, identify a deep freshwater bigyran clade, and reveal a radically new position for the actinophryid *Actinosphaerium* as being derived from raphidophytes; it is neither a pedinellid (Davidson 1982; Mikrjukov and Patterson 2001) nor an opalozoan (Cavalier-Smith and Chao 2006) as previously supposed. We argue that *Ciliophrys azurina* (Mikrjukov and Patterson 2001) is also not a pedinellid but a likely evolutionary link between the non-ciliate actinophryids and typical photosynthetic raphidophytes, so transfer it to a new genus *Heliorapha* within Actinophryida and Raphidomonadea; we revise the classification of Raphidomonadea to include Actinophryida and discuss the major cellular transformations involved in the origin of Actinophryida. (4) To revise higher classification of the heterokont phylum Bigyra (Cavalier-Smith 1997a; Cavalier-Smith and Chao 2006) in light of our phylogeny and other recent data that has revealed a considerable expansion of zooflagellate diversity in the subphylum Opalozoa.

In seeking to identify *I. marina* we critically considered three previously described uniciliate gliding heterotrophic genera (*Kiitoksia*, *Clautriavia*, *Rigidomastix*) that contain somewhat similar species to which our new flagellate might potentially have been related (all hitherto inadequately classified: Vørs 1992; Tong 1997; Tong et al. 1997; Schouteden 1907). We conclude that all three genera are excessively heterogeneous and probably include unrelated species; so we revised their circumscription, placing one formerly *Rigidomastix* species also in *Incisomonas* and another in *Rhizomastix*, transferring *Clautriavia parva* (Schouteden 1907) to *Kiitoksia*, and excluding the biciliate *K. kaloista* (Tong et al. 1997) from that genus.

Material and Methods

The culture of *Incisomonas marina* was obtained from a sandy littoral marine sample taken from Boutilliers Point, Nova Scotia, Halifax, Canada in May 2011. Serial dilution (8 series) was used to isolate it into marine medium in which the only food was bacteria derived from the inoculum; it is placed in the Culture Collection for Algae and Protozoa, Oban, UK as CCAP 997/1. We used the soil extraction protocol of the MOBIO UltraClean™ Soil DNA Kit to extract DNA from half the cells in one Petri dish; the other half was fixed for electron microscopy (EM). PCR and DNA sequencing used the same eukaryote-wide primers; 25F (forward: 5′-CATATGCTTGTCTCAAAGATTAAGCCA-3′), 1801R (reverse: 5′-TGATCCTTCTGCAGGTTCACCT-3′) with a third internal primer at the sequencing stage 3NDF (forward: 5′-GGCAAGTCTGGTGCCAG-3′). A single 25 μl PCR (using Invitrogen™ reagents) was subject first to a denaturation step (5 min at 95 °C), then to 35 cycles as

follows: 95 °C for 32 s; 60 °C for 30 s; 72 °C for 2 min. Then a final extension of 7 min at 72 °C completed the PCR. The single band viewed after 1% agarose gel electrophoresis under UV after ethidium bromide staining was cut from the gel and cleaned using a GE Healthcare GFX^{TM} extraction kit. PCR products were sequenced using dye terminators and separated on an automated ABI-377 sequencer. Editing used Sequence Scanner v. 1.0 (http://www.appliedbiosystems.com); contig assembly was by BioEdit, CAP Contig Assembly Program (Hall 1999). Transmission EM samples were fixed by adding 25% glutaraldehyde to a final concentration of 2.5% for a minimum of 1 h and then rinsed with distilled water via filtering and the vial allowed to settle to concentrate cells before applying them to the grid. The grid was washed in distilled water again. Samples were viewed as unstained whole mounts on formvar-coated 200 mesh copper grids with an FEI Tecnai 12 electron microscope.

Phylogenetic analysis

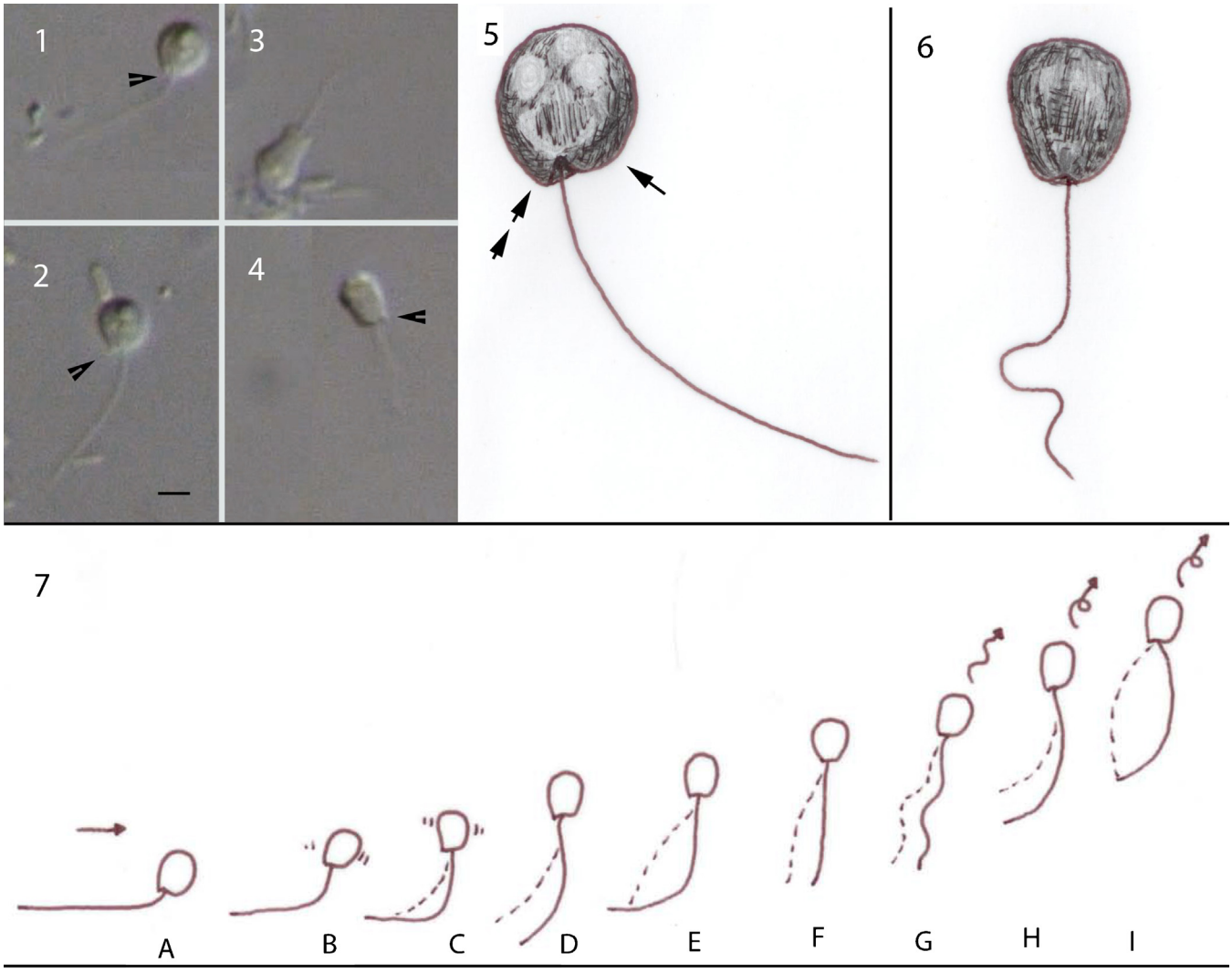
We manually substantially improved the 18S rDNA alignment used by Gómez et al. (2011), added many more sequences manually (using MACGDE v. 2.4: Linton macgde.bio.cmich.edu), giving an alignment of 457 heterokont sequences, made preliminary distance trees and then selected for thorough phylogenetic analysis 328 heterokont sequences giving a balanced and broad sampling of all lineages plus 27 broadly representing the closest outgroups Alveolata and Rhizaria (rich and representative sampling of relatively close outgroups is important for correct rooting within Heterokonta; many published trees use so few outgroup sequences that the root position may be erroneous). We analysed 1654 unambiguously aligned nucleotide positions (more than any previous study; we included the largest number of sites judged by eye to be reliably aligned across Heterokonta and harosan outgroups, using MacGDE v. 2.4: http://macgde.bio.cmich.edu/) by ML using the rapid bootstrap option (1000 resamplings) of RAxML v. 7.0.3 (Stamatakis 2006) with the GTRMIX model and evaluating the optimal tree by GTR + GAMMA with empirical base frequencies and 25 per site rate categories. The alignment of the 355 harosan sequences used for the trees is available as electronic supplementary material (extended Phylip format). To look at heterokont topology without outgroups to see how taxon sampling affects the position of *Actinosphaerium* and other critical findings we applied the same method to produce trees for the 327 Heterokonta alone (not shown) and for the 128 Ochrophyta alone (1655 nucleotide positions), as well as for reduced data sets of 140, 188, 224, 268, 281, 300, 307, 327, 333, 342 and 354 sequences of Harosa (i.e. the chromist subkingdom comprising Heterokonta, Alveolata and Rhizaria: Cavalier-Smith 2010a), which removed most Ochrophyta and varying numbers of others by excluding more closely related sequences. To see which groupings were stable irrespective of method we also ran neighbor joining (NJ) distance trees for these and other taxon samples using the F84 gamma model of Phylip v. 3.68 (Felsenstein http://evolution.genetics.washington.edu/phylip).

Results

Incisomonas morphology and behaviour

The new flagellate can both glide on surfaces and swim using its posterior cilium, which emerges from a slight ciliary pocket. It spends most time stationary on the bottom of the dish, attached by its non-ciliated end, often in clumps and exhibiting either a single cilium (which may undulate or be held rigid) or no cilium. Figs 1–4 show both the stationary forms that we suspect are feeding on suspended bacteria and gliding ones that appear to be a temporary state for dispersal. In motile stages the cilium is several times the cell length. No trace of a second anterior cilium was seen by light or electron microscopy. Its most distinctive feature is the asymmetry of the cell at the end bearing the cilium; on one side of this is the indentation from which the solitary cilium emerges beside a narrow overhanging tooth-like projection or lip (Figs 1, 2, 4, 5; not visible from all angles). The indentation and tooth-like projection is also seen in the sessile form. A minority of cells are pyriform with the lip raised all around the base of the cilium as a bilaterally symmetric oval collar (Fig. 3).

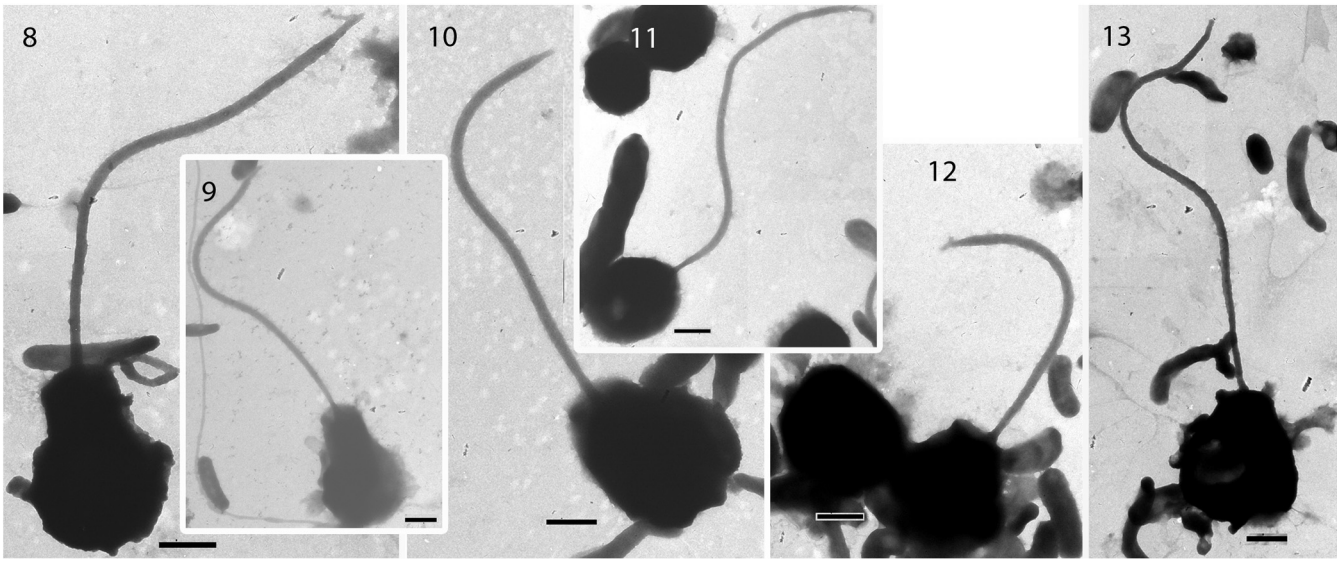
Ciliary motility is extremely versatile. Gliding cells mostly have the non-undulating cilium in contact with the substratum throughout its length (Figs 2, 5), but sometimes the basal portion is raised from it (Fig. 1). It may be held straight or gently curved (often with a more pronounced curve about one cell length from the cell surface). They usually glide in one direction for only a few seconds and then detach and undulate their cilium to change direction or swim over the surface in a straight line with slowly undulating cilium (held posteriorly); they sometimes rest without moving. Gliding is at an even rate; the cell body can be essentially free of jiggling motion or may vibrate slightly from side to side – typically it jiggles much more rapidly immediately prior to lift off when changing over to swimming (Fig. 7). Elongate cells and swimming cells are more common in well-fed cultures. Cells also swim to the meniscus layer of the Petri dish and slowly descend, not changing orientation. Most often when swimming freely in the medium the cilium is held in a gentle curve behind the cell and its distal two thirds beats so rapidly that only its asymmetric beat envelope can be seen, with the basal third rigid; in some cells the beat envelope extends to the base of the cilium or is restricted to only the distal half. Such cells do not swim in straight lines but in a broad spiral whose pitch varies. In attached ciliated cells the beat is much slower and the asymmetric beat pattern of its distal two thirds can be readily seen (Figs 3, 6); the wave is then of greater amplitude and even more asymmetric than in free-swimming cells and passes from base to tip.



Figs 1–7. Morphology and ciliary behaviour of *Incisomonas marina*: (**1–4**) DIC images (arrowheads mark the asymmetrically protruding lip); scale bar 2 μm; (**5–7**) drawings. (**1**) Gliding cell, cilium trailing; cell body slightly raised. (**2**) Gliding cell; cell body and cilium in contact with substratum. (**3**) Feeding/benthic cell with unusually elongate collar, attached to detritus. (**4**) Swimming cell viewed from side and slightly tilted towards view. (**5**, **6**) Pencil and ink interpretations of the cell form. (**5**) Gliding cell: single arrow indicates the typically more retracted side of the cell anterior away from the ciliary pocket; double arrow the overhanging lip. (**6**) A typical feeding benthic cell and ciliary beat pattern of distal half only. *Incisomonas marina* is not perfectly rounded but has smooth indentations and curves around its body. (**7**) Range of cell and ciliary movements from gliding/swimming cells. (**A**) The cell glides smoothly (arrow indicates direction); it then stops and becomes agitated and vibrates (**B**, **C**). (**D**–**H**) During lift off from the substratum when it begins to swim the cilium often beats irregularly and can beat in a range rhythms and patterns. (**I**) continuous vibration too rapid to see the cilium is the most common pattern when fully free, when the cell spirals; it looks as though there are two cilia at the extreme edge of the beat envelope. (**G**) In the most sedate and slow pattern it is continuously visible, resembling an undulating fish tail.

Electron microscopy shows that the cell surface is devoid of scales and the cilium lacks hairs (Figs 8–13), consistent with it being the smooth posterior heterokont cilium. The cilium has a short pointed acroneme (Figs 8, 10). Both cells with an asymmetric ciliary end (possibly gliders as relatively free of bacteria) and pyriform cells with symmetric collar (Figs 10, 12, 13 often clumped with bacteria) were noted, as were non-ciliate sedentary cells in clumps with numerous bacteria. Dried sedentary cells often preserve a similarly asymmetric ciliary wave to live ones.

The closest previously described organism to *Incisomonas marina* is *Rigidomastix devoratum* (Tong 1997). Both are uniciliates with one cilium emerging from an asymmetrically placed apical notch. Cell size and shape are almost identical, but the *R. devoratum* cilium is significantly longer (up to 20 μm); it could be retracted into its pocket, and became shorter and eventually no longer visible – we have not observed this directly in *I. marina* but the sedentary stages with sometimes shorter cilia than gliding cells or often none suggest a similar ciliary retraction. Two key differences



Figs 8–13. Whole mount transmission electron micrograph of *Incisomonas marina*. Scale bar 1 μm.

between Tong's strain and ours prevent their treatment as the same species: (1) her cells swam cilium first, but the opposite is generally true for *I. marina*; (2) she noted a thin thread attached to the base of the cilium, not seen in *I. marina*. She also did not mention the characteristic erratic shaking of the cell body before and after swimming so obvious in *I. marina*. Tong's species has precisely the same asymmetric cell shape as most *I. marina*; though she did not mention pyriform cells with a bilaterally symmetric collar, her Fig. 9g seems to be an example; *I. marina* and *Rigidomastix devoratum* appear to glide similarly: with cell body slightly above the substratum. Overall they are sufficiently similar to be in the same genus, but as explained below we do not agree that either is closely related to the type species of *Rigidimastix* (*R. coprocola*), so establish the new genus *Incisomonas* for both.

Taxonomy

The new taxa described here are all phagotrophic and non-algal and so are treated under the International Code of Zoological Nomenclature (ICZN), except for the new photosynthetic algal class Aurophyceae and subclasses Schizocladiophycidae and Raphidophycidae plus their new families that are treated under the International Code of Nomenclature for algae, fungi, and plants (ICN). Now that so many former protozoan groups are added to Chromista (Cavalier-Smith 2010a) it is no longer appropriate to treat all Chromista under the International Code for Botanical Nomenclature (ICBN, now replaced by ICN) as originally recommended (Cavalier-Smith 1981).

New class Nanomonadea Cavalier-Smith

Diagnosis: Phagotrophic and non-photosynthetic free-living heterokont zooflagellates phylogenetically with 18S rDNA sequences more closely similar to those of the phagotrophic unicilates *Solenicola* and *Incisomonas* than to those of the non-phagotrophic gut-dwelling Opalinata. **Etymol**: *Nanos* Gk dwarf; *monad* Gk unit. **Comment**: embraces the entire MAST-3 clade. Gómez et al. (2011) applied the unranked name Solenicolida to MAST-3 but it was not accompanied by a diagnosis or published as a taxon; it is preferable to use such genus-based names only for a family (as we do below) and better to choose a class name that is descriptively apt for the whole group – Nanomonadea achieves this by emphasizing that all are tiny flagellates. It is placed in phylum Bigyra and subphylum Opalozoa (see Table 1) and at present includes only one order:

New Order Uniciliatida Cavalier-Smith. **Diagnosis**: Heterotrophic heterokonts with a single hairless posterior cilium but no anterior cilium. **Etymol**: *Uni* L. one; *cilium* L. lash. Comprising families Incisomonadidae (non-amoeboid gliders) and Solenicolidae (partially amoeboid):

New family Incisomonadidae. **Diagnosis**: Rigid non-amoeboid, colourless marine uniciliate cells, usually slightly elongated and rounded; glide on substratum by the long non-acronematic, hairless cilium protruding from an asymmetrically placed notch or pocket that has slender lip-like overhang; gliding cilium several times body length, points to posterior, most often contacts substratum throughout its length; predominantly stationary, attached to substratum by non-cilium end, often in clumps, sometimes non-ciliated. Can also swim. **Etym**: *Inciso* L. notched + *monas* Gk unit. Type genus ***Incisomonas*** Scoble and Cavalier-Smith. **Diagnosis**: as for family Incisomonadidae. **Type species**: *Incisomonas marina* sp. n. **Diagnosis**: Ciliated end of cell usually with asymmetric lip only on one side of ciliary pocket, but cells sometimes pyriform with raised oval collar completely surrounding the ciliary base. Glides smoothly and briefly, sometimes with cell body raised or a gentle lateral jiggling of

Table 1. Revised classification of the bigyran subphylum Opalozoa Cavalier-Smith, 1991 stat. n. 2006 em.

Infraphylum 1. Bikosia Cavalier-Smith infraphyl. n.
- **Class Bikosea**[a] Cavalier-Smith nom. n. pro Bicosoecea Cavalier-Smith, 1986, p. 341
  - **Subclass 1. Bicosidia** Cavalier-Smith, 2006 orth. em.
    - **Superorder 1. Cyathobodoniae** Cavalier-Smith, 1993 em. stat. n. 2006
      - **Order 1. Bicoecida** Grassé, 1926
        - Family 1. Bicosoecidae Ritter von Stein, 1878 (as Bikoecida) (*Bicosoeca*, *Stephanocodon*, *Poteriodendron*)
        - Family 2. Labromonadidae Cavalier-Smith, 2006 (*Labromonas*)
      - **Order 2. Anoecida** Cavalier-Smith, 1997a em. 2006
        - Family 1. Cafeteriidae Moestrup, 1995 em. (*Cafeteria*, *Anoeca*, *Symbiomonas*)
        - Family 2. Caecitellidae Cavalier-Smith, 2006 (*Caecitellus*, *Halocafeteria*)
      - **Order 3. Pseudodendromonadida** Hibberd, 1985 em. Cavalier-Smith, 2006
        - Family 1. Pseudodendromonadidae Hibberd, 1985 (*Pseudodendromonas*, *Cyathobodo*)
        - Family 2. Siluaniidae Karpov, 1998 (*Siluania*, *Adriamonas*)
        - Family 3. Paramonadidae Cavalier-Smith, 2006 (*Paramonas*)
        - Family 4. Nanidae Cavalier-Smith fam. n. (*Nanum* = *Nanos* Kim et al., 2010)
        - Family 5. Neradidae Cavalier-Smith, 2006 (*Nerada*)
        - Family 6. Filidae Cavalier-Smith fam. n. (*Filos*)
    - **Superorder 2.** Borokiae Cavalier-Smith, 2006
      - **Order Borokida** Cavalier-Smith, 2006
        - Family Borokidae Cavalier-Smith, 2006 (*Boroka*)
  - **Subclass 2. Rictidia** Cavalier-Smith subcl. n.
    - **Order Rictida** Cavalier-Smith ord. n.
      - Family Rictidae Cavalier-Smith fam. n. (*Rictus*)

Infraphylum 2. Placidozoa Cavalier-Smith infraphyl. n.
- **Superclass 1. Wobblata**[b] Cavalier-Smith, 2006 supercl. n.
  - **Class 1. Placididea** Moriya et al., 2002
    - **Order Placidida** Moriya et al., 2002
      - Family Placidiidae Moriya et al., 2002 (originally misspellt Placidae: *Placidia*, *Wobblia*)
  - **Class 2. Nanomonadea** Cavalier-Smith cl. n. (clade MAST-3)
    - **Order Uniciliatida** Cavalier-Smith ord. n.
      - Family 1. Solenicolidae Cavalier-Smith fam. n. (*Solenicola*)
      - Family 2. Incisomonadidae Cavalier-Smith and Scoble fam. n. (*Incisomonas*)
  - **Class 3. Opalomonadea**. Cavalier-Smith cl. n. (clade MAST-12)
- **Superclass 2. Opalinata** Wenyon, 1926 em. Cavalier-Smith, 1996 stat. n. 2006
  - **Class 1. Opalinea** Wenyon, 1926 stat. n. Cavalier-Smith, 1993 em.
    - **Order 1. Proteromonadida** Grassé, 1952 em. Cavalier-Smith, 1993
      - Family Proteromonadidae Grassé, 1952 (*Proteromonas*)
    - **Order 2. Opalinida** Poche, 1913 stat. n. Hall, 1953 em. Cavalier-Smith
      - Family 1. Karotomorphidae Travis, 1934 (*Karotomorpha*)
      - Family 2. Opalinidae Claus, 1874 (e.g. *Cepedea*, *Protoopalina*, *Opalina*)
  - **Class 2. Blastocystea** Zierdt et al., 1967
    - **Order Blastocystida** Zierdt, 1978
      - Family Blastocystidae Jiang and He, 1988. Type genus *Blastocystis* Aléxéieff, 1911. The fossil echinoderm family Blastocystidae Jaekel, 1918 based on the junior homonym *Blastocystis* Jaekel, 1918 is invalid.

Opalozoa incertae sedis: *Pendulomonas* Tong, 1997; **Bordnamonadidae** Cavalier-Smith fam. n. **Diagnosis**: pliable naked swimming biciliates with anterior cilium held in an arc, posterior trailing; rounded preciliary rostrum used for ingesting bacteria excludes if from all other heterokont families. Type genus *Bordnamonas* Larsen and Patterson (1990). *Bordnamonas* a raptorial feeder, is possibly related to *Rictus*, but it might be a euglenozoan instead.

Bigyra incertae sedis: Pseudobodonidae Cavalier-Smith fam. n. (*Pseudobodo tremulans* Griessmann, 1913 and *P. minuta* Ruinen, 1938)

[a] Replaced because this is a better modification of the original class name Bicosoeca (Cavalier-Smith 1986; improperly formed from Greek) than was Bicoecea (Cavalier-Smith 1989), which means 'inhabiting a vase' (from *bikos* Gk vase, bowl, drinking jar, plus *oekein* Gk inhabit; also *oikos* Gk is a house or household – as in economy and ecology), thus misleadingly refers to the lorica that is absent from four of the orders now included in the class and present only in Bicoecida. Instead we can regard *bikos* (bowl) as a reasonable description of cell shape in many Bikosea, which is quite similar from *Rictus* to *Cafeteria* and somewhat different from Placididea. Using a k not a c in the changed name reduces potential confusion between the class and order and recognises the huge expansion in composition of the class from the single genus *Bicosoeca* in 1986. We use the vernacular bikosean for class Bikosea, bicosid for subclass Bicosidia, and bicoecid for order Bicoecida.

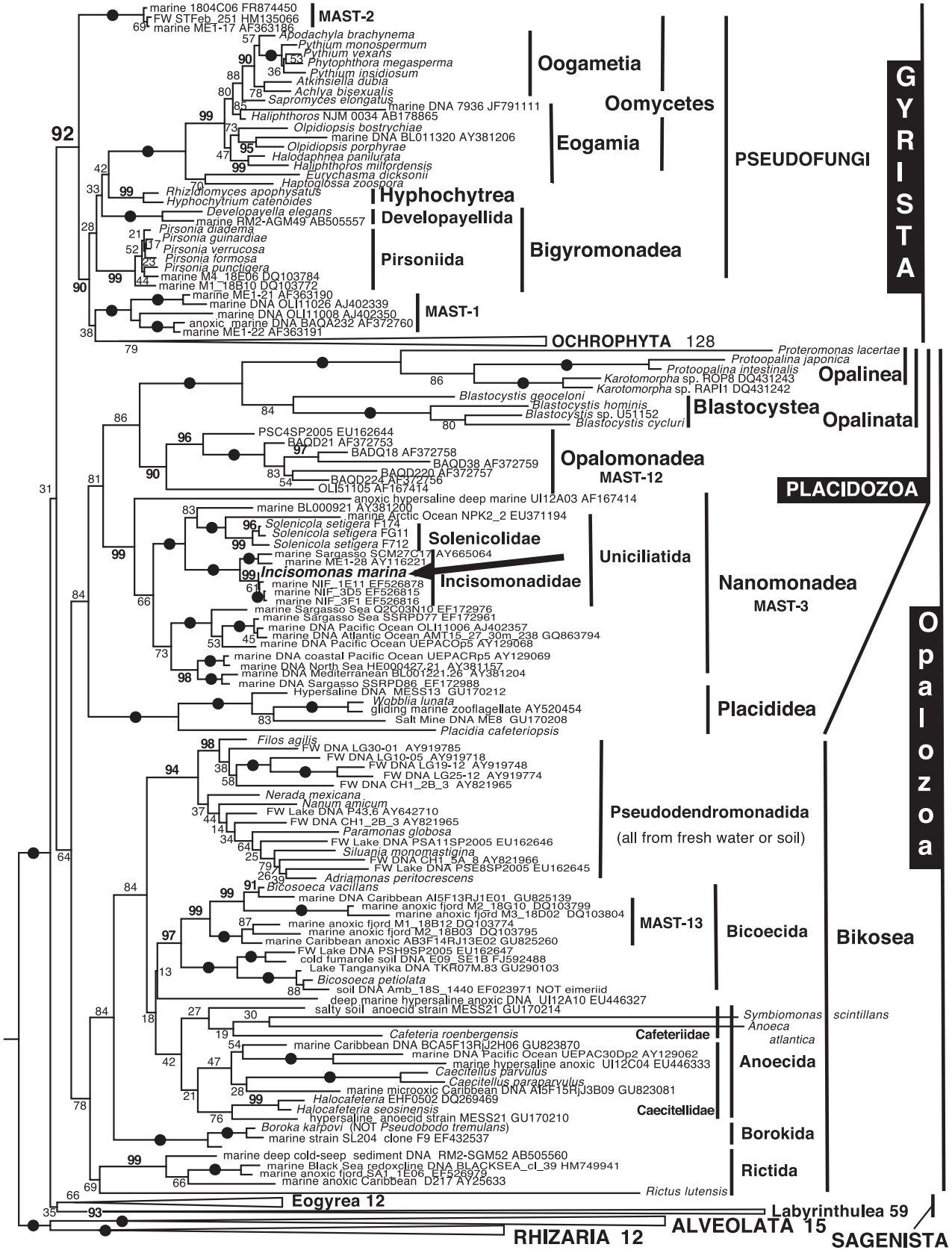
[b] Ancestral or paraphyletic taxa; all others believed to be holophyletic. Contrary to mistaken dogma (see Cavalier-Smith 2010b) there is nothing intrinsically wrong with ancestral (paraphyletic) taxa, but to prevent possible misunderstanding it is important to flag them when that status is known.

cell body. Gliding cells often swim briefly, erratically vibrating cell body beforehand and upon landing. Swims in spirals with cilium trailing behind; cilium beats asymmetrically, usually so fast that only beat envelope is visible (predominantly of distal two thirds); also beats slowly with asymmetric higher amplitude distal undulations when swimming in a straight line close to substratum. Cell size 3.2–3.6 μm × 3.6–4.1 μm. Cilium hairless; length, 11.4–13.6 μm ($N = 11$). Gliding or swimming cells less common than sedentary cells. Sedentary cells round to elongate and irregular, especially when clustered; aggregate often in large numbers within a film of attached bacteria, lone cells rarer. In sedentary cells cilium beats variously, often with distal undulation from base to tip; rhythm slow sometimes fast, movement originating midway on cilium. Sometimes cilium does not beat, but held rigid straight out in front of cell, periodically becoming limp and partially bent. Sedentary cells 3.2–4.1 μm × 3.6–5.0 μm ($N = 11$); cilium lengths 5.5–13.6 μm, but most often absent. Cell often has bright refractile granule/s. **Type strain**: CCAP 997/1. **Type sequence**: GenBank JN848814. **Sampling site**: sandy shoreline sediment near Boutillier's Point, Nova Scotia, Canada.

Incisomonas devorata Scoble and Cavalier-Smith comb. n. **Basionym**: *Rigidomastix devoratum* Tong, 1997. **Comment**: *Rigidomastix coprocola* (Aléxéieff 1929), type species of the genus *Rigidomastix*, was not a marine swimming or gliding cell but a stationary one isolated from horse dung. Its cilium was rigid and did not move, at least whilst he observed it. Most importantly, the original *Rigidomastix* cells were ovoid with a symmetric ciliated end devoid of a ciliary depression or lateral lip and larger (up to 8 μm) and with a much longer entirely rigid cilium. Unlike *Incisomonas*, some cells were embedded in mucus traversed by the cilium. There was therefore no reason to have placed *R. devoratum* in the same genus as *R. coprocola*. Being heterotrophic and uniciliate were the only shared characters – insufficient even to place them in the same phylum. Tong might have been misled by an erroneous statement (Patterson and Zölffel 1991) that *Rigidomastix coprocola* glides. Aléxéieff also observed a non-flagellate palmelloid stage in the same dung samples that he thought might be either an *Oikomonas* species or a stage in the life history of *Rigidomastix*. Confusingly, Patterson and Zölffel (1991) redrew Aléxéieff's palmella illustration, incorrectly adding cilia – making the cells resemble *Rigidomastix* more closely than they actually did, and omitted his caveat that he had no evidence that the palmella was from *Rigidomastix* not another genus.

New Family Solenicolidae Cavalier-Smith. **Diagnosis**: Naked, colourless, uniflagellate, non-swimming epiphytes of filamentous diatoms anchored to substratum by a broad spreading pseudopodial base or by mucilage investment; long cilium hairless with pointed end. **Type genus**: *Solenicola* Pavillard (1916). **Comment**: We do not agree that *Solenicola* is a junior synonym of *Rhizomonas* Saville Kent (1880) (Patterson et al. 1993, 2002). All they have in common are a single long cilium, a sedentary habit, and ability to make pseudopodia. These features are also found in Amoebozoa in Phalansteriida (Cavalier-Smith et al. 2004; Smirnov et al. 2011), which are phylogenetically extremely distant from heterokonts. It is relatively rare for the same genus to inhabit both marine and freshwater and many substantial protist clades have uniformly either marine or freshwater/soil distribution (Bass et al. 2009; Howe et al. 2011a; Nitsche et al. 2011; von der Heyden and Cavalier-Smith 2005; von der Heyden et al. 2004). It is likely that *Rhizomonas verrucosa* (Saville Kent 1880) from a hay infusion was a soil protozoan belonging to Phalansteriida, which are all from soil or freshwater. By contrast all known Nanomonadea sequences are marine. We therefore place *Rhizomonas* in the order Phalansteriida in a new family:

Rhizomonadidae Cavalier-Smith. **Diagnosis**: sedentary heterotrophic uniciliate cells with permanent pointed conical pseudopods over entire non-attached surface (not temporary filose pseudopods as in *Phalansterium filosum*). Type genus *Rhizomonas* Saville Kent, 1880. **Comment**: We do not accept the combination *Rhizomonas setigera* Patterson et al., 1993 or that the marine organism figured under that name (Patterson et al. 1993, 2002) is the same species as *Solenicola setigera* Pavillard (1916), the only previously named *Solenicola*. *Solenicola setigera* had 15–20 μm spindle-shaped cells, basally flattened and spreading across the siliceous diatom tube, with a low central hump from which or beside which the cilium emerged; in contrast the cells of the illustrated '*R. setigera*' are quasispherical, 6–8 μm in the illustration (though stated in text to reach 20 μm, was that either a conflation with the original decription of *S. setigera* or else based on the other non-illustrated specimen that might have been more like *S. setigera*?) and all basally rounded and not spread out laterally along the tubular substratum. We regard it as an undescribed *Solenicola*: ***Solenicola mucosa*** Cavalier-Smith sp. n. **Diagnosis**: sedentary marine uniciliate heterotroph with rounded cell base attached to substratum (diatom tubes or other filaments) by mucilage investment not a spreading cytoplasmic base as in *S. setigera*: sometimes with finger-like subapical pseudopodia. 6–8 μm cell body; cilium 14–25 μm, emerging from non-attached end (sometime slightly pointed or depressed) of near-spherical cell. Type illustration: Fig. 10 Patterson et al. (1993) p. 89. **Etymol**: *mucosa* emphasises mucilage attachment. **Comment**: in contrast all five *S. setigera* samples in Gómez et al. (2011) were spindle-shaped with cilium emerging from the central hump, so clearly belong to the morphospecies *S. setigera*; as they found three significantly different sequences their morphologically similar samples probably included three different sibling species (Fig. 14). Possibly *S. mucosa* is represented by or related to one of the two deeper branching lineages (NPK2_2; BL000921.38) in that subclade; however a referee informed us that NPK2_2 is chimaeric, only nucleotides 620–1800 being MAST-3 (1–620 a cryptophyte, explaining its wrongly much longer branch).


marine 1804C06 FR874450
FW STFeb_251 HM135066
marine ME1-17 AF363186
MAST-2
Apodachyla brachynema
Pythium monospermum
Pythium vexans
Phytophthora megasperma
Pythium insidiosum
Atkinsiella dubia
Achlya bisexualis
Sapromyces elongatus
marine DNA 7936 JF791111
Haliphthoros NJM 0034 AB178865
Olpidiopsis bostrychiae
marine DNA BL011320 AY381206
Olpidiopsis porphyrae
Halodaphnea panilurata
Haliphthoros milfordensis
Eurychasma dicksonii
Haptoglossa zoospora
Rhizidiomyces apophysatus
Hyphochytrium catenoides
Hyphochytrea
Developayella elegans
marine RM2-AGM49 AB505557
Develapayellida
Pirsonia diadema
Pirsonia guinardiae
Pirsonia verrucosa
Pirsonia formosa
Pirsonia punctigera
marine M4_18E06 DQ103784
marine M1_18B10 DQ103772
Pirsoniida
Oogametia
Eogamia
Oomycetes
Bigyromonadea
PSEUDOFUNGI
GYRISTA
marine ME1-21 AF363190
marine DNA OLI11026 AJ402339
marine DNA OLI11008 AJ402350
anoxic marine DNA BAQA232 AF372760
marine ME1-22 AF363191
MAST-1
OCHROPHYTA 128
Proteromonas lacertae
Protoopalina japonica
Protoopalina intestinalis
Karotomorpha sp. ROP8 DQ431243
Karotomorpha sp. RAPI1 DQ431242
Opalinea
Blastocystis geoceloni
Blastocystis hominis
Blastocystis sp. U51152
Blastocystis cycluri
Blastocystea
Opalinata
PSC4SP2005 EU162644
BAQD21 AF372753
BADQ18 AF372758
BAQD38 AF372759
BAQD220 AF372757
BAQD224 AF372756
OLI51105 AF167414
Opalomonadea
MAST-12
anoxic hypersaline deep marine UI12A03 AF167414
PLACIDOZOA
marine BL000921 AY381200
marine Arctic Ocean NPK2_2 EU371194
Solenicola setigera F174
Solenicola setigera FG11
Solenicola setigera F712
Solenicolidae
marine Sargasso SCM27C17 AY665064
marine ME1-28 AY116221
Incisomonas marina
marine NIF_1E11 EF526878
marine NIF_3D5 EF526815
marine NIF_3F1 EF526816
Incisomonadidae
Uniciliatida
Nanomonadea
MAST-3
marine Sargasso Sea Q2C03N10 EF172976
marine Sargasso Sea SSRPD77 EF172961
marine DNA Pacific Ocean OLI11006 AJ402357
marine DNA Atlantic Ocean AMT15_27_30m_238 GQ863794
marine DNA Pacific Ocean UEPACOp5 AY129068
marine DNA coastal Pacific Ocean UEPACRp5 AY129069
marine DNA North Sea HE000427-21 AY381157
marine DNA Mediterranean BL001221-26 AY381204
marine DNA Sargasso SSRPD86 EF172988
Hypersaline DNA MESS13 GU170212
Wobblia lunata
gliding marine zooflagellate AY520454
Salt Mine DNA ME8 GU170208
Placidia cafeteriopsis
Placididea
Opalozoa
Filos agilis
FW DNA LG30-01 AY919785
FW DNA LG10-05 AY919718
FW DNA LG19-12 AY919748
FW DNA LG25-12 AY919774
FW DNA CH1_2B_3 AY821965
Nerada mexicana
Nanum amicum
FW Lake DNA P43.6 AY642710
FW DNA CH1_2B_3 AY821965
Paramonas globosa
FW Lake DNA PSA11SP2005 EU162646
Siluania monomastigina
FW DNA CH1_5A_8 AY821966
FW Lake DNA PSE8SP2005 EU162645
Adriamonas peritocrescens
Pseudodendromonadida
(all from fresh water or soil)
Bicosoeca vacillans
marine DNA Caribbean AI5F13RJ1E01 GU825139
marine anoxic fjord M2_18G10 DQ103799
marine anoxic fjord M3_18D02 DQ103804
marine anoxic fjord M1_18B12 DQ103774
marine anoxic fjord M2_18B03 DQ103795
marine Caribbean anoxic AB3F14RJ13E02 GU825260
FW Lake DNA PSH9SP2005 EU162647
cold fumarole soil DNA E09_SE1B FJ592488
Lake Tanganyika DNA TKR07M.83 GU290103
MAST-13
Bicoecida
Bikosea
Bicosoeca petiolata
soil DNA Amb_18S_1440 EF023971 NOT eimeriid
deep marine hypersaline anoxic DNA UI12A10 EU446327
salty soil anoecid strain MESS21 GU170214
Symbiomonas scintillans
Anoeca atlantica
Cafeteria roenbergensis
Cafeteriidae
marine Caribbean DNA BCA5F13RJ2H06 GU823870
marine DNA Pacific Ocean UEPAC30Dp2 AY129062
marine hypersaline anoxic UI12C04 EU446333
Caecitellus parvulus
Caecitellus paraparvulus
marine microoxic Caribbean DNA AI5F15RJJ3B09 GU823081
Halocafeteria EHF0502 DQ269469
Halocafeteria seosinensis
hypersaline anoecid strain MESS21 GU170210
Caecitellidae
Anoecida
Boroka kaipovi (NOT Pseudobodo tremulans)
marine strain SL204 clone F9 EF432537
Borokida
marine deep cold-seep sediment DNA RM2-SGM52 AB505560
marine Black Sea redoxcline DNA BLACKSEA_cl_39 HM749941
marine anoxic fjord SA1_1E06_EF526979
marine anoxic Caribbean D217 AY25633
Rictus lutensis
Rictida
Eogyrea 12
Labyrinthulea 59
ALVEOLATA 15
RHIZARIA 12
SAGENISTA

Potentially similar uniciliate species

We consider that *Rigidomastix coprocola* is not a heterokont, and place it in the zooflagellate order Phalansteriida within phylum Amoebozoa, because its radially symmetric anterior cell shape closely resembles that of the uniflagellate soil-dwelling amoebozoans *Phalansterium solitarium* and *P. filosum* (Sandon 1927; Smirnov et al. 2011). But its cilium is rigid, unlike the asymmetrically beating *Phalansterium* cilium, so we do not put it in Phalansteriidae but in a new family **Rigidomastigidae** Cavalier-Smith. **Diagnosis**: Coprophilic non-amoeboid uniflagellates with a single rigid cilium stemming from a slightly pointed radially symmetric cell apex. Type genus *Rigidomastix* Aléxéieff, 1929. **Comment**: The presence of mucilage around some cells through which the cilium protrudes is also characteristic of *Phalansterium*, but not Uniciliatida. There is no reason to assign *Rigidomastix* to Uniciliatida or to Cercozoa. It would clearly have been easier for a horse-dung flagellate like *Rigidomastix* to evolve (by losing ciliary motility) from a symmetric *Phalansterium* in the soil than from an asymmetric marine ancestor like *Incisomonas*. The only *Rigidomastix* species we accept is *R. coprocola*. The third nominal species is clearly misclassified:

Rigidomastix scincorum (Bovee and Telford 1962) is an amoeboflagellate from reptile (skink) dung, and apart from being uniciliate almost totally unlike Aléxéieff's non-amoeboid motionless cells from horse dung; its cilium was much shorter. However, *R. scincorum* looks much like *Rhizomastix* of which six species inhabit amphibian and insect intestines (Čepička 2011) – one has been found in soil (Foissner 1991); it similarly has a rhizostyle and elongate cell with amoeboid posterior and a separate round nonflagellate form. Bovee and Telford (1962) considered *R. scincorum* to be midway between *Rhizomastix gracilis* and *Rigidomastix coprocola* but oddly assigned it to the much less similar *Rigidomastix* on the spurious grounds that it 'is a coprophile, has several forms amoeboid and flagellate', asserting that it resembles *Rigidomastix coprocola* (which was not amoeboid) more than any other protozoan; they wrongly said that *Rigidomastix* are amoeboflagellates. We regard it as a sixth *Rhizomastix* species and make the new combination: *Rhizomastix scincorum* Cavalier-Smith. **Basionym**: *Rigidomastix scincorum* Bovee and Telford, 1962. **Comment**: We place *Rhizomastix* in phylum Amoebozoa beside the order Phalansteriida of the class Variosea because the asymmetric placement of its rhizostyle appears to differ from the symmetric microtubular cone of Archamoebea, and we therefore do not agree with Čepička (2011) that it is more like Archamoebea than Breviatea; we cannot rule out the possibility that it belongs instead in Breviatea as its asymmetric rhizostyle might be related to the sagittal ribbon root of *Breviata* (Walker et al. 2006; Cavalier-Smith et al. 2004) now placed in the sulcozoan subphylum Apusozoa (Cavalier-Smith 2012). However, pseudopodial structure of *R. scincorum* is rather different from that of the two known breviates *Breviata* and *Subulatomonas* (Katz et al. 2011; Minge et al. 2009; Walker et al. 2006) and of Phalansteriida (Smirnov et al. 2011); pseudopodia have not been well characterised in other *Rhizomastix* species (Čepička 2011). As the long curved rhizostyle differs from the cytoskeleton of both orders Breviatida and Phalansteriida we place *Rhizomonas* in the old order Rhizomastigida Doflein (1916) which originally applied especially to what are now Mastigamoebida (class Archamoebea) but has not been used recently because it was excessively heterogeneous through lumping unrelated taxa such as cercomonads and pedinellids (even *Rhizomastix* itself was ignored by recent reviews of flagellate diversity: Patterson and Zölffel 1991; Lee et al. 2002); it is better to revive Rhizomastigida for *Rhizomastix* only than to invent a superfluous new ordinal name. Any family restricted to *Rhizomastix* must be called Rhizomastigidae; that name exists, being often attributed to Calkins (1926), but he did not include *Rhizomastix*, placing it instead in Oicomonadidae, and it actually dates with that spelling at least back to Stokes (1888) who included *Mastigamoeba* and various pseudoheliozoans and attributed it to Bütschli; Bütschli (1885) had named the family Rhizomastigina. As Copeland (1956) pointed out, that old family name Rhizomastigidae is invalid under ICZN (founded only in 1895) being not originally based on an included genus: *Rhizomastix* was discovered much later (Aléxéieff 1911). We therefore establish a new family **Rhizomastigidae** Cavalier-Smith with *Rhizomastix* Aléxéieff, 1911 the type genus. **Diagnosis**: anaerobic amoeboflagellates with a single motile cilium and a long rhizostyle stemming from the centriole and curving round the nucleus towards the cell posterior.

Another colourless uniflagellate considered when identifying *Incisomonas* is *Kiitoksia ystava* (Vørs 1992), a tiny

Fig. 14. Maximum likelihood tree for 328 heterokonts using 27 Rhizaria and Alveolata as outgroups and 1654 nucleotide positions. Rooted between Rhizaria and Alveolata/Heterokonta in accord with multigene trees (Burki et al. 2009). *Incisomonas* sequence shown in bold and by arrow. For better display of the topology for the 92 bigyran sequences, outgroup branches, Labyrinthulea, and Ochrophyta are collapsed (the number beside their names indicates the number of taxa included for each); their internal branching is shown for a separate analysis of Ochrophyta only in Fig. 15 and for Labyrinthulea, Eogyrea and the outgroups in Fig. 16. Bootstrap supports for bipartitions are based on 1000 resamplings using GTRMIX option of RAxML. Black blobs indicate 100% bootstrap support. To avoid congestion the phylum Bigyra (Opalozoa plus Sagenista is not labeled). New oomycete subclasses are defined thus: **Eogamia** Cavalier-Smith subcl. n. (paraphyletic). **Diagnosis:** holocarpic marine parasitic oomycetes. Orders Eurychasmales, Haptoglossales, Haliphthorales, Olpidiopsidales. **Etym:** *Eos* Gk dawn; *gam* Gk marriage; to emphasize their sexual primitivenes. **Oogametia** Cavalier-Smith subcl. n. (holophyletic). **Diagnosis:** oogamous terrestrial or freshwater parasites or saprophytes. With superorders Saprolegniidae and Peronosporidae (previously subclasses). **Etym:** *Oos* Gk egg; *gam* Gk marriage; to signify their oogamy.

near-spherical gliding marine protist with one trailing cilium that is entirely applied to the substratum as in both *Incisomonas* species. Its body is only 1–2(–3) μm, typically half the size of *Incisomonas*. More importantly, it lacks the asymmetric anterior notch of *Incisomonas*, its only cilium stemming from its convex ventral surface; unlike *Incisomonas* it did not swim or form sessile aggregates. Therefore our strain cannot be included in *Kiitoksia*. Though *K. ystava* is clearly different from *Incisomonas* it might be related and also belong in Uniciliatida; but is perhaps more likely to belong to the phylum Cercozoa that has many more gliding zooflagellates than Heterokonta and includes three phylogenetically distinct groups that contain posteriorly gliding uniflagellates: Glissomonadida, Metromonadea, and the imbricate *Clautriavia* (Howe et al. 2011a). It is unlikely that *Kiitoksia ystava* is related to *Clautriavia*, as the only phylogenetically well-placed species is huge and has a pronounced ventral groove (Chantangsi and Leander 2010); it is equally unlikely to belong in Glissomonadida as most are biciliate and virtually all species (including the only uniflagellate genus *Allantion*) inhabit freshwater (Howe et al. 2009, 2011b). *Kiitoksia ystava* was recently placed in order Metromonadida (Cavalier-Smith and Oates 2012) within the cercozoan class Metromonadea (all its previously characterised species are biciliate marine gliding phagotrophs: Howe et al. 2011a,b). We now establish the new family **Kiitoksiidae** Cavalier-Smith. **Diagnosis**: marine uniciliate heterotrophs that glide on a posterior cilium and do not swim; unlike *Metromonas* lack a second stubby cilium and do not feed whilst tethered to the substratum and vigorously oscillating the cilium. Type genus *Kiitoksia* Vørs, 1992. **Comment**: we doubt that *K. kaloista* from freshwater (Tong et al. 1997) is a *Kiitoksia* or a metromonad; its habitat and lack of oscillating behaviour makes it more likely to be a glissomonad from one of the uncharacterised environmental clades in the *Bodomorpha* part of the tree (Howe et al. 2011b). *K. kaloista* has a short posterior pointing second cilium like the freshwater glissomonad *Bodomorpha* (Howe et al. 2009), but as it lacks an anterior beak it is not a *Bodomorpha*.

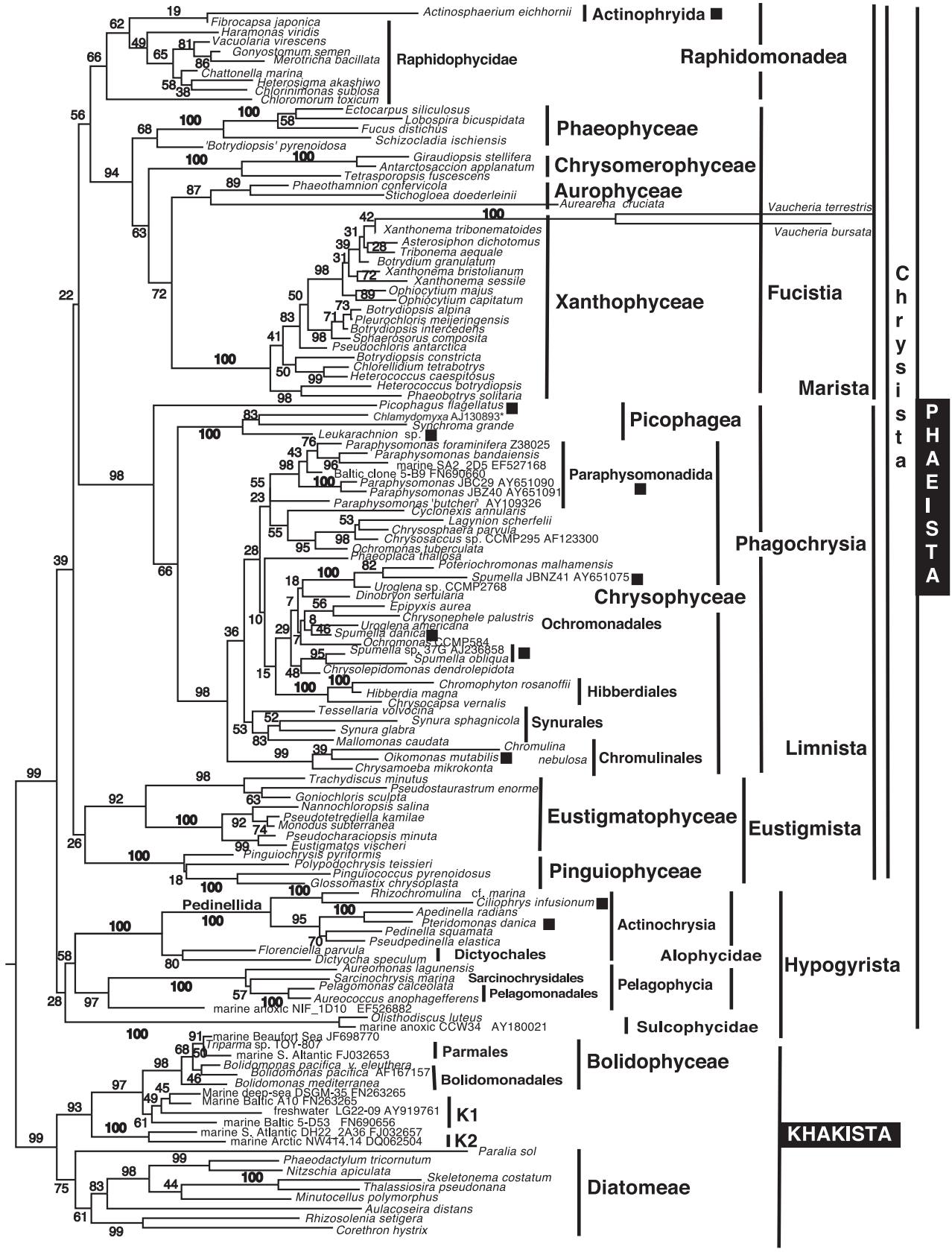
Another small gliding zooflagellate with only a posterior cilium that might be confused with *Incisomonas* is *Clautriavia parva* from a brackish ditch (Schouteden 1907); unlike the much larger type species, the freshwater *Clautriavia minus* (Massart 1900), and phylogenetically well characterised marine *C. biflagellata* (Chantangsi and Leander 2010), it lacks a ventral groove or evidence for a rigid pellicle, and so was incorrectly placed in *Clautriavia*. In fact *C. parva* is much less similar to *C. minus* than it is to *K. ystava*, from which it differs significantly only by being larger (about 8 μm instead of 1–3 μm, not 10 μm as stated by Lemmermann (1914), if we accept Schouteden's figure legend noting that *C. minus* is reduced by 9/10 even though his text says both were drawn to the same scale). Therefore we transfer *C. parva* to *Kiitoksia*: ***Kiitoksia parva*** Cavalier-Smith comb. n. **Basionym**: *Clautriavia parva* Schouteden, 1907. We do not accept the suggestion that *C. parva* may be a stage in the life cycle of *Metromonas* (Patterson and Zölffel 1991) as it lacks the tiny anterior cilium and posterior ciliary oscillation of that genus, but agree with the similarity they noticed sufficiently to include it in the same order but in a separate family.

As no family is available for *Clautriavia* a new one is needed: **Clautriaviidae** Cavalier-Smith fam. n. **Diagnosis**: cercozoan flagellates with only one long posterior gliding cilium and in some species also a short backwards pointing anterior one; rigid dorsoventrally flattened cells with mid-ventral groove. Type genus: *Clautriavia* Massart, 1900. **Comment**: as the marine *C. cavus* has a ventral groove, albeit somewhat indistinct (Lee and Patterson 2000), we accept it as one of only three authentic *Clautriavia* species (*minus*, *cavus*, *biflagellata*).

Phylogenetic analysis

Our maximum likelihood (ML) tree of Heterokonta 18S rDNA (Fig. 14) shows that the *Incisomonas marina* sequence is almost identical to three sequences amplified from a Norwegian fjord, and forms a mini-clade with two other environmental sequences which is robustly sister to a subclade of environmental sequences that also includes *Solenicola setigera* (Gómez et al. 2011); thus *Incisomonas* and *Solenicola* and their related environmental DNAs together form a large subclade within MAST-3 (now Nanomonadea) that corresponds to the new order Uniciliatida (100% bootstrap support (BS)). We suspect that most of the differences between the *Incisomonas* and Norwegian rDNAs are sequencing errors; if allowance is made for likely errors in the environmental sequences we regard the *Incisomonas* sequence as identical to 'uncultured marine eukaryote clone' (GenBank accession number EF526878; Behnke et al. 2010) and therefore probably the same species. MAST-3 clade (Nanomonadea) has stronger support (99%) than in some previous studies (e.g. Massana et al. 2004 (no BS) or Gómez et al. 2011 – only 67%), as does its position as sister to Opalinata plus MAST-12 (81%), but both closely agree with Yubuki et al. (2010). MAST-12 is strongly holophyletic (BS 87–96%); and strongly sister to Opalinata (BS 86–89%), agreeing with Gómez et al. (2011; 98%, probably higher because only *Blastocystis* was used, the long branch Opalinea being excluded), and contradicts some previous studies that placed MAST-3 not MAST-12 as sister to Opalinata (e.g. the Bayesian but not ML or distance trees of Kolodziej and Stoeck (2007)) which now seems likely to be erroneous. Placidida being sister to these three clades is strongly supported (79–87%) as Yubuki et al. (2010) also found; these four clades constitute infraphylum Placidozoa (Table 1). Subphylum Opalozoa as now expanded to include Bikosea (Table 1) is holophyletic with moderate support.

The most striking novelty in our ML trees is that the pseudoheliozoan *Actinosphaerium* groups robustly within Ochrophyta (BS 80%), specifically within Raphidomonadea (Fig. 15) and is no longer sister to the bigyran Opalinata


Actinosphaerium eichhornii
Actinophryida
Fibrocapsa japonica
Haramonas viridis
Vacuolaria virescens
Gonyostomum semen
Merotricha bacillata
Chattonella marina
Heterosigma akashiwo
Chlorinimonas sublosa
Chloromorum toxicum
Raphidophycidae
Raphidomonadea
Ectocarpus siliculosus
Lobospira bicuspidata
Fucus distichus
Schizocladia ischiensis
'Botrydiopsis' pyrenoidosa
Phaeophyceae
Giraudiopsis stellifera
Antarctosaccion applanatum
Tetrasporopsis fuscescens
Chrysomerophyceae
Phaeothamnion confervicola
Stichogloea doederleinii
Aurophyceae
Aureareha cruciata
Vaucheria terrestris
Vaucheria bursata
Xanthonema tribonematoides
Asterosiphon dichotomus
Tribonema aequale
Botrydium granulatum
Xanthonema bristolianum
Xanthonema sessile
Ophiocytium majus
Ophiocytium capitatum
Botrydiopsis alpina
Pleurochloris meijeringensis
Botrydiopsis intercedens
Sphaerosorus composita
Pseudochloris antarctica
Botrydiopsis constricta
Chlorellidium tetrabotrys
Heterococcus caespitosus
Heterococcus botrydiopsis
Phaeobotrys solitaria
Xanthophyceae
Fucistia
Marista
Chrysista
PHAEISTA
Picophagus flagellatus
Chlamydomyxa AJ130893*
Symphroma grande
Leukarachnion sp.
Picophagea
Paraphysomonas foraminifera Z38025
Paraphysomonas bandaiensis
marine SA2_2D5 EF527168
Baltic clone 5-B9 FN690660
Paraphysomonas JBC29 AY651090
Paraphysomonas JBZ40 AY651091
Paraphysomonas 'butcheri' AY109326
Paraphysomonadida
Cyclonexis annularis
Lagynion scherfelii
Chrysosphaera parvula
Chrysosaccus sp. CCMP295 AF123300
Ochromonas tuberculata
Phaeoplaca thallosa
Phagochrysia
Poteriochromonas malhamensis
Spumella JBNZ41 AY651075
Uroglena sp. CCMP2768
Dinobryon sertularia
Epipyxis aurea
Chrysonephele palustris
Uroglena americana
Spumella danica
Ochromonas CCMP584
Spumella sp. 37G AJ236858
Spumella obliqua
Chrysolepidomonas dendrolepidota
Chrysophyceae
Ochromonadales
Chromophyton rosanoffii
Hibberdia magna
Chrysocapsa vernalis
Hibberdiales
Tessellaria volvocina
Synura sphagnicola
Synura glabra
Synurales
Mallomonas caudata
Chromulina nebulosa
Oikomonas mutabilis
Chrysamoeba mikrokonta
Chromulinales
Limnista
Trachydiscus minutus
Pseudostaurastrum enorme
Goniochloris sculpta
Nannochloropsis salina
Pseudotetrediella kamilae
Monodus subterranea
Pseudocharaciopsis minuta
Eustigmatos vischeri
Eustigmatophyceae
Eustigmista
Pinguiochrysis pyriformis
Polypodochrysis teissieri
Pinguiococcus pyrenoidosus
Glossomastix chrysoplasta
Pinguiophyceae
Rhizochromulina cf. marina
Ciliophrys infusionum
Pedinellida
Apedinella radians
Pteridomonas danica
Pedinella squamata
Pseudpedinella elastica
Actinochrysia
Florenciella parvula
Dictyocha speculum
Dictyochales
Alophycidae
Aureomonas lagunensis
Sarcinochrysis marina
Sarcinochrysidales
Pelagomonas calceolata
Aureococcus anophagefferens
Pelagomonadales
Pelagophycia
Hypogyrista
marine anoxic NIF_1D10 EF526882
Olisthodiscus luteus
marine anoxic CCW34 AY180021
Sulcophycidae
marine Beaufort Sea JF698770
Triparma sp. TOY-807
marine S. Atlantic FJ032653
Parmales
Bolidomonas pacifica v. eleuthera
Bolidomonas pacifica AF167157
Bolidomonas mediterranea
Bolidomonadales
Bolidophyceae
Marine deep-sea DSGM-35 FN263265
Marine Baltic A10 FN263265
freshwater LG22-09 AY919761
K1
marine Baltic 5-D53 FN690656
marine S. Atlantic DH22_2A36 FJ032657
marine Arctic NW414.14 DQ062504
K2
KHAKISTA
Paralia sol
Phaeodactylum tricornutum
Nitzschia apiculata
Skeletonema costatum
Thalassiosira pseudonana
Minutocellus polymorphus
Aulacoseira distans
Rhizosolenia setigera
Corethron hystrix
Diatomeae

as on earlier NJ trees (Cavalier-Smith and Chao 2006). It is weakly sister to *Fibrocapsa* (BS 19–29%). Its earlier grouping with Opalinata was presumably a long-branch artefact, which can be hard to avoid with NJ which necessarily first groups shorter branches together and thus excludes exceptionally long branches from their correct position. Both the Harosa-wide and 128 Ochrophyta-only ML trees put *Actinosphaerium* as sister to *Fibrocapsa*; but support for monophyly of all Raphidomonadea including *Actinosphaerium* was higher (66%) on the Ochrophyta-only tree (33–36% on Harosa-wide trees). Fig. 15 also shows greater support for the *Actinosphaerium*/*Fibrocapsa* clade being sister to all other Raphidomonadea except the deepest branching *Chloromorum* (62% not 24–47% as on heterokont-wide trees). With our extensive taxon sampling and more extended alignment even GTR gamma-corrected NJ trees can also group *Actinosphaerium* with Ochrophyta, but unlike for ML this grouping is unstable to differences in taxon sampling; many taxon samples group *Actinosphaerium* instead with Oomycetes (or rarely Opalinata as before, or with both plus *Rictus*), so NJ does not clearly assign it to a phylum. ML invariably put *Actinosphaerium* within subphylum Phaeista of Ochrophyta when taxon number was varied; only in the 327 and 342 taxon trees was it sister to Eustigmatophyceae, not to *Fibrocapsa* within Raphidomonadea as in all others. The NJ Ochrophyta-only tree put *Actinosphaerium* within raphidophytes, as sister to the deepest-branching *Chloromorum* not *Fibrocapsa*.

The even longer branch bikosean *Rictus* was more consistently misplaced as sister to Opalinata on all NJ trees, but with ML *Rictus* was sister to a small short-branch environmental DNA clade (65–71% BS) and this joint clade sister to other Bikosea (74–77% BS) in agreement with the ML and Bayesian analyses of Yubuki et al. (2010), but now with higher BS. Subclass Bicosidia (classical aerobic bikoseans) is strongly holophyletic (83%) and there is strong support for Borokida being its deepest branch prior to the ill-resolved separation of the other three bicosid orders. We identified for the first time environmental DNA sequences specifically related to the bicosid *Boroka* and the pseudofungal flagellate *Developayella* (Fig. 14).

MAST-13 is not a novel clade; all its sequences group robustly within the well-known loricate genus *Bicosoeca*; indeed, all are more closely related to *B. vacillans* (BS 99–100%) than to *B. petiolata* and not even a clade when *B. vacillans* is included in the tree, some being closer to it than others (Fig. 14). That was overlooked by Zuendorf et al. (2006) as their tree had no Bicoecida, only anoecids, but its bicoecid nature was independently discovered by del Campo and Massana (2011); inadequate taxon sampling has often led to similar erroneous claims of novel clades (see Cavalier-Smith 2004). Unlike in Cavalier-Smith and Chao (2006), Anoecida (a distinct non-loricate order of Bikosea) is holophyletic (more weakly supported than other major bikosean orders: the robustly holophyletic Bicoecida and Pseudodendromonadida), an improvement stemming from the better, more richly sampled alignment (notably including *Halocafeteria* and an environmental clade sister to *Caecitellus*; not to our present use of ML, less subject to long-branch exclusion than NJ, for Anoecida are now holophyletic with NJ also).

MAST-5 is firmly within Labyrinthulea and thus also no longer altogether unidentified (Fig. 16). In Labyrinthulea three other major new clades of mainly environmental DNAs are now evident. Two (Diplophryidae sensu stricto and the clade containing '*Diplophrys marina*') contain both marine and freshwater sequences and are sisters to the entirely marine Thraustochytriidae (Fig. 16). The freshwater filose protist *Diplophrys* sp. previously grouped weakly with *Labyrinthula* (Cavalier-Smith and Chao 2006), but on our now much better sampled tree groups robustly (BS 100%) with a very dissimilar freshwater environmental sequence; these two group equally robustly with a marine one to form a long-branch clade that is firmly excluded from Labyrinthulida and groups strongly (BS 82–89%) with MAST-5, '*Diplophrys marina*' and Thraustochytriidae. Previously the partial sequence of *Diplophrys marina* grouped weakly with Aplanochytriidae (Cavalier-Smith and Chao 2006); now it is robustly (BS 100% both for the whole clade and the subclade to which it belongs) part of a substantial new even longer branch clade of freshwater (oddly not marine) environmental DNAs that is sister to Thraustochytriidae with moderate support (63%). It is strongly sister to sequence RT5iin25 from an acid-polluted river (Amaral Zettler et al. 2002) and is thus within clade

Fig. 15. Maximum likelihood tree for the same 128 Ochrophyta (only) as in the 355-taxon tree shown in Fig. 14. Bootstrap support for bipartitions are based on 1000 resamplings using GTRMIX option of RAxML. The topology of this tree based on 1655 nucleotide positions is identical to the collapsed Ochrophyta part of Fig. 14 except for insignificantly supported minor changes within Chrysophyceae, Xanthophyceae and Pinguiophyceae; bootstrap values were identical or only slightly lower or higher, except within Raphidomonadea where some were significantly higher on this tree. The black squares mark the non-photosynthetic taxa; these plus one not shown within diatoms show that Ochrophyta lost photosynthesis at least 11 times independently, compared with single losses only at the bases of Pseudofungi and Bigyra, making 13 losses in all in Heterokonta. K1 and K2 are two environmental DNA clades within Khakista to which no phenotypes are yet attached; their position makes them potentially especially interesting for testing ideas on early ochrophyte evolution. This tree implies that *Triparma* nests within *Bolidomonas*, suggesting that Bolidomonas is ancestral not a sister (Guillou 2011) to Bolidomonadales; alternatively if clade K1 also consists of Parmales, Bolidomonadales (Guillou et al. 1999) might be derived from Parmales by scale loss or simply another phase in their life cycle and thus a junior synonym. We consider that the organism from which sequence AJ130893 (asterisk) came was misidentified and is not *Chlamydomyxa labyrinthuloides* (which is not an alga and probably belongs in Labyrinthulida) and is an undescribed species in a novel algal genus within Synchromales.

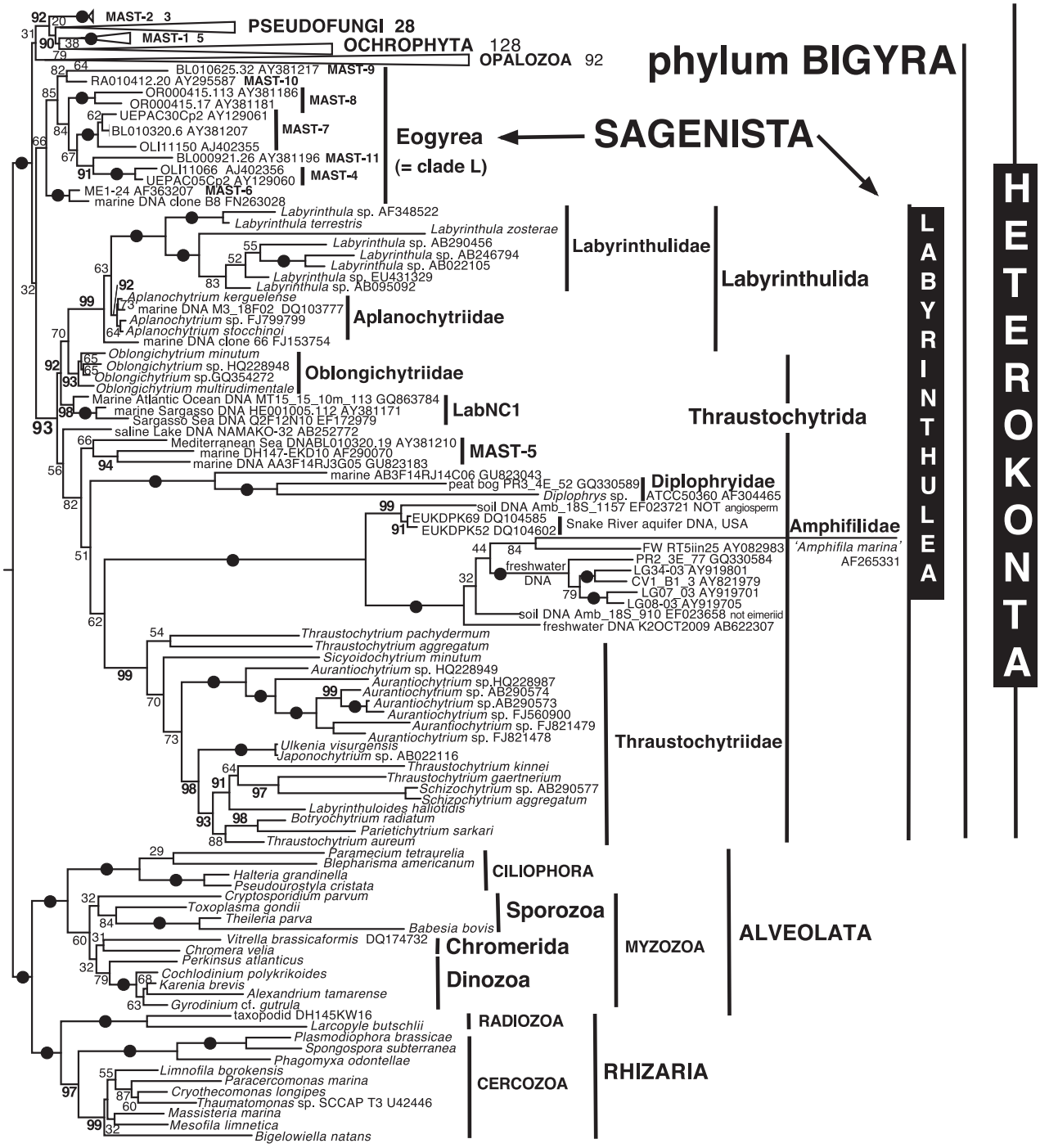


Fig. 16. Maximum likelihood tree for 328 heterokonts emphasizing Labyrinthulea and Eogyrea (subphylum Sagenista of Bigyra) and outgroups. Internal branches for other groups are collapsed but shown on Figs 14, 15. Bootstrap supports for bipartitions are based on 1000 resamplings using GTRMIX option of RAxML. Black blobs indicate 100% bootstrap support. The root is arbitrarily placed beteween heterokonts and ougroups, but should probably be between alveolates and Rhizaria. Note that sequence DH147-EKD10 (AF290070) originally alone used to define MAST-5 is a chimaera (Worden and Not 2008); only the first part (nucleotides 1–1010) is from a labyrinthulid. Had we been aware of that earlier we would have removed the 3′ terminal portion (nt. 1010–1800) from a marine alveolate (MALV-II dinoflagellate) before running the tree; that should be done in future heterokont trees. Familial classification of Labyrinthulea follows Anderson and Cavalier-Smith (2012), where the new name '*Amphifila marina*' and a new species for *Diplophrys* sp. are formally published.

LG-B (Richards et al. 2005) that was extended to an even larger uncharacterised environmental clade by Lara et al. (2011). As '*D. marina*' is not closely related to *Diplophrys* sp. and differs morphologically from freshwater strains of *Diplophrys* it is now placed in a new genus and family as the only morphologically characterised member of this huge long-branch clade (Anderson and Cavalier-Smith 2012); its members from soil are grossly misannotated in GenBank – EF023721 as a flowering plant and EF023658 and many others as an eimeriid (sporozoan parasite); eukaryotic annotations from the soil study generating EF023658 (Lesaulnier et al. 2008) must be treated with extreme caution as we and others (Cavalier-Smith and Chao 2010, 2012; Howe et al. 2011b; Kudryavtsev et al. 2011; Lara et al. 2010) found it also includes sequences from apusomonads, glissomonads, three different groups of Amoebozoa, and MAST-12 all mislabelled as eimeriids and a heliozoan called a fungus! The third new environmental clade (LabNC1) of 3 sequences is sister to *Oblongichytrium* on NJ trees but to Labyrinthulida/*Oblongichytrium* (Fig. 16) or all Labyrinthulea (some other taxon samples) by ML.

Seven MAST clades (4, 6–11) form a single moderately supported (BS 66%; 52–72% in other taxon samples) superclade (clade L of Cavalier-Smith and Chao 2006) that in many taxon samples weakly sister to Labyrinthulea as in Cavalier-Smith et al. (2006) but on a few of the taxonomically most richly sampled ones (including Fig. 14) was weakly sister to all other Opalozoa. With ML clade L was never paraphyletic as in the ML tree of Gómez et al. (2011) and Bayesian tree of Kolodziej and Stoeck (2007) or the trees of Yubuki et al. (2010), which seem likely to be erroneous, perhaps because of misrooting through their using too few outgroups or less extensive or imperfect alignments. Thus there are only three deep-branching MAST clades within Bigyra: MAST-12 (here called Opalomonadea as they are invariably sister to Opalinata: see below); Nanomonadea (MAST-3) sister to Opalinata plus Opalomonadea; and clade L, the earliest branching clade of heterokont zooflagellates.

MAST-2 branches as sister to Pseudofungi plus Ochrophyta with strong support; Kolodziej and Stoeck (2007) placed MAST-2 in Pseudofungi, which might be correct, but to decide firmly whether they really should be there rather than in Bigyra probably requires culturing, sequencing other genes and morphology. MAST-1 with only one prominent cilium (Massana et al. 2006) is positionally unstable: in the 268 taxon dataset it branched weakly with Pirsoniida (as it did substantially more strongly in Gómez et al. 2011) within Pseudofungi, but in Fig. 14 it is weakly sister to Ochrophyta. However on the corresponding distance trees MAST-1 and MAST-2 both form a single clade with Pseudofungi.

In most other respects topology of previously well-supported major groupings on the heterokont tree is similar to earlier trees (Cavalier-Smith and Chao 2006; Gómez et al. 2011; Massana et al. 2004). We found strong support (80–88%) for holophyly of phylum Ochrophyta, which includes all algal heterokonts plus a few heterotrophs (e.g. Actinophryida), but only weak support for holophyly of phylum Pseudofungi. Ochrophyta plus Pseudofungi (i.e. superphylum Gyrista: Cavalier-Smith 1998) form a robust clade (90–94%) that also includes MAST-1, and which is robustly sister to MAST-2 (BS 92–98%). Fig. 14 weakly implies paraphyly of phylum Bigyra, as in previous 18S rDNA trees (Cavalier-Smith and Chao 2006; Gómez et al. 2011) and even a recent 7-gene tree (Riisberg et al. 2009), with Opalozoa holophyletic and Labyrinthulea (usually plus Eogyrea, a new name for the deepest branching phenotypically uncharcterised clade L) the deepest branching heterokont group. But on two ML trees (with 327 or more sequences) Bigyra were holophyletic. Bikosea are invariably sisters (BS 60–71%) to the large clade comprising Placidida, Nanomonadea, Opalinata and MAST-12, as found with much stronger support (91%) by Gómez et al. (2011) who used fewer nucleotide positions and only a few Rhizaria as outgroup, but surprisingly not found at all by Yubuki et al. (2010).

Strongly supported supra-class clades within Ochrophyta (Fig. 15) include Khakista (BS 99%; 98% in 355 taxon tree: Fig. 14), Fucistia (94%), and Phagochrysia, the clade comprising Chrysophyceae and Picophagea (Fig. 14: 97%; Fig. 15: 98%) (Grant et al. 2009; Patil et al. 2009). A significant new finding is that *Olisthodiscus* (traditionally most often regarded as a raphidophyte) does not group with raphidophytes plus *Actinosphaerium*, but is instead sister to Alophycidae, albeit with weak support. Moreover, the *Pleurochloridella*/*Phaeobotrys* clade (Pleurochloridellales) groups with Xanthophyceae with 100% support indicating that combining Pleurochloridellales and Phaeothamniales as the class Phaeothamniophyceae (Bailey et al. 1998) was erroneous; *Tetrasporopsis* and *Botrydiopsis* also were clearly misplaced in 'Phaeothamniophyceae', *Tetrasporopsis* grouping strongly (BS 100%) with Chrysomeridales (Chrysomerophyceae), three *Botrydiopsis* species in multiple places within Xanthophyceae (100% BS for its holophyly), and '*Botrydiopsis pyrenoidosa*' with Phaeophyceae (BS 68% in Fig. 15). Pinguiophyceae and Alophycidae (previously both in class Hypogyristea: Cavalier-Smith 2004; Cavalier-Smith and Chao 2006) group together with NJ but with ML usually do not: Pinguiophyceae then weakly goes with Eustigmatophyceae – positions of both groups were always unstable on published 18S rDNA trees (Cavalier-Smith and Chao 2006) – poorly supported and inconsistent among methods even on 7-gene trees (Riisberg et al. 2009), but now clearer on a 5-gene tree including four chloroplast proteins (Yang et al. 2012) and a 4-gene tree (Grant et al. 2009); the latter groups Pinguiophyceae with Eustigmatophyceae as in Fig. 14. With 327 or 342 taxa ML placed Pinguiophyceae within Hypogyristea (between Alophycidae and *Olisthodiscus* as on most NJ trees). Raphidomonadea are consistently weakly sister to Fucistia (56% BS in Fig. 15), which is more strongly supported in the 5-gene tree (Yang et al. 2012),

but deeper branches within Phaeista are insignificantly supported.

Revision of Opalozoa and Raphidomonadea

Our improved trees make it necessary to transfer Actinophryida to Ochrophyta. The former placement of Actinophryida in Opalozoa stemmed solely from their apparent position in the distance rRNA tree of Cavalier-Smith and Chao (2006), here considered an artefact of the NJ algorithm. As that spurious grouping with the non-phagotrophic Opalinata had no morphological rationale, we transfer Actinophryida from Bigyra to Ochrophyta. We also expand Actinophryida by adding *Ciliophrys azurina*, a heterotrophic uniflagellate with similar axopodia (Mikrjukov and Patterson 2001) here transferred from Pedinellales to the new actinophryid genus *Heliorapha*. We now group raphidophytes and Actinophryida in the same class under the name Raphidomonadea (previously used as the class name in preference to Raphidophyceae: Cavalier-Smith 1986); we down-rank the previously separate class Raphidophyceae (photosynthetic raphidophytes) and establish Raphopoda' for Actinophryida and Commatiida) as subclasses of Raphidomonadea:

Raphidophycidae Cavalier-Smith subcl. n. Diagnosis as for Raphidophyceae Chadefaud ex Silva (1980); **Raphopoda** Cavalier-Smith subcl. n. **Diagnosis**: Heterotrophic phagotrophic protists with either a single microtubule-supported proboscis/axopodium and kinetocyst extrusomes or numerous broad tapering axopodia containing mitochondria, conspicuous amorphous extrusomes, and axonemes nucleated on the nucleus or nuclei; uni- or multinucleate; non-ciliate or with single tinsel cilium; nuclei very large with central nucleolus and peripheral heterochromatin; peripheral cytoplasm often strongly vacuolated; in Actinophryidae at least axopodia of two interdigitating spirals of microtubules with prominent cross links. Orders Commatiida (Cavalier-Smith 1997b) with new family Commatiidae Cavalier-Smith (**Diagnosis:** comma-shaped gliding cells with single axopodium and temporary cilium. Type genus *Commation* Thomsen and Larsen (1993) and Actinophryida Hartmann, 1913 with two new suborders: **Actinosphaerina** Cavalier-Smith subord. n. **Diagnosis**: no cilium; many nuclei, no more than one axopodial axoneme associated with each; sometimes with conical dense material around their bases; some nuclei and some axonemes may not be mutually attached. Sole family **Actinosphaeriidae** Cavalier-Smith fam. n. Diagnosis as for suborder. Type genus *Actinosphaerium* Ritter von Stein, 1857. **Actinophryina** Cavalier-Smith subord. n. **Diagnosis**: with single nucleus having multiple attched axonemes with no conical dense material around their bases. Two families Actinophryidae Dujardin, 1841 (no cilium; one large nucleus nucleates numerous axopodia: *Actinophrys*), and **Helioraphidae** Cavalier-Smith fam. n. **Diagnosis**: with similar tapering axopodia to Actinophryidae, but unlike Actinophryidae has a single tinsel cilium; nucleus single. Type and sole genus *Heliorapha* Cavalier-Smith gen. n. Diagnosis as for Helioraphidae. Type and sole species *Heliorapha azurina* Cavalier-Smith comb. n. **Basionym**: *Ciliophrys azurina* Patterson (Mikrjukov and Patterson 2001, pp. 15–16).

Comment: *H. azurina* differs from *Ciliophrys* and all other Pedinellales by having tapering axopodia that are so broad at the base that it is not credible that their axonemes could be simply a triad of microtubules as in all Pedinellales. It was therefore assigned to the wrong genus and order. Mikrjukov and Patterson (2001) considered *H. azurina* to be related and a clade that they called heliomonads; however, that name is confusing and undesirable for this clade as an order Heliomonadida had been proposed earlier in a different sense (Cavalier-Smith 1993), which is now placed within Granofilosea in Cercozoa (Bass et al. 2009) and revised to contain only Acinetactidae and Tetradimorphidae (Yabuki et al. 2012). We regard Raphidophycidae as comprising one order, Chattonellales; the deep-branching '*Chloromorum*' originally misidentified as a *Chattonella* has not been validly published as a genus or species – when described properly it might deserve being put in a third raphidomonad order. As explained in the Discussion, photosynthetic raphidophytes (Chattonellales) are sufficiently diverse to merit three families. We therefore segregate two new ones from the existing sole family, Vacuolariaceae: **Haramonadaceae** Cavalier-Smith fam. n. **Diagnosis**: rhizostyle of two nested trough shaped microtubule bands, an adnuclear band of 7 relatively widely spaced microtubules separated from a more deeply tough shaped band of 10 more closely spaced by a single layered trough shaped sheet closely associated with the 10-mt band and a less densely stained lattice associated with the adnuclear band (Vacuolariaceae have many more microtubules and more extensive dense fibrillar material giving rhizostyle a more deeply U-shaped cross section: Heywood 1978a; Mignot 1967, 1976; Vesk and Moestrup 1987; Yamaguchi et al. 2010). Dense material associated with the anterior microtubular root not obviously laminated as it is in Vacuolariaceae (Vesk and Moestrup 1987; Yamaguchi et al. 2010). Extrusomes small mucocysts or absent. Type and sole genus *Haramonas* Horiguchi 1996. **Fibrocapsaceae** Cavalier-Smith fam. n. anterior microtubular root or rhizostyle not reported; singlet microtubules radiate in fan from posterior centriole to nucleus and penetrate envelope; extrusomes large elongated trichocysts with dense rod like core (not mucocysts as in most Vacuolariaceae). Type and sole genus *Fibrocapsa* Toriumi and Tokano, 1975.

Within phylum Bigyra we revise Opalozoa, as summarised in Table 1:

Opalozoa: new class Opalomonadea Cavalier-Smith. **Diagnosis**: Biciliate phagotrophs with anterior hairy cilium, often from anaerobic habitats (Kolodziej and Stoeck 2007); have 18S rRNA sequences that group within the MAST-12 clade on phylogenetic trees as the sister group to Opalinata. **Etymol**. Opalo refers to their relationship to

Opalinata; *monad* Gk unit, refers to their being flagellates (monads). **Comment**: Same as clade O of Cavalier-Smith and Chao (2006). Two small swimming biciliates from anaerobic marine sediments in Australia that are not yet assigned to higher taxa are candidates for consideration as possible opalomonads (*Barthelona vulgaris* and *Quasibodo loughtoni* (Bernard et al. 2000)) but it is premature to place them in this or any other group until this is tested by culturing and rDNA sequencing.

New infraphylum Placidozoa Cavalier-Smith. **Diagnosis**: Biciliate, uniciliate or multiciliate phagotrophic or anaerobic osmotrophic heterokonts symbiotic in animal guts; double ciliary transition helix; unlike most Bikosia, lack a discrete cytopharynx, posterior ventral singlet root, and root R2-associated microtubule X; phylogenetically all members are cladistically closer to *Opalina* than to *Bicosoeca.* **Etymol**: 'Placid' is derived from *Placidia* a free-living member of the taxon with one of the more ancestral phenotypes; 'zoa' (*zoon* Gk animal, organism) stresses that they are heterotrophic, not algae. **Comment**: Placidida were previously treated as subclass Placidae within Bicoecea because they grouped weakly together on NJ trees (Cavalier-Smith and Chao 2006). That grouping was misleading, as shown by the much stronger grouping on our ML trees with the clade comprising Nanomonadea, Opalomonadea and Opalinata, as recognised here by treating the robust clade comprising these three taxa and Placididea as an infraphylum of an expanded subphylum Opalozoa, thereby excluding Placidida from Bikosea.

Revisions to Bikosea (formerly Bicoecea): (1) new pseudodendromonad families. As Fig. 14 shows that *Nanos* and *Filos* are robustly within the order Pseudodendromonadida but do not group specifically with any existing families, we establish new families for each. As *Nanos*, though valid under ICBN, is a junior synonym of the scarab beetle genus *Nanos* Westwood, 1847, we propose a replacement name *Nanum* that is valid under ICZN. **Nanidae** Cavalier-Smith fam. n. **Diagnosis**: Uniciliate, monadiform, non-gliding, phagotrophic heterokonts with anterior hairy cilium, cytostome/cytopharynx, and tubulicristate mitochondrion attached to posterior end of nuclear envelope. Type genus: *Nanum* Cavalier-Smith; replacement name for *Nanos* Kim et al. (2010, p. 192) with the same diagnosis. The sole species is now *Nanum amicum* Cavalier-Smith comb. n. (ICZN) = *Nanos amicus* Kim et al. (2010, p. 193) (ICBN). **Filidae** Cavalier-Smith fam. n. **Diagnosis**: Uniciliate, monadiform, non-gliding, phagotrophic heterokonts with anterior hairy cilium, cytostome/cytopharynx, and anterior tubulicristate mitochondrion attached to the centriole. Type genus: *Filos* Kim et al., 2010. We simplified classification of Pseudodendromonadida by accepting the inclusion of *Adriamonas* within Siluaniidae (Karpov 2000; but not of the very different *Caecitellus*: presence of a cytopharynx is probably ancestral for Bikosea so an inadequate reason for placing it in Siluaniidae) as the loss of the posterior cilium by *Siluania* and of ciliary hairs by *Adriamonas* now seem insufficient reasons for a separate family Adriamonadidae (Cavalier-Smith and Chao 2006). (2) **Familial revisions in Anoecida.** We simplify anoecid classification by transferring *Symbiomonas* and *Anoeca* to Cafeteriidae (from separate monogeneric families: Cavalier-Smith and Chao 2006, who hinted that *Anoeca* might belong with *Cafeteria*), making Cafeteriidae a broader family for all Anoecida lacking a cytopharynx (needs checking by electron microscopy for *Anoeca*; moreover if *Cafeteria marsupialis* is an anoecid, the generalisation that they lack a cytopharynx may not be correct as it apparently has one at the hind end of its groove: Larsen and Patterson 1990); their grouping together and with *Cafeteria* on all our ML trees largely dispels earlier worries that their original clustering might have been a long-branch artefact. We transfer *Acronema sippewissettensis* (Teal et al. 1998) to *Cafeteria* as it was insufficiently distinct to merit a separate genus; moreover *Acronema* is invalid for this species under ICBN being preoccupied by a genus of umbelliferous flowering plants (*Acronema* Falconer ex Edgeworth, 1846): *Cafeteria sippewissettensis* (Teal et al. 1998) Cavalier-Smith comb. n. is valid under both ICZN and ICN. We place *Halocafeteria* (previously unassigned to a family) within Caecitellidae as it groups with *Caecitellus* on all our and some earlier trees (Park et al. 2006; Yubuki et al. 2010; but not in Park and Simpson 2010, though Anoecida are holophyletic on their tree) and both genera lack ciliary hairs unlike Cafeteriidae (Table 1); presumably hairs were lost in their common ancestor. (3) **New subclass Rictidia and order Rictida** Cavalier-Smith: Diagnoses as for sole family **Rictidae** Cavalier-Smith fam. n. **Diagnosis**: Biciliate raptorially feeding bikoseans with cytopharynx, but no ciliary hairs or lorica. May tolerate anaerobic conditions and have mitochondria with dense matrix and reduced cristae. Cytostome rim supported by a broad microtubular band and adjacent singlet microtubular that loop round it to return almost to their point of origin at the posterior centriole.; swim; do not glide. Type genus *Rictus* Yubuki et al., 2010 (p. 274).

New superclass Wobblata Cavalier-Smith. **Diagnosis**: Ancestrally aerobic phagotrophic biciliates (or derived uniciliates that have lost the anterior cilium) with tubular mitochondrial cristae and hairy anterior cilium (both unlike Opalinata); split right microtubular root; no cytopharynx; double transition helix distal to transition zone transverse plate. A phylogenetically basal group comprising Placididea, Nanomonadea, and Opalomonadea. **Etymol**: named after *Wobblia* the first discovered member of the best characterised constituent class Placididea. **Comment**: Our extremely well sampled ML trees strongly support the grouping of Placididea with Opalinata seen by Moriya et al. (2002; then statistically insignificant) rather than the earlier position of *Wobblia* at the base of Heterokonta (Moriya et al. 2000) or the weak grouping of Placididea with Bikosia on NJ trees (Cavalier-Smith and Chao 2006), so we now accept them as a distinct opalozoan class. The clade comprising

Opalinata and Wobblata is robust (Fig. 14 and Yubuki et al. 2010).

Corrrecting confusions over *Pseudobodo*. Numerous heterokonts have been misidentified as *Pseudobodo tremulans* (ranging from *Boroka*, through *Labromonas*, *Cafeteria*, to *Caecitellus*: see Cavalier-Smith and Chao 2006), and the true *P. tremulans* Griessmann (1913), which also attached to substrates by its shorter posterior ciliary tip, has not been sequenced. Following Larsen and Patterson (1990) most recent descriptions of it seem to refer to *Labromonas* (e.g. Patterson and Zölffel 1991; Patterson 2002). The original *P. tremulans* however had no lateral feeding pouch, nor a 'collar' around the cilia, was laterally not apically biciliate, and had a much longer anterior cilium than *Boroka* or *Labromonas fencheli* – four times its body length. Moreover it was reported to ingest food over its entire body surface (though pseudopodia were not seen: Griessmann 1913) making it doubtful that it even belongs in Bikosea where it has long been placed; its cell lacks the D-shape widespread in Bikosea. It might belong anywhere in Heterokonta and ought to be considered a possible member of any biciliate MAST clade; for example its unusually long anterior cilium makes it a candidate for membership of MAST-12 (Kolodziej and Stoeck 2007). We put it in Bigyra incertae sedis until a sequence is available. It is different enough from all existing families to merit a new one: **Pseudobodonidae** Cavalier-Smith. **Diagnosis**: laterally biciliate non-photosynthetic, non-amoeboid rounded (non-D shaped) heterokonts that ingest food anywhere on body surface and lack a collar around its ciliary bases; often feeds while attached by ciliary tip (without mucus strand, unlike *Labromonas*); anterior cilium much longer than posterior one or cell body. Type genus *Pseudobodo* Griessmann (1913): includes both *P. tremulans* the type species and *P. minuta* Ruinen, 1938. **Comment**: *P. minuta* was wrongly transferred to *Cafeteria*, which it does not resemble in shape, on the mistaken view that a 'collar' is 'characteristic and diagnostic of *Pseudobodo*': Larsen and Patterson 1990). The idea of a 'collar' around both ciliary bases in *Pseudobodo tremulans*, the type species, appears to have been introduced in a drawing (Larsen and Patterson 1990, Fig. 49e) and much reproduced later. However, no such collar is evident in the only light micrograph they included of '*P. tremulans*' (Fig. 48b); instead there is a large triangular projection well away from the ciliary insertion point, which seems to be the feeding pouch of a *Labromonas*; we conclude that the idea of a periciliary collar is a misinterpretation of the right lateral feeding pouch of *Labromonas* and the term 'collar' should be avoided for either *Pseudobodo* or *Labromonas*.

Revisions to Ochrophyta: (1) Aurophyceae Cavalier-Smith cl. n. **Diagnosis**: coccoid unicells or capsoid, filamentous or pseudofilamentous golden-brown algae with biciliate zoospores; laminate cell walls; multicells divide by eleutheroschisis (unlike Melanophycidae) and unicells only in naked flagellate phase; dense cortical physodes (unlike Xanthophyceae); chloroplasts with fucoxanthin, diadinoxanthin and diatoxanthin; two centrioles orthogonal to strongly divergent, with dense central plug in distal lumen; posterior right centriolar root R2 of 2–4 mts exceptionally short (Kai et al. 2008 used the older R3 label); cilia with 4–6 gyred transition helix distal to distal transition zone plate (unlike Melanophycidae). **Etym**: *aurum* L. gold refers to their golden brown colour. **Comment**: Phaeophyceae was recently expanded to include *Schizocladia* (here placed in **Subclass Schizocladiophycidae** Cavalier-Smith subcl. n. Diagnosis as for order Schizocladiales (Kawai et al. 2003, p. 224)) and Phaeothamniales (Cavalier-Smith and Chao 2006). Our ML analysis strongly supports including *Schizocladia* in Phaeophyceae, but confirms that *Phaeothamnion* and *Stichogloea* (here treated together with *Phaeoschizochlamys* as Phaeothamniales sensu stricto) are much more closely related to the unicellular brownish alga *Aurearena* than to multicellular Phaeophyceae, as Kai et al. (2008) first showed. We therefore establish this new class for the robust clade (BS 81%) comprising Aurearenales (Kai et al. 2008) and Phaeothamniales sensu stricto (Fig. 15). Several differences between *Aurearena* and Phaeothamniales are simply differential losses of ancestral characters for Fucistia (e.g. loss of chlorophyll c and heteroxanthin and the girdle lamella by *Aurearena* (independently of Eustigmatophyceae; it was also lost by Chromerida within Alveolata) and loss of the rhizoplast and violaxanthin, antheraxanthin and zeaxanthin by Phaeothamniales; there are no innovations in either sufficiently major to merit separate class status. Expansion of Phaeophyceae to include *Schizocladia* and also the unicellular '*Botrydiopsis*' *pyrenoidosa* (Fig. 15) and grouping the unicellular flagellate *Aurearena* and Phaeothamniales as Aurophyceae exemplify the classic principle that algal classes often embrace the whole gamut of growth forms from the ancestral flagellate unicell to complex filaments or multicells (Fritsch 1948, p. 1), and thus complete the integration of algal and flagellate classification initiated by Klebs (1893).

(2) Sulcophycidae Cavalier-Smith subcl. n. **Diagnosis**: naked, unicellular, biciliate heterokont algae with TR of two indistinct stacked dense rings below the ciliary transition zone dense plate; anterior cilium with two rows of tripartite mastigonemes without side hairs; posterior cilium lies in a ventral groove running the length of the cell (unlike all other Ochrophyta); no ciliary eyespot; 2–7 chloroplasts with fucoxanthin, girdle lamella, and projecting pyrenoids with no traversing thylakoids (unlike Raphidophycidae). Cortical alveoli, axopodia, ciliary paraxial rod, and extrusomes absent. **Etymol**: *sulcus* L. groove (non-typified name). Includes **Olisthodiscales** Cavalier-Smith ord. n. and **Olisthodiscaceae** Cavalier-Smith fam. n. **Diagnosis**: cells highly flattened; 6–13 peripheral plastids; major longitudinal microtubule bands not on the rim of the groove as in *Sulcochrysis* but on the flanks of the cell. Type genus *Olisthodiscus* Carter, 1937. **Comment**: placed in class Hypogyristea because our trees weakly suggest that it is related to them and more strongly indicate that it probably does not belong in Raphidomonadea where formerly placed. Possibly

Sulcochrysis (Honda et al. 1995) belongs in this subclass not Chrysophyceae.

Discussion

Nanomonadea: a novel heterokont phenotype

Incisomonas is the first phylogenetically well-characterised heterotrophic heterokont to lack an anterior cilium and glide on its posterior cilium. *Incisomonas* is the third kind of gliding heterokont zooflagellate discovered in recent years. Placidida (i.e. *Placidia* and *Wobblia*; and possibly *Pendulomonas*: Moriya et al. 2000, 2002; Tong 1997) are the most ancient more deeply branching group of gliding phagotrophic heterokonts, but all retain the ancestral anterior cilium complete with tripartite hairs, unlike *Incisomonas*. The bicosid *Caecitellus* is also a biciliate glider but its anterior cilium has lost hairs and beats laterally (O'Kelly and Nerad 1998), unlike most heterokont anterior cilia that undulate from base to tip. As *Incisomonas* is closely related to *Solenicola*, which also has a single hairless cilium that beats asymmetrically while the cell is sedentary, it is highly probable that the anterior tinsel cilium was lost in a common ancestor of *Incisomonas* and *Solenicola*, not independently. We suggest that it was lost in association with the evolution of a sedentary habit and use of ciliary currents generated by the asymmetrically beating smooth posterior cilium to bring bacteria to the cell for ingestion. Uniciliatida is only the second group of heterokonts known to have lost the anterior tinsel cilium that is the ancestral synapomorphy for the infrakingdom Heterokonta. Its loss was clearly independent of that of the marine sand-dwelling pinguiophyte algae *Glossomastix* (O'Kelly 2002) and *Polypodochrysis* (Kawachi et al. 2002) that belong in Ochrophyta (Fig. 15). Interestingly both these genera (probably independently) gave up swimming (the ancestral condition exemplified by *Phaeomonas* the only known biciliate pinguiophyte: Honda and Inouye (2002); other genera are non-ciliate coccoids) and are vegetatively sessile, in mucilage or lorica respectively, and their uniciliate zoospores exhibit gliding motility and an unusual anterior pseudopodium/axopodium. A fourth gliding heterotrophic genus, *Commation*, was believed to have tripartite hairs on its single anterior cilium and thus considered a heterokont (Thomsen and Larsen 1993); despite earlier scepticism of that interpretation (Cavalier-Smith 1997b), the presence of a single axopodium/proboscis, extrusomes and a rhizoplast suggests that *Commation* are colourless raphidomonads; they glide by means of their proboscis like uniciliate pinguiophytes, not by their cilium, so we consider them not closely related to *Incisomonas*.

It is necessary to cultivate more-deeply branching nanomonads in order to determine whether some retain the ancestral anterior cilium or whether the uniciliate condition extends to the base of the clade (if it does, Uniciliatida should be extended to embrace them all). The anterior cilium was clearly lost after nanomonads diverged from their sister groups, as Opalinata were undoubtedly ancestrally biciliate (Cavalier-Smith and Chao 2006; though some later multiplied cilia) and at least some MAST-12 flagellates (Opalomonadea) have two cilia (Kolodziej and Stoeck 2007). Culturing early-branching nanomonads will also show if they were ancestrally gliders and *Solenicola* lost gliding or whether gliding evolved in *Incisomonas* only after it diverged from *Solenicola*. As our trees suggest that Placidida are sisters to the clade comprising Nanomonadea and Opalomonadea plus Opalinata, it is possible that gliding evolved prior to their last common ancestor and was lost by Opalinata when they became gut symbionts and independently by *Solenicola* when it became an epiphyte. As *Caecitellus* nests relatively shallowly within the predominantly non-gliding Bikosea (which seem to have less reason for losing gliding than gut parasites or epiphytes) it perhaps evolved gliding independently of Placidida and *Incisomonas*.

Nanomonadea are clearly abundant in most marine habitats. The only marine environments where MAST-3 sequences were not found were shallow anoxic sediments and hydrothermal vents, suggesting that most are aerobic zooflagellates. *Incisomonas marina* came from an intertidal sandy shoreline in Nova Scotia in the spring; its virtual sequence identity with one from Norway shows that the species occurs right across the North Atlantic.

Algal origins of Actinophryida

The raphidophyte placement of *Actinosphaerium* on our ML tree could not have been seen on the original ML 18S rDNA tree of Nikolaev et al. (2004) who sequenced it, because they included only two ochrophytes not 128 as we did (and no Raphidophycidae); nonetheless following Davidson (1982) and Mikrjukov and Patterson (2001) they assumed it to be related to pedinellids even though it did not group specifically with *Ciliophrys* the sole pedinellid on their tree. Our trees provide convincing evidence that *Actinosphaerium* is related neither to pedinellids nor to Opalinata (Cavalier-Smith and Chao 2006) but belongs in the ochrophyte subphylum Phaeista, most likely within Raphidomonadea. Raphidophycidae, *Actinophrys* and *Actinosphaerium*, and *Heliorapha* all share several cellular characters that are unusual or unique in Heterokonta, thus supporting their inclusion in Raphidomonadea. Unlike any other heterokonts, all three have unusually large nuclei with strongly developed peripheral chromatin; their mitosis is unique in heterokonts – in both *Actinophrys* and raphidophytes spindle microtubules enter through tiny pores in an almost intact nuclear envelope which persists though telophase when it divides by furrowing and two new daughter envelopes are assembled on the chromatin surface within the old envelope without any direct continuity with it (Heywood 1978b; Mignot 1984). This form of mitosis is otherwise extremely rare, occurring

in one excavate and in a ciliate macronucleus (Heywood 1978a). Secondly, Raphidomonadea as a whole, unlike other members of the ochrophyte subphylum Phaeista apart from some Pinguiophyceae (sparse cortical alveoli are widespread in Hypogyristea), have strongly developed cortical alveoli, which could be related to the cortical silica deposition vesicles of diatoms and cortical alveoli of alveolates, Telonemea, Haptophyta, and Glaucophyta. Thirdly, most Raphidomonadea have well developed amorphous cortical extrusomes that differ from the ellipsoidal extrusomes of pedinellids, the kinetocysts of Placidida (Moriya et al. 2000, 2002) and *Boroka* (the deepest branching aerobic bikosean), or the concentric extrusomes of Pseudodendromonadidae, the only other heterokont groups with extrusomes; those of Actinophryida are quite similar morphologically to the mucocysts of most Raphidophycidae and trichocysts of the raphidophyte *Fibrocapsa* (Hara and Chihara 1985; Yamaguchi et al. 2008, 2010); the presence of extrusomes would have preadapted them for evolving predation by axopodia. Therefore a position of Actinophryida within Raphidomonadea is evolutionarily much more plausible than one within pedinellids, still less as sisters of the anaerobic gut parasitic Opalinata. *Heliorapha* provides a no-longer-missing evolutionary link between Raphidophycidae and *Actinophrys*, making it clear that axopodia evolved and both photosynthesis and the posterior cilium were lost prior to loss of the anterior cilium. The vegetatively walled Eustigmatophyceae that lack extrusomes, cortical alveoli or large nuclei are implausible candidates for ancestors or close relatives of Actinophryida so we discount as an aberration the single ML tree showing this.

The microtubular centriolar roots of Raphidophycidae differ sharply from those of all other Ochrophyta; instead of four roots as is usual in Chrysista, there is only one anterior root and one posterior root (the axostyle) that in *Haramonas* at least is so profoundly modified as to be preadapted for evolving into an actinophryid axopodial axoneme. In families Vacuolariaceae and Haramonadaceae the axostyle consists of one or two concave microtubule bands associated with dense fibrous material that run backwards in a groove in the nucleus to the cell posterior; in contrast to all other heterokonts, the rhizostyle runs forwards between the centrioles to end at the cell surface in the ciliary groove well anterior to the centrioles. The axostyle is probably radically modified from the standard heterokont R2, as explained below, making it the only heterokont root that is a feasible direct ancestor of the actinophryid axopodial axoneme. Its attachment both to the nucleus and to the cell surface uniquely resembles that of actinophryid axonemes. We suggest that the raphidophyte axostyle evolved directly into a single actinophryid axopodial axoneme by being polymerised at its distal end and pushing the cell surface outwards to form a broad extension analogous to the projecting 'pseudopodia' supported by just one or four microtubules in *Glossomastix* and *Polypodochrysis* (Pinguiophyceae). As in these two algae, where the pseudopodium is axopodium-like in containing microtubules, we suggest that a single anterior-pointing axopodium evolved prior to the loss of chloroplasts and that the posterior cilium was lost at this stage. These two uniciliate, unipseudopodial pinguiophyte algae exemplify how a similar uniciliate derivative of a raphidophyte alga could have evolved a single axopodium. However as raphidophytes had extrusomes, unlike pinguiophytes, they could be moved up the axopodial axoneme to help trap prey. As soon as such prey trapping and ingestion became efficient photosynthesis was probably lost.

Even the precise actinophryid axopodial pattern of two offset spiral rows of closely apposed microtubules can be readily derived from the known structure of raphidophyte axostyles. We suggest that the intermediate alga that evolved an axopodium had an axostyle quite similar to that of *Haramonas pauciplastida* with two laterally attached concave microtubule bands of closely attached microtubules (Yamaguchi et al. 2008, Fig. 18). We suggest that the longer connectors between the two spiral rows of microtubules in actinophryid axonemes evolved from the lattice that clothes the groove-facing surface of the adnuclear band of the *Haramonas* axostyle. If the intervening dense trough-like lamina were lost, the distal ends of the lattice elements could attach instead directly to the second band of microtubules (mt). If in this putative intermediate alga the second mt band of the axostyles also bore a lateral lattice this would in a single step generate the core structure of two adjacent curved rows of microtubules that forms the nucleus of the double spiral structure; addition of extra microtubules to the sides of each and slightly accentuating the cross-sectional curvature of the two offset mt bands would generate a structure essentially identical to that shown in Fig. 13 of Jones and Tucker (1981) which constitutes the morphogenetic core of actinophryid axopodia. The close spacing of the mts within each row is essentially the same as in the axostyle bands (nearer the slightly closer spacing of the outer band of *Haramonas* than that of the inner band), whereas the markedly greater spacing of the two mt bands in axopodia is essentially identical to the thickness of the axostyle lattice. Thus the core features of the actinophryid axonemes are prefigured in the detailed structure of the *Haramonas* axostyle. Unfortunately, ciliary root structure is poorly known in *Fibrocapsa*, the raphidophyte that is consistently sister to *Actinosphaerium* on our trees. As the only microtubules reported in *Fibrocapsa* are singlet mts (Hara and Chihara 1985) it is unclear whether or not it has an axostyle or anterior root; however, such structures might have been overlooked.

A more detailed study of ciliary roots in *Fibrocapsa* and the even deeper branching but not yet formally described '*Chloromorum*' is important for better understanding early raphidomonad evolution and testing our deductions concerning the origin of actinophryids and the striking divergence in raphidomonad root structure from other Chrysista (see below). It is desirable to establish the polarity of the rhizostyle microtubules. In *Gonyostomum* the R2 component of the rhizostyle appears to start beside the posterior centriole and to have a normal loukozoan-style I fibre (see discussion of loukozoan roots in Cavalier-Smith 2012) with obviously

double lamina at its base (Mignot 1967, Fig. 6), and it is possible that the second mt band present in the *Haramonas* rhizostyle is absent in *Gonyostomum*, so rhizostyle structure may vary markedly across Raphidophycidae – if it does it would accentuate the differences among the three raphidophycid families recognised here. It seems that the adnuclear component of the *Haramonas* rhizostyle may also be a relatively normal R2 starting at that point (Horiguchi and Hoppenrath 2003, Fig. 14). It is unclear whether the second rhizostylar mt band that appears to be continuous well forward of this point in *Haramonas* (Horiguchi and Hoppenrath 2003, Fig. 14; Yamaguchi et al. 2008, Figs 15, 16) is nucleated at the nucleus (and thus antiparallel to R2), which would predispose it for evolving into an axopodial axoneme component) or at the cell surface in the groove, which would make it necessary to relocate the nucleating centre for it to become part of the axoneme (similarly necessary if the axoneme nucleating centre evolved from that of R2).

We suggest that *Actinosphaerium* and *Actinophrys* diverged independently from the putative uniciliate and uniaxopodial intermediate. As soon as efficient food capture evolved there would be strong selection for multiplying axopodia that could be done in two contrasting ways represented by the two suborders we established above. Perhaps mechanistically simplest would have been simply increasing the number of nuclei and attached axopodia to generate a multinucleate cell with one axopodium per nucleus (retaining the ancestral state of one axostyle per nucleus) as in *Camptonema* and *Echinosphaerium* – lumping these genera into *Actinosphaerium* (Mikrjukov and Patterson 2001) was unwise in our view) (suborder Actinosphaerina). This lineage later relaxed the connection of axopodium to nucleus which appears to be mandatory in *Camptonema* so that some or most axopodia could be formed unattached to the nucleus as in *Echinosphaerium* and most or all nuclei lack attached axopodia as in *Actinosphaerium*. As soon as nuclei and axopodia multiplied, as in *Camptonema* that is intermediate in size and number of nuclei between *Heliorapha* and *Echinosphaerium*, retention of cilia would have been pointless and wasteful. An alternative way of multiplying axopodia was to modify their nucleating centre from its ancestral lateral attachment to the nucleus (retained by Actinosphaeriidae) to basal attachment of a form allowing the nucleation of many axopodia per nucleus as in *Actinophrys* and *Heliorapha* (suborder Actinophryina). The *Actinosphaerium* lineage probably made its nuclei smaller than in the ancestral raphidophyte whereas the *Actinophrys*/*Heliorapha* lineage made them larger, allowing an increase in cell volume without multiplying nuclei. As *Heliorapha* is relatively small (body 15 μm) retention of the cilium was advantageous, as the water current it generates would help pull prey onto its axopodia. But *Actinophrys* lost the cilium and grew much larger (12–114 μm depending on species plus up to another 200 μm in each direction for the axopodia). The multiple nuclei of Actinosphaeriidae allowed even larger cell sizes (up to 400 μm cell body or rarely more than double that).

Camptonema is a clear but previously unrecognised evolutionary intermediate between raphidophytes and *Echinosphaerium* because all its roughly 20 (not 10 as Mikrjukov and Patterson 2001 stated) oval nuclei have a single axoneme and all axonemes are attached to a single nucleus and are subtended at their base by a distinctive dense cone that is indistinguishable in the light microscope (Schaudinn 1894) from the dense fibrillar cone that attaches the rhizostyle to the nucleus in all raphidophytes (Mignot 1967, 1976; Yamaguchi et al. 2008) including *Fibrocapsa* (Hara and Chihara 1985, Fig. 10) where it is less strongly developed than in others, but in no other ciliated heterokonts. Uniform retention of this dense ancestral raphidophyte cone by *Camptonema* shows that in Actinosphaerina the cilium was lost before this cone. Conversely its absence in *Heliorapha* shows that in Actinosphaerina the cone was lost before the cilium. This means that there was never a direct evolutionary connection between the multinucleate actinosphaeriids and uninucleate *Actinophrys*; they lost cilia independently after the axoneme evolved. Mikrjukov and Patterson (2001) mention cone-like aggregations of material around the base of axopodia of *Actinosphaerium* as a reason for synonymising *Camptonema nutans* with *Actinosphaerium eichhornii* but their sole electron micrograph of *A.* '*eichhornii*' (Fig. 20) showed no evidence of such a cone or even of attachment to the nucleus and they cited no references in support of dense cones. That micrograph might not even be of *A. eichhorni* but could have been another undescribed *Actinosphaerium* species, the only reason given for the identification being the central nucleolus, which is so widespread in protists that it is not even a genus-specific character, still less species-specific, and the juxtaposition of nucleus and axonemal base, which shows no evidence of actual attachment, may be pure coincidence. The freshwater *Actinosphaerium eichhornii* is 200–300 μm with up to hundreds of spherical nuclei (13–17 μm) that are generally unattached to axonemes; it differs so greatly from the marine *Camptonema* (only 120–180 μm, with only 20 or so oval nuclei always attached to axonemes by a dense cone) that they cannot be the same species; synonymising them (Mikrjukov and Patterson 2001) was wrong. Though Ritter von Stein (1857) when establishing the genus *Actinosphaerium* and combination *A. eichhornii* did not discuss axonemal attachment, subsequent authors for over a century prior to Mikrjukov and Patterson (2001) all regarded the axonemes of *A. eichhornii* as terminating basally independently of nuclei, confirmed by the first ultrastructure with reasonable preservation (Hovasse 1965). Penard (1904) indicated that the nuclei form a monolayer internal to the endoplasm ectoplasm boundary and axonemes terminate somewhat outside that boundary; Bütschli (1882) drew some overlap between the bases of the axonemes and nuclear layer but showed them as structurally independent, both contrary to the schematic diagram of Mikrjukov and Patterson (2001, Fig. 1b).

The ultrastructural study of Anderson and Beams (1960) on *Echinosphaerium* (=*Actinosphaerium*) *nucleofilum* was

on two different strains: *A. nucleofilum* from Barrett its describer, and primarily one originally called *Actinosphaerium eichhornii* from Carolina Biological Supply, but the authors do not indicate which micrographs were from each or give evidence that both were definitely *nucleofilum*, as they assumed. Though we cannot exclude the possibility that their data refer to a mixture of the two genera, their finding that axonemes terminated either freely or in close association with nuclei agrees with Barrett (1958) for *A. nucleofilum* and their schematic drawing is consistent with his. Neither their diagram nor the micrographs of Tilney and Porter (1965) of Carolina *E. nucleofilum* axopodia originating directly from nuclei show a dense cone, unlike in *Camptonema* – instead axopodial bases are lodged in a depression in the nuclear envelope and cemented to it and each other only by very small amounts of medium density cement (similar sparse cement was at the base of a nucleus-free axopodium). The dense nucleation centres of another *E. 'nucleofilum'* strain were also not conical but independent of nuclei, though within 1.5 μm of them (Jones and Tucker 1981) so perhaps it was misidentified or an undescribed species: their Fig. 1 resembles *A. eichhornii* as depicted by Barrett (1958) more than it does his *nucleofilum*.

In our view most of the varieties described by Penard (1904) under the name *A. eichhornii* are likely to be separate species and there has probably been excessive lumping of actinophryids as in most other protist genera and species. Many more actinophryid strains need to be isolated and studied simultaneously by sequencing and light and electron microscopy, so that species and generic distinctions can be better defined; we predict that this will reveal far more genuine species than recognised by Mikrjukov and Patterson (2001) – it was premature to lump the two new '*Echinosphaerium*' species described by Shigenaka et al. (1980) in *Actinosphaerium* and to suppress *A. arachnoideum* (Penard 1904). If *Commation* really belongs in Raphopoda (a sequence to test this is needed) its single proboscis could be an homologous relic of the hypothetical intermediate; if so, photosynthesis was lost before axopodia multiplied. Lumping *E. ikachiensis* with *E.* (=*A.*) *nucleofilum* was especially unjustified as its nucleolar structure was more like that of *A. eichhornii* than *nucleofilum* and it did not have dense masses attached to the inside of the nuclear envelope as does *E. nucleofilum* (contrary to Mikrjukov and Patterson 2001); its larger nuclei (mean diameter 2×) imply an eight times higher genome size than for *nucleofilum* (Cavalier-Smith 2005) or a high degree of polyploidy, so it is most unlikely to be the same species. To a lesser degree the contradictory synonymisation with *A. eichornii* (Siemensma 1991), with much larger nuclei than *E. nucleofilum* but only slightly larger nuclei than *ikachiensis*, is also flawed, though we agree that *ikachiensis* is an *Actinosphaerium*, not *Echinosphaerium*. Synonymisation of *E. akamae* with *A. eichornii* (Mikrjukov and Patterson 2001; Siemensma 1991) is arguable, but its averagely smaller nuclei than for *eichornii* and other differences favour retention as a separate species. As Shigenaka et al. (1980) misconstrued as total name replacement Hovasse's (1965) separation of *Echinosphaerium nucleofilum* from *Actinosphaerium*, which we consider valid, and mistakenly abandoned the name *Actinosphaerium*, we transfer their species to *Actinosphaerium* as two new combinations: *Actinosphaerium ikachiensis* Cavalier-Smith (Basionym *Echinosphaerium ikachiensis* Shigenaka et al. (1980, p. 117) and *Actinosphaerium akamae* Cavalier-Smith (Basionym *Echinosphaerium akamae* Shigenaka et al., 1980, p. 117).

The highly divergent nature of actinophryid rDNA means that this order will be systematically underrepresented in environmental rDNA surveys; a sequence needs to be obtained from *Actinophrys* or *Heliorapha* to test our argument that they are deeply divergent from Actinosphaeriidae and to enable design of an actinophryid-specific primer so their genetic diversity in nature can be rapidly surveyed.

As Actinophryida nest well within Raphidophycidae on all but one ML tree they ought to retain leucoplasts for the synthesis of lipids like many other chromists, e.g. the colourless heterokont pedinellids (Actinochrysia) and chrysomonad *Paraphysomonas*, the cryptomonad *Cryptomonas paramecium*, and the alveolates *Perkinsus* and most Sporozoa such as malaria parasites, coccidians, and piroplasms (Ferguson et al. 2007; Robledo et al. 2011; Roos et al. 2002; Sekiguchi et al. 2002). Leucoplasts would easily have escaped detection in electron micrographs of their highly vacuolate cytoplasm and even now would be hard to find, as the cells are so large and only few might be present; PCR studies of plastid rRNA genes should detect them more easily. Likewise the two non-photosynthetic orders of Picophagea (Picophagales and Leukarachnida) are likely to have leucoplasts that should be similarly sought likewise in *Commation*.

Unity of Opalozoa and Placidozoa

Clearly, the probably predominantly free-living zooflagellate Opalomonadea (MAST-12) are the real sisters of the anaerobic endosymbiotic Opalinata, not Actinophryidae. Removal of Actinophryida from Opalozoa substantially increases its phenotypic homogeneity; with the addition of Bikosea (Table 1), Opalozoa now includes all zooflagellates of the phylum Bigyra except the uncharacterised Eogyrea. With the sole exception of the parasite *Blastocystis* that lost cilia, infraphylum Opalozoa now consists entirely of heterotrophic flagellates, which seem to have diverged early on into two clades: Placidozoa and Bikosia, with somewhat distinct cytology. The consistent grouping of Placididea with Opalinata, Opalomonadea, and Nanomonadea as the clade Placidozoa is concordant with the presence of a double ciliary transition helix in all Opalinea and Placidida. Thus Placididea are currently the best guide as to the phenotype of the free-living zooflagellates from which the gut-symbiotic Opalinata evolved. When opalomonads are cultivated they will become even more relevant to this question.

Evolutionary significance of Opalomonadea

A majority of opalomonad sequences came from anaerobic habitats (anoxic marine sediments), raising the possibility that many are anaerobes with unusual respiration-deficient mitochondria. Although some are from the intertidal zone, a few are from the open sea (OL151105) and one is from fresh water (KKTS_D3: Kolodziej and Stoeck 2007), it remains possible that many more might be from anaerobic microenvironments. We placed Opalomonadea in the basal superclass Wobblata (Table 1) on the conservative assumption that at least some are aerobic zooflagellates like Placididea, Nanomonadea and Bikosea. But if perchance all Opalomonadea turn out to be anaerobes or microaerophilic, their ancestors would have been preadapted for evolving into gut parasites, and they should be transferred to Opalinata instead. At least some are biciliates with hairy anterior cilium (Kolodziej and Stoeck 2007), which supports the view that tubular hairs were transferred from cilium to cell body of an ancestor of the opalinate flagellate *Proteromonas* after, not before it became a gut symbiont (Cavalier-Smith 1998). Cultivating opalomonads would enable molecular and physiological studies that would throw much light on how the symbiotic Opalinata evolved from free-living ancestors. It will be especially interesting to know whether opalomonads and *Incisomonas* have a cytopharynx; we expect them not to, for if our tree topology is correct, the most parsimonious inference is that a cytopharynx evolved in the last common ancestor of Bikosea but was never in Placidozoa.

Conclusion

We have shown that opalozoan flagellates are phenotypically more diverse than previously supposed and established two new bigyran classes and many new lower taxa. We also clarified diversification of Ochrophyta by showing *Actinosphaerium* to belong in Raphidomonadea, thereby making Opalozoa more uniform (now all Opalozoa except *Blastocystis* are heterotrophic flagellates), and improved higher classification of both groups. Our analyses show that MAST clades represent many fewer really major novel lineages than is sometimes implied. The phylogenetic positions of MAST-3 (Nanomonadea) and -12 (Opalomonadea) are now firm within Placidozoa; but we need to determine the phenotype of opalomonads and deeper-branching nanomonads. Characterising Eogyrea is the most essential task for understanding the earliest evolutionary divergences within Bigyra, especially the origin of Labyrinthulea from zooflagellate ancestors, whilst MAST-1 and -2 are crucial for understanding evolution at the base of the Pseudofungi/Ochrophyta clade (Gyrista).

Acknowledgements

JMS thanks BBSRC for a studentship, Alastair Simpson for hospitality in his Halifax laboratory and taking her to the sampling site, as well as using his NSERC grant for supporting her visit and the Canadian Institute for Advanced Research (CIFAR) Integrated Microbial Biodiversity Program and Green Templeton College, Oxford for meeting travel costs, and Mike Shaw for help with electron microscopy. TCS thanks NERC for grant support and D. Moreira for the Gómez et al. alignment. We thank a referee for telling us of two chimaeric MAST sequences.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejop.2012.09.002.

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Protist, Vol. 162, 332–372, April 2011
http://www.elsevier.de/protis
Published online date 4 February 2011

Protist

ORIGINAL PAPER

Novel Cultured Protists Identify Deep-branching Environmental DNA Clades of Cercozoa: New Genera *Tremula*, *Micrometopion*, *Minimassisteria*, *Nudifila*, *Peregrinia*

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Submitted April 25, 2010; Accepted September 6, 2010
Monitoring Editor: Michael Melkonian

We describe three new orders of filosan Cercozoa, five new deep-branching genera, eight new species of *Thaumatomonas*, *Reckertia*, *Spongomonas*, *Rhogostoma*, *Agitata*, *Neoheteromita* and *Paracercomonas*, sequence their 18S rDNA, and construct 18S rDNA trees for 148 Cercozoa. Our phylogeny indicates that Filosa were ancestrally gliding flagellates; non-flagellate filose amoebae evolved from them five times independently. The new genera are more closely related to environmental DNA sequences than cultured organisms. *Tremula longifila*, a zooflagellate glider on both flagella (unlike other Cercozoa), is the most divergent filosan (Tremulida ord. n.). *Micrometopion nutans* is a eukaryote-eating gliding zooflagellate like *Metopion* and *Metromonas*. *Minimassisteria diva* is a widespread trimorphic marine amoeboflagellate granofilosan. *Peregrinia clavideferens*, a non-testate, scale-bearing, filose amoeba, branches deeply in Thaumatomonadida, which are probably sisters to Spongomonadida. *Nudifila producta* is a filose amoeboflagellate related to *Clautriavia* and Marimonadida (ord. n., e.g. *Pseudopirsonia, Auranticordis*). We substantially revise Imbricatea, now including Spongomonadida, and Thecofilosea to include Phaeodaria. Thecofilosea and Imbricatea and Thecofilosea are sisters, both arguably ancestrally rigid gliding flagellates with ventral pseudopod-emitting grooves. Scale-free *Ovulinata parva* is sister to *Paulinella*, so imbricate silica scales can be lost. Internal hollow silica skeletons evolved twice in Thecofilosea (Ebriida, Phaeodaria) or were multiply lost. *Protaspa* replaces preoccupied '*Protaspis*'.
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Key words: 18S rDNA phylogeny; *Peregrinia*; *Protaspa*; *Rhogostoma*; *Tremula*; Ventricleftida.

Introduction

Cercozoa is a diverse, increasingly well-characterised phylum of protists (Cavalier-Smith 1998a,b) founded mainly on the basis of 18S rDNA

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doi:10.1016/j.protis.2010.10.002

phylogenies that revealed a phylogenetic relationship between a variety of gliding zooflagellates and filose amoebae and the plasmodiophorid plant parasites (Cavalier-Smith 1996/7; Cavalier-Smith and Chao 1997). Its unity was later confirmed by actin trees (Keeling 2001), molecular synapomorphies (Archibald and Keeling 2004; Archibald et al. 2003; Bass et al. 2005; Cavalier-Smith 2002; Cavalier-Smith and Chao 2003a), morphological synapomorphies (Cavalier-Smith et al. 2008a, 2009), and combined 28S and 18S rDNA phylogenetic analyses (Moreira et al. 2007). Together with Amoebozoa, Cercozoa have by far the greatest biomass of any protist phyla in soil (Urich et al. 2008) and are also abundant in every marine habitat, and so must be quantitatively significant players in the carbon and mineral cycles by preying on bacteria and other protists. Cercozoa probably diversified from an early phagotrophic, rhizopodial biflagellate (Cavalier-Smith and Chao 2003b) to contain a vast array of phenotypes (Bass et al. 2009a,b; Cavalier-Smith 1998a,b), all but overwriting and obscuring ancestral morphological characters.

This diversity of cercozoan body forms includes filose testate amoebae (both scale-bearers like *Euglypha* and non-silicified ones like *Pseudodifflugia*), amoeboflagellate algae (e.g. *Chlorarachnion*), zooflagellates with pseudopodia and siliceous scales (e.g. *Allas*), diverse parasites (e.g. Ascetosporea, the syncytial *Plasmodiophora, Pseudopirsonia*), naked reticulose amoebae (e.g. *Filoreta*), testate reticulose amoebae (*Gromia*), colonial flagellates (e.g. some *Spongomonas*), planktonic swimming flagellates (e.g. *Ebria*), numerous small gliding flagellates (e.g. *Sandona*, *Bodomorpha*, *Allapsa*, *Cavernomonas*), helioflagellates (e.g. *Aurigamonas*), soft-bodied amoeboflagellates (e.g. *Agitata, Cercomonas, Eocercomonas* and *Paracercomonas*), and thecate or scaly amoeboflagellates that emit ventral pseudopods from a marked groove or cleft (e.g. Thaumatomonadida, '*Protaspis*' (here renamed *Protaspa*), *Ventrifissura*), and even protists with axopodia (desmothoracids and phaeodarians). Recent studies show that many hitherto completely unknown organisms as well as those of previously uncertain affinity are actually Cercozoa (e.g. Bass et al. 2009a,b; Burki et al. 2002; Chantangsi et al. 2008; Chantangsi and Leander 2010a,b; Hoppenrath and Leander 2006a,b; Kühn et al. 2000; Longet et al. 2004; Vickerman et al. 2005). Despite this great expansion of the group, phylum-specific environmental gene libraries reveal that many Cercozoa remain to be described (Bass and Cavalier-Smith 2004; Bass et al. 2009a,b; Howe et al. 2009). Targeted surveys within major cercozoan groups have shown some genera, notably the bacterivorous *Cercomonas* (Bass et al. 2009b) and *Sandona* (Howe et al. 2009), to be astoundingly speciose and genetically diverse. Others, such as predators on other eukaryotes like *Aurigamonas* and *Metopion* and the divergent flagellates *Sainouron* (Cavalier-Smith et al. 2008a) and *Helkesimastix* (Cavalier-Smith et al. 2009), are relatively rarely detected in either environmental or culture surveys, although they may be more common than current indications suggest.

This paper uses light microscopy, electron microscopy, 18S rDNA sequencing and phylogenetics to describe species representing five new genera: the basal filosan *Tremula longifila*, and four divergent but later branching filosans: *Micrometopion nutans*; *Minimassisteria diva*; *Nudifila producta*; and *Peregrinia clavideferens*. We also describe eight new species in existing genera: 1) a new solitary spongomonad, *Spongomonas solitaria;* 2) a novel group B2 paracercomonad (Bass et al. 2009b), *Paracercomonas baikali*; 3) a new gliding pansomonad, *Agitata vibrans* (and make the new combination *Agitata agilis*); 4) the first clearly marine glissomonad, *Neoheteromita tolerans*; 5) three thaumatomonads: *Reckertia filosa*, recognizing the genus *Reckertia* Conrad, 1920 as distinct from *Thaumatomastix; Thaumatomonas oxoniensis*; and *Ts vancouveri*; 6) a filose thecate amoeba, *Rhogostoma micra*. Where possible we use ITS2 rDNA sequences to barcode the new species at comparably high resolution and describe scale ultrastructure for the four new thaumatomonads. We also identify a novel scale type in *Allas* that distinguishes it more clearly from *Thaumatomonas* and revise existing taxonomy where necessary using the new morphological and phylogenetic data and critical evaluation of the literature, establishing three new orders and three families and several new combinations for species previously incorrectly assigned to genera. Finally we discuss evolutionary and taxonomic implications of our trees, the most comprehensive so far for Cercozoa, which particularly illuminate the evolution of Thecofilosea and Imbricatea and the nature of the earliest filosan lineages.

Results

Phylogenetic Analyses

Figure 1 shows the phylogenetic positions of the new isolates described below. The most striking phylogenetic novelties are the deep position of the

gliding zooflagellate *Tremula*, the deepest branching filosan of known phenotype; the somewhat less deep branching of the new predatory gliding zooflagellate genus *Micrometopion*; a novel granofilosean *Minimassisteria* that is highly divergent from *Massisteria*; and the position of the non-gliding filose amoeboflagellate *Nudifila* as sister to novel clade (NC) 2 plus the gliding flagellate *Clautriavia*. The latter grouping defines a new clade of entirely marine flagellates or amoeboflagellates related to the filose testate Euglyphida (characterised by silica scales) and new order Marimonadida. The position of *Ovulinata* robustly within Euglyphida shows that its ancestor lost scales. Numerous new sequences substantially expand the known phenotypic diversity of Thaumatomonadida, all having silica scales of a different form from euglyphids, and Cryomonadida. We also ran several other trees with different taxon samples and many more environmental sequences that had to be excluded from this figure for clarity, which confirmed the broad picture shown. Supplementary Figures S1 and S2 are just two examples of these extra trees.

Figure S1 is similar to Figure 1 but is rooted on Endomyxa rather than Radiozoa, and omits several long-branch taxa and/or those of very uncertain branching position: e.g. Phaeodaria, Metromonadea, in response to a referee's concern that long branches like Metromonadea with weak support for the position of its constituent taxa might be distorting the overall tree. As Figure S1 shows, removal of Metromonadea, the environmental clade just below Thecofilosea, Phaeodaria, some *Paracercomonas* species, and the long branch radiozoan outgroup (and replacement of the longer-branch environmental sequence in NC2 by a much shorter one) strongly increases support for the holophyly of Monadofilosa and for Granofilosea and Chlorarachnea branching substantially more deeply, as well as for NC11/12 of unknown phenotype being the deepest branching filosan clade of all (see Bass et al. 2009a). As in Figure 1 there is maximal support for Tremulida being the deepest branching cultured Filosa. Both trees also show Imbricatea and Thecofilosea as holophyletic and as sisters, but with weak support, and place all our new non-metromonad sequences in precisely the same position relative to the closest previously known sequences (most within these two classes). Both also put Spongomonadida within Imbricatea in conformity with the revised classification proposed below and group Spongomonadida with Thaumatomonadida; however the Figure S1 Bayesian tree places Spongomonadida within Thaumatomonadida, making Thaumatomonadida paraphyletic, which cell evolution arguments (see Discussion) make less likely than a sister position – but if it were correct it would mean that spongomonads evolved from thaumatomonads by scale loss and flagellar reoreintation. The same applies to Cercomonadida and Granofilosea, which are paraphyletic in Figure S1 but holophyletic in Figure 1. None of these differences is statistically supported.

The tree in Figure S2 includes only Imbricatea, Thecofilosea, and Sarcomonadea; by omitting more divergent taxa, this restricted monadofilosan dataset allowed us to include more nucleotide positions in the analyses in an attempt to better resolve the topology of this part of the cercozoan tree. This increased support for the grouping of Nudifila and *Clautriavia* and for the holophyly of the major imbricate subgroup comprising Euglyphida, NC3, Marimonadida and NC2, and for NC3 being sister to Euglyphida. It also increased support for the holophyly of Glissomonadida and for their being sisters of Pansomonadida. Both supplementary figures weakly suggest that NC2 may be sister to Marimonadida plus Euglyphida and NC3, rather than to Marimonadida alone as in Figure 1. It also made *Rhogostoma* clearly holophyletic to the exclusion of the related soil sequence and swapped the position of *Reckertia* and the related marine sequence. Both supplementary trees moved environmental sequence AY620281 to become sister to the other environmental sequences within Protaspidae, suggesting that both belong to *Protaspa*. In neither supplementary tree did removal of the long-branch Phaeodaria affect the grouping of Ventricleftida as sister to Tectofilosida, Ebriida, and Cryomonadida or the relative position of these three orders. However, this tree placed Spongomonadida as sister to Thaumatomonadidae (both Bayesian and ML, with no support), rather than to *Peregrinia* (Fig. S1 Bayes) or to Thaumatomonadida as a whole (Fig. 1 both methods and Fig. S1 ML).

Microscopic Observations, Diagnoses of New Taxa, and Taxonomic Revisions

New species are described by differential interference contrast microscopy. We apply existing names only if the culture closely matches original descriptions, establishing new ones if one or more critical characters differ. Figures 2–6, 9, 12, and 14 show light micrographs of each strain extracted from digital videos of multiple live individuals. For brevity we combine light microscopic observations with new taxon diagnoses. Abbreviations used: cv = contractile vacuole; n = nucleus; AF = anterior flagellum; PF = posterior flagellum; X BL = times

body length. Strain and sequence details, and cell and flagellar dimensions (mean followed by range in brackets), precede qualitative descriptions in a standard order. Necessary nomenclatural or other comments, e.g. on phylogeny and for some strains electron microscopic observations, follow each diagnosis. Throughout this paper type cultures (available from public or author collections as specified), type 18S and ITS rDNA sequences, and type illustrations are to be regarded as part of the syntype for new species (fixed specimens would not preserve diagnostic characters); see Cavalier-Smith and Chao (2010) for the merits of this mode of typification for protists.

We start with a gliding soil flagellate that is the most deeply branching zooflagellate on the cercozoan 18S rDNA tree, and which has a phenotype previously unknown for Cercozoa, i.e. habitual gliding on surfaces by both flagella. Its deep phylogenetic divergence and behavioural uniqueness merit a new order and genus:

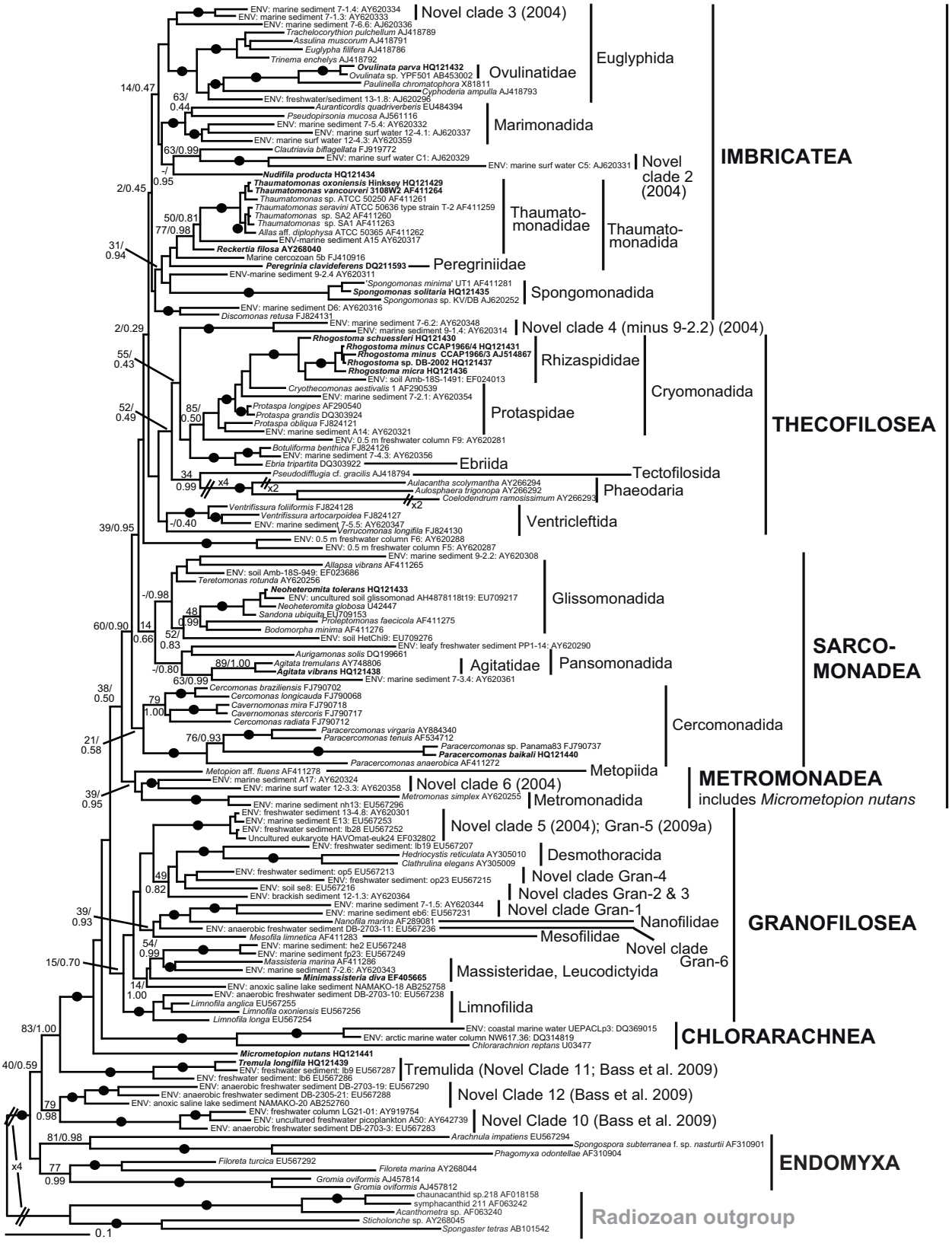
New Order Tremulida Cavalier-Smith and Howe. **Diagnosis:** Biflagellate heterotrophic and phagotrophic flagellates with long anterior and posterior flagella; glide on surfaces by means of both flagella, one pointing forwards and one backwards. Without light microscopically visible theca, scales or cytostome.

New Genus *Tremula* Howe and Cavalier-Smith. **Diagnosis:** Anterior flagellum inserts at anterior end of cell but, unlike in *Glissandra*, not in a groove or notch; posterior flagellum inserts on ventral side at a mid-cell depression or notch, where cell narrows, or apically. Often vibrates energetically during movement. Able to survive in dry soil. Sometimes with an amoeboid phase that retains both flagella. **Etym.** *tremulans* L. trembling. **Type species:** *Tremula longifila*.

Tremula longifila Howe and Cavalier-Smith sp. n. Fig. 2A. Type strain ATCC50530 (Year unknown; dry soil, Mexico City, Mexico; T. Nerad via J. Martinez-Cruz). **Diagnosis:** as for the genus, plus: 18S rDNA sequence, GenBank HQ121439; ITS2 rDNA GenBank HQ176332; cell size 13.5 μm (12-14.5 μm). AF and PF: 40-45 μm; heterodynamic, AF active, rapid waves flicker along its entire length. PF passive, trailing or anchoring cell to substratum. AF emerges at cell anterior, PF from a notch midway on ventral side (indicated by arrow). Cell rounded with tapered anterior and posterior ends or elongated and somewhat spindle-shaped; medium notch variably pronounced. Cell and flagella usually flush with substratum, cell vibrates during gliding, sending vibrations along PF; AF flickers independently. Cells occasionally rise above substratum, anchored by PF and oscillate slowly to very rapidly. Cells usually travel, though pause frequently to pivot, twirl or vibrate in one location. No pseudopodia. N usually central; cv in posterior half of cell, anterior cv sometimes visible. Cysts not observed (those observed by ATCC may be of the heterokont prey *Adriamonas* sp., which does make cysts), but it grew well on just Volvic® and grain, suggesting it can eat bacteria instead. **Etym.** *longus* L. long *filum* L. thread. Another strain (BCKV), isolated from dried freshwater stream bed sediment at Beaver Creek, Montezuma Castle Monument, Arizona, USA by KV, had identical 18S rDNA and ITS1 sequences.

Comments: ATCC50530 was originally misidentified as "*Cercomonas vibrans* Sandon". Sandon (1927) described *Cercobodo vibrans* as "broader in shape than *C. agilis*, sometimes quite spherical; length about 15 μm; movements vibratory, not spiral; amoeboid phase often with fine, sometimes branching, pseudopodia" and found it in 41 of 148 soils examined. He thought it similar in many respects to *Cercomonas*, but differed "in the freedom of the posterior flagellum and in the greater distinctness of the amoeboid and swimming stages". In the "flagellate condition short, finger-like pseudopodia are not infrequently found on all parts of the body, but most often at the posterior end. The amoeboid phase sometimes has a single broad pseudopodium, but at other times is surrounded by long, finger-like processes, often branched". Although ATCC50530 is similar in size and position of nucleus and contractile vacuole to Sandon's *C. vibrans*, one obvious difference is that *T. longifila* is never amoeboid. A second key difference is the position and lengths of the flagella: in *C. vibrans* both were inserted anteriorly, the anterior flagellum "a little longer than the body, and the . . . [posterior flagellum] usually twice the body-length"; but in ATCC50530 the posterior flagellum inserts midway down the cell; both are approximately three times body-length. These differences make *C. vibrans* a different species from *T. longifila*; we consider it another *Tremula* species with shorter flagella and more amoeboid behaviour. We base this on Sandon's description of its 'very characteristic' 'swimming' with **both** flagella being '*straight and almost rigid*, moving with a *vibratory motion* which throws the whole body into vibration' and 'progression is jerky but fairly rapid, and takes place *along the line of the two flagella*, the body of the organism being turned at a small angle to this line.' [our italics]. Given that there was probably no conception of flagellar gliding motion in Sandon's day, his use of the word 'swimming' may be misleading and should probably be discounted as his description of its motion appears rather similar to our observations of its otherwise unique gliding moltility.

However, if *T. filosa* and *C. vibrans* are congeneric, the name *Cercobodo* is inapplicable as it is probably a junior synonym for *Cercomonas* (Karpov et al. 2006) and ATCC50530 is phylogenetically entirely distinct from Cercomonadida, differing also in gliding on both flagella. *Tremula filosa* differs from all cercomonads but *Cavernomonas* (Bass et al. 2009b) in not obviously forming pseudopodia. The difference in posterior flagellar insertion between *T. longifila* and *C. vibrans* is insufficient to require separate genera, especially as Sandon


ENV: marine sediment 7-1.4: AY620334
ENV: marine sediment 7-1.3: AY620333
ENV: marine sediment 7-6.6: AJ620336
Trachelocorythion pulchellum AJ418789
Assulina muscorum AJ418791
Euglypha filifera AJ418786
Trinema enchelys AJ418792
Ovulinata parva HQ121432
Ovulinata sp. YPF501 AB453002
Paulinella chromatophora X81811
Cyphoderia ampulla AJ418793
ENV: freshwater/sediment 13-1.8: AJ620296
Auranticordis quadriverberis EU484394
Pseudopirsonia mucosa AJ561116
ENV: marine sediment 7-5.4: AY620332
ENV: marine surf water 12-4.1: AJ620337
ENV: marine surf water 12-4.3: AY620359
Clautriavia biflagellata FJ919772
ENV: marine surf water C1: AJ620329
ENV: marine surf water C5: AJ620331
Nudifila producta HQ121434
Thaumatomonas oxoniensis Hinksey HQ121429
Thaumatomonas vancouveri 3108W2 AF411264
Thaumatomonas sp. ATCC 50250 AF411261
Thaumatomonas seravini ATCC 50636 type strain T-2 AF411259
Thaumatomonas sp. SA2 AF411260
Thaumatomonas sp. SA1 AF411263
Allas aff. diplophysa ATCC 50365 AF411262
ENV-marine sediment A15 AY620317
Reckertia filosa AY268040
Marine cercozoan 5b FJ410916
Peregrinia clavideferens DQ211593
ENV-marine sediment 9-2.4 AY620311
'Spongomonas minima' UT1 AF411281
Spongomonas solitaria HQ121435
Spongomonas sp. KV/DB AJ620252
ENV: marine sediment D6: AY620316
Discomonas retusa FJ824131
ENV: marine sediment 7-6.2: AY620348
ENV: marine sediment 9-1.4: AY620314
Rhogostoma schuessleri HQ121430
Rhogostoma minus CCAP1966/4 HQ121431
Rhogostoma minus CCAP1966/3 AJ514867
Rhogostoma sp. DB-2002 HQ121437
Rhogostoma micra HQ121436
ENV: soil Amb-18S-1491: EF024013
Cryothecomonas aestivalis 1 AF290539
ENV: marine sediment 7-2.1: AY620354
Protaspa longipes AF290540
Protaspa grandis DQ303924
Protaspa obliqua FJ824121
ENV: marine sediment A14: AY620321
ENV: 0.5 m freshwater column F9: AY620281
Botuliforma benthica FJ824126
ENV: marine sediment 7-4.3: AY620356
Ebria tripartita DQ303922
Pseudodifflugia cf. gracilis AJ418794
Aulacantha scolymantha AY266294
Aulosphaera trigonopa AY266292
Coelodendrum ramosissimum AY266293
Ventrifissura foliformis FJ824128
Ventrifissura artocarpoidea FJ824127
ENV: marine sediment 7-5.5: AY620347
Verrucomonas longifila FJ824130
ENV: 0.5 m freshwater column F6: AY620288
ENV: 0.5 m freshwater column F5: AY620287
ENV: marine sediment 9-2.2: AY620308
Allapsa vibrans AF411265
ENV: soil Amb-18S-949: EF023686
Teretomonas rotunda AY620256
Neoheteromita tolerans HQ121433
ENV: uncultured soil glissomonad AH4878118t19: EU709217
Neoheteromita globosa U42447
Sandona ubiquita EU709153
Proleptomonas faecicola AF411275
Bodomorpha minima AF411276
ENV: soil HetChi9: EU709276
ENV: leafy freshwater sediment PP1-14: AY620290
Aurigamonas solis DQ199661
Agitata tremulans AY748806
Agitata vibrans HQ121438
ENV: marine sediment 7-3.4: AY620361
Cercomonas brazilliensis FJ790702
Cercomonas longicauda FJ790068
Cavernomonas mira FJ790718
Cavernomonas stercoris FJ790717
Cercomonas radiata FJ790712
Paracercomonas virgaria AY884340
Paracercomonas tenuis AF534712
Paracercomonas sp. Panama83 FJ790737
Paracercomonas baikali HQ121440
Paracercomonas anaerobica AF411272
Metopion aff. fluens AF411278
ENV: marine sediment A17: AY620324
ENV: marine surf water 12-3.3: AY620358
Metromonas simplex AY620255
ENV: marine sediment nh13: EU567296
ENV: freshwater sediment 13-4.8: AY620301
ENV: marine sediment E13: EU567253
ENV: freshwater sediment: lb28 EU567252
Uncultured eukaryote HAVOmat-euk24 EF032802
ENV: freshwater sediment: lb19 EU567207
Hedriocystis reticulata AY305010
Clathrulina elegans AY305009
ENV: freshwater sediment: op5 EU567213
ENV: freshwater sediment: op23 EU567215
ENV: soil se8: EU567216
ENV: brackish sediment 12-1.3: AY620364
ENV: marine sediment 7-1.5: AY620344
ENV: marine sediment eb6: EU567231
Nanofila marina AF289081
ENV: anaerobic freshwater sediment DB-2703-11: EU567236
Mesofila limnetica AF411283
ENV: marine sediment: he2 EU567248
ENV: marine sediment fp23: EU567249
Massisteria marina AF411286
ENV: marine sediment 7-2.6: AY620343
Minimassisteria diva EF405665
ENV: anoxic saline lake sediment NAMAKO-18 AB252758
ENV: anaerobic freshwater sediment DB-2703-10: EU567238
Limnofila anglica EU567255
Limnofila oxoniensis EU567256
Limnofila longa EU567254
ENV: coastal marine water UEPACLp3: DQ369015
ENV: arctic marine water column NW617.36: DQ314819
Chlorarachnion reptans U03477
Micrometopion nutans HQ121441
Tremula longifila HQ121439
ENV: freshwater sediment: lb9 EU567287
ENV: freshwater sediment: lb6 EU567286
ENV: anaerobic freshwater sediment DB-2703-19: EU567290
ENV: anaerobic freshwater sediment DB-2305-21: EU567288
ENV: anoxic saline lake sediment NAMAKO-20 AB252760
ENV: freshwater column LG21-01: AY919754
ENV: uncultured freshwater picoplankton A50: AY642739
ENV: anaerobic freshwater sediment DB-2703-3: EU567283
Arachnula impatiens EU567294
Spongospora subterranea f. sp. nasturtii AF310901
Phagomyxa odontellae AF310904
Filoreta turcica EU567292
Filoreta marina AY268044
Gromia oviformis AJ457814
Gromia oviformis AJ457812
chaunacanthid sp.218 AF018158
symphacanthid 211 AF063242
Acanthometra sp. AF063240
Sticholonche sp. AY268045
Spongaster tetras AB101542
0.1
Novel clade 3 (2004)
Euglyphida
Ovulinatidae
Marimonadida
Novel clade 2 (2004)
Thaumato-monadidae
Thaumato-monadida
Peregriniidae
Spongomonadida
IMBRICATEA
Novel clade 4 (minus 9-2.2) (2004)
Rhizaspididae
Cryomonadida
Protaspidae
Ebriida
Tectofilosida
Phaeodaria
Ventricleftida
THECOFILOSEA
Glissomonadida
Agitatidae
Pansomonadida
Cercomonadida
SARCO-MONADEA
Metopiida
Novel clade 6 (2004)
Metromonadida
METROMONADEA
includes Micrometopion nutans
Novel clade 5 (2004); Gran-5 (2009a)
Desmothoracida
Novel clade Gran-4
Novel clades Gran-2 & 3
Novel clade Gran-1
Nanofilidae
Mesofilidae
Novel clade Gran-6
Massisteridae, Leucodictyida
Limnofilida
GRANOFILOSEA
CHLORARACHNEA
Tremulida (Novel Clade 11; Bass et al. 2009)
Novel Clade 12 (Bass et al. 2009)
Novel Clade 10 (Bass et al. 2009)
ENDOMYXA
Radiozoan outgroup
MONADOFILOSA

(1927) noted that 'sometimes it passes for a short distance through the body and thus appears to be inserted laterally'; thus this rare condition in *C. vibrans* appears to be the norm for *T. longifila*. In view of these similarities, especially in their shared unusual movement, we place *C. vibrans* also in Tremulidae. As the range of cell shape in *T. longifila* is very similar to the non-amoeboid version of *C. vibrans*, it seems unwise to establish two separate genera for them just because of the absence of amoeboid behavior in *T. longifila* and its much longer flagella. Accordingly we place *C. vibrans* in *Tremula* as *T. vibrans* Cavalier-Smith comb. n. (Basionym *Cercobodo vibrans* Sandon 1927 pp 73-76).

Tremula longifila groups robustly within NC11; perhaps *T. vibrans* is one of the other three NC11 lineages. This soil or freshwater clade was first identified in Bass et al. (2009a), where it associated with two other novel clades, 10 and 12, as sister to Endomyxa. However, *Tremula longifila* has the commoner of the two filosan sequence signatures in rDNA helix 49 (AU base-pair replacing the GC seen in all eukaryote groups except opisthokonts; Cavalier-Smith et al. 2008a), whereas NCs 10 and 12 have the ancestral GC signature, as do other Endomyxa. Therefore *Tremula* is the most basal filosan identified so far. Figure 1 is rooted between Filosa, which ancestrally had the AU signature sequence, and all Cercozoa that have the more widespread GC; until the phenotype of largely freshwater clade NC10/12 is known we shall not know if they are morphologically filosan or endomyxan.

Both *Tremula* species differ substantially from *Glissandra innuerende* (Patterson and Simpson 1996), the only other zooflagellate known to glide with both flagella held tightly to the substratum; it is larger, with longer flagella, more elongated; its posterior flagellum inserts differently: *Glissandra* flagella emerge close together from a small anterior groove associated with an apical notch, both absent in *Tremula*; the *T. longifila* posterior flagellum emerges far away from the cell apex from a lateral notch. *G. innuerende* is smaller (5-8 μm), with equal flagella ~3.5X BL. The flagella move differently: AF of *Glissandra* moves only at its tip, the remainder held stiffly in the direction of movement; the *T. longifila* AF is much less rigid, waves passing along its entire length. *Glissandra* has many cytoplasmic granules, *Tremula* does not. *Glissandra* is marine; contrastingly ATCC50530 and BCKV were from dry soil or dry stream sediment, respectively. *T. vibrans* from soil made large, very distinctive cysts. No sequences exist for *Glissandra*, so we do not know if it is a cercozoan. From its morphology and behaviour *Glissandra* is most likely a filosan cercozoan and might belong in Tremulida, but without a sequence (or evidence for any marine members of NC11) we do not place it there but as Filosa incertae sedis.

Class Granofilosea Cavalier-Smith and Bass, 2009. Naked non-gliding biflagellate or non-flagellate heterotrophs with granular filopodia ancestrally stretched across substratum.

Order Leucodictyida Cavalier-Smith, 1993. **Family Massisteriidae** Cavalier-Smith 1993.

New genus *Minimassisteria* Arndt and Cavalier-Smith. **Diagnosis:** Trimorphic naked filose rhizopodial biflagellate. Trophic phase feeds by non-anastomosing filose pseudopodia that bear obvious granules at intervals along their length. With distinct swimming form and (unlike *Massisteria* its closest relative) a crawling form with a thicker non-granular anterior tractional pseudopodium. Heterotrophic. **Etym.** *mini* small, plus *massisteria*, for its similarity to *Massisteria.* Type species: *Minimassisteria diva.*

Minimassisteria diva Arndt and Cavalier-Smith sp. n. Fig. 2B. Two strains were isolated independently by the Oxford and Cologne laboratories; as they have identical 18S sequences we publish together to avoid assigning different names to one species. Type strain CCAP 1947/1 isolated by Ema Chao (Oxford) as a contaminant in a marine culture of *Oxnerella* sp. that was cultured by DB from muddy coastal sand (Walney Island, Cumbria, UK) (2009; GenBank HQ176324). **Diagnosis:** Sequences GenBank EF405665 (18S rDNA) EF405698 (ITS1) from the Cologne strain HFCC380. Trimorphic, with stationary feeding forms with slender granular filopodia stretched in all directions over the substratum; crawling forms that creep with thicker non-granular filopodia; and swimming monads. **Feeding form:** flattened body attached to the substrate. Cell body 2.3-5.0 μm (average 3.2 ± 0.5 μm, n = 33). 1-15 radiating delicate filopodia with up to about 11 granules (probably extrusomes); length from a few to 52 μm (average 30.5 ± 12.8 μm, n = 10). Aggregations of cells on substratum, where attached cells multiplied by fission. **Swimming form:** attached cells change into sausage-shaped swimming stages, more slender than trophonts (2 by 4.5 μm in size), have one anterior and one trailing flagellum, each ~4 μm long, and move relatively fast. Within a few minutes, swimming stages may attach to the substratum reforming trophonts. Flagella are partly resorbed and

Figure 1. Bayesian analysis of 18S rDNA for the novel Filosa in the context of core Cercozoa and Endomyxa, rooted on Radiozoa (148 sequences, 1539 positions). Bipartition support values are shown as RAxML bootstraps/Bayesian posterior probabilities when more than or equal to 85%/0.85, although values below this threshold but relevant to the discussion are also provided. Black dots represent support above 95%/0.95. The novel taxa discussed in this paper are emboldened. *Rhogostoma* DB is an additional strain not illustrated (but it had reticulopodia) or mentioned in the text as it died before high quality micrographs could be obtained.

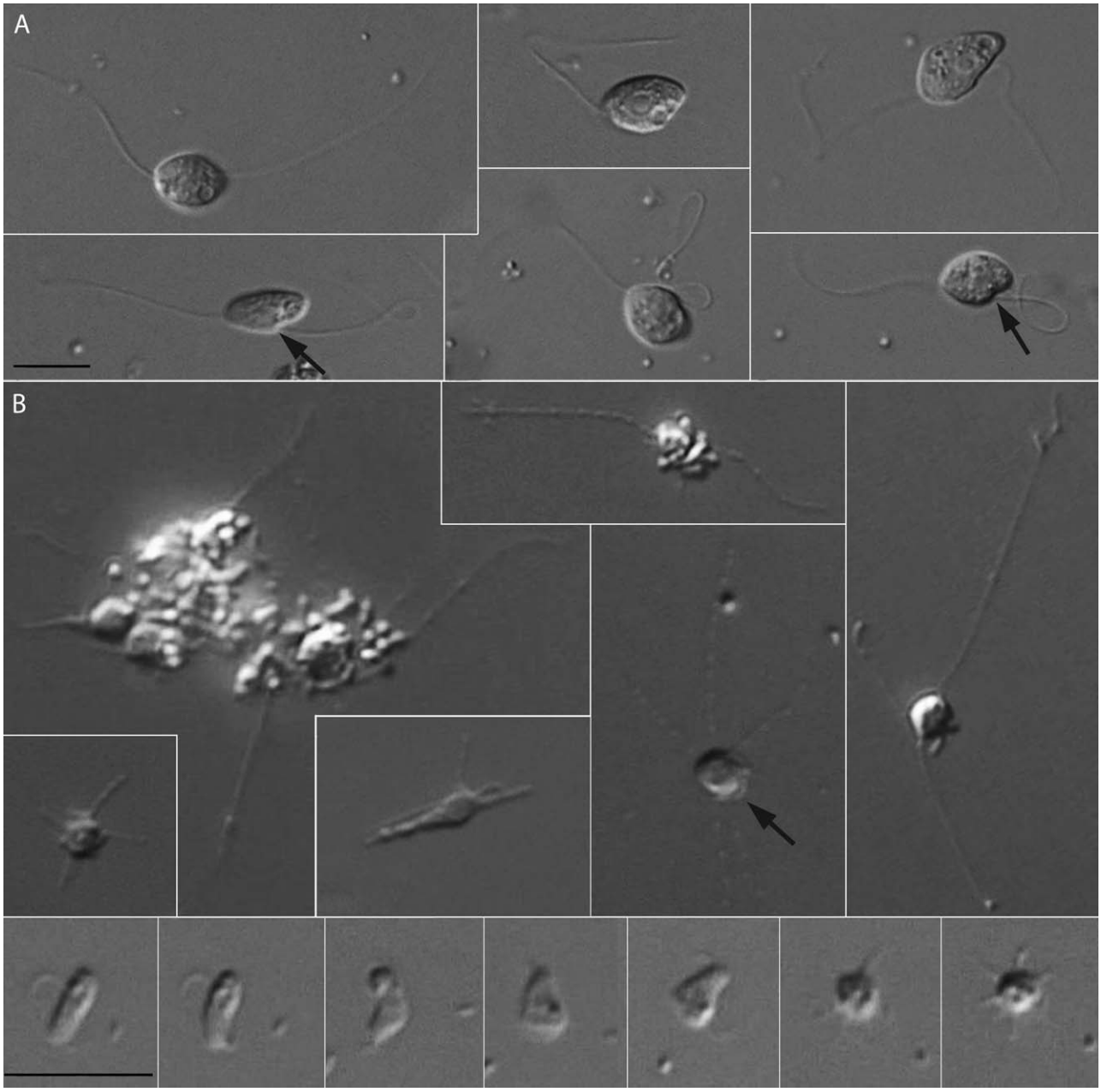


Figure 2. Light micrographs of living cells of **A:** *Tremula longifila* (DIC X60); **B:** *Minimassisteria diva* (DIC X40 and X60). Arrow indicates flagellum. Scale bar 10 μm for both.

shorter than in swimming stages (<2 μm). Generally, trophont flagella are motionless and hard to see. Phagotrophic, capture bacteria. **Cysts:** 2.5–3.0 (mean 2.7) μm diameter. The foregoing described the Cologne strain. The Oxford strain trophonts had similar cell body size, 3 μm (2.5–3.5 μm); the two flagella, approximately 0.5 to <1X BL emerge at (thus defined) cell anterior; usually held curved towards the posterior flush with the surface (indicated by arrow) either on the same or one on each side of cell; PF straight and usually non-motile. AF curved and occasionally slowly extends perpendicular to the cell then returns with rapid flicking motion. Cells globose, with several long granular non-anastomosing filose pseudopodia in contact with substratum at all points; very little cell activity seen; though most granules stationary, some move up or down filopodia. Lone cells, or those collected in groups, often surrounded by and embedded in debris. Swimming form morphologically like Cologne strain, except flagella unequal; swims shorter AF first, spinning/rotating on longitudinal axis and sinuously through water column. Grows in artificial seawater with mixed bacteria to high density. Cysts not observed in Oxford strain. No cv; n central. **Crawling form:** one major pseudopodium (thicker and shorter than in trophonts, so granules invisible), posterior flagellum trails, typically with other thick pseudopods on either side. **Etym.** *diva* L. goddess, but named after deep-sea expe-

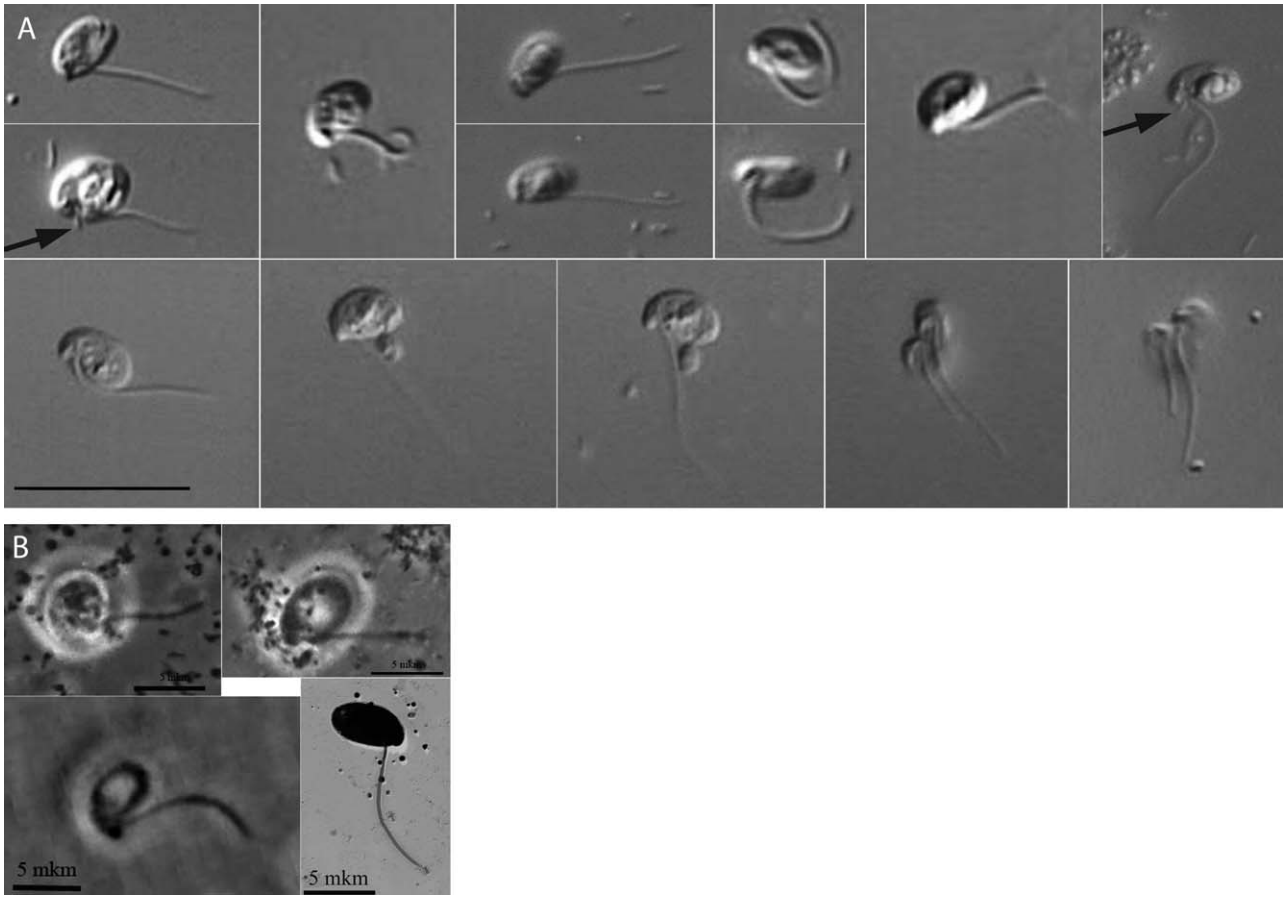


Figure 3. Light micrographs of living cells of **A:** *Micrometopion nutans*; arrow indicates anterior flagellum (both DIC X40 and X60). Scale bar 10 μm; **B:** *Metopion* aff. *fluens* (phase contrast and a shadowed transmission electron micrograph, by courtesy of A. P. Mylnikov). Scale bars show size.

dition DIVA on which HFCC380 was collected [Northern Cape Abyssal Plain (28°6.8'S, 7°20.8'E) at depth of 5036 m; strain isolated March 4th, 2005 (R/V "Meteor", Cruise M63/2, station #38)].

Minimassisteria diva 18S rDNA sequence from both our isolates is identical to marine sediment environmental clone he17 (Bass et al. 2009a), which in most datasets including many other Granofilosea is a long-branch member of a robustly supported marine clade including *Massisteria marina*. In Figure 1 with fewer Granofilosea it is sister to the *Massisteria* clade with strong Bayesian but weak ML support.

Infraphylum Monadofilosa Cavalier-Smith 1997 stat. n. Ancestrally amoeboflagellates that glide on posterior flagellum only.

Class Metromonadea Cavalier-Smith, 2007. Here emended by adding Metopiida because metopiids resemble metromonads in two characters absent from sarcomonads with which they were formerly classified (and with which they do not group on trees): a dense extracellular surface coat and highly elongated extrusomes. **Revised diagnosis:** Non-pseudopodial marine gliding biflagellate predators on other eukaryotes; non-thecate but with a dense single or double layered surface coat that may extend up flagella. Extrusomes highly elongated.

New Genus *Micrometopion* Cavalier-Smith. **Diagnosis:** Rigid non-amoeboid, non-metabolic marine zooflagellates that glide on the substratum by a non-acronematic posterior flagellum, which emerges from a ventrolateral groove (on cell's left) a fifth of body length from anterior. Typically uniflagellate, strongly compressed, with a slightly beaked anterior rostrum that is less obvious and less bulbous than in *Metopion* and lacks its two rows of granules. **Etym.** *micro* Gk + *Metopion*, the zooflagellate described by Larsen and Patterson (1990), as it resembles but is smaller than *Metopion fluens*. Type species: *Micrometopion nutans.*

Micrometopion nutans Howe et al. sp. n. Fig. 3A. Type strain CCAP 1904/1 (2002, littoral sample from White Sea (salinity 12‰), nr Kartesh, Russia; AP Mylnikov). **Diagnosis:** 18S rDNA sequence, GenBank HQ121441; cell size 6.1 μm (5–7.5 μm). PF longer than in *M. fluens* (Larsen and Patterson, 1990) at 11.9 μm (8.5–14 μm) 2-2.75X BL, not acronematic; second

flagellum stubby, rarely visible or rarely present (marked by arrow), its posterior-pointing and position in the same groove as PF is best distinction from *Planomonas* (where AF comes from anterior tunnel and is usually very thin: Cavalier-Smith et al. 2008b). Both flagella equally thick. Cells flattened, quasi-oval; less convex on left, which is slightly indented at flagellar insertion, making left anterior appear as a slightly projecting rostrum. Cells compressed laterally; rigid, non-metabolic; lie on their side flush with substratum. PF trails alongside, from point of attachment under rostrum. Cell pivots in nodding, knocking motion, raising and lowering its posterior while gliding along substratum. Cells usually nod constantly and energetically, often travelling fairly directly at variable speed. Bud-like projections, approximately a quarter of cell size, seen on several cells near posterior; probably result of misdivision. Culture medium half-strength artificial seawater; consumes eukaryotes; fed on *Procryptobia sorokini.* Flagellated resting stage; no cysts. Large cv, usually central, occasionally in posterior half. Cells divide parallel to plane of compression (Fig. 3A). **Etym.** *nutans* L. nodding.

Comments: the second short flagellum that projects laterally from the groove in some cells and beats actively might be a growing posterior flagellum prior to division. Mylnikov gave us this strain as "*Metopion fluens* small form". Earlier he had given us a larger one designated simply *Metopion fluens*, described ultrastructurally by Mylnikov et al. (1999) without light micrographs. Its 18S rDNA sequenced previously (Cavalier-Smith and Chao 2003b) so radically differs from that now found for *Micrometopion nutans* (see below) that we cannot treat them as the same genus, despite morphological similarity. That larger-celled culture was isolated from a marine aquarium in the Zoology of Invertebrates Department, Moscow State University (salinity 35‰), but it is long dead and no high quality light micrographs exist. However, Mylnikov kindly supplied micrographs of that culture from his files, some shown as Figure 3B. Comparison with the type micrograph (figure 52 (f) of Larsen and Patterson) gives no strong reason to question their identification as *M. fluens*, but their lack of clarity makes identification somewhat uncertain, so we now call that strain *Metopion* aff. *fluens*. Comparison of the text of Larsen and Patterson (1990) and Mylnikov et al. (1999) initially raised doubts about their identity, as the former gave 10 μm and the latter 15-17 μm for PF length; however, in the nine micrographs Mylnikov sent us the length is 11-12 μm, not significantly different from our measurements of the micrographs in Larsen and Patterson (11-12.5 μm). Cell length for the Moscow strain (Mylnikov et al. 1999) and in the type diagnosis were both 6-9 μm; so no measurement discrepancies really exist. Descriptions of behaviour and morphology are also concordant. The angle of PF to the cell may differ subtly, but they are obviously much more similar than either is to *Micrometopion*. *Micrometopion* differs in five ways: (1) cells on average are smaller than *M. fluens* and *M.* aff. *fluens*; (2) PF length is >2X BL, but closer to 1.5X BL in *M. fluens* and *M.* aff. *fluens*; (3) the position of the PF and the angle at which it trails with respect to the cell differ subtly; (4) it has a less bulbous rostrum with no obvious granules; (5) the cell appears narrower than *M. fluens*. These slight phenotypic differences would not require a different genus were it not that they differ so greatly in sequence that some trees do not even group them, one or other often grouping distantly with *Metromonas* (see below). *Micrometopion nutans* usually occupies somewhat more basal positions in 18S rDNA phylogenies than our *Metopion* aff. *fluens* sequence, differing among methods and taxon samples (none significantly supported). It is not associated with any known environmental sequence, having only 89% similarity to any 18S rDNA sequence in GenBank; this lack of close neighbours using 18S rDNA probably exacerbates its positional instability. However, 28S rDNA analyses suggest a closer relationship to *Metromonas*. Polyubiquitin was sequenced from the Moscow *Metopion* aff. *fluens* strain (Bass et al. 2005). We place *Micrometopion* in Metromonadea for reasons given in the discussion.

Class Sarcomonadea Cavalier-Smith, 1993. **Revised diagnosis:** gliding, ancestrally (and almost always) biflagellate gliding zooflagellates without theca, often amoeboid; extrusomes usually spherical or shortly ellipsoid. Bacterivores, except for *Aurigamonas*. Mainly inhabit freshwater and soil.

Order Cercomonadida Poche, 1913 emend. Grassé, 1952 and Karpov et al., 2006

Family Cercomonadidae Saville Kent, 1880 *sensu* Karpov et al., 2006

Paracercomonas baikali Howe et al. sp. n. Fig. 4A. Type strain B148 (2007; G8, grassland at edge of Listvyanka village, 0.5 km from Lake Baikal shore, Russia; AH. Strain no longer alive.) **Diagnosis:** 18S rDNA sequence, GenBank HQ121440; ITS2 rDNA GenBank HQ176333; cell size: 10.5 μm (8.5-12 μm). AF ~1X BL; PF 1.5-2X BL, acronematic. Cell metabolic, pliable. Wider at anterior than posterior while travelling, somewhat teardrop-shaped. Cell movement can be quite rapid; moderately frequent changes of direction. AF constantly flickers rapidly along entire length. Unlike all other paracercomonads cells occasionally vibrate and flicker *both* AF and PF while static. Pseudopodia project from anywhere. Frequently extend and retract cytoplasmic tail to >1BL; cysts slightly angular, thick-walled. N anterior; cvs in various positions, including anterior to n. **Etym.** *baikali* from Baikal, the type locality. Also isolated: B113 (2007; Sample 4, grassland nr Lake Baikal, Russia; AH). Flagellar lengths are given as a proportion of BL: Bass et al. (2009b) found that cell length and flagellar length are correlated in cercomonads, and relative values are more informative than absolute values.

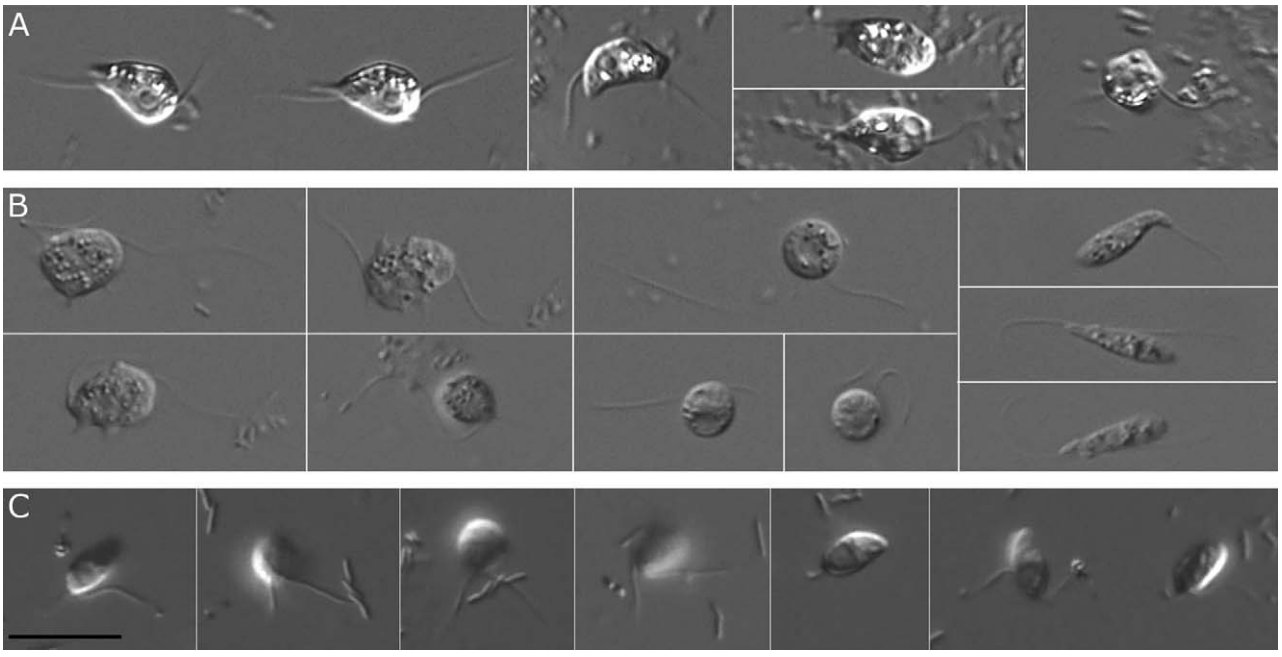


Figure 4. Light micrographs of living cells of **A:** *Paracercomonas baikali* (DIC X40); **B**: *Agitata vibrans* (DIC X60); **C:** *Neoheteromita tolerans* (DIC X40). Scale bar 10 μm for all.

Paracercomonas baikali is the first described aerobic representative of a very divergent lineage within *Paracercomonas*, Group B2, branching just above the previously most divergent described species, *P. anaerobica* (Bass et al. 2009b). As the *P. baikali* lineage is rarely isolated it is interesting that another of the same 18S-type was isolated from a different site in the same sampling area near Lake Baikal, suggesting a local bloom of this paracercomonad.

Order Pansomonadida Vickerman, 2005

Family Agitatidae Cavalier-Smith and Bass, 2009 (in Bass et al. 2009b)

Genus *Agitata* Cavalier-Smith and Bass, 2009

Agitata vibrans Howe and Cavalier-Smith sp. n. Fig. 4B. Type strain ATCC50407 (Year, place, isolator unknown; depositor TK Sawyer). **Diagnosis:** 18S rDNA sequence, GenBank HQ121438; ITS2 rDNA GenBank HQ176334; cell size 12 μm (6.5-17 μm). AF: c. 17 μm, PF: c. 33.5 μm. Cell larger, on average, than type species *A. tremulans*, but flagellar proportions similar. Anisokont flagella flicker actively and heterodynamically, neither associated with cell along its length. Arise from same point; PF usually beneath cell, appears to emerge from opposite end. When stationary and attached to substratum, cell usually rounded; lamellar and filose pseudopodia extend and retract from all around cell, bulbous and finger-like pseudopodia less common than in *A. tremulans*. Flagella wave independently and infrequently in same plane. Cell occasionally wobbles liquidly and gently on substratum or vibrates frenetically. Swimming cells round to highly elongated, or oval; tapered anterior and posterior ends (spindle-shaped); cells very plastic: when elongated, bend sinuously along their length. Swimming cell vibrates energetically while rotating or swims smoothly in swirling circles. Culture medium Volvic® and grain. 1-2 central or posterior cvs; nucleus usually in anterior half of cell. Cysts not observed. Differs from *Agitata* (=*Dimastigamoeba*) *agilis* comb. n. (below) only by somewhat longer AF (1.4X BL not 0.7-1X BL) and much longer PF (2.8X BL, not 1.2X BL). **Etym.** *vibrans* L. vibrating.

Comment: We bought ATCC50407 as *Cercomonas agilis* OC-2 but its rDNA sequence shows that it is not a *Cercomonas* but is closely related to *Agitata tremulans* (Bass et al. 2009b), itself also originally misidentified as *Cercobodo agilis* (another synonym for *Dimastigamoeba agilis*), so the name *Cercomonas* is inapplicable. ATCC50407 is morphologically much closer to *D. agilis* than was *A. tremulans*, but differs enough in posterior flagellum length to merit a new species. *Cercomonas agilis* was originally described as *D. agilis* by Moroff (1904); his organism was so similar to *Agitata vibrans* except for its shorter flagella that we now place it in the genus *Agitata*. The name *Dimastigamoeba* is unsuitable, being a junior synonym for *Cercomonas*. *Cercobodo* used for this species by Lemmermann (1914) is also unsuitable as the identity of its type species is obscure (Bass et al. 2009b) and it was not very similar to *D. agilis*.

Agitata agilis Cavalier-Smith comb. n. Basionym: *Dimastigamoeba agilis* Moroff (1904). Synonyms *Cercobodo agilis* Lemmermann, 1914; *Cercomonas agilis* Mylnikov and Karpov, 2004. There are now three *Agitata* species (*A. tremulans*, *A. vibrans*, *A. agilis*).

Aurigamonas solis Vickerman, 2005

We isolated one culture from grassland at the edge of Listvyanka village, 0.5 km from the Lake Baikal shore (Siberia), and another from volcanic soil from Costa Rica, with an extremely similar 18S rDNA sequence to the three *A. solis* strains of Vickerman et al. (2005). Our strain AurBaikal (GenBank HQ176323) is available as CCAP 1909/1. Our observations confirm and supplement those of Vickerman et al. (2005): cell size 8.2 μm (6-10 μm); shorter AF c. 4 μm difficult to observe, often curved; PF 38.2 μm (32 - 48 μm). PF flickers rapidly along its length occasionally, while tethering cell body to substratum by its tip. Cell spherical or slightly elongated; radiating filose spine-like haptopodia all over periphery, 1.5-8.5 μm long (mean 4.1 μm), usually slightly bulbous at distal tips. Much variation in cell and haptopodia length. Pseudopodia not observed. Cell and PF often unattached to substratum, PF movement makes cell rapidly yo-yo up and down in medium; occasionally its distal end tethers cell to substratum. Culture medium Volvic® and grain; grows best in company of other eukaryotes (probably prey; feeding not observed). Cv at anterior end of cell where flagella emerge. Globular cysts readily formed.

Order Glissomonadida Howe et al. 2009

Family Sandonidae Howe et al. 2009

Neoheteromita tolerans Bass and Cavalier-Smith sp. n. Fig. 4C. Type strain: Marine glisso (found as contaminant in culture ATCC50344 *Paracercomonas marina*). **Diagnosis:** 18S rDNA sequence, GenBank HQ121433; ITS2 rDNA GenBank HQ176335; cell size: 6 μm (5.5–7.5 μm); AF 1.5-2X BL; PF 1.75-2.25X BL. AF flickers, whip-like; PF trails passively. Posterior end of cell raised, not in contact with PF (except at insertion point) or substratum. Cell plastic: shape varies from round to tear-drop with acuminate posterior, most often cell posterior wide and blunt narrowing towards anterior. Cell jiggles energetically, vibrates as AF beats; during travel, cell flops side to side of its longitudinal axis. Cell usually travelling, changes of direction frequent. Adhesive posterior tail, can extend >1X BL. N in anterior half of cell. Marine, unlike all other glissomonads; maintained in artificial seawater. **Etym.** *tolerans* L., referring to its ability to grow in a range of salinities.

Within glissomonads, the 18S rDNA sequence of *Neoheteromita tolerans* is very closely related to *N. globosa*, and effectively identical to the soil-derived environmental clone AH48p78p11 (Howe et al. 2009). However, the type strain of *N. tolerans* was unable to grow in a medium less salty than 40% Artificial Seawater (CCAP recipe) in Volvic®. These findings together provide further evidence that 18S-types can be insufficient for resolving species-level differences in morphology or ecology among strains.

Class Thecofilosea Cavalier-Smith, 2003 em. **Revised diagnosis:** ancestrally with robust organic extracellular theca (unlike most other Cercozoa which are usually naked or with scales), ventral filose pseudopodia, and two flagella with divergent centrioles; ancestrally benthic flagellates gliding on posterior flagellum only, emitting filose pseudopodia from ventral groove, but some secondarily planktonic swimmers amongst which ebriids have lost pseudopodia; flagella secondarily lost in Rhizaspididae and the tectofilosid testate amoebae (i.e. Amphitremidae Poche, 1913; Pseudodifflugiidae De Saedeleer, 1934; Chlamydophryidae De Saedeleer, 1934; Psammonobiotidae Golemansky, 1974; Volutellidae Sudzuki, 1979), and restricted to zoospores in phaeodarians; theca with two perforations for flagella in the flagellates, one perforation for pseudopodia in the thecate and most testate amoebae, two opposite perforations for pseudopodia in Amphitremidae, and three perforations in phaeodaria (thus also called Tripylea Hertwig, 1879), which have surrounded it by a pseudopodial net containing a pigmented phaeodium, thus converting it into a 'central capsule' (not homologous with that of Polycystinea of phylum Radiozoa). Silica scales absent (unlike many Imbricatea), but hollow silica endoskeleton in all ebriids and most phaeodarians.

Comment: We hereby emend the class by including a new order of gliding zooflagellates, Ventricleftida (see discussion for reasons), *Botuliforma* which has a robust theca like most Thecofilosea and ventral furrow (Chantangsi and Leander 2010b) and its sister group Ebriida (where the 'theca' is only tenuous as in *Cryothecomonas aestivalis* and a groove is absent) (both having condensed interphase chromosomes like *Protaspa*), and also including Phaeodaria at subclass rank:

Phaeodaria Haeckel, 1879 stat. n. Cavalier-Smith **Diagnosis:** with 'central capsule' (modified theca) perforated by large astropylum and typically lateral parapylae; dark pigmented phaeodium in extracapsular protoplasmic region; usually with hollow silica endoskeleton. (Silicofilosea (Adl et al. 2005) was a pointless name change; and misleading as most Thecofilosea are non-silicified.)

Order Cryomonadida Cavalier-Smith, 1993 emended by adding Rhizaspididae because rDNA trees show *Rhogostoma* within the cryomonad zooflagellates, so they evolved after the flagellate common ancestor of Ebriida and Cryomonadida by loss of flagella and are unrelated to *Pseudodifflugia*.

Family Rhizaspididae Skuja, 1948 orth. em. (Skuja mistakenly put *Rhizaspis* within euglenoids, even though euglenoids never have filopodia, and invariably have flagella, both unlike *Rhizaspis*, treating it nomenclaturally as a plant, and overlooked the much greater similarity to *Rhogostoma* noted here for the first time). **Revised diagnosis:** thecate amoebae with ventral cleft that emits filopodia, which anastomose as reticulopodia in one species; theca thin, flexible, laterally compressed, adherent throughout to cell surface, consisting of single smooth dense layer outside and scarcely thicker than the plasma membrane; thus with bilateral symmetry, unlike Chlamydophryidae De Saedeleer, 1934 and other Tectofilosida which are typi-

cally radially symmetric. Ventral cleft contrasts with the circular or oval ventral orifice of Tectofilosida. Type genus *Rhizaspis* Skuja, 1948; other genera *Rhogostoma* Belar, 1921; *Capsellina* Penard, 1909. Electron microscopy by Simtzis and Le Goff (1981); we accept their evidence that *C. timida* is a *Rhogostoma* and establish *R. timida* Cavalier-Smith comb. n. (Basionym *Capsellina timida* Brown, 1911).

Comment: De Saedeleer (1934) did not include any of these in his Chlamydophryinae, placing *Capsellina* in Lobosa and ignoring *Rhogostoma*. Meisterfeld (2002) put *Rhogostoma* and *Capsellina* in Chlamydophryidae, but ignored *Rhizaspis,* which prior to our present recognition of its close similarity to *Rhogostoma*, was placed with *Protaspis* (now *Protaspa*) in the invalid family Thaumatomastigaceae (Patterson and Zölffel 1991). Our trees show that *Rhogostoma* (and by implication *Rhizaspis*) is robustly related to *Protaspa* but that neither belongs in Thaumatomonadida, which is not even in the same class.

Genus *Rhogostoma* Belar, 1921 (Type species *R. schuessleri*). We isolated six new strains of this thecate amoeba from soil or freshwater (only four were used in this study because two were identical to existing strains), videoed them and sequenced their 18S and ITS2 rDNA; they belong to *R. schuessleri* or *R. minus*, the only two known species, or to a new species. First we show that a previously sequenced strain misidentified as *Lecythium* sp. (Nikolaev et al. 2003) is also *R. minus*.

Rhogostoma minus Belar, 1921. Figure 5A shows freshwater strain (CCAP 1966/3) isolated by AP Mylnikov (his strain code Le-2) from a waste treatment plant at Borok, Yaroslavskaya Oblast, Russia, in 1993 (as '*Lecythium* sp.'), whose actin and 18 rDNA sequences were reported (Nikolaev et al. 2003) without photographic documentation of its morphology. We restudied it to check its identity because we are also sequencing other genes from it (Lewis et al. in prep.) and isolated six related strains. Belar (1921) described two *Rhogostoma* species that differed from freshwater *Lecythium* in four respects: (1) having a narrow crack-like, not a large round, aperture; (2) being filose not reticulose; (3) being several times smaller (*R. minus* 10-12 μm, *R. schuessleri* 15 μm); and (4) in thecae having bilateral not radial symmetry. In all four respects Mylnikov's strain matches *R. minus* and differs from *L. hyalinum*. It was thus misidentified and is *Rhogostoma minus* not a *Lecythium*. Its correct identity may have been overlooked as it was unaccountably omitted from the standard work (De Saedeleer 1934). Nikolaev et al. (2003) noted cell length as 8-10 μm and width as 5-8 μm, but wrongly wrote that it 'closely resembles the type species, *Lecythium hyalinum* (Hertwig *et* Lesser, 1874), as illustrated by Cash and Wailes (1915; Plates 49 and 51).' In fact, the original description and illustration of Hertwig and Lesser (1874) shows pseudopods sometimes anastomosing (probably not so in this strain) and that the nucleus alone is 10-15 μm across, which would make cell width (seen end on from aboral pole) ~30-45 μm, i.e. 3-4 times Mylnikov's strain. Moreover *L. hyalinum* (Ehrenberg) Hertwig and Lesser is depicted with perfect radial symmetry; all these features are clear in Cash and Wailes (1915) except that they do not describe or depict anastomosis, so perhaps studied a different species from Hertwig and Lesser (1874). The other freshwater species in Cash and Wailes (1915) (*Lecythium spinosum* (=*Trinema spinosum* Penard, 1890); *Lecythium granulatum* (=*Gromia granulata* Schulze, 1875); *Lecythium mutabile* (=*Pamphagus mutabile* Bailey, 1853)) are comparably large or even larger and do not resemble Mylnikov's strain. De Saedeleer (1934) described a marine *Lecythium minutum* (3-11 μm) that is similar to *R. minus* except for its obvious circular aperture.

Description: Flexible, smooth inorganic test, with aperture from which radiating lamellar, finger-like, non-granular, branching filopodia constantly extend and retract at all angles; shape a strongly laterally flattened prolate ellipsoid projecting vertically from substratum; cell broader diameter seen from dorsal end: 7.3 μm (6.5-8.5 μm) in our living cultures when rapidly growing (can be 10-12 μm in old stationary cultures). Nikolaev et al. (2003) do not make it clear whether their measurements were from its oral to aboral end (what length should mean) or at right angles to that axis as ours are. Cell fills test completely. Cleft-like aperture beneath the cell hard to see in life, opening towards substratum as in figure G1,2 of Belar (1921); pseudopodia emerge through the cleft, can extend many times body length and pull the cell along the substratum; cell pulled over leading pseudopodia as trailing pseudopodia retract, progress smooth. Refractile granules; several small vesicles; large lateral cv; n usually central. Form groups of several cells. Divide by binary fission. Freshwater. Heterotrophic. Freshwater species of *Lecythium* are 3-10 times larger. ITS2 rDNA sequence: GenBank HQ176325. **Other strains:** Fig. 5B. A slightly larger strain (RhogFloat CCAP 1966/4 (GenBank 18S rDNA HQ121431): isolated in 2009 by DB from soil from Oxford University Harcourt Arboretum, Nuneham Courtenay, UK; cell size: width 7-14; length 9-15 μm) had an identical ITS2 sequence to CCAP 1966/3; so we treat it as the same species, even though its

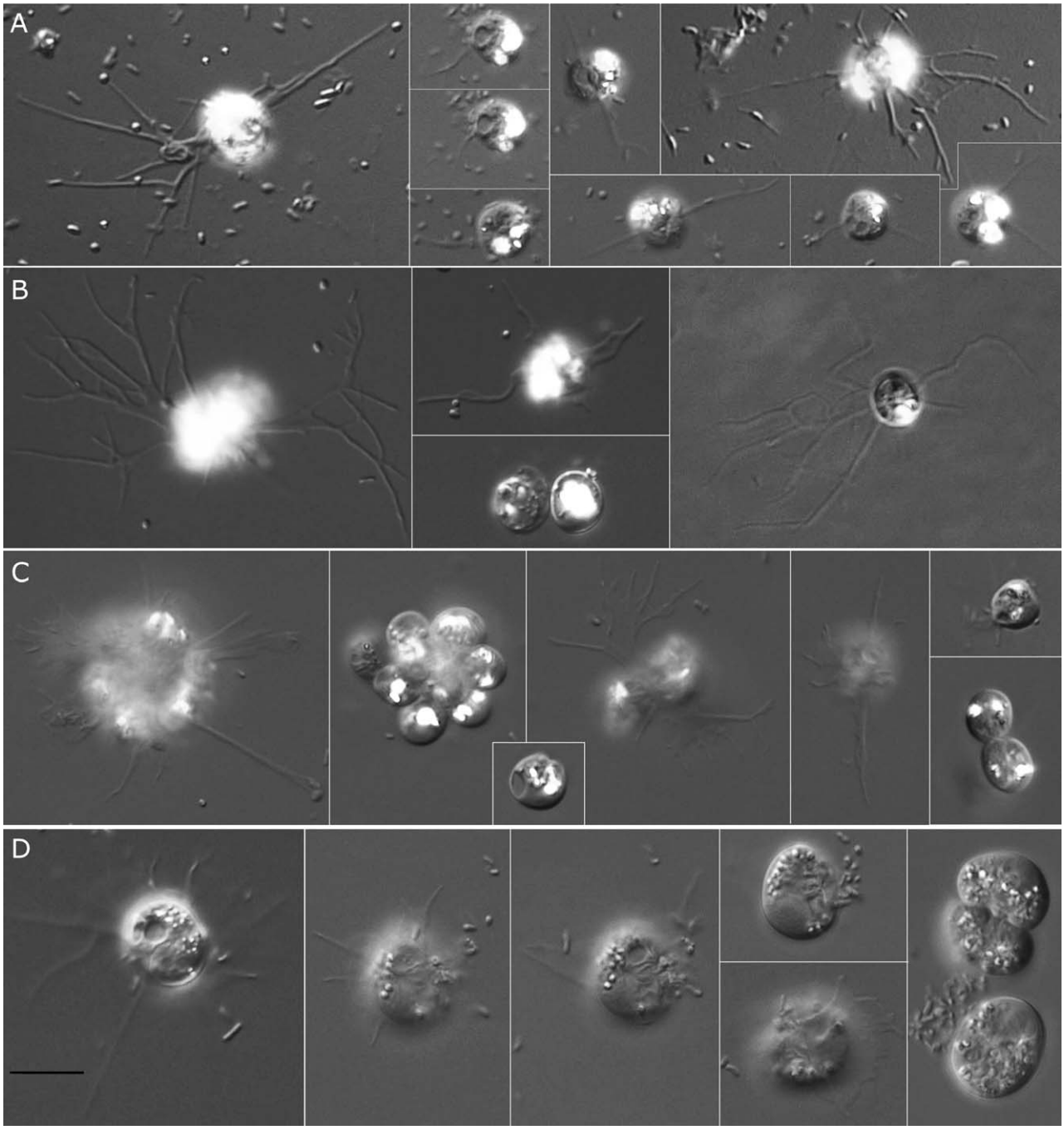


Figure 5. DIC light micrographs of *Rhogostoma*. **A:** *R. minus* CCAP1966/3; **B:** *R. minus* strain FLOAT CCAP1966/4; **C:** *R. micra* PML2A CCAP 1966/2; **D:** *R. scheussleri* strain Arb CCAP1966/1. Scale bar 10 μm for all.

filopodia often fuse into a definite reticulum or reticula form by lacunae opening within lamellipodia (unlike *R. schuessleri*, *minus*, and *micra*). Given this ITS2 identity, the apparent differences in 18S DNA sequences of CCAP 1966/3 (AJ514867) and CCAP 1966/4 (HQ121431) are all attributable to PCR and/or sequencing errors. The errors are probably all in the CCAP 1966/3 sequence (AJ514867: Nikolaev et al. 2003) making it a longer branch on our trees, because we independently isolated three other strains that all yielded identical 18S rDNA sequences to HQ121431 of CCAP 1966/4.

As these came from California, USA (freshwater stream sediment near Monterey), freshwater stream sediment near Porlock Harbour, Somerset (JMS), and Hinksey Lake, Oxford, UK (DB) this *R. minus* genotype is widespread in north temperate continents in both freshwater and soil.

Rhogostoma micra Cavalier-Smith and Scoble sp. n. Fig. 5C. Type strain Rhog PML2A CCAP 1966/2 (2009; still stream sediment, Port Meadow, Oxford, UK; JMS). **Diagnosis:** 18S rDNA sequence, GenBank HQ121436; ITS2 rDNA GenBank HQ176327; cell size: 7.5 μm (6.5-10 μm). Morphology as *R. minus*, from which reliably distinguishable only by distinct 18S rDNA sequence (Fig. 1). Test more often associated with debris and bacteria. Behaviour very similar, but pseudopodia more often, but not restricted to, trailing and leading bifurcating strands extruded from beneath the cell; cells can be less regular in shape than in *R. minus*. Finger-like pseudopodia more frequently exhibited, and lamellar pseudopodia less frequent, than in *R. minus*; when travelling, cell occasionally tilts to point cleft in direction of travel, perhaps to facilitate or as a result of extension of leading pseudopodia for locomotion – cells then appear slightly narrowed at the aperture end (light-bulb-shaped; compare figure G3 of Belar (1921) for *R. minus*). Freshwater. Division by binary fission. **Etym.** *micros* Gk small.

Rhogostoma schuessleri Belar, 1921. Strain LArb CCAP 1966/1 (Fig. 5D). (2009; isolated from Oxford University Harcourt Arboretum soil, Nuneham Coutenay, UK. JMS). Cell size 15 μm. Indistinguishable from the original description (Belar 1921). 18S rDNA sequence, GenBank HQ121430; ITS2 rDNA GenBank HQ176326. Larger than the three preceding species. Forms conspicuous lamellipodium broader than the cell, from which filopodia extend.

Comment: *R. minus/micra* clade is a complex of closely related small filose or sometimes reticulose organisms distinct from the larger lamellar/filose *R. schuessleri*. The fact that *R. minus* has both non-reticulose strains (like the other two species) and a reticulose strain CCAP 1966/4 suggests that the latter evolved its ability to fuse pseudopodia relatively recently, suggesting that in this group it is a fairly easy evolutionary transition (yet one rarely made in Filosa, apart from in Chlorarachnea and very few cercomonads, unlike in the probably ancestrally reticulose Endomyxa and Retaria: Bass et al. 2009a).

Family Protaspidae Cavalier-Smith, 1993. Constituent genera *Protaspa* Cavalier-Smith nom. n. (replacement name for *Protaspis* Skuja, 1939) and *Cryothecomonas* Thomsen, Buck, Bolt & Garrison, 1991.

Protaspis and three species were validly described under the International Code of Botanical Nomenclature (ICBN) by Skuja (1939) as was his later (fourth) species (Skuja 1948), but the genus name is invalid under the International Code for Zoological Nomenclature (ICZN) as it was preoccupied by the Devonian fish *Protaspis* Bryant, 1933 (family Protaspididae), yet since Cavalier-Smith (1993) it has been treated under the ICZN (ICZN, now used for all Cercozoa). The species names of Larsen and Patterson (1990) were described under both codes but are valid only under ICBN. All subsequently described species names (Hoppenrath and Leander 2006a; Chantangsi and Leander 2010b) are illegitimate under ICBN as they lacked Latin diagnoses, and invalid (like those of Larsen and Patterson 1990) under ICZN as junior synonyms of *Protaspis* Bryant. Since the root of *Protaspis* is protaspid- the protist zoological family name (Cavalier-Smith 1993) ought to have been Protaspididae; to allow continued use of a zoological generic name very close to Skuja's *Protaspis*, to validate all existing species names under ICZN, and to retain Protaspidae as the valid family name, we propose *Protaspa* as a replacement name for *Protaspis* Skuja (1939) under ICZN and make 14 new combinations:

Protaspa glans Cavalier-Smith comb. n. basionym *Protaspis glans* Skuja, 1939; *Protaspa major* Cavalier-Smith comb. n. basionym *Protaspis major* Skuja, 1939; *Protaspa metarhiza* Cavalier-Smith comb. n. basionym *Protaspis metarhiza* Skuja, 1939; *Protaspa obovata* Cavalier-Smith comb. n. basionym *Protaspis obovata* Skuja, 1948; *Protaspa tanyopsis* Cavalier-Smith comb. n. basionym *Protaspis tanyopsis* Norris,1961; *Protaspa gemmifera* Cavalier-Smith comb. n. basionym *Protaspis gemmifera* Larsen and Patterson, 1990; *Protaspa obliqua* Cavalier-Smith comb. n. basionym *Protaspis obliqua* Larsen and Patterson 1990; *Protaspa tegere* Cavalier-Smith comb. n. basionym *Protaspis tegere* Larsen and Patterson, 1990; *Protaspa verrucosa* Cavalier-Smith comb. n. basionym *Protaspis verrucosa* Larsen and Patterson, 1990; *Protaspa simplex* Cavalier-Smith comb. n. basionym *Protaspis simplex* Vørs, 1992; *Protaspa grandis* Cavalier-Smith comb. n. basionym *Protspis grandis* Hoppenrath and Leander, 2006; *Protaspa rotunda* Cavalier-Smith comb. n. basionym *Protaspis rotunda* Chantangsi and Leander 2010; *Protaspa obaniformis* Cavalier-Smith comb. n. basionym *Protaspis obaniformis* Chantangsi and Leander, 2010; *Protaspa oviformis* Cavalier-Smith comb. n. basionym *Protaspis oviformis* Chantangsi and Leander, 2010; and *Protaspa longipes* Cavalier-Smith comb. n. basionym *Cryothecomonas longipes* Schnepf and Kühn, 2000. We also formally transfer Protaspidae from Thaumatomonadida (Cavalier-Smith 1993) to Cryomonadida and place *Cryothecomonas* in it. In view of evidence that *Protaspa* and *Cryothecomonas* are very similar morphologically and rather closely related genetically (Hoppenrath and Leander 2006a), we no longer need the separate family Cryothecomonadidae Cavalier-Smith, 1993, here regarded as a junior synonym of Protaspidae by virtue of its being later in the paper that initiated both names (Cavalier-Smith 1993) and *Protaspa* being discovered before *Cryothecomonas*.

New Order Ventricleftida Cavalier-Smith. **Diagnosis:** Strongly flattened oval zooflagellates with rigid theca without scales; two unequal flagella emerge subapically, often from apical notch, posterior flagellum used for gliding on surfaces; ventral cleft from which branched filose pseudopods emerge for

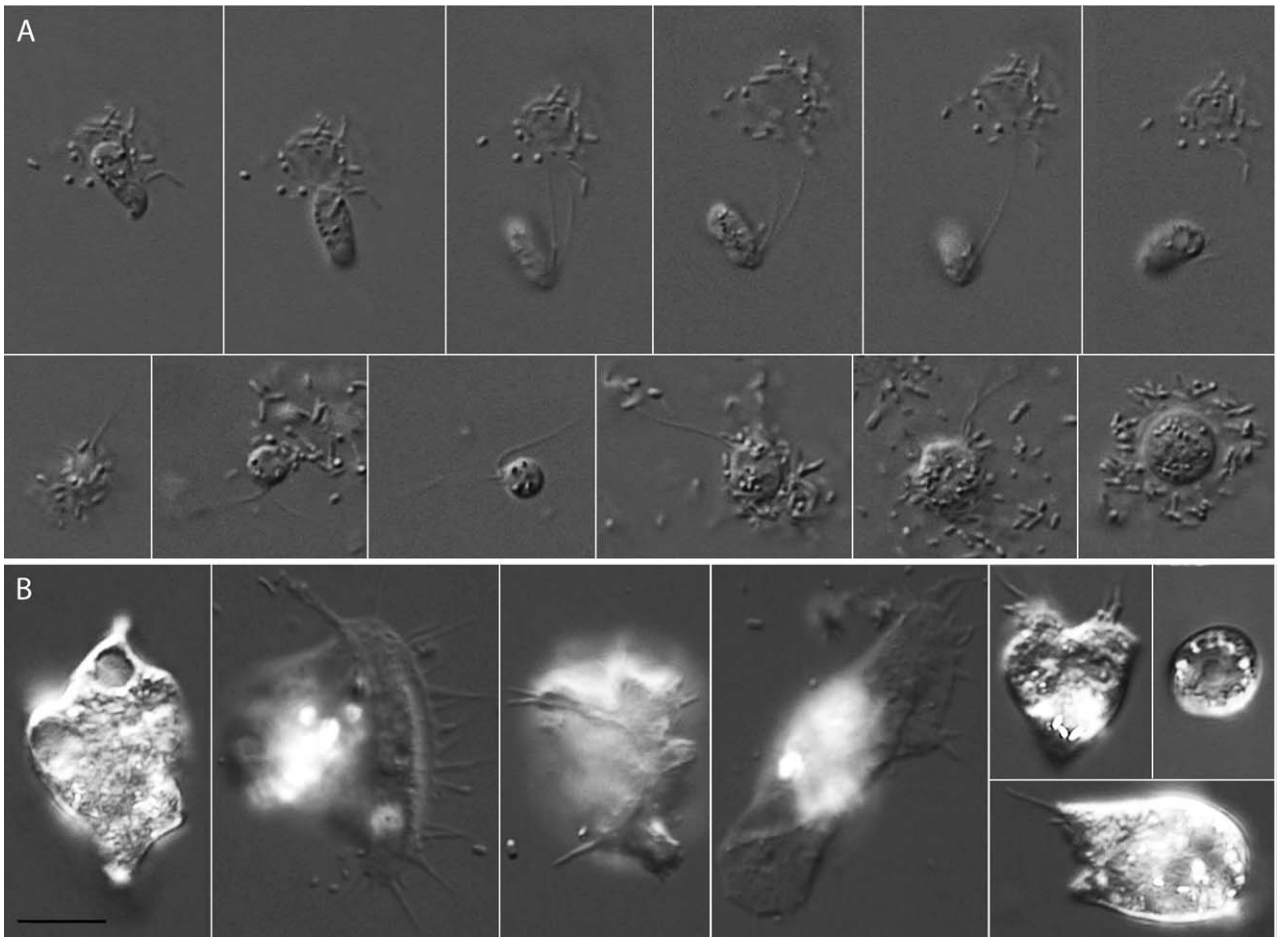


Figure 6. Light micrographs of living cells of **A:** *Spongomonas solitaria*, including a series of frames of a stationary cell extricating itself from the surrounding debris (DIC X60); **B:** *Peregrinia clavideferens* (DIC X40). Scale bar 10 μm for both.

feeding separate from and posterior to flagellar groove (unlike Thaumatomonadida and *Auranticordis*). Extrusomes. **Etym.** *venter* L. belly; plus *cleft* E.; because of their ventral cleft. Contains *Ventrifissura* Chantangsi and Leander, 2010 and *Verrucomonas*, Chantangsi and Leander, 2010.

Comment: Our Bayesian tree (Fig. 1) shows these genera as a clade that is sister to all previously known Thecofilosea; on both Bayesian and ML trees neither branches within Tectofilosida as stated but not demonstrated by Chantangsi and Leander (2010b). Therefore we do not accept their placement within Tectofilosida or suggested modification to its diagnosis. Nor are they sisters of Imbricatea and Glissomonadida respectively as in the undersampled tree that used too few nucleotides of Chantangsi and Leander (2010b). Because trees conflict on their precise position it is premature to decide whether this order is ancestral to or sister of other thecofilosans. Nonetheless, this order is a morphologically defined monophyletic taxon and whether it is paraphyletic or holophyletic (as weakly on Fig. 1) is immaterial to its taxonomic validity (see Cavalier-Smith (2010) for a refutation of the widespread but mistaken antipathy to paraphyletic taxa).

Class Imbricatea Cavalier-Smith, 2003. **Revised Diagnosis:** Cercozoa with often imbricate silica scales and their closest non-scaly relatives. Ancestral condition probably a gliding zooflagellate with two flagella with divergent centrioles, and relatively rigid pellicle (unlike most Sarcomonadea) that helped define a distinct ventral groove from which filose pseudopods extended, but without the dense extracellular theca of Thecofilosea or internal silica skeleton of ebriids and Phaeodaria. Flagellar transition region longer than in cercomonads and sainouroids; unlike them with dense distal plate but without the internal dense aggregates and elaborate extra structures opposite the thecal contact zone in cryomonads. Groove and flagella secondarily lost by euglyphids, centrioles multiplied and reoriented to make four posteriorly directed gliding flagella in *Auranticordis*, which also lost pseudopodia; centrioles independently made parallel in the thaumatomonad/spongomonad subclade.

Comment: Imbricatea, originally defined as comprising only Thaumatomonadida and Euglyphida, the two cercozoan orders with silica scales (Cavalier-Smith and Chao 2003), is hereby redefined phylogenetically to comprise the last common ancestor of these two groups plus all its multiform descendants. This revision of Imbricatea includes for the first time the non-scaly order Spongomonadida (see Discussion for some ultrastructural similarites to Thaumatomonadida), a new non-scaly order Marimonadida (including the enigmatic amoeba *Rhabdamoeba* with a posteriorly biflagellate gliding phase (Rogerson et al. 1998), *Auranticordis*, and *Pseudopirsonia*) and two new genera (*Peregrinia* with and *Nudifila* without silica scales).

Order Spongomonadida Hibberd, 1983

Family Spongomonadidae Karpov, 1990

Spongomonas solitaria Vickerman, Howe, and Cavalier-Smith sp. n. Fig. 6A. Type strain SpSpA, CCAP 1971/1 (2002; dried freshwater creek sediment, Montezuma Castle Monument, Arizona, USA; KV). **Diagnosis:** 18S rDNA sequence: GenBank HQ121435; ITS2 rDNA GenBank HQ176337; cell 6.5 μm in diameter (4-9 μm). Two heterodynamic flagella of roughly equal length, 13–26 μm, emerge from or just below a small tapering anterior rostrum. Cell shape irregular-spherical or spherical when stationary, elongated along antero-posterior axis when swimming. Stationary cells surrounded and covered by varying amounts of particle matter, resembling a cocoon; flagella project above the cell, and flicker often using only their distal 4/5ths, distal half strongly acronematic. Cysts readily form, also surrounded by debris; look much like active cell but without flagella. Pseudopodia not observed. Unlike *S. intestinalis* Cienkowski, *S. uvella* Ritter von Stein, *S. discus* Ritter von Stein and *S. sacculus* Saville Kent it has not been seen to form colonies by building up extracellular granular material so as to project from the substratum; instead cells remain close to the substratum. **Etym.** L. *solitarius* solitary, for its predominantly solitary life.

Comment: Cultured in Volvic® and grain, bacterivorous. During division cells enlarge and two pairs of flagella emerge from different locations before fission**.** Occasionally flagellar movement causes cell to vibrate somewhat. Swimming cells most often twirl and vibrate energetically, flagella extended. Dislodged cells twirl and vibrate above substratum attached to it by their flagella (with or without debris associated), often move a short distance before returning to rest. Cells can also swim smoothly and very rapidly with only slight vibrations. At other times, cells project upwards into the medium, attached by their flagella to the substratum, and oscillate. During the transition between resting and swimming, the cell begins to rotate within the centre of the debris, before emerging, wormlike, flagella extend, and the cell begins to rapidly vibrate and swim up into medium (upper line of frames in figure). Isolated four more times, all by KV: (2002) one strain from the same location as type; (2004) two strains from an infusion of un-dried meadow grass leaves, nr River Kelvin, Glasgow Botanic Gardens, UK; (2007) sample of recycled horticultural waste from Scottish Water plant at Deerdykes, UK. All have identical 18S and ITS sequences, showing that this genotype is present in both Europe and North America. *Spongomonas solitaria* is the first formally named *Spongomonas* strain with associated sequence data, providing clear evidence that *Spongomonas* is cercozoan. A morphologically indistinguishable solitary strain from soil (7A) was studied ultrastructurally (Cavalier-Smith and Chao 2003b) showing that this morphospecies is also present in South Africa, but no authentic 18S rDNA sequence was obtained (that mislabelled '*Spongomonas* 7A' in their trees and in Cavalier-Smith (2000) was actually from *Metromonas simplex*, as Bass and Cavalier-Smith (2004) and Cavalier-Smith et al. (2004) explained). *Spongomonas minima* (Dangeard 1910; Tschermak-Woess 1950) is another solitary particulate-cocoon-forming species that had no flagella; it was called a *Spongomonas* just because it has a cocoon of granules like both *S. solitaria* and the unrelated *Phalansterium solitarium* (Sandon 1924), now obviously a polyphyletic character; it might be related to one or other or to neither. It is unclear why strains ATCC50404/5 were identified as *Spongomonas minima*. We sequenced their 18S rDNA; UT-1, remarkably, is a *Spongomonas* (it does not look like one in the light microscope, yet does by electron microscopy), but is not *S. minima*; AZ-3 is a glissomonad (*Neoheteromita*): see Cavalier-Smith and Chao 2003b; Cavalier- Smith et al. 2004; Howe et al. 2009). The statement in Adl et al. (2005) that *Spongomonas* 7A 'was not correctly identified' is wrong; it was microscopically certainly a *Spongomonas* indistinguishable from *S. solitaria*. Their statement that UT-1 was misidentified is correct, but misleading with respect to its position on the rDNA tree because it is a *Spongomonas* but an undescribed species, not *S. minima*.

Order Thaumatomonadida Shirkina, 1987

Thaumatomonads are flagellates with a largely rigid surface covered by silica scales formed in vesicles attached to mitochondria, and which often emit ventral pseudopodia for feeding. We have discovered a novel largely non-flagellate freshwater amoeba that possesses two-tiered siliceous scales similar to but not identical with those of *Gyromitus limax*, a creeping pear-shaped cell with two anterior flagella arising from a small anterior depression

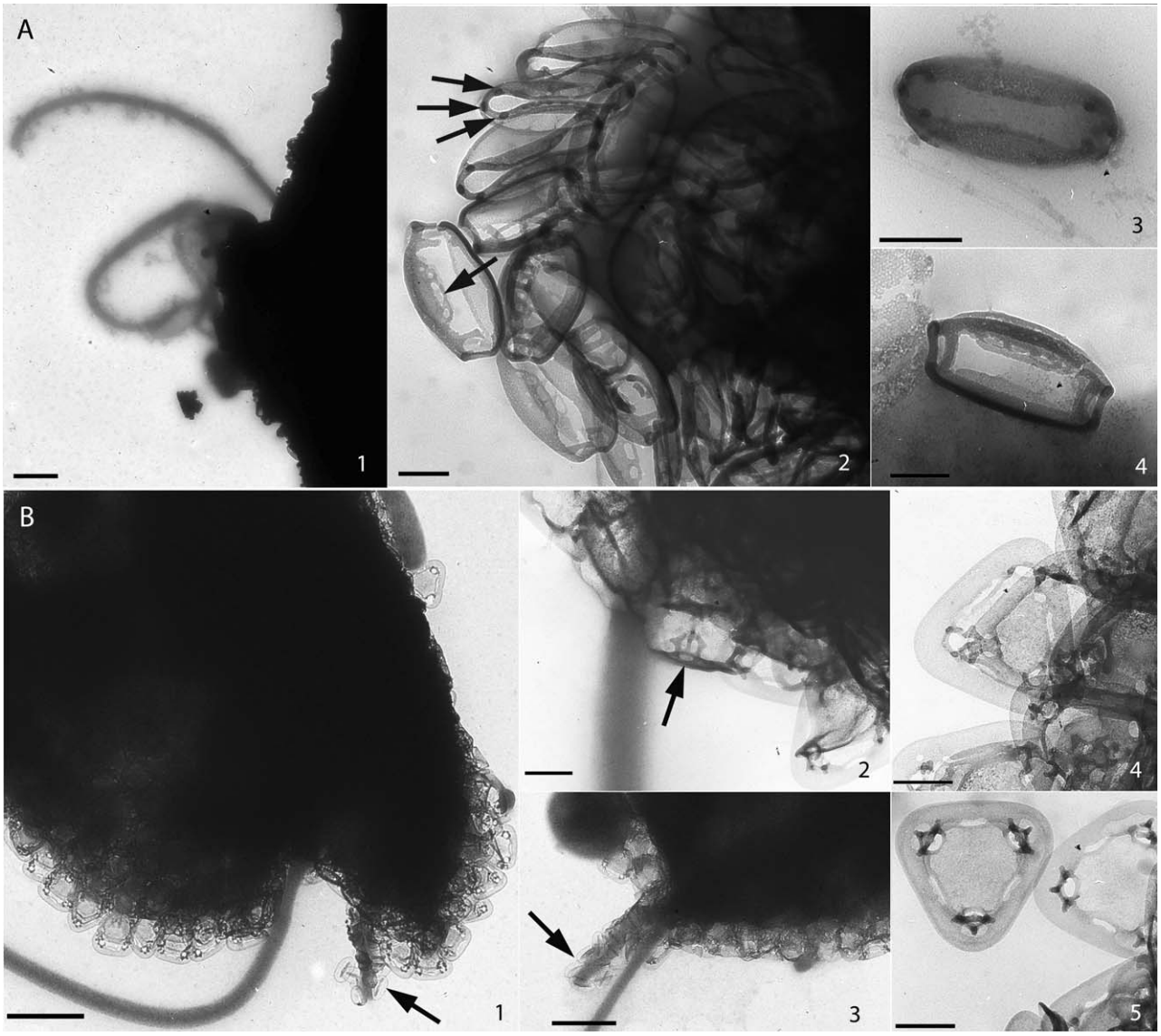


Figure 7. Transmission electron micrographs of unstained whole mounts of new thaumatomonads and their scales. **A:** *Peregrinia clavideferens*. **1** Two probable flagella without scales. **2** Cell surface with overlapping barrel-like scales viewed in different orientations; side view with perforated lateral flange projecting downwards from the upper plate (lower arrow); and top view of the pinched apertures of several scales with three dense spots indicating where struts attach (upper arrows). **3** Scale aperture (less pinched in than in A2) with three struts at each end. **4** Side view of scale. **B:** *Reckertia filosa*: **1** Cell with large triangular scales covering the cell body and much smaller oval scales on the short anterior flagellum (arrow); the longer posterior flagellum is naked. **2** Overlapping triangular body scales in situ on cell surface; arrow indicates a side view of a three-pointed corner strut adjoining an oval corner hole**. 3** Anterior flagellum covered in small oval scales (arrow), with smooth posterior flagellum below**. 4** Face view of overlapping triangular triangular body scale. **5** Face view of detached non-overlapping body scales, showing distinctive three-strut arrangement beside the corner holes. Scale bars: A1 and B1 1 μm; A2-4 and B2-5 200 nm.

and delicate pseudopodia arising from a posterior groove (Swale and Belcher 1975). When first isolated in Germany our new strain also showed two flagella in some cells but after months of culture these were no longer visible by light microscopy, though by whole mount electron microscopy we occasionally saw images suggestive of two short non-scale-bearing flagella (Fig. 7A1). The largely

amoeboid nature of our strain (now CCAP 1956/1), its usually different shape, and distinguishable scales make it a separate species from *G. limax*, but in the same genus given its homologous scale architecture. However, the scales of *G. limax* and our new species differ so substantially from those of the *Gyromitus* type species (*G. disomatus*) that we separate them both from *Gyromitus* as a new genus, *Peregrinia*.

Gyromitus 'disomatus' scales resemble an oval serviette ring: a laterally compressed, latticed hollow cylinder with strongly outrolled upper and lower rims and a sieve-like sheet across its lower surface (Nicholls 1979; Swale and Belcher 1974), markedly different from all other thaumatomonad scales. In contrast, those of *Peregrinia* (=*Gyromitus*) *limax* comb. n. (Swale and Belcher 1975) and our new species *Peregrinia clavideferens* have two dissimilar upper and lower tiers connected by three vertical struts at each end, somewhat as in certain *Thaumatomonas* species. *Peregrinia* and *Gyromitus* differ in scale structure and in locomotion (being amoeboid creepers or planktonic swimmers respectively not flagellar gliders) from the core Thaumatomonadidae for which scale structures and sequences are now both known (i.e. *Thaumatomonas*, *Allas*, *Thaumatomastix* and *Reckertia*). We therefore establish a new family Peregriniidae to embrace *Peregrinia* and *Gyromitus*. Thaumatomonadidae are holophyletic on our trees and collectively sister to *Peregrinia* (Fig. 1 and Scoble et al. unpublished). This grouping of the new scaly amoeba *Peregrinia* as deep sister to Thaumatomonadidae shows that two-tier thaumatomonad scales are phylogenetically much more ancient than previously realised, going back in time close to when thaumatomonads separated from other imbricate lineages. *Peregrinia* and *Reckertia* sequences show that most of basal group T of Bass and Cavalier-Smith (2004) comprises deep branching thaumatomonads.

New family Peregriniidae Cavalier-Smith. **Diagnosis:** thaumatomonads with only oval two-tiered body scales and no flagellar scales; absence of spine scales and flagellar scales distinguishes them from *Thaumatomastix*; scales either symmetric ovals with heavily outturned upper and lower rims (*Gyromitus*) or asymmetric ovals with concave to flat lower surface and convex upper surface with rims more strongly laterally inrolled than at the ends (*Peregrinia*). In contrast to *Thaumatomonas*, flagellar pit apical not subapical and ventral, or cells so amoeboid as to lack defined shape; flagella not clearly differentiated into anterior and posterior; unlike Thaumatomonadidae no evidence for flagellar gliding; locomotion by swimming or slow amoeboid creeping. **Type genus:** *Peregrinia* Cavalier-Smith gen. n. Other genus *Gyromitus* Skuja (1939).

Peregrinia Cavalier-Smith gen. n. **Diagnosis:** large freshwater non-thecate amoeboflagellates; move by amoeboid creeping, not swimming or flagellar gliding; surface covered in single layer of two-tiered siliceous scales visible only by electron microscopy; scales oval in surface view with inrolled sides to the upper tier, oblong in lateral view with more convex upper surface; highly metabolic flattened amoebae with lamellar and finger-like pseudopodia. Cysts unknown. **Etym.** *peregrinus* L. wanderer for its creeping behaviour. **Type species:** *Peregrinia clavideferens*.

Peregrinia clavideferens Howe, Arndt and Cavalier-Smith sp. n. Type illustrations: Figs 6B and 7A. Type strain CCAP 1956/1; HFCC94 (2001; freshwater, Schoehsee, nr Ploen, Germany; C. Wylezich; =*Gyromitus* sp. of Wylezich et al., 2007). **Diagnosis:** type 18S rDNA sequence, GenBank DQ211593; ITS2 rDNA GenBank HQ176336; cell size very variable, 25.4 μm (14-40 μm); cell flattened (disc-like), irregular-shaped, very amoeboid, constantly changing. Highly metabolic; flagella usually not visible. Finger-like and long tapering filose, sometimes branching, pseudopodia all over cell. Often a lamellar sheet projects from leading edge, from which filose and finger-like pseudopodia extend and contract as cell creeps along substratum. Thickly covered with numerous scales of bilaterally asymmetric, rounded three-dimensional oblong shape, tapering somewhat like a squashed elongated but bilaterally asymmetric barrel, with bowed sides, top and bottom (0.21-0.25 x 0.51-0.62 μm); oval in surface view with lateral lips typically more strongly inrolled than in *P. limax* often so as to virtually touch each other (Fig. 7A2), but sometimes less pinched in laterally (Fig. 7A3); scales with a fenestrated flange on the more curved side (unlike *P. limax*) and with slightly indented ends with three thick struts connecting the two bowed surfaces (Fig. 7: A2 and A4). Numerous large and small vacuoles throughout cell. Culture medium Volvic® and grain; bacterivorous. Vacuolate cysts frequent, round, 12.3 μm (11-14 μm) diameter. Two flagella visible in light microscope only when culture first isolated. **Etym.** *clavus* L. nail, rudder; *deferens* L. bearing; for its filose pseudopodia.

Comment: HFCC94 was identified as *Gyromitus* sp. when its 18S rDNA was sequenced (Wylezich et al. 2007). To clarify the difference between *Peregrinia* and *Gyromitus* we summarize here past knowledge of *Gyromitus* strains and scale morphology. The *Gyromitus* type species is *G. disomatus* (Skuja 1939), a free-swimming monad, barely metabolic with two flagella emerging from an anterior depression, 15-22 μm long, 11-19 μm wide, but with an ability to emit pseudopods from a posterior notch; it differed from *Peregrinia* by not becoming an amoeba with no visible flagella. The biflagellate strain of Swale and Belcher (1974) emitted pseudopods from its posterior while swimming and would also settle and transform into an amoeba with thin branching pseudopods similar to those of *P. clavideferens* and no apparent flagella (unlike Skuja's organism, so might not be the same). Two other *Gyromitus* species are known: *G. cordiformis* (Skuja 1948), whose scale type is unknown, and *G. limax* (Swale and Belcher 1975) that we now treat as a second *Peregrinia* species because its

scales are similar to those of *P. clavideferens*. The *Gyromitus* strains studied by Nicholls (1979) from six Ontario lakes were definitely not *P. limax*; he thought most of them identical to those of Swale and Belcher's strain and called them all *G. disomatus*, but recognised that some were clearly different. However, none of those he illustrated are 'identical' to those of Swale and Belcher; his five micrographs show at least four distinct scale types and, if each species has only one, may represent five species; as he saw some forms with pseudopodial tails but never a fully amoeboid stage like that of Swale and Belcher (1976), at least some of his strains may be closer to the original *G. disomatus* than was theirs. Nicholls seems to have been unaware of *G. cordiformis* or the need to observe characters such as the position of contractile vacuoles (which he did not record) to distinguish the two species. However, as *G. cordiformis* (Skuja 1948) produced pseudopods from the middle of a marked ventral groove (not a terminal notch as in *disomatus*), Nicholls' mention of 'tails' suggests that at least some of his were more *disomatus*-like.

Nicholls noted that the latticed cylinder wall of the Canadian strains had 4-8 rows of pores; by contrast in the single UK strain (probably the only one in clonal culture) the scales seem very uniform with four rows of medium sized pores of equal size in the cylinder wall (Swale and Belcher 1974). Unfortunately Nicholls did not mention which micrographs came from different samples; his Figure 5 shows scales much deeper in proportion to their length than the UK strain or any others he illustrates; as they apparently have eight rows of pores of equal size it cannot be the same species. By contrast his other scales are much shallower, somewhat more like those of the UK strain in depth. But their pores are clearly different; his Figures 8 and 9 might be one species as both show two prominent central rows of very large holes; as he mentions four rows, he presumably includes also the somewhat smaller pores visible in the dense rims of these scales – if we had included these rim pores for the UK strain the pore rows would number six, not four; thus the different number of pore rows and much larger pore size of the two main rows compared with the UK strain means they are probably separate species. His Figure 7 differs radically from the other four and from that of Belecher and Swale in that the lower rim with the sieve-like plate has a much smaller diameter than the upper rim, so this is yet another species. His Figure 6 has more equal sized rims but they are much more delicate than the heavily inrolled rim of the UK strain. Thus, there is evidence for at least five different species of *Gyromitus* with scales of this oval sieve type, which Nicholls (1979) demonstrated contain silica; the non-scale phenotype of these strains embraced that of *G. disomatus* but as their scales all differ we shall never know exactly which scale type was present in Skuja's original. It is therefore best to call all these strains *G.* aff. *disomatus* until a new culture becomes available suitable for defining a neotype. Nicholls convincingly demonstrates that *Hymenomonas prenanti* (Lecal 1965) was not a new species of haptophyte but a misidentified *Gyromitus*. But we do not agree that it was the same species as that of Swale and Belcher (1974) as unlike theirs it appears to have at least two kinds of scales (if those in his Figure 1 really came from one cell): tall ones with about six rows of small holes (similar to those of Nicholls' figure 5) and squat ones about half as tall, more like other strains of Nicholls or Swale and Belcher but apparently not precisely the same as any of them. Until a *G. disomatus* neotype of known scale type is established we cannot say whether *H. prenanti* is a superfluous synonym for *G. disomatus* as Nicholls argued or whether *prenanti* is instead a fourth species of *Gyromitus* (most likely).

What is clear is that *P. clavideferens* scales differ from all the *G.* aff. *disomatus* strains (Nicholls 1979; Swale and Belcher 1974) so radically that it cannot be *G. disomatus* and must be a separate genus, whereas '*H.*' *prenanti* scales (Lecal 1965) are sufficiently like *G.* aff. *disomatus* to be congeneric. Scales of both *Peregrinia* species resemble those of Thaumatomonadidae in having an upper plate, unlike all *G.* aff. *disomatus*, which fits the position of *Peregrinia* as sister to Thaumatomonadidae on our tree, so we assign *Pereginia* to Thaumatomonadida. However, as the upper plate of *Peregrinia* has a central cleft with inrolled margins, we exclude *Peregrinia* from Thaumatomonadidae, whose scales are bilaterally symmetric ovals or triangular with an upper plate having an entire (cleft-free) centre (*Thaumatomastix* additionally makes long spine scales with round or triangular bases).

Peregrinia limax Cavalier-Smith comb. n. Basionym *Gyromitus limax* Swale and Belcher, 1975

Gyromitus **Skuja, 1938. Revised diagnosis:** Cordiform phagotrophic heterotrophic biflagellates with two equal forward-pointing flagella issuing from apical depression; flagella used for swimming, not gliding; cell body but not flagella covered by siliceous oval scales with microporous sieve-like base and cylindrical sides bearing 4-8 rows of pores, inrolled upper and lower rims, and no upper plate; scales made in vesicles associated with mitochondria with tubular cristae; can emit pointed branching filose pseudopods from rear or ventral surface; some strains may transform completely into an amoeba.

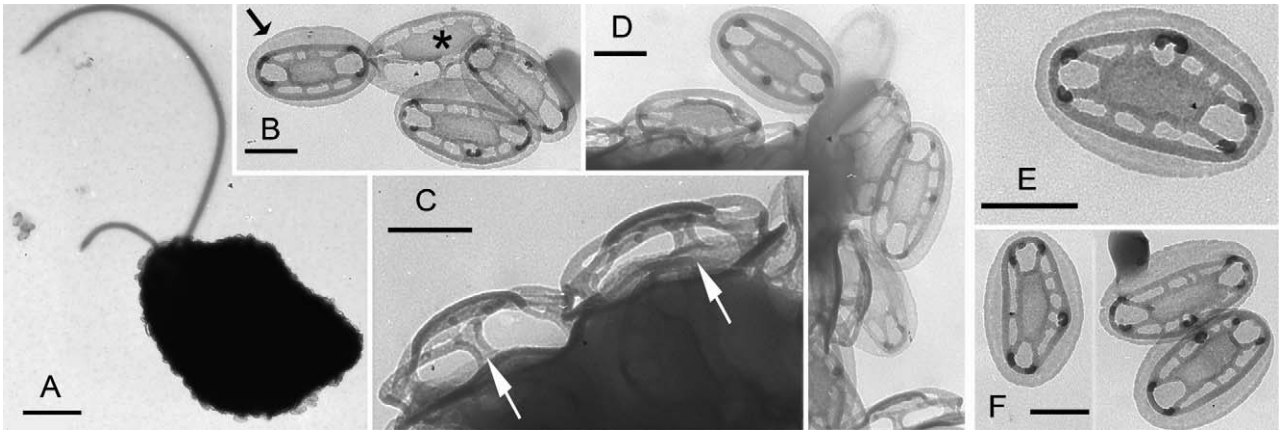


Figure 8. Transmission electron micrographs of unstained whole mounts of *Allas* aff. *diplophysa.* **A**: Whole cell showing both scale-free flagella and a layer of bodyscales. **B**: Four detached scales showing the main variants: the bottom two are the typical and common form with a dense U-shaped end perforations and lateral strut. The top two are atypical; one minor variant devoid of the lateral strut (arrow), and a malformed atypical scale (asterisk). **C**: Side view of attached scales showing lateral struts (arrows). Note convex upper plate. **D**: Normal oval scales with no bulge at the side of lateral strut. **E**: Asymmetric scale with prominent lateral bulge, fairly common scale type, giving an intermediate shape between oval and triangular. **F**: Additional examples of perforations being fewer on the side without the lateral strut. Scale bars 2um, except **B-F** 200 nm.

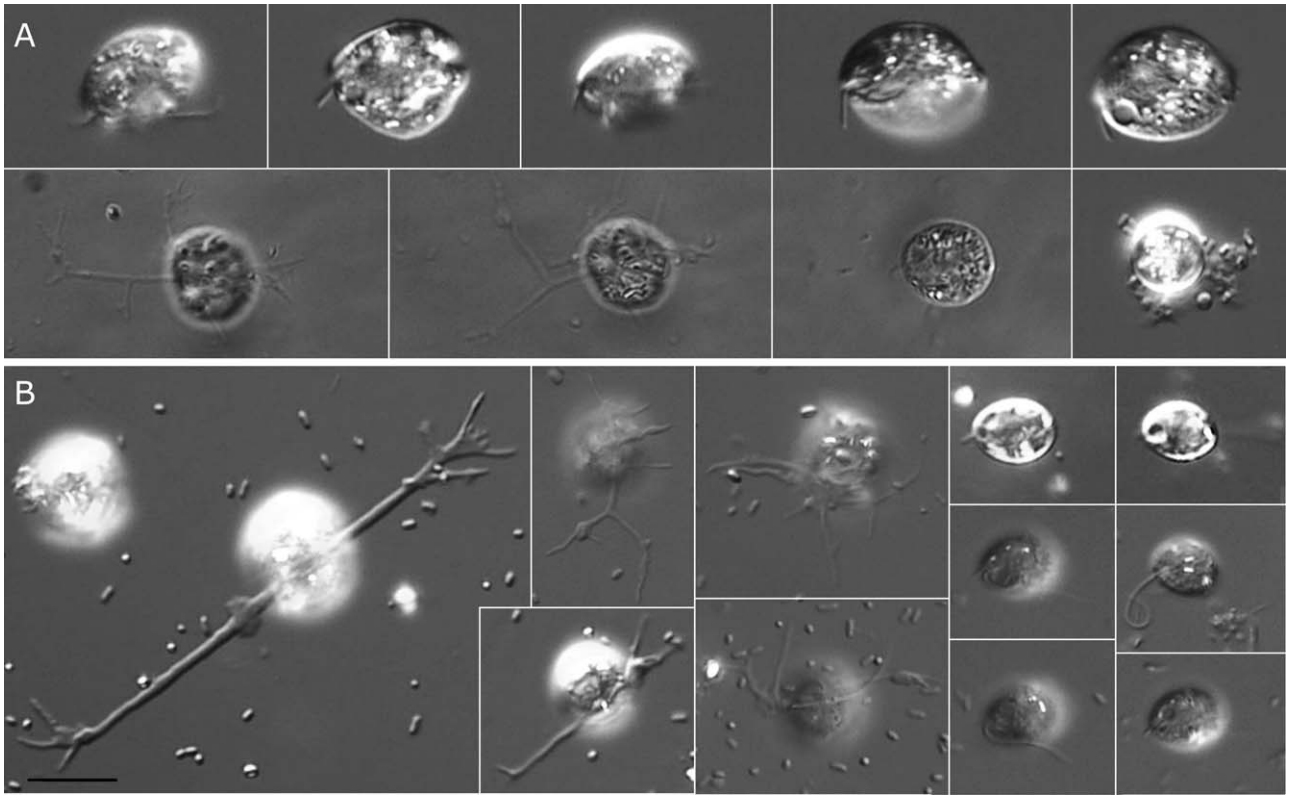


Figure 9. Light micrographs of living cells of thaumatomonad species in their motile phase (short anterior and long posterior flagella) and stationary phase (branching filopodia). **A:** *Allas aff. diplophysa*; **B:** *Thaumatomonas oxoniensis***.** Scale bar 10 μm and DIC both X40 and X60.

Family Thaumatomonadidae Hollande, 1952 (The name Thaumatomastigaceae (Patterson and Zölffel 1991), is illegitimate under ICBN as as it lacked a Latin diagnosis and invalid under both ICBN and ICZN (which we apply to all Cercozoa) as it was a nomenclaturally superfluous synonym (equally invalid is Thaumatomastigidae: Vørs 1992); Zhukov (1971) correctly attributed the family to Hollande but Ekebom et al. (1995/6) wrongly gave Patterson and Zölffel as authority (their use of Thaumatomonadidae was not incorrect, as Patterson et al. (2002) wrongly thought).

Revised diagnosis: Biflagellate cells with ventral pseudopodia, long ventral posterior pointing flagellum used for gliding on surfaces (unlike Peregriniidae) and a much shorter anterior flagellum; siliceous scales formed in vesicles attached to mitochondria cover the rigid cell surface except for a ventral zone that emits pseudopodia for feeding; unlike Peregriniidae do not transform completely into an amoeba; all have oval or triangular two-tiered body scales, with an upper plate bearing species-specific perforations supported at the oval ends or triangle corners by discrete struts (unlike *Gyromitus*); upper plate lacks central cleft with inrolled sides (unlike *Peregrinia*); *Thaumatomastix* only additionally has long spine scales with near-circular or rounded triangular bases. Anterior flagellum naked (*Thaumatomonas*, *Allas*) or with small scales (*Reckertia*, *Thaumatomastix*). **Type genus:** *Thaumatomonas* De Saedeleer, 1931. **Other genera:** *Thaumatomastix*, *Allas*, *Reckertia*, *Hyaloselene*.

Distinguishing *Allas* from *Thaumatomonas*

Nomenclatural confusion surrounds the morphologically similar genera *Allas* Sandon, 1927 and *Thaumatomonas* De Saedeleer, 1931. The main difference between *Al. diplophysa* of Sandon (1927) and *Thaumatomonas lauterborni* of De Saedeleer (1931), who was probably unaware of Sandon's work, is the larger size of *Allas* (10-24 μm not 7-11 μm for the flagellate phase (De Saedeleer 1931); later authors gave 8.3-16.5 μm as the range for the T1 *Ts lauterborni* clone: Mylnikov and Karpov 1993) and the fact that Sandon did not observe syncytia in older cultures. However, the presence or absence of syncytia is insufficient on its own for generic distinction, as in cercomonads some species within the same genus readily make syncytia whereas close relatives do not (Bass et al. 2009b) and there is no reason to think that thaumatomonads differ in this respect. Thus syncytia (not of uniform morphology) were reported in *Ts lauterborni*, *Ts seravini* (Mylnikov and Karpov 1993), and *Ts zhukovi* (Mylnikov and Mylnikov 2003), but not in *Ts coloniensis* (Wylezich et al. 2007). The redrawing of De Saedeleer's *Thaumatomonas lauterborni* in Patterson et al. (2002) is at the wrong scale and made the cell much too big and also the anterior flagellum proportionally far too long (~12 μm instead of the correct 1.8-3.1 μm), thus exaggerating the relatively small differences between it and *Allas* and the *Thaumatomonas* species described here. Thus there are no light microscopic morphological grounds for retaining *Thaumatomonas* as a genus distinct from *Allas*, which agrees with their close similarity in 18S rDNA sequence.

The tight clustering and interspersion of sequences previously published for strains identified as *Allas* and *Thaumatomonas* also calls into question their distinctness (Cavalier-Smith and Chao 2003b; Wylezich et al. 2007). Previously we thought that this interspersion, and the light microscopic differences between *Allas* aff. *diplophysa* and *Thaumatomonas* being not significantly greater than those among previously named species of *Thaumatomonas*, meant that no valid distinction could be made between *Thaumatomonas* and *Allas*, and that the younger name *Thaumatomonas* must be suppressed as a synonym. Supression would require a change to the family but not the ordinal name, and as *Thaumatomonas* is much more often used than *Allas* it would be ideal if it can be avoided. We achieve this by using the novel scale type here discovered in ATCC50365 *Allas diplophysa* (sequenced by Cavalier-Smith and Chao 2003) as a distinguishing feature for *Allas* (Fig. 8). We do not know what kinds of scales were present in the type species of either genus, as both were described before electron microscopy (the 'delicate cuticle' sometimes seen by Sandon (1926) was probably the scale layer). But purely for nomenclatural stability, we now treat ATCC50365 as a reference strain of *Allas* and likewise treat strain T-1 of Mylnikov (scales described by Karpov and Zhukov 1987 and Mylnikov and Karpov 1992) as correctly identified as *Thaumatomonas lauterborni* (the type species) even though its cells were slightly larger and its anterior flagellum slightly shorter than in De Saedeleer (1931). Accepting both avoids having to change the younger name *Thaumatomonas* to *Allas* by using scale morphology to distinguish the genera. Though we do not know for sure that the original *Thaumatomonas lauterborni* even had scales, the possibility that it was a *Protaspa*, some of which are superficially similar, is excluded by its clearly parallel centrioles (De Saedeleer 1931) – divergent in *Protaspa* (Hoppenrath and Leander 2006), making it most likely to be genuinely related to later described *Thaumatomonas* species, all of

which have scales; unfortunately Sandon (1927) did not show whether centrioles are parallel in *Al. diplophysa* or not, as they apparently are in *Al.* aff. *diplophysa*. *Allas* aff. *diplophysa* scales are all oval as in *Thaumatomonas coloniensis* (Wylezich et al. 2007), but unlike them come in two forms; a few are bilaterally symmetric like those of *Thaumatomonas*, but most are a novel type of oval scale with a single dense strut at the middle of one side only (Fig. 8). Thus we retain both genera even though they are very closely related and can be distinguished only by scale ultrastructure or sequences.

Genus *Allas* Sandon, 1927

Allas* aff. *diplophysa Sandon, 1927. Figs 9A and 10 of strain ATCC50365 "*Allas diplophysa"* (1991; light brown sandy soil, Tuscon, AZ, USA; TK Sawyer). **Description:** 18S rDNA sequence, GenBank AF411262, ITS2 rDNA sequence HQ176331. Distinguished from *Thaumatomonas oxoniensis*, *Ts vancouveri* and *Reckertia filosa* by larger cell-size: 16 μm (13-20 μm, our observations, though 10-24 μm in Sandon 1927). In contrast to *Ts oxoniensis* and *R. filosa,* PF is much shorter, usually <1-1X BL, rarely extends beyond cell. AF short, but longer than in *R. filosa* and *Ts oxoniensis.* Motile cell shape oval, flattened, disc-like. Long, branching filose and lamellar pseudopodia produced from beneath stationary cells; project in all directions. Scales mostly oval, usually with 2 (rarely 3) terminal struts at each end and one lateral strut with U-shaped cross section midway on one side; 5-6 lateral perforations on that side and 3-4 on the opposite side (Fig. 8). Some scales lack the lateral strut and then have the same number of holes on each side (3-4) (Fig. 8B arrow). Those with struts may be bilaterally symmetric in overall shape (especially the lower plate, the upper one typically being flatter and less convex on the side away from the strut) but usually bulge out more on the strutted side making the whole shape asymmetric; rarely the bulge may be more pronounced making the scale somewhat triangular (Fig. 8E). Bacterivorous. Anterior cv. Cysts smooth, round, ~10 μm. Freshwater, cultured in Volvic® with grain. **Comments:** The type species, *Allas diplophysa* Sandon 1927 (p. 113, Plate III fig. 15) was originally described as "Sp. epsilon" (Sandon 1924). *Allas diplophysa* was described as "a flattened, more or less oblong organism with rounded ends, rather metabolic and sometimes with ragged pseudopodia especially at the hind end; length 10-24 μm" though usually it is 18 μm, its "width about half the length; two flagella arising at the anterior end of the body, one, slightly longer than the body, being trailed passively behind, and the other which is very short (about 2 to 3 μm long) moving actively. The animal glides rapidly forwards very like *Allantion tachyploon*, but sometimes with a slight vibration owing to the active lashing of the short flagellum." ATCC50365 is close to Sandon's description. We therefore designated it *Al.* aff. *diplophysa* Sandon. We did not make it a neotype as its cell size is smaller (average 16 μm, with an upper limit of 20 μm). Although cell size can depend on culture conditions, the PF of this strain is usually 1X BL and sometimes not seen extending beyond the cell, whereas Sandon's was just over 1X BL. Therefore it is not strictly concordant with Sandon's description. Accepting it as a genuine *Allas* (unlike the other strains previously called *Allas* which are even smaller and thus still less like *Al. diplophysa*) its novel scale type becomes the first clear morphological distinction from *Thaumatomonas*; accordingly, in Figure 15 we renamed as *Thaumatomonas* the other '*Allas*' strains that lack this scale type. We do not know if another sequenced '*Allas'* strain (formerly *Allas* sp. SA) has scales, but its position on the tree makes that likely; from its position, with no contrary evidence, we treat it also as a *Thaumatomonas***.**

Two new *Thaumatomonas* species

Thaumatomonas vancouveri and *oxoniensis* probably have identical 18S rDNA sequences (setting aside a few possible errors in the previously published one), yet have clearly different ITS2 and are morphologically distinct:

Thaumatomonas oxoniensis Bass and Cavalier-Smith sp. n. Fig. 9B. Type strain Hinksey - CCAP 1903/2 (2002; Hinksey Park lake benthos, Oxford, UK; DB). **Diagnosis:** 18S rDNA sequence, GenBank HQ121429; ITS2 rDNA GenBank HQ176328. Cell size 9.5 μm (9–10.5 μm) PF trailing, longer than *Al. diplophysa*: approximately 2X BL, 18.5 μm (17-19 μm); AF little more than a stub, 2 μm (1.5–2.5 μm), shorter than *Al.* aff. *diplophysa*, unlike *R. filosa* moves very little. When motile, cell shape a rigid oval, anterior and posterior ends sometimes acuminate; spherical during stationary phase. Long, branching filose and lamellar pseudopodia emerge from beneath stationary cell, project in all directions. Light sensitive: observation causes stationary cells to become motile. During the transition pseudopodia retract, PF is manipulated, cell rotates and begins gliding. Cell movement swift; glides smoothly with little or no vibration, unlike *Al. diplophysa* Sandon and *R. filosa*. Changes direction fairly frequently. Anterior cv, n in anterior half of cell. Single layer of two-tiered scales, bilaterally symmetric ovals of rather variable structure; (4-)5 terminal struts at each end as in *Ts coloniensis* and four lateral perforations each side of upper plate (not 4-6 as in *Ts coloniensis*). Cysts not observed. Freshwater, bacterivorous, cultured in Volvic® and grain. **Etym.** *oxoniensis* of Oxford, the type locality.

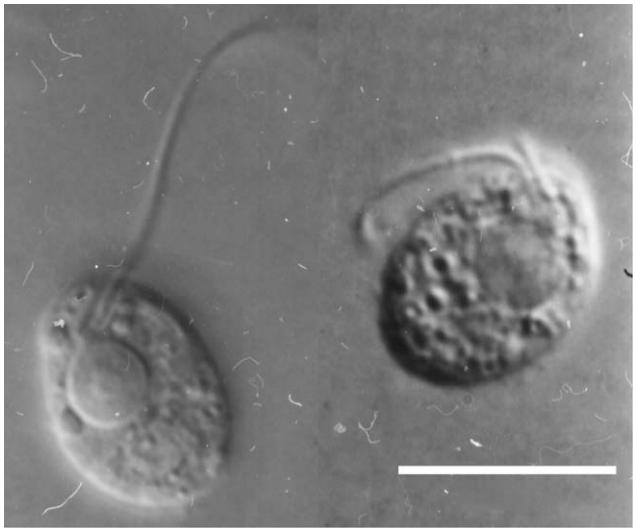


Figure 10. DIC light micrographs of *Thaumatomonas vancouveri* fixed in osmium vapour. Zeiss Axioplan X100 oil immersion NA 1.3 by courtesy of Brian Oates. Scale bar 10 μm.

Thaumatomonas vancouveri Cavalier-Smith and Chao sp. n. Type illustrations Fig. 10. **Diagnosis:** 18S rDNA sequence, GenBank AF411264; ITS2 rDNA GenBank HQ176329; the original strain 3108W2 (compost heap; Vancouver, Canada; isolator Ema Chao, 1996) is dead. Cell size ~8 μm (measured on fixed cells, not live ones as for other species). Motile cell morphologically similar to *Ts oxoniensis*, but with proportionately longer PF (1.2X BL). AF ~1 μm, shorter than in *A. diplophysa*. Large anterior *n* with prominent nucleolus; centrioles long, parallel; contact *n* at their base. No pseudopods observed in life. ITS2 sequence differs from *Tx oxoniensis* in at least 16 nucleotides including two pairs of compensatory base changes and four hemicompensatory substitutions. Oval two-tier scales with broad upper plate supported by about 4 struts at each end and several lateral perforations (all different from *Ts lauterborni*, *seravini* and *zhukovi,* but fairly similar to *oxoniensis* and *coloniensis*). **Etym.** *vancouveri* from Vancouver, BC, the type locality.

Strain 3108W2 was originally identified as *Allas* sp. (Cavalier-Smith and Chao 2003) as its cell shape and flagellar proportions were closer to *Allas diplophysa* than to *Thaumatomonas lauterborni* of De Saedeler (1931) and were even less like *Thaumatomonas seravini,* the only two *Thaumatomonas* species then known, both of which also had plasmodial stages not observed in *Ts vancouveri* or reported for *Allas*.

Electron microscopy (Fig. 11): a pronounced flagellar pocket over 1 μm deep is supported on its sides by numerous cortical microtubules (Fig. 11A). A ventral groove contains numerous filopodia (Fig. 11C, G); we refer to this cavity as a 'groove' for consistency with the terminology for *Thaumatomonas lauterborni* (Karpov and Zhukov 1987), but our few pictures suggest that it may really be a cytopharynx with only a terminal opening rather than groove open along its length. The entire cell surface except for the flagella, flagellar pocket and ventral groove is covered with a layer of oval scales with a double structure in cross section, which resemble those of *Ts coloniensis* (Wylezich et al. 2007) in having lateral perforations in the upper plate, unlike *Ts lauterborni* that does not (Karpov and Zhukov 1987), but are probably at least slightly distinct from all those previously described for thaumatomonads. Scales develop in vesicles attached to the mitochondria, which have tubular cristae (Fig. 11A). The two centrioles are parallel and connected by fibrillar material (Fig. 11A). Dense fibrillar material is attached to the basal two thirds of the centriole and below their proximal end (Fig. 11F), and at some sectioning angles appears as a V-shaped base to the centriole with one arm of the V attached to opposite sides of the centriole (Fig. 11G). The flagellar transition region is long and has a dense proximal plate, which is probably a homologue of the hub-lattice structure of other Cercozoa (Cavalier-Smith et al. 2008a), and a dense distal plate. No scales or hairs were seen on the flagellar membranes.

Untangling muddled *Thaumatomonas* sequence names

Whole mount scale structure was previously used to divide *Thaumatomonas* into four species, two with oval scales (*Ts lauterborni*: Mylnikov and Karpov 1993; *Ts coloniensis*: Wylezich et al. 2007), one with triangular scales (*Ts seravini*: Mylnikov and Karpov 1993), and one having both oval and triangular scales (*Ts zhukovi*: Mylnikov and Karpov 2003). Because we did not use whole mounts we cannot directly compare those of *Ts vancouveri*, but one image in almost surface view (Fig. 11G) shows that they are distinct from those of *Ts lauterborni* in strut pattern, and distinct from the other three in strut pattern and/or shape; they are probably oval scales most like those of *Ts coloniensis*. *Ts vancouveri* groups with *Ts coloniensis* and *Ts lauterborni* on the rDNA tree (Fig. 13) not with ATCC *Ts seravini* that has triangular scales.

Such mapping of scale structure onto the tree was previously hampered by incorrect labelling of all three *Thaumatomonas* 18S rDNA sequences obtained by Ekelund et al. (2004). We were alerted to this mix up by '*Ts seravini*' being in two contradictory places on the tree (Fig. 12) even though only one strain of this species has ever been isolated (Mylnikov and Karpov 2003). The ATCC50636

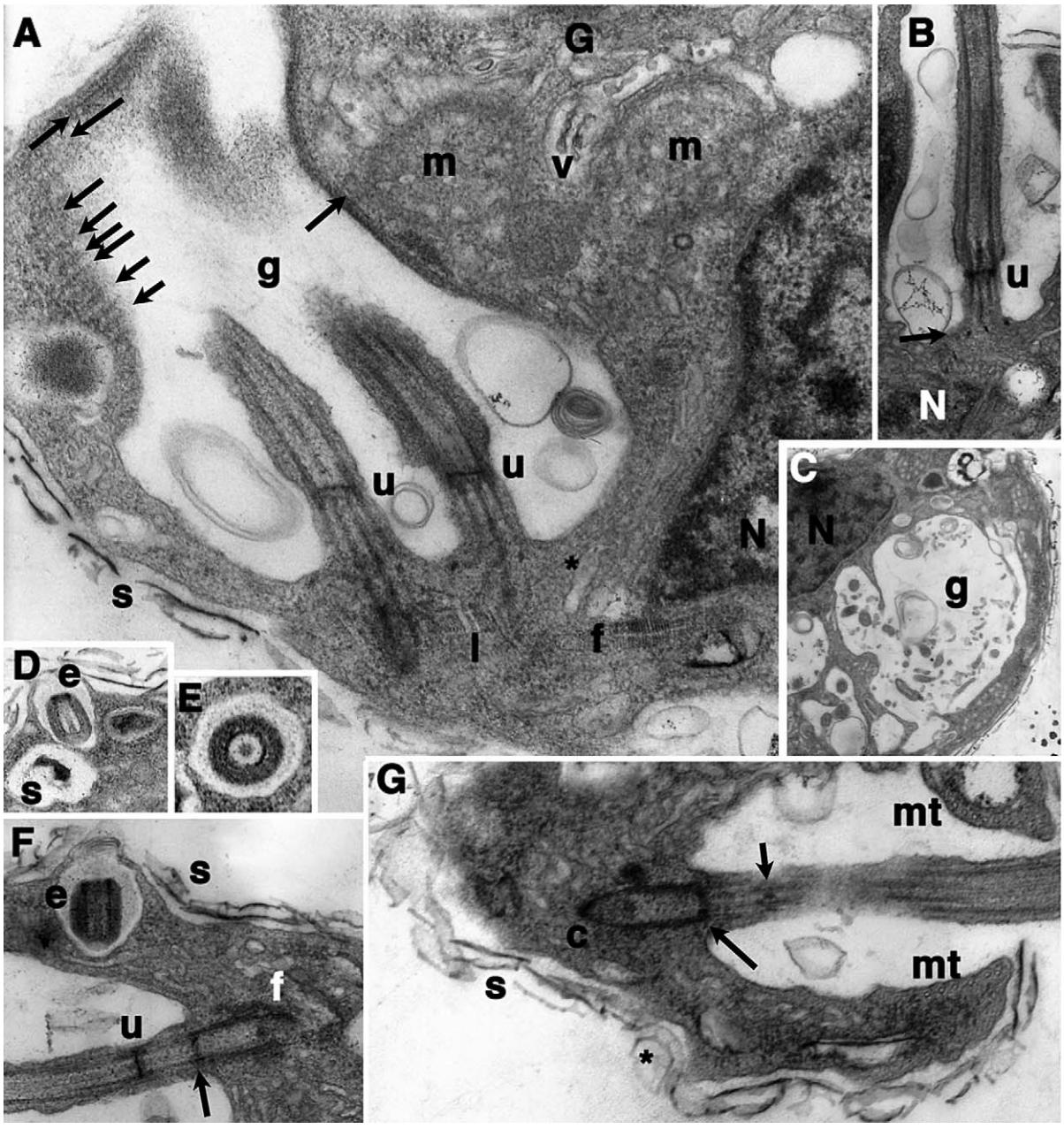


Figure 11. Electron micrographs of *Thaumatomonas vancouveri.* **A:** Longitudinal section through the two flagella and flagellar pocket, showing cortical microtubules (arrows) and the distal flagellar transition region plate (u), the fibrillar linker (l) between the centrioles, a striated fibrillar flagellar root (f), and a flagellar root microtubule (asterisk). N = nucleus, m = mitochondrion with attached developing scale vesicle (v), G = Golgi apparatus. **B:** Longitudinal section through a flagellum showing distal flagellar transition region plate (u), and cross sections through three transitional fibres (arrow). **C:** Cross section through the ventral 'groove'/cytopharynx (g). **D:** Longitudinal section through an extrusome (e) and putative scale vesicle (s). **E:** Transverse section through an extrusome. **F:** Median longitudinal section through flagellum showing distal transition region plate (u), hub-lattice structure (arrow), and dense fibrillar material (f) alongside and at the base of the centriole. **G:** Peripheral longitudinal section of flagellum, showing the hollow cone-like dense fibrillar material (c) at the centriolar base. Large arrow marks the hub-lattice structure and small arrow the edge of the distal flagellar transition region plate. * = scale sectioned almost parallel to its broad face. mt = transverse sections through cortical microtubules near mouth of flagellar pocket.

Ts seravini is derived from the type strain T-2 given by Mynlikov in 1994 to TCS who subsequently deposited it in ATCC; 18S rDNA sequence AF411259 of Cavalier-Smith and Chao (2003) was from that strain; we recently re-obtained it from ATCC and independently from Mylnikov, and have checked microscopically that both versions are still morphologically *Ts seravini*, not *lauterborni* (whose cells are proportionally narrower or more elongated) as Ekelund et al. (2004) wrongly stated. Yoon et al. (2008) re-sequenced 18S rDNA from ATCC50636 and found virtually the same sequence as Cavalier-Smith and Chao (2003: EF455776; the three differences must be errors), which shows that the nearly identical '*Ts lauterborni* T2*'* AY496045 sequence of Ekelund et al. (2004), which differs from the two authentic *Ts seravini* sequences by only 5 nucleotides (but not the same 5 for each) was really from *Ts seravini*; thus its strain designation was correct but the species name wrong. Figure 13 shows this as *Thaumatomonas seravini* 'SCCAP T2' AY496045. Ekelund et al. (2004) do not say where or by whom strains T1-T3 were isolated, but that they 'are kept in the Scandinavian Culture Collection of Algae and Protozoa (SCCAP)'. However, the SCCAP curator informed TCS that they have no record of their being received. Ekelund et al. (2004) wrote that T1 was a soil strain provided by Vickerman (who has no record of sending one, but might have donated one at a meeting) and Ekelund told TCS that T2 and T3 were given to him by Karpov, and came ultimately from Mylnikov, but had not been put in SCCAP. Designations T1-T3 are clearly from Mylnikov, who states that T-1 was his *lauterborni*, T-2 his *seravini* type strain, and T-3 his *zhukovi* type strain. If Ekelund's T2 (AY496045) was indeed T-2 it must have been mislabelled *lauterborni* and have really been *seravini*, which would make it agree with the *seravini* sequences from the other two laboratories and with the distribution of scale structures on the tree. To verify this inference JMS sequenced ITS of both type strain T-2 freshly provided by Mylnikov and ATCC *seravini*; they are identical.

We cannot directly check which of the other two sequences was *lauterborni* as Mylnikov's T-1 is dead, but have relabelled Ekelund's AY496046 as *Thaumatomonas*? *lauterborni* T-1 'SCCAP T1' on the assumption that it was indeed Mylnikov's T-1. Consistently with this JMS (unpub.) has isolated new cultures with scales almost indistinguishable from *Ts lauterborni* (Karpov and Mylnikov 1987) and 18S rDNA sequences placing them in this part of the tree. That leaves Ekelund's AY496044 (originally mislabeled *seravini* T3); it cannot be *Ts zhukovi* T-3, whose 18S rDNA sequence (unpublished, kindly provided by D. Tikhonenkov from Mylnikov's laboratory) is quite different, being instead identical except for one nucleotide to that of South African *Thaumatomonas* sp. SA2 (AF411260); thus the strain number and species name in Ekelund et al. (2004) for AY496044 must both be wrong. We cannot confidently identify it with a named strain so relabel it *Thaumatomonas* sp. 'SCCAP T3' AY496044; however, if the labels T1 and T3 had become interchanged at some stage, it would be *lauterborni* instead and AY496046 would be *Thaumatomonas* sp. as originally labelled. JMS (unpub.) has isolated two new cultures with *lauterborni*-type scales and 18S rDNA sequence almost identical to AY496044, so scale structure is compatible with either identification.

As the very same *Ts seravini* T-2 strain that is now ATCC50636 has been sequenced three times independently in different laboratories all differences between sequences AF411259, EF455776, AY496045 are PCR or sequencing errors: the correct sequence is obtainable by replacing each of the few nucleotides found uniquely in one lab by the consensus from the other two. Re-labeling these sequences correctly (Fig. 12) shows that *Ts lauterborni* falls in the same subclade as *Ts coloniensis, oxoniensis*, and *vancouveri*, which makes sense as all four have only oval scales. By contrast *Ts seravini*, which has only triangular scales, falls in the other main *Thaumatomonas* clade (close to *Ts zhukovi*, not shown). Although this *seravini/zhukovi* clade is sister to *Allas diplophysa* in Figure 12 this is unreliable as 18S rRNA is so conservative that very few nucleotide differences govern this position. In a tree including many more *Thaumatomonas* strains isolated by JMS after this paper was submitted, and using both 18S rDNA and ITS sequences, the two *Thaumatomonas* clades strongly group together, with *Allas diplophysa* as their sister. Thus *Thaumatomonas* as here defined by excluding *Allas* aff. *diplophysa* is probably holophyletic.

Vickerman isolated *Thaumatomonas* sp. (Thau M1) sequenced by Cavalier-Smith and Chao (1997) (U42446) from the moss *Rhytidiadelphus squarrosus* on a lawn in Glasgow, Scotland. It is now dead but Vickerman et al. (1991), Vickerman (1998 fig. 1.4) illustrated it; it had oval strutted scales only (most like those of *Ts lauterborni*) and showed plastogamy like *Ts lauterborni*. It groups in the same subclade as *Ts? lauterborni* and also the sequence of Ekelund et al. (2004) we now call *Ts* sp. 'SCCAP T3' (AY496044); though close

it is apparently not the sister of either and is probably a separate species. Our labelling corrections show that the *lauterborni* scale type is quite widespread in the upper *coloniensis*/*lauterborni* clade of Figure 12, but absent from the lower *seravini*/*zhukovi* clade, which contains all the strains with triangular scales (or a mix of triangular and oval). Thus scale morphology now maps rationally onto the *Thaumatomonas*/*Allas* sequence tree. *Thaumatomonas lauterborni* and *T. coloniensis* with oval scales only are very closely related; *T. seravini* and *zhukovi*, both with triangular scales, are very closely related but more distant from *T. lauterborni*/*seravini*.

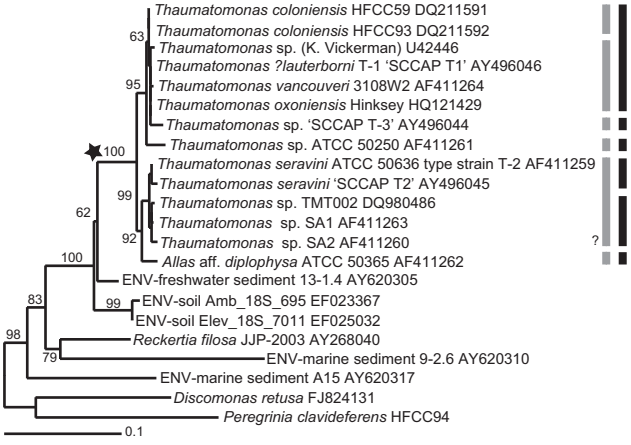


Figure 12. RAxML tree of Thaumatomonadida plus close outgroups. 22 sequences, 1744 18S rDNA positions. Because sequences in the main non-marine *Thaumatomonas* clade (marked with a star) are very similar, differences between some of them may be caused by PCR/sequencing errors, therefore not representing distinct strains. To eliminate such ambiguities, vertical lines to the right of the branch labels join strains with identical sequences in the most variable part of the V4 (grey vertical lines) and the V9 (black lines) regions. Note that the V4 region is not the most variable 18S region in all genotypes. AF411260 is marked by '?' as its V4 sequence differs from the four above it at only one position and is not unambiguously different from them. Thus there may only be seven genuinely different 18S rDNA types in the freshwater *Ts oxoniensis/vancouveri/seravini/lauterborni/coloniensis/Allas* cluster (star). RAxML bootstrap support values are given for the main bifurcations. A Bayesian analysis for the same dataset gave the same topology within the starred clade, with comparable support values. ENV- denotes an environmental clone sequence with the habitat type specified, followed by its sequence identity code. The identity of all three *Thaumatomonas* sequences from Ekelund et al. (2004) was incorrect in that paper and in GenBank (their 'SCCAP' designation is misleading; they are not in that collection); their names and those of several originally misidentified cultures have been corrected on this tree as explained in the text.

A further muddle is the misidentification of ATCC50250 '*Thaumatomastix* sp.'. Thomas Nerad (pers. comm.) has told us that his microscopical examination shows that strain ATCC50250 '*Thaumatomastix*' sp. (isolated from horse dung infusion) was misidentified and is really a *Thaumatomonas*, although no published micrographs exist for it. If it were really a *Thaumatomastix*, its placement within the tight *Thaumatomonas* clade (Cavalier-Smith and Chao 2003) would contradict any sharp distinction between *Thaumatomonas* and *Thaumatomastix*. However, a genuine *Thaumatomastix* branches as sister to *Reckertia* plus *Thaumatomonas*/*Allas* (Scoble, Yabuki, Ishida and Cavalier-Smith in prep.), which confirms that ATCC50250 is not a *Thaumatomastix* and shows that *Thaumatomastix* is phylogenetically distinct from both *Thaumatomonas*/*Allas* and *Reckertia*. We relabelled ATCC50250 accordingly on Figure 12.

Reckertia is a separate genus from *Thaumatomastix*

Wujek et al. (2008) called *Thaumatomastix* 'an unwieldly, artificial conglomeration of forms that produce scales of two morphologies'. When they synonymised *Reckertia* with *Thaumatomastix*, Beech and Moestrup (1986) apparently overlooked that *Thaumatomastix setifera* of Lauterborn (1896, 1899) had spines and no pseudopodia (though *Tx setifera* of Skuja (1939) did have extensive branched ventral pseudopods), whereas *Reckertia* had pseudopodia but no spines (Conrad 1920). Though *Tx salina* and *Reckertia sagittifera* both have scales on the anterior flagellum (Beech and Moestrup 1986; Moestrup 1982) that is insufficient reason to group them together. Because of the absence of spine scales in *Reckertia sagittifera* and *filosa* and because a spiny *Thaumatomastix* sp. does not group with the non-spiny *R. filosa* on our 18S rRNA tree (Scoble et al. in prep.) we treat *Thaumatomastix* and *Reckertia* as distinct genera, thus solving the problem noted by Wujek et al. (2008). Nine nominal species of *Thaumatomastix* lack separate spine scales and are here segregated in the genus *Reckertia*, which is easier to confuse in the light microscope with *Allas* or *Thaumatomonas* than are *Thaumatomastix* sensu stricto, whose spine scales would usually

distinguish them. Most *Thaumatomastix* and *Reckertia* have a relatively short anterior flagellum as in *Allas/Thaumatomonas* (or apparently none: Mikrjukov 2002; Thomsen et al. 1993). In all *Reckertia* species listed below the anterior flagellum bears small scales (Thomsen et al. 1993, 1995, 1997), except that no anterior flagellum was observed in *R. nigeriensis* (Wujek et al. 2008).

Revised diagnosis of *Reckertia* Conrad, 1920: Thaumatomonads with triangular, basket, or irregular two-tier siliceous body scales; separate spine scales absent; PF longer than body, lacks scales; AF much shorter than body, covered in smaller round or oval scales with pointed or rounded ends (AF possibly sometimes absent); pseudopodia in at least two species (*R. sagittifera* and *filosa*); marine except for *R. nigeriensis* (Wujek et al. 2008). **Type species:** *R. sagittifera* Conrad, 1920.

Comment: The new amoeboflagellate *Reckertia filosa* is the first cultured member of the genus and first genuine *Reckertia* of known sequence:

Reckertia filosa Howe, Scoble, and Cavalier-Smith sp. n. Type Figures 13A and 7B. Type strain CCAP 1903/1 (isolation date unknown; Russia; AP Mylnikov; originally identified as *Allas* sp.). **Diagnosis:** Type 18S rDNA sequence, GenBank AY268040 (JJP-2003); ITS2 rDNA GenBank HQ176330; cell size 11.4 μm (11-12.5 μm). PF gliding, 17.8 μm (17-20 μm), 1.75-2X BL, trails; AF stubby, 2.5 μm (2-3.5 μm), constantly beats. Cell rigid; oval, flattened, disc-like; usually slightly wider at posterior than anterior. Long, branching filose and lamellar pseudopodia (contrasting with the broad finger-like pseudopods of *R. sagittifera*: Conrad 1920) emerge beneath stationary cells, project in all directions. Two-tiered triangular scales cover cell surface (Fig. 7B1); these body scales (at their broadest 0.50-0.59 μm) have prominent oval holes set back from each corner, not circular holes as in *R. formosa* (Thomsen et al. 1993), unlike any other named species; unlike in *formosa,* the denser rim of each hole is supported by three dense outward-projecting struts (Fig. 7B5); also unlike in *formosa,* the edge of the scale is homogeneously thin and has an elongated bean- to oval-shaped hole in the upper tier between each corner hole (Figs 7B4 and B5). PF naked; anterior flagellar scales rounded ovals (0.33-0.37 x 0.20-0.22 μm) (Figs 7B1 and B3), not circular as in *R. formosa*, *dysboena* and *spinosa*; unlike in *R. sagittifera* the central linear density of flagellar scales is straight not sinuous. Unlike *Al.* aff. *diplophysa*, and *Ts oxoniensis* and *vancouveri,* has multinucleate phase and thrives in saltwater. Cell movement swift, smooth gliding, slight vibrations caused by movement of AF, as in Sandon's *Al. diplophysa*. Changes of direction fairly frequent. Cultured in artificial seawater with grain; bacterivorous. Anterior cv. Cysts not observed. **Etym.** *filum* L. thread, for its filopodia.

Nine other *Reckertia* species: we here transfer all nine named *Thaumatomastix* that lack separate pointed/spined body scales (the key defining character of the type species *Tx setifera*) in addition to two-tiered plate scales to *Reckertia*. As the single type of body scale of *Tx splendida*, despite having a long central spine, seems not to be homologous with those of *Thaumatomastix* but an independent secondary modification of triangular two-tier plate scales like those of *R. fusiformis*, we include it in the transfers to *Reckertia*, making 11 species of *Reckertia* altogether.

New combinations: *Reckertia spinosa* Cavalier-Smith comb. n. Basionym *Thaumatomastix spinosa* Thomsen et al., 1993

Reckertia dybsoena Cavalier-Smith comb. n. Basionym *Thaumatomastix dybsoena* Thomsen et al., 1993

Reckertia formosa Cavalier-Smith comb. n. Basionym *Thaumatomastix formosa* Thomsen et al., 1993

Reckertia fragilis Cavalier-Smith comb. n. Basionym *Thaumatomastix fragilis* Thomsen et al., 1995

Reckertia fusiformis Cavalier-Smith comb. n. Basionym *Thaumatomastix fusiformis* Thomsen et al., 1995

Reckertia splendida Cavalier-Smith comb. n. Basionym *Thaumatomastix splendida* Thomsen et al., 1995

Reckertia igloolika Cavalier-Smith comb. n. Basionym *Thaumatomastix igloolika* Thomsen and Ikävalko, 1997

Reckertia groenlandica Cavalier-Smith comb. n. Basionym *Thaumatomastix groenlandica* Thomsen and Ikävalko, 1997

Reckertia nigeriensis Scoble and Cavalier-Smith comb. n. Basionym *Thaumatomastix nigeriensis* Wujek, Pershon and Kadiri, 2008.

Revised diagnosis of *Thaumatomastix* Lauterborn, 1899: Unicellular, non-colonial, biflagellate thaumatomonads with long spine scales (no flagella observed in *Tx patelliformis* or *tauryanini*, two of three species known only from very few specimens on electron microscope whole mounts); triangular or oval two-tiered body scales also present (except type species ultrastructure unknown, so no evidence for plate scales). PF longer than body; after emerging from apical flagellar pocket bent backwards, used for gliding on surfaces; AF rather short except in *Tx setifera* (in *Tx salina* bears small elongated oval flagellar scales, often somewhat pointed at ends); pseudopodia reported only in *Tx setifera* and *tripus*. Phagotrophs, some algivorous. Type species *Tx setifera* Lauterborn, 1899.

Comment: This restricts *Thaumatomastix* to six species (four marine, two freshwater) with spine scales (at least two more unnamed ones exist with unique oval scales: Thomsen et al. 1993): *Tx setifera, salina*, *tripus*, which all have ventral grooves and glide on their long smooth posterior flagellum (Beech and Moestrup 1976), and *Tx patelliformis*, *bipartita* and *tauryanini*, which have never been seen alive and whose locomotory mode is unknown. Transfer of the biflagellate freshwater colonial *Chrysophaerella triangulata* (Balanov 1980) to *Thaumatomastix* (Beech and Moestrup 1976) was wrong; Balonov called its longer anterior flagellum pinnate, feathery or plumose (in both Latin and Russian; i.e. evidently a hairy or tinsel heterokont flagellum) and described a profoundly dissected single chromatophore and stigma, but no groove. All four characters decisively contradict assignment to *Thaumatomastix*; its lateral flagellum is much shorter (opposite to *Thaumatomastix*) and Balanov observed neither gliding nor pseudopods.

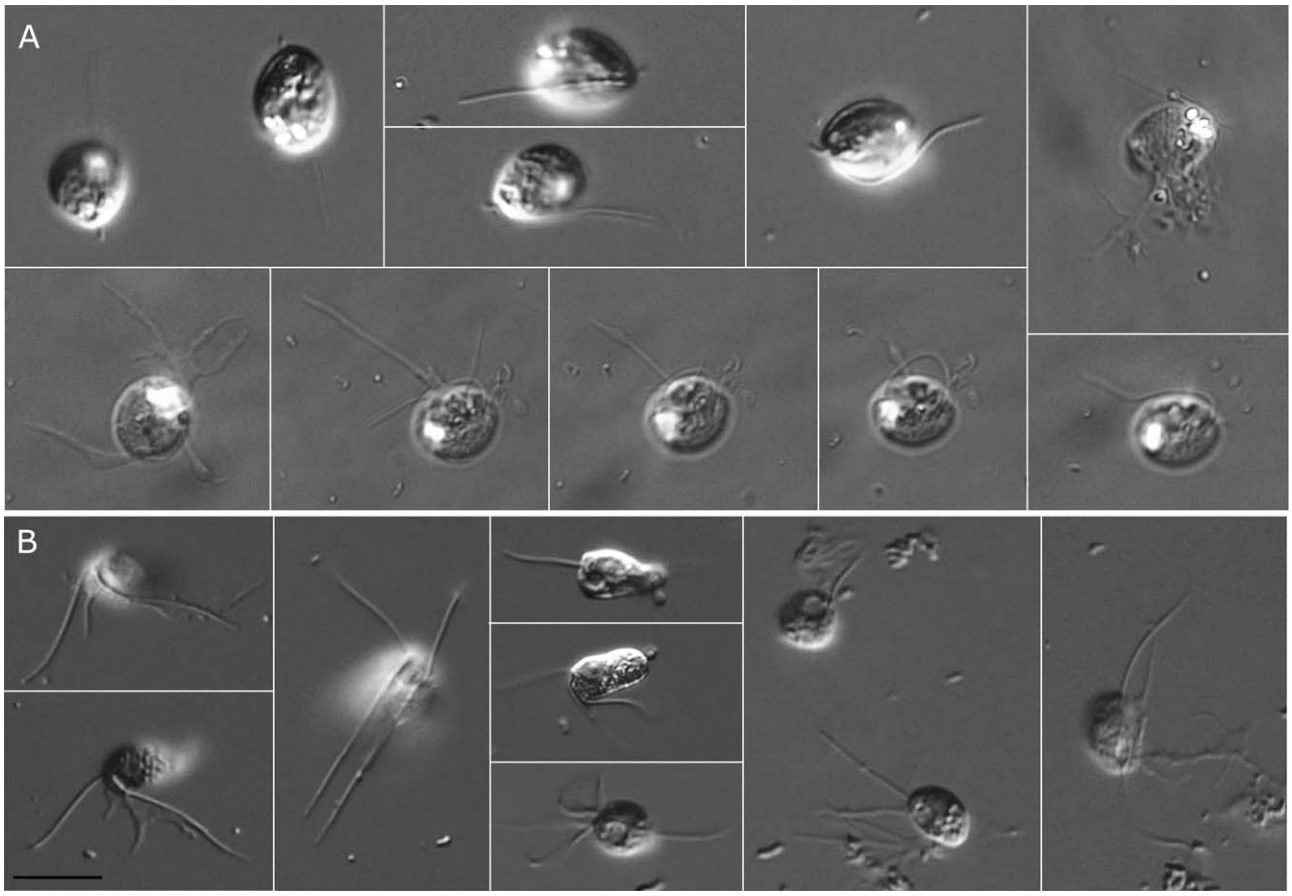


Figure 13. Light micrographs of living cells of **A:** *Reckertia filosa* in its motile and stationary phases; **B:** *Nudifila producta* including a dividing cell. Scale bar 10 μm and DIC both X40 and X60 for both.

He studied body scales and flagella by electron microscopy (reporting no AF scales); spine scales have a basal bobbin-like structure more similar to that of *Chrysosphaerella brevispina* Beech and Moestrup (1976) than the less symmetric one of C. *longispina*, with a much broader distal disc than the narrow distal discs of *Tx salina*, *tripus*, *bipartita* and *T. patelliformis* (Beech and Moestrup 1976), or *Tx tauryanini* (Mikrjukov 2002). As there are nine unambiguous differences from *Thaumatomastix, C. triangulata* was clearly a synurid chrysomonad with novel triangular plate scales as well as spine scales, just as Balonov thought; its coloniality and basal spine structure both make it a *Chrysosphaerella* not a *Spiniferomonas*.

Thaumatomonadidae now include five genera: *Thaumatomastix* Lauterborn, 1899; *Reckertia* Conrad, 1920; *Allas* Sandon, 1927; *Thaumatomonas* De Saedeleer, 1931; *Hyaloselene* (Skuja, 1956). *Rhizaspis* and *Protaspa*, formerly lumped with them (Patterson and Zölffel 1991), are now in separate families in Cryomonadida within Thecofilosea (see below). *Hyaloselene* (including *Synoikomonas*) has been assigned to 'Thaumatomastigaceae' (Patterson and Zölffel 1991), but as there is no evidence that it had scales it might be a protaspid instead (though it is freshwater and most protaspids are marine). Against that, *Hyaloselene* has parallel centrioles as in all Thaumatomonadida and Spongomonadida, not divergent ones as in Cryomonadida, so is probably a thaumatomonad, not a protaspid; as it has a trailing flagellum and subapical flagellar pocket (both unlike Peregriniidae) we place it in Thaumatomonadidae. Our exclusion of *Gyromitus* Skuja, 1939, formerly in the invalid Thaumatomastigaceae (Patterson and Zölffel 1991) and earlier still entirely misclassified within green algae (Skuja 1939), makes Thaumatomonadidae a homogeneous family of rigid gliding thaumatomonads, in contrast to the swimming or amoeboidly creeping amoeboflagellate Peregriniidae.

New Order Marimonadida Cavalier-Smith and Bass. **Diagnosis:** non-scaly and non-thecate marine zooflagellates.

Biflagellate swimming cells (*Pseudopirsonia*: diatom parasites) or interstitial gliding zooflagellates with somewhat deformable, semi-rigid pellicle underlain by muciferous bodies and four posterior flagella associated with ventral cleft (*Auranticordis*), or amoeboflagellates with two gliding posterior flagella and a non-flagellate feeding stage with broad lobose fan like pseudopods (*Rhabdamoeba*). Differ from Euglyphida by the absence of silica scales and presence of flagella, and from thaumatomonads (the only other gliding imbricates) by absence of scales. **Etym.** *mare* L. sea, *monas* Gk unit, because all known are marine zooflagellates. Includes *Pseudopirsonia* Kühn et al., 2004; *Auranticordis* Chantangsi et al., 2008, and *Rhabdamoeba* Dunkerley, 1921.

Comment: The name Auranticordida (invalid as a taxon, being rankless and devoid of diagnosis) was used to label NC1 (i.e. *Auranticordis*, *Pseudopirsonia* and two environmental sequences) on a tree (Chantangsi et al. 2008), but none of these taxa was previously assigned to an order. Their distinctive characters and marked differences from Euglyphida, Thaumatomonadida, and Spongomonadida merit ordinal recognition irrespective of whether they are holophyletic sisters of euglyphids, as weakly shown on Figure 1, or paraphyletic ancestors of euglyphids. *Rhabdamoeba* was omitted from Lee et al. (2002, pp 1302-1328).

Incertae sedis within Imbricatea

New family Nudifilidae Cavalier-Smith and **new genus *Nudifila*** Howe and Cavalier-Smith. **Diagnosis:** soft-bodied heterotrophic biflagellates with two prominent flagella, forming flattened and/or filose, branching, and reticulose pseudopodia that project from all around cell. **Etym.** *nudus* L. naked, because cell is a naked, not a testate amoeba; *filum* L. thread, for its filopodia. **Type species:** *Nudifila producta.*

Nudifila producta Howe and Cavalier-Smith sp. n. Fig. 13B. Type strain CS-4a, CCAP 1911/1 (found contaminating an aged culture of *Cercomonas plasmodialis* CS-4 isolated by AP Mylnikov in 1980 from soil, Borok, Russia). **Diagnosis:** 18S rDNA sequence, GenBank HQ121434; cell size 9.3 μm (5–15 μm); AF 1–1.5X BL; PF 2–2.5X BL. PF not always associated with the cell along its length. AF and PF both emerge anteriorly from slight sub-apical depression. Cell shape round, very metabolic, usually somewhat irregular. Web-like filose, branching and reticulose pseudopodia usually project all around cell. Almost always with bulbous, finger-like and lamellar pseudopodia. Cytoplasmic tail usual: filose, finger-like or lamellar. Cell movement slow creeping, usually in contact with substratum; projects and retracts pseudopodia during progress. Freshwater; cultured in Volvic® and grain; bacterivorous. Large cv in anterior half of cell. N usually central, adjacent and posterior to cv. Cell fission longitudinal, splits right to left (Fig. 13B). Cysts not observed. **Etym.** *productus* L. stretched out, extended; like its pseudopodia.

Nudifila producta groups weakly with environmental DNA NC1 and 2 (Bass and Cavalier-Smith 2004), *Clautriavia*, and Marimonadida. Our unpublished environmental DNA data show that this part of the cercozoan tree is extremely diverse with many unidentified and relatively deeply-branching lineages. These lineages reveal only a very small proportion of an unappreciated imbricate diversity as there are many unidentified environmental DNAs in this region of the tree (most not shown).

Order Euglyphida Copeland, 1956, emend. Cavalier-Smith, 1997

New family Ovulinatidae Cavalier-Smith. **Diagnosis:** filose amoebae with ovoid organic test, lacking scales or mineral particles, aperture round to oval; pseudopodia hyaline, sometimes branched, long, tapering to a point, arising directly from aperture or from web of hyaline cytoplasm emergent from it. Differs from its sister family Paulinellidae by lacking silica scales or plastid-like enslaved cyanobacterium. Type genus: *Ovulinata* Anderson, Rogerson and Hannah, 1997. **Comment:** This new family is essential because our trees show that *Ovulinata* was previously wrongly classified in Pseudodifflugiidae (Thecofilosea) and must be transferred to Imbricatea and Euglyphida. It differs so radically from *Paulinella* that it cannot be included in the same family.

Genus *Ovulinata* (=*Ovulina* Anderson, Rogerson and Hannah, 1996**)** Anderson, Rogerson and Hannah, 1997. Type strain CCAP 1554/1 (1993; seawater from Ballochmartin Bay, Isle of Cumbrae, Scotland, UK; A. Rogerson). We add to its morphological description the first DIC micrographs (Fig. 14), which confirm its identity and serve as vouchers for our 18S sequence of the type strain (GenBank HQ121432).

Our phylogenetic analysis shows that *Ovulinata parva* was previously incorrectly classified in Pseudodifflugiidae. Yoon et al. (2009) recently revealed a novel marine clade that is sister to *Paulinella* (their figure 2A). *Ovulinata* was not included in their analysis; however, these environmental sequences (EF526891, FJ456918, AB275059, AB275100; not shown in Fig. 1) are close relatives of *Ovulinata parva.* Neither electron microscopy by Anderson et al. (1996) nor our DIC studies detected silica scales on *Ovulinata parva*, making it the only known euglyphid testate amoeba lacking them. A second *Ovulinata* strain isolated in 2005 from a marine environment (Ashiya-hama, Ashiya Hyogo, Japan) by A. Yabuki (available from culture collection NIES-2377 (strain YPF501)) was identified as *Ovulinata parva* Anderson, Rogerson and Hannah, 1996. As its 18S rDNA sequence (GenBank AB453002) differs significantly from ours for the type strain, the Japanese strain is probably a second, undescribed *Ovulinata* species.

Discussion

Our numerous new cultures and sequences have yielded the most comprehensive phylogenetic analysis of Cercozoa yet, which clarifies many aspects of evolution in this most diverse phylum:

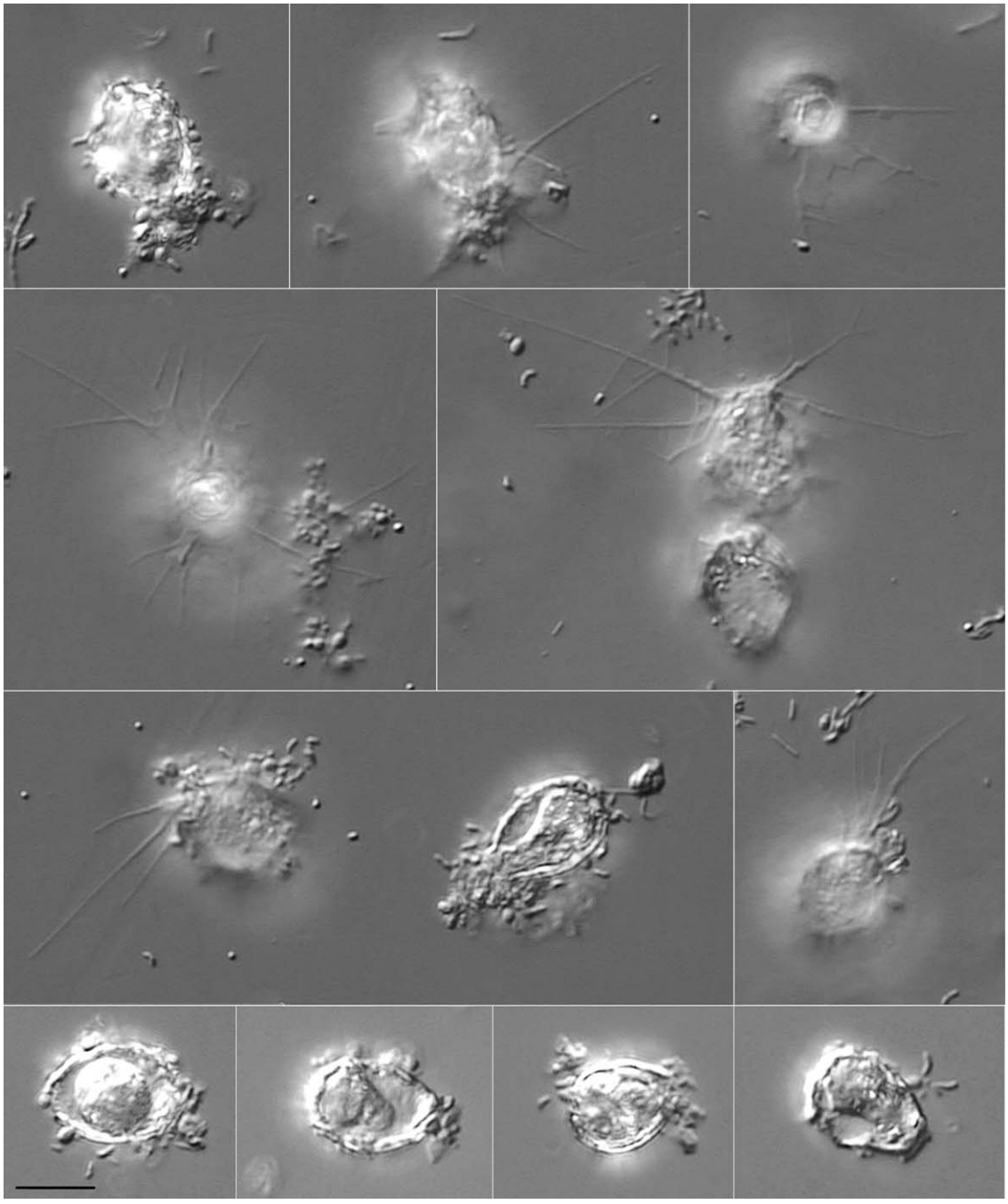


Figure 14. Light micrographs of living *Ovulinata parva* type strain (DIC X40). Scale bar 10 μm.

Tremulida, the Earliest Diverging Gliding Clade of Novel Phenotype

Our discovery that the novel freshwater *Tremula longifila* belongs to an early diverging lineage previously known only from environmental DNA sequences (NC11 of Bass et al. 2009a) reveals an organism in this clade for the first time. It is especially interesting that it is a novel phenotype for Cercozoa: a zooflagellate that glides on both of its exceptionally long flagella, which are simultaneously in contact with the substratum. Among protists this phenotype of gliding on both flagella was known previously only for the marine *Glissandra innuerende* (Patterson and Simpson 1996) and, if our interpetation of his description is correct, for

Ta (=*C.*) *vibrans* of Sandon (1927). The environmental sequences grouping with *Tremula* are also from soil, so may represent similar flagellates. It is unlikely that either is *G. innuerende* because identical zooflagellate genotypes almost never inhabit both soil and marine sediments; though one or other might be another species of that genus, *Glissandra* is probably a more distantly related cercozoan (less likely in another phylum).

As *Tremula* has the filosan rDNA signature sequence discussed before (Bass et al. 2009a), it is the deepest known filosan lineage; previously, because of uncertainty of rooting the Cercozoa and because the NC11 environmental sequences excluded that region of the molecule, it was unclear whether NC11 was a deep branching filosan or deep endomyxan clade. That uncertainty still applies to NC10 and NC12 (which on Fig. 1 branch together but not with Tremulida). Both NC10 and NC12 lack the 'filosan' signature; discovering their morphology is now crucial for determining whether they are Filosa or Endomyxa and clarifying the ancestral phenotype for Cercozoa. Even now, showing that tremulids are Filosa is important for two reasons. It shows for the first time that gliding phenotypes extend to the very base of known Filosa; previously the three deepest branching filosan groups were Chlorarachnea, Metromonadida/Metopiida, and Granofilosea, of which only Metromonadida/Metopiida are gliders, but as their relative branching order was unclear the possibility existed that Granofilosea and/or Chlorarachnea might branch more deeply than any gliding group. Now it is clear that the deepest divergence so far within Filosa is between Tremulida that glide on both flagella and a huge clade comprising Granofilosea, Chlorarachnea, and Monadofilosa (comprising all four classes that contain gliders on the posterior flagellum only: Metromonadea, Sarcomonadea, Thecofilosea, Imbricatea, which branch in that order on our tree). The second point to note is that, even with the characterization here of *Tremula* and three other highly divergent gliding genera (*Micrometopion*, *Nudifila, Reckertia*) and of five recently described novel gliding genera from marine sandy interstitial habitats (Chantangsi et al. 2008; Chantangsi and Leander 2010a,b), all gliding Cercozoa belong to Filosa, none to Endomyxa.

Reticulopodia or filopodia were not seen in *Tremula*, but because one or other occurs in most other cercozoan clades, this may be a derived loss in *Tremula*. The position of insertion of the flagella is another likely derived character: unlike in most other flagellated Cercozoa, both flagella of *Tremula longifila* do not insert close together at the anterior of the cell. Most likely the two centrioles are adjacent as in other Cercozoa, but with the proximal part of the posterior axoneme intracellular as in *Ta vibrans*. Thin-section electron microscopy of *Tremula* is important to test this and determine its cytoskeletal organization; this would allow inference of the ancestral intracellular phenotype for Filosa.

Expansion of Metromonadea

The phenotypic similarity of *Micrometopion nutans* and *Metopion fluens* despite their extreme genetic distance provides yet another example of morphotypes containing many genetically diverse lineages (Bass et al. 2009b; Boenigk et al. 2005; Howe et al. 2009). As in the previously taxonomically most comprehensive tree (Bass et al. 2009a), our analyses weakly but consistently group together the two predatory, non-amoeboid gliding zooflagellates *Metromonas* and *Metopion.* However the position of the highly diverging *Micrometopion* sequence is unstable. It was never sister to the morphologically similar *Metopion*; either it was deep within the clade that includes both *Metopion* and *Metromonas* or on its own as sister to all Filosa but tremulids, or even with Granofilosea in some taxon samples. The latter positions could be long-branch artefacts to which fast evolving singleton sequences like *Micrometopion* are particularly prone. In view of this and preliminary 28S rDNA sequence evidence that *Micrometopion* is related to *Metromonas*, we place *Micrometopion* in Metromonadea. The ultrastructural phenotypes of *Metopion* (Mylnikov et al. 1999) and *Metromonas* (Mylnikov and Mylnikov 2008) are similar. Both strains, unusually for Cercozoa, and specifically unlike Sarcomonadea, have similar dense extracellular material outside the plasma membrane. In *Metopion* this investment is double, with filaments projecting from the outer layer, and it extends over the flagellar membrane, unlike in any other Cercozoa. In *Metromonas* the layer is single, resembling the inner layer of *Metopion*, but we do not know if it also covers the flagellum. Both genera have very similar highly elongated extrusomes, entirely different from any in Sarcomonadea where they are short ellipsoids, often spherical. Elongated extrusomes occur in Cryomonadida (Hoppenrath and Leander 2006a) and can be inferred from their extruded forms in Ventricleftida (Chantangsi and Leander 2010b), but as those orders belong to Thecofilosea this may be convergence. In the past Metopiida have been either included in Sarcomonadea (Cavalier-Smith and Chao 2003b; Howe et al.

2009) or excluded (Bass and Cavalier-Smith 2004; Bass et al. 2005; Cavalier-Smith et al. 2009).

As the grouping of *Metopion* and *Metromonas* on our trees and those of Bass et al. (2009a), albeit weak, is consistent with ultrastructural resemblances now apparent for both genera (Mylnikov et al. 1999; Mylnikov and Mylnikov 2008), which imply a closer mutual relationship than either has to Sarcomonadea (i.e. Cercomonadida, Pansomonadida, Glissomonadida), we now place Metopiida with Metromonadida in class Metromonadea. It is noteworthy also that both metromonadean orders are currently exclusively marine and comprise only gliding flagellates that are predators on other protozoa. By contrast Sarcomonadea are almost exclusively freshwater and predators on bacteria, and all three sarcomonad orders have amoeboid or pseudopodial propensities, especially strong in Cercomonadida. Metromonadea as here revised contrast strongly in external morphology, ultrastructure, locomotory, and feeding behaviour with sarcomonads, but are internally cohesive in all these respects. Thus this taxonomic revision makes sense from all viewpoints. Although we suspect that Metromonadea as revised are probably holophyletic, multigene trees are needed to test this and also whether Sarcomonadea are paraphyletic (as on most 18S rDNA trees including our present ones) or holophyletic; for neither class is current evidence against holophyly compelling.

The relative branching order of Metromonadea, Chlorarachnea, and Granofilosea remains unclear; our trees weakly support previous evidence that they all diverged before Sarcomonadea, Thecofilosea and Imbricatea; and that Monadofilosa are holophyletic with Granofilosea their sisters. As NC5 and NC6 branch in this region of the tree they might belong to Metromonadea; if like other metromonads they eat eukaryotes, this would explain why they have escaped our culturing efforts that have focused on bacterivores.

Minimassisteria: a New Granofilosean

Granofilosea comprise naked amoeboflagellates or non-flagellates with granular filopodia or axopodia for feeding (Bass et al. 2009a). We have discovered a new marine genus *Minimassisteria*. Like *Massisteria* it has a distinct flagellate phase with two flagella, but unlike *Massisteria* also has a crawling phase with a thick anterior pseudopodium that pulls it forward. Its discovery defines the large marine environmental DNA clade to which it and *Massisteria* belong as Massisteriidae. Our tree shows each of the four sequenced orders of Granofilosea as holophyletic, with Limnofilida diverging first, but emphasizes that many granofilosean environmental DNA subclades still lack known phenotypes.

Sarcomonadea

Now that Metopiida is more firmly excluded, Sarcomonadea comprise only Cercomonadida, Pansomonadida, and Glissomonadida. A sister relationship between Pansomonadida and Glissomonadida noted in other phylogenies (Bass et al. 2009a; Cavalier-Smith et al. 2009; Howe et al. 2009; Vickerman et al. 2005) has low support in Fig. 1. This clade is morphologically uniform with a dense distal plate in the flagellar transitional region, unlike Cercomonadida, which also differ greatly in flagellar root structure, in having a shorter transition region, and in being more generally amoeboid. As before, Glissomonadida and Pansomonadida are each holophyletic. As in previous trees, clades A and B of Cercomonadida may weakly group together (Fig. 1), or sometimes not, when the long-branch clade B may branch more deeply (Fig. S2); probably a long-branch artefact, which might even be causing Cercomonadida as a whole to branch too deeply; single gene trees cannot exclude the possibility that Sarcomonadea are holophyletic. Superfamily Sainouroidea was excluded from the present analysis because their extreme branch-lengths on rDNA trees prevent accurate placement (Cavalier-Smith et al. 2009); they may be structurally highly divergent relatives of glissomonads.

Our two new sarcomonads significantly expand their known diversity. Until we discovered *Neoheteromita tolerans*, glissomonads were thought to be restricted to soil and freshwater (Howe et al. 2009). Morphological and behavioural characteristics of *Neoheteromita tolerans* are remarkably like those of soil and freshwater species, despite its adaptation to life in saltwater. Its nesting within freshwater glissomonads shows that at least one lineage can live in marine environments. Its 18S rDNA sequence differs only about 0.5% from AH48p78p118t19, an environmental sequence from soil at Wytham, near Oxford and it is the closest cultured relative of the type species, *N. globosa*. Although numerous protist groups contain marine and non-marine clades, this lineage is unusual in having physiologically adapted to a marine environment without major genetic divergence from non-marine lineages.

Our new *Paracercomonas*, *P. baikali,* is closely related to *Paracercomonas* 'Tempisque' from Costa Rica and *Paracercomonas* sp. Panama 83, both

now dead, belonging to the very long-branch, basal *Paracercomonas* group B2 (Karpov et al. 2006). This group is rarely detected in culture; our present trees, like that of Bass et al. (2009b), suggest that B2 may be paraphyletic and not a clade as in Karpov et al. (2006) since the facultative anaerobe, *P. anaerobica*, branches more deeply than *P. baikali* or clade B1. Comparing *P. baikali* and *anaerobica* might clarify the origin of anaerobiosis in the latter, the only known anaerobic cercozoan.

Since the original strain of the pansomonad *Aurigamonas solis* is dead, our new strain of *As solis* will enable study of its unique haptopodia. This and the availability now of cultures of two *Agitata* species (strongly sisters on our tree, but not trivially close) will facilitate multigene and other studies of Pansomonadida.

Expansion of Class Imbricatea

Originally Imbricatea were restricted to Euglyphida and Thaumatomonadida, which share the character of often imbricate silica scales (Cavalier-Smith and Chao 2003b). Bass and Cavalier-Smith (2004) discovered several novel clades (NC1-3; also others within basal group T) of marine environmental DNA that are sister to one or other of these taxa. They also clarified an earlier confusion about *Spongomonas*, providing the first evidence that Spongomonadida, which lack silica scales, may be related to Thaumatomonadida and several environmental clades. Since then several of these environmental clades have been partially associated with phenotypes, either from cultured material or single cells collected from nature and subjected to rDNA sequencing and microscopy. Thus NC1 includes the marine diatom parasite *Pseudopirsonia* (Kühn et al. 2004) and the large grooved gliding marine interstitial zooflagellate *Auranticordis* (Chantangsi et al. 2008). The position of NC2 and NC3 has been less constant. On the original distance tree they grouped weakly with spongomonads (Bass and Cavalier-Smith 2004) but Bayesian analysis grouped them together as sister to Euglyphida and NC1 (Cavalier-Smith et al. 2009). Our present analyses place NC3 as sister to Euglyphida, and NC2 quite strongly as sister to the gliding zooflagellate *Clautriavia* and this clade weakly as sister to *Nudifila*. Marimonadida might also prove to include *Clautriavia* as these together form a well-supported clade in the tree of Chantangsi and Leander (2010a), with that clade a weak sister to Euglyphida (as in Fig. 1); though on some of our present trees they branch as sister to Marimonadida plus Euglyphida instead, there is no bootstrap support for either position.

We include the peculiar marine *Rhabdamoeba*, which alternates between a non-feeding flagellate with two mutually adherent posterior gliding flagella and a feeding non-flagellate amoeba, in Marimonadida because *Rhabdamoeba* and *Auranticordis* are the only known protists with two or four mutually adhering posterior gliding flagella, and *Rhabdamoeba* previously had no likely relatives and was totally unclassified (Rogerson et al. 1998). Its ultrastructure with tubular cristae and well-developed Golgi is consistent with this assignment. The fact that its feeding pseudopodia are very broad is no obstacle, as a mixture of flagellates with broad flat pseudopods and filopodia is long established in the other major order of naked cercozoan flagellates, Cercomonadida, which by contrast to the exclusively marine marimonads appear to be solely terrestrial or freshwater. Marimonads are very different and cannot to be confused with cercomonads. The robustness of the grouping of parasitic *Pseudopirsonia* and *Auranticordis* leaves little doubt that the parasite evolved from a phagotrophic gliding flagellate ancestor by abandoning gliding, making the gliding phenotype ancestral for Marimonadida. As one forward and one backward pointing gliding flagellum was also ancestral for Thaumatomonadida, and arguably for the outgroup Thecofilosea, it follows that Spongomonadida (*Spongomonas*, *Rhipidodendron*) reoriented their posterior flagellum when abandoning gliding and becoming sessile bacterivores with vibratile parallel highly acronematic flagella for catching food. The feeding trophosome of *Pseudopirsonia* (Kühn et al. 2004) has some similarity to the large feeding pseudopod of *Rhabdamoeba* (Rogerson et al. 1998), suggesting they might be related.

Our demonstration that the testate amoeba *Ovulinata*, which lacks scales, is sister to *Paulinella*, which has them, and that this clade in turn is robustly sister to *Cyphoderia*, which also has them, shows that scales were lost once within Euglyphida, in an ancestor of *Ovulinata*. Our discovery that *Ovulinata* is much more closely related to *Paulinella* than are any other heterotrophic euglyphids provides for the first time a close heterotrophic outgroup to the photosynthetic *Paulinella*, now available in culture for researchers who might wish to use comparative genomics to study the changes that took place during the conversion of a cyanobacterium into a chloroplast-like organelle, independently of the primary symbio-

genesis that created Plantae (Nakayama and Ishida 2009; Nowack et al. 2008; Yoon et al. 2009).

Determining the ultrastructure of *Nudifila* is important for clarifying imbricate evolution; preliminary examination shows it lacks scales. If the Figure 1 topology is correct only two additional losses of scales (in the ancestors of Marimonadida and of Spongomonadida), apart from that we demonstrated for *Ovulinata*, need be postulated for the tree to be compatible with the idea that the common ancestor of euglyphids and thaumatomonads had scales (Cavalier-Smith and Chao 2003b); if the supplementary figure topology were correct three losses would be needed. The two-tier structure that is probably ancestral for thaumatomonad scales (unless *Gyromitus* which has a different type diverged before *Peregrinia*) is never seen in euglyphids, so it must have arisen after they diverged; whether independently from a naked ancestor similar to *Nudifila* or from a common ancestral single-tier siliceous scale might be clarified by discovering the phenotype of other deep-branching environmental clades of imbricates.

Spongomonads and thaumatomonads, which are closer to each other on our tree (and that of Cavalier-Smith et al. 2009) than either is to any other ultrastructurally well characterised flagellates, have a rather similar flagellar pouch supported at its rim by two or more microtubules, and enclosing the two subparallel centrioles (Hibberd 1976; Karpov 1987; Karpov 1993; Karpov and Zhukov 1987); these three shared derived characters point to a fairly close evolutionary relationship between spongomonads and thaumatomonads, despite the latter's gliding on a posterior flagellum, scaliness, and having pseudopodia contrasting with the two parallel non-gliding flagella of the non-pseudopodial non-scaly spongomonads. We therefore now place Spongomonadida also within Imbricatea; formerly it was in a class on its own (Cavalier-Smith 2000; Cavalier-Smith and Chao 2003b) because its true affinities were obscured by the '*Spongomonas*' sequence on the trees in those papers actually being from *Metromonas*. Now that we have sequences from three independent *Spongomonas* isolates, two with typical light microscope morphology of two parallel strongly acronematic flagella and the third with ultrastructural characters of *Spongomonas* (as well as two independent mutually corroborating *Metromonas* sequences), we are confident that the branch labelled Spongomonadida on Figure 1 really represents *Spongomonas* and that its placement in Amoebozoa (Adl et al. 2005) is mistaken.

Given that Marimonadida and the *Nudifila*/*Clautriavia* clade also lack scales, their absence in Spongomonadida is no obstacle to placing spongomonads in Imbricatea. Either scales were lost by these three groups or they evolved independently in Thaumatomonadida and Euglyphida. The likelihood that scales were of great antiquity within imbricates is increased by our discovery of the scaly filose amoeba *Peregrinia*, which groups not with the silica-scaled euglyphids but (weakly) as a very deep sister to Thaumatomonadidae. The broad resemblances between the scales of *Peregrinia* and Thaumatomonadidae indicate that scales may be even more ancient in the thaumatomonad lineage than in euglyphids. We must determine whether scales are present in *Discomonas*, the sister (with environmental sequence D6) on our tree to Spongomonadida plus Thaumatomonadida, not to Sarcomonadea as in Chantangsi et al. (2010a). If *Discomonas*, which we here place in Imbricatea, has scales, that would be direct evidence that spongomonads lost them.

Our revision of the thaumatomonad genera *Allas, Thaumatomonas*, *Reckertia*, *Gyromitus*, and *Thaumatomastix* correcting previous mixups or misidentifications makes scale structure and distribution congruent with the sequence trees. Our erection of two new species of *Thaumatomonas* and one of *Reckertia* pave the way for further studies of this group of gliding flagellates, which is more diverse than hitherto realised. We are doing further culturing, electron microscopy of scales, and rDNA sequencing to put thaumatomonad taxonomy on an even sounder footing.

Our present tree identified environmental sequence D6 (one of many within paraphyletic basal group T of Bass and Cavalier-Smith 2004) as related to the gliding flagellate *Discomonas* (Chantangsi and Leander 2009a); their joint clade weakly groups as sister to Thaumatomonadida plus Spongomonadida. The Bayesian tree in Chantangsi and Leander (2010b) instead grouped it with poor support (and none by ML) with glissomonads instead, but as it had inadequate taxon sampling (including only 35 sequences) that must be discounted. Their tree based on just 923 nucleotides did group it with D6 (firmly by both methods) but as sisters to thecofilosean cryomonads; however, as that tree also had few taxa, and no Thaumatomonadida, Spongomonadida or Glissomonadida, it was uninformative about the wider position of *Discomonas.* The present position of *Discomonas*/D6 as weakly sister to Thaumatomonadida/Spongomonadida suggests

that this whole clade ancestrally consisted of gliding zooflagellates, since *Discomonas* glides on its posterior flagellum like Thaumatomonadida, but unlike them lacks a ventral groove or flagellar pocket (its two flagella emanate just from an anterior notch). Therefore the microtubule-supported flagellar pocket of Thaumatomonadida and Spongomonadida and the parallelism of their centrioles are probably synapomorphies for these orders jointly, which might be appropriately grouped as a superorder.

Evolution of Thecofilosea

We described a new species of the thecate amoeba *Rhogostoma* and studied both existing species. These three plus an environmental sequence form a robust clade within Cryomonadida, to which we have now transferred them. The species cluster including *R. minus* suggests that still more will be discovered and confirms the view that these amoebae were under-split taxonomically (Simitzis and Le Goff 1981); as Belar (1921) noted, some can grow remarkably fast in the laboratory and could be useful experimental systems. They are non-flagellate close relatives of the biflagellates *Protaspa* and *Cryothecomonas*, both of which have ventral clefts from which filose pseudopods emerge. The aperture of *Rhogostoma* is also a cleft, not a round orifice as in most genera of Chlamydophryidae, so it is easy to visualize how they evolved from protaspid-like flagellates by flagellar loss. We refer to them as thecate not testate as a theca is normally closely adherent to the protoplast whereas a test is typically looser. Another filose non-flagellate, *Rhizaspis* (Skuja 1948), has been suggested to be related to *Protaspa* and thaumatomonads (Patterson et al. 2002); we consider it to be a close relative of *Rhogostoma*, which we have placed in the same family (Rhizaspididae). Our results confirm that rhizaspidids are related to *Protaspa*, from which they probably evolved by loss of flagella, but show they are not thaumatomonads; the idea that *Protaspa* belongs to the invalid family Thaumatomastigaceae (Patterson and Zölffel 1991**)** was refuted by Hoppenrath and Leander (2006a).

Our trees strongly show that the testate amoeba *Pseudodifflugia* is not sister to *Rhogostoma*, but probably is to Phaeodaria instead. Ebriida are probably closer to Cryomonadida than to Phaeodaria, even though both ebriids and most Phaeodaria share the character of hollow internal silica skeletons. Placement of the deep-branching new ventricleftid flagellates (*Ventrifissura* and *Verrucomonas*) at the base of Thecofilosea (much deeper than *Protaspa*, which like them has a ventral groove from which pseudopods are generated), although weakly supported, suggests that gliding and rigid zooflagellates with two flagella and a ventral groove plus pseudopods were the ancestral phenotype for Thecofilosea and that swimming planktonic flagellates like Ebriida and *Cryothecomonas*, as well as benthic thecate and testate amoebae and Phaeodaria, are all secondarily derived from such zooflagellate ancestors. NC4, partly contrary to Bass and Cavalier-Smith (2004), appears to be deeply within Thecofilosea (9-2.2 remains associated with Glissomonadida in Fig. 1). If like *Ventrifissura* and *Verrucomonas* lineages 7-6.2 and 9-1.4 (both NC4) feed on eukaryotes this would explain why they have escaped our bacterivore-focused culturing efforts.

As Phaeodaria are clearly related to Thecofilosea and probably branch within them, we have now formally placed them as a subclass within Thecofilosea, not as separate class Phaeodarea. *Pseudodifflugia* is the only representative on our tree of Tectofilosida, as here revised by the transfer of *Rhogostoma* and *Capsellina* from the tectofilosid family Chlamydophyridae into Rhizaspididae within Cryomonadida. Just before going to press a sequence (GQ37676) became available for a '*Capsellina* sp.' (ATCC50039), which we find is sister to the clade containing *Rhogostoma micra* and *R. minus* with RAxML bootstrap support of 86%. Therefore this *Capsellina* may be a misidentified *Rhogostoma* (not unlikely as such an error has been made before (Simitzis and Goff 1981) and many ATCC protist cultures have been misidentified). If it is a true *Capsellina* it raises the possibility that *Capsellina* evolved directly from a *Rhogostoma* by evolving a depression around a wider slit and attaching brown debris to the test. In any case this finding provides support for our grouping both genera in the same family, Rhizaspididae. Removal of *Rhogostoma* and *Capsellina* from Chlamydophryidae makes that family substantially more homogeneous; nonetheless, that homogeneity is lessened by the continued inclusion of *Lecythium*, which unlike the other remaining genera does not have a clearly separated test and protoplast; we suspect that *Lecythium* also may actually belong elsewhere (though probably not in Rhizaspididae). Other genera of Chlamydophryidae need molecular study to test its monophyly.

The grouping of *Pseudodifflugia* and Phaeodaria is weak, but might be correct and evolutionarily significant. Although most Phaeodaria have an internal hollow silica skeleton, the aberrant order Phaeogynocellida does not (or has only a cup

over the oral pole); those with no skeleton at all (families Phaeosphaeridae, Phaeodinidae) collect shells of other protists and phaeodium granules in their peripheral cytoplasm instead; possibly this is a more primitive state than an internal skeleton which could have been transitional from a testate ancestor like *Pseudodifflugia* that makes its test by collecting foreign particles. Of the Phaeodaria on the tree two have connected internal skeletons, but the most divergent, *Aulacantha*, instead has unconnected radial silica scales; these radially arranged scales might be transitional between the putatively still more primitive Phaeogynocellida and typical Phaeodaria with connected silica skeletons. These considerations make the inclusion of Phaeodaria within Thecofilosea evolutionarily reasonable.

The non-grouping of *Pseudodifflugia* (family Pseudodifflugiidae) and *Rhogostoma* (family Rhizaspididae) means that these two families of testate and thecate filose amoebae are not directly related - here placed in separate orders. Thus there have been at least two separate origins of testate or thecate amoebae within Thecofilosea, compared with only one of testates – Euglyphida – within Imbricatea (though *Peregrinia* represents a separate origin of a scaly filose amoeba, and one might regard its scaly investment as a theca, it has not totally lost flagella, unlike the others). Molecular data are needed for the other non-euglyphid testate amoebae (Psammonobiotidae, Amphitremidae, Volutellidae) to verify that they belong to Tectofilosida within Thecofilosea. Our unpublished environmental DNA studies have revealed a vast array of new environmental clades within Thecofilosea that may include such amoebae as well as other known filose genera not even classified into families (Bass et al. in prep.). The fact that all three genera of non-euglyphid filose testate/thecate amoebae (*Pseudodifflugia*, *Rhogostoma*, *Peregrinia*) group with other organisms in two classes, not with each other, emphasizes that polyphyly may be more rampant for this broad morphotype than suspected; some might even belong in other filosan classes, as flagellar loss is not unusual.

Relationship of Imbricatea and Thecofilosea

Including Phaeodaria within Thecofilosea and Spongomonadida within Imbricatea simplifies cercozoan classification by reducing the classes by two. On our trees Imbricatea and Thecofilosea as now revised are each consistently holophyletic and always each other's sister, even though both conclusions have trivial bootstrap support. The likely sister relationship between Imbricatea and Thecofilosea suggests that their common ancestor was probably a biflagellate gliding zooflagellate with semi-rigid pellicle or theca and a ventral cleft emitting branching filose pseudopods, since these characters are present in both classes, especially in deeper branching members. Furthermore, since Imbricatea have two orders that make silica scales (Thaumatomonadida, Euglyphida) and Thecofilosea have two taxa (Ebriida, Phaeodaria) with internal skeletons (and *Aulacantha* even has silica scales), but no other Cercozoa are known to manipulate silica in either way, it is possible that this common ancestor might also have used silica structurally; investigation of silica-manipulating proteins might reveal common features among them that differ from those of other silica-secreting phyla like Ochrophyta (diatoms, chrysophytes, silicoflagellates) among the heterokonts, Radiolaria, and loricate choanoflagellates (Choanozoa).

Conclusions

A new array of morphologically, behaviourally, and phylogenetically diverse lineages is shown here to be Cercozoa. We have identified representatives of several clades previously known only from environmental DNA. However, others clades of key importance for cercozoan deep phylogeny are still unidentified, notably NC5, 6 and 10/12 and many in Endomyxa (Bass et al. 2009a). Our new filosan sequences have yielded more comprehensive and reliable trees and allowed improvements in higher-level cercozoan taxonomy, notably a reduction in the number of classes and establishment of three phenotypically and genetically distinctive new orders: Tremulida, Ventricleftida and Marimonadida. Especially significant are the discovery of *Tremula*, with its two gliding flagella; the recognition of Marimonadida, an order of marine naked ancestrally gliding amoeboflagellates and flagellates entirely distinct from the essentially terrestrial Sarcomonadea; and the new insights into common ancestral features underlying the deep diversity of form within Filosa - notably the conclusion that the common ancestor of Thecofilosea and Imbricatea was a gliding zooflagellate emitting ventral filose pseudopodia (probably from a marked groove), from which three different lineages of filose testate or thecate amoebae ultimately independently evolved, by the convergent evolution of tests and loss of flagella (the thecate Rhizaspididae probably simply inherited their theca from their protaspid flagellate ancestor).

Methods

Isolation of cultures: Cultures from culture collections were maintained in concordance with the strain-specific instructions provided. The others were isolated from the environment: samples were collected from soil, freshwater, and marine sediments in sterile Eppendorf or Falcon tubes. Samples were hydrated with Volvic® water (Danone) or dilutions of CCAP Artificial Sea Water Medium and grown at room temperature, with or without enrichment (a boiled wheat grain or an aliquot of the enriched water after boiling the grains), for a few days to a few weeks. Depending on the concentration of organisms, a 10-100 μl aliquot was then serially diluted across eight or twelve wells of 250 μl of enriched Volvic in a 96-well cell culture plate (Nunclon). The plate was then parafilmed and incubated at room temperature for a few days to a couple of weeks. Two or three rounds of serial dilution were carried out for each isolate, using only apparently pure strains to seed the final round. In cases where rDNA sequencing suggested that more than one strain was present, further scrutiny of the cultures, and an additional round of serial dilution were carried out before re-extracting DNA and re-sequencing the strain. Wells apparently containing a single morphospecies were transferred into a 90 mm Petri dish with nutritive medium; other wells required repeated serial dilution to obtain pure cultures. The boiled grain or the resulting enriched water is the food source for bacteria on which the protists feed. Culture collection accession numbers are given individually in the results section; where these are not given the cultures were difficult to maintain and died before they could be submitted.

Differential interference contrast high definition video microscopy and electron microscopy: Live cultures were filmed and photographed using a Nikon Eclipse 80i microscope, with a x40 differential interference contrast water immersion lens (NA 0.6) of a X60 water immersion lens (NA 1.0) and a Sony HDV 1080i Handycam®. Films were analysed on Final Cut Express HD 3.5.1, and digital images were exported and transferred to Adobe Photoshop 10.0 CS3 Extended for processing. The Russian strain *Metopion* aff. *fluens* was imaged separately, on a Biolam-I microscope equipped with a video camera MC-1009/S and phase contrast water immersion 70X NA 1.25 objective (KF-5 device). Culture characteristics were observed on a Panasonic NVHS850 video recorder. The videotape recording was then digitized. Digital images were transferred to Photoshop CS3 (Adobe) for processing. For thin sectioning transmission electron microscopy fixation was in glutaraldehyde followed by osmium tetroxide as detailed previously (Cavalier-Smith et al. 2004). For examining scales in whole mounts samples were fixed with 2.5% glutaraldehyde; formvar-coated 200 mesh copper grids were used for viewing unstained cells in an FEI Tecnai 12 electron microscope.

DNA extraction, purification, and sequencing: Most of the culture medium was decanted off, and using a sterile scraper, cells were collected from the bottom of the culture dish, then concentrated by centrifugation at 24 x g for 15 min at 5 °C. Total DNA was extracted from the pellet following the Maximum Yield Protocol of the UltraClean Soil DNA Isolation Kit (MoBio Laboratories, CamBio, UK). 18S rDNA from cultures was PCR-amplified using one or two of the following primer sets: 1) 25F & 1256R and 1259F & 369R (Cercozoa-specific: Karpov et al. 2006) 2) 25F & 1801R 3) 25F & 28SR1 (Bass and Cavalier-Smith 2004). All primers were ordered from Invitrogen (UK). PCR conditions were: initial denaturation at 95 °C for 5 min, 35 cycles (denaturation at 95 °C for 32 s, annealing at 70 °C (primer set 1) or 68 °C (primer sets 2 and 3) for 36 s, extension at 72 °C for 2.5 min), and final extension at 72 °C for 7 min. PCR products were run on a 1% agarose gel. Bands of appropriate size were excised, and cleaned following the protocol of the GFX PCR DNA Gel Band Purification Kit (GE Healthcare, UK). Alternatively, an aliquot of the PCR products were run on a 1% agarose gel and checked for a single band of correct size. If present, PCR products were cleaned using the polyethylene glycol (PEG) protocol: for a 12.5 μl PCR reaction, 12.5 μl (20%PEG/2.5 M NaCl) PEG was added to each tube and mixed by vortexing. They were left to incubate for 30 min at room temperature; then centrifuged at 1500 x g for 30 min to pellet the PCR products. Supernatant was discarded by pulse-spinning the inverted tubes. The pellet was washed with ice cold 70% ethanol, spun for ten minutes at 1500 x g, again inverted and pulse-spun to remove the supernatant. The ethanol wash was repeated; the PCR pellet was then re-suspended in de-ionised water, and stored at -20 °C until use. PCR products were sequenced using dye terminators and separated on an automated ABI-377 sequencer. The sequencing primers were the same as the initial PCR primers plus an internal sequencing primer, Pre3NDF (Karpov et al. 2006), used in order to obtain a complete sequence. All sequence chromatograms were examined by eye and edited using the trace editor BioEdit (Hall 1999) and Finch TV (http://www.geospiza.com/finchtv/).

Sequence analysis and construction of alignments: Edited sequences were added to our previously existing alignment of Cercozoa containing sequences from all known clades of Cercozoa including all cultured and environmental clades defined in Bass and Cavalier-Smith (2004), Bass et al. (2009a), and Howe et al. (2009). A representative selection of cercozoan sequences were chosen to provide a stable phylogeny for the phylogenetic analysis of new taxa found in this study. Sequences were aligned using MAFFT (Katoh 2008) and the alignment refined by eye using BioEdit or MacGDE (http://www.msu.edu/∼lintone/macgde/) (Smith et al. 1994). Alignments were masked to omit ambiguously aligned positions from the analyses. Alignments are available upon request from the corresponding author. Genbank accession numbers are given for individual species in Results.

Construction of phylogenetic trees: A Bayesian consensus tree was constructed using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) in parallel mode (Altekar et al. 2004). Two separate MC^3 runs with randomly generated starting trees were carried out for 5 M generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 100 generations. 1 M generations were discarded as burnin (trees sampled before the likelihood plots reached a plateau) and the consensus tree constructed from the remaining sample. Maximum-likelihood (ML) analyses used RAxML-7.0.4 (Stamatakis 2006). 1000 bootstrap replicates were performed using the novel rapid bootstrap algorithm (Stamatakis, unpublished) using the GTR model with CAT approximation (all parameters estimated from the data). Bootstrap support values were mapped onto the tree with the best likelihood and the MrBayes consensus tree. Alignments used for phylogenetic analyses are available on request from DB.

New 18S rDNA sequences are deposited in GenBank; accession numbers are shown in Figures 1 and 13. The following are already present in GenBank but given a different name: *Peregrinia clavideferens,* previously *Gyromitus* sp. HFCC94 - DQ211593; *Reckertia filosa*, previously *Allas* sp. JJP-2003 - AY268040; *Thaumatomonas vancouveri*, previously *Allas* sp. 3108W2 – AF411264; *Allas* aff. *diplophysa*, previously *Allas*

diplophysa ATCC50365 – AF411262; *Rhogostoma minus* previously *Lecythium* sp. AJ514867.

Acknowledgements

We thank A. P. Mylnikov (Borok) for cultures of *Micrometopion nutans* (as *Metopion fluens* small form), *Reckertia filosa* (via J. Pawlowski as *Allas* sp. JJP-2003) and *Rhogostoma minus* (as *Lecythium* sp.), light micrographs of *Micrometopion nutans*, and information about his *Thaumatomonas* strains, B. Oates for *Thaumatomonas vancouveri* electron micrographs, T. Nerad for advice on an ATCC strain, F. Ekelund for information about the *Thaumatomonas* strains he sequenced, Claudia Wylezich for assistance with describing *Peregrinia clavideferens*, and Ema Chao for purifying *Minimassisteria*. TC-S thanks NERC and the Canadian Institute for Advanced Research Evolutionary Biology Program for Fellowship support and NERC for research grant NE/C510975/1, which supported DB in part and ATH. RL was supported by a NERC research studentship. JMS is supported by a BBSRC research studentship and thanks M. Shaw (William Dunn School of Pathology, Oxford University) for help with the electron microscope. KV thanks NERC for a grant under its Soil Biodiversity Thematic Programme and the Leverhulme Foundation for an Emeritus Fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2010.10.002.

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