Taxonomy, biodiversity, and ecology of Apusozoa (Protozoa)

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Contents

Authorship, Abstract, Acknowledgements, and Figures

Chapter 1
General introduction

Chapter 2
The novel marine gliding zooflagellate genus *Mantamonas* (Mantamonadida ord. n.: Apusozoa)

Chapter 3
Phylogeny and evolution of Planomonadida (Apusozoa): nine new species and new genera *Fabomonas* and *Nutomonas*

Chapter 4
A PCR-based environmental survey and mining of publically available metagenomic databases to explore the diversity and ecology of Mantamonadida (Apusozoa)

Chapter 5
A PCR-based environmental survey of free-living flagellates Apusomonadida and Planomonadida

Chapter 6
Closely related protest strains have different grazing impacts on natural bacterial communities

Chapter 7
General discussion, conclusions, and future perspectives

Chapter 8
General references

Appendices
I : Conference participation
II : POSTnote: Biodiversity Offsetting

General declaration of authorship and author contributions

Declaration of authorship
In accordance with the University of Oxford's Notes of Guidance for Biological Sciences (GSO.21, 6v), the following declarations are made: *I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any other university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.*

Permission to submit a thesis in the format of publications/manuscripts
This thesis includes two original papers published in peer-reviewed journals (Ch. 2, 6), one manuscript that has already gone through two rounds of review (Ch. 3), and two data chapters nearly ready for submission (Ch. 4, 5). Permission to submit a thesis by publications and manuscripts was granted by John Iles, Director of Graduate Studies, Department of Zoology, by email, on June 13, 2011.

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Contribution to each chapter
The ideas, development, and writing of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Zoology, under the supervision of Professor Tom Cavalier-Smith (TCS) with guidance from David Bass (DB). In the case of the five data chapters in this thesis, my contribution was:

Chapter 2

Glücksman E., Snell, E.A., Berney, C., Chao, E.E., Bass, D., Cavalier-Smith, T. The novel marine gliding zooflagellate genus *Mantamonas* (Mantamonadida ord. n.: Apusozoa). *Protist* **162**: 207-221.

My contribution (**roughly 70% of total**): maintenance of cultures, observation by microscope and photography, extraction of DNA, PCR amplification and sequencing of SSU and ITS1 molecules, editing and concatenation of sequences, co-curating 18S alignment with TCS, 18S phylogenetic analyses, creation of all micrograph figures, labelling of all phylogenetic trees, submission of new sequences to online GenBank database, writing of initial manuscript draft and further editing of paper with TCS, submission of manuscript to *Protist*, co-editing manuscript and amending phylogenetic analyses based on reviewers' comments with TCS. I was the majority contributor to all sections except the Discussion (50% by TCS).

Chapter 3

Glücksman, E., Snell, E.A., Cavalier-Smith, T. Phylogeny and evolution of Planomonadida (Apusozoa): nine new species and new genera *Fabomonas* and *Nutomonas*.

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Chapter 4

A PCR-based environmental survey and mining of publically available metagenomic databases to explore the diversity and ecology of Mantamonadida (Apusozoa)

My contribution: **90%**, with guidance and some editing by DB

Chapter 5

A PCR-based environmental survey of free-living flagellates Apusomonadida and Planomonadida

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Chapter 6

Glücksman E., Bell, T., Griffiths, R.I., Bass, D. (2010) Closely related protist strains have different grazing impacts on natural bacterial communities. *Environmental Microbiology* **12**: 3105-3113.

A majority of the lab work and analyses featured here were carried out as part of an MSc project (Integrative Biosciences, 2006-2007) in the Department of Zoology, University of Oxford. However, with permission from my departmental supervisory committee (Tom Bell, Angus Buckling, Alex Kacelnik), it is included here since the manuscript preparation and answering of reviewers' comments was done during the course of my DPhil.

My contribution (**roughly 50% of total**): all molecular and culturing lab work, initial write-up, and creation of figures as an MSc thesis. Tom Bell led much of the follow-up work, including the creation of figures for publication. DB provided the cultures and helped with the experimental design.

Signed:

Edvard Glücksman

Date: November 18, 2011

General abstract

Taxonomy, biodiversity, and ecology of Apusozoa (Protozoa)
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Apusozoa (Protozoa) is a phylum of heterotrophic gliding zooflagellates of unknown taxonomic affiliation, commonly observed in environmental samples. Almost nothing was previously known about the diversity and ecology of apusozoan species though, as bacterivores, they are probably important functional constituents within microbial assemblages.

We explored apusozoan morphological and genetic diversity, ecology, and related methodological questions. By culturing environmental material from a range of habitats, we isolated and maintained monocultures of both previously described apusozoan orders, Apusomonadida (apusomonads) and Planomonadida (planomonads). For planomonads, we present a revised taxonomy based on morphology, ultrastructure, and 18S rDNA genetic differences. We describe nine new species and new genera *Nutomonas* and *Fabomonas*, and demonstrate ITS2 rDNA secondary structure analysis for species delineation.

During our culturing effort, we also isolated two genotypes of a previously unknown flagellate group, shown here to belong to a novel third apusozoan order, Mantamonadida. We designed molecular probes specific to all three orders and applied them to environmental DNA, detecting novel 18S and ITS1 rDNA lineages in a range of habitats.

We mined publically available metagenomic and metatranscriptomic sequence databases using 18S rDNA of described species as seeds, identifying hundreds of sequences with affinities to all three orders. Phylogenies featuring newly retrieved lineages with previously described species suggest that direct sequencing of transcriptomic material is more effective than amplification-dependent methods at detecting rare cells in mixed microbial assemblages.

Finally, to test potential future applications of our newly isolated strains, we ran microcosm experiments examining the effect of protozoan (Cercozoa) grazing on the structure of bacterial assemblages, demonstrating that closely related and morphologically similar species can have different impacts on their prey base.

Taken together, by combining traditional culturing and modern molecular methods, this thesis drastically improves our understanding of apusozoan diversity and sets the scene for future work using next-generation sequencing and ecologically driven functional experiments.

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Summary of Figures

Page

1-15 Eukaryotes and the tree of life

1-16 Scanned drawings of *Ancyromonas sigmoides* by Kent (1880-82)

2-4 General morphology of living *Mantamonas plastica* type strain

2-5 Anterior cilium of living *Mantamonas plastica* type strain

2-5 Locomotion of living *Mantamonas plastica* type strain

2-7 Position of Mantamonadida on 28S-rRNA gene phylogeny

2-8 Position of Mantamonadida on 18S/28S-rRNA concatenated two-gene phylogeny

2-9 Maximum likelihood (RaxML) 18S rRNA gene phylogeny for 76 eukaryotes

3-57 18S rRNA gene phylogeny of Planomonadida and other Apusozoa

3-58 Morphology of *Fabomonas tropica*

3-59 Transmission electron micrograph of *Fabomonas tropica*

3-60 Transmission electron micrograph of *Fabomonas tropica* type strain nyk4

3-61 Differential interference contrast light micrographs of living *Nutomonas longa*

3-62 Ribosomal RNA Internal Transcribed Spacer 2 (ITS2) secondary structure of selected new strains from each major planomonad clade

3-63 Morphological diversity amongst nine novel planomonad isolates

3-64 18S rRNA gene phylogeny of Planomonadida and other unikonts

3-65 Optimal folds for all 3 *Planomonas* species (marine)

3-66 Optimal folds for 3 *Nutomonas* species of subgenus *Kentomonas*

3-67 Optimal folds for 2 *Nutomonas* species of subgenus *Nutomonas*

4-35 New *Mantamonas plastica* ITS1 rDNA lineages

4-36 18S rDNA gene phylogeny of Planomonadida and other Apusozoa

4-37 18S rDNA gene phylogeny of Apusomonadida and other Apusozoa

5-24 18 rDNA gene phylogeny of Planomonadida, including new lineages found with primers specific to all putatively freshwater nutomonads

5-25 ITS1 rDNA gene phylogeny of the putatively marine nutomonad clade, including new lineages

5-26 18S rDNA gene phylogeny of Apusomonadida, including new lineages

5-27 Heat map showing PCR amplifications by environmental DNA sample and group-specific primer pair

6-4 Hierarchical clustering of the protozoan strains according to their (A) phylogentic distance, (B) trait distance, (C) mean bacterial tRFLP distance

6-5 Relationship between (A) phylogenetic distance and trait distance, (B) phylogenetic distance and tRFLP distance, (C) trait distance and tRFLP distance

6-6 Heat map of relative abundance of each bacterial taxon (columns) in each microcosm (rows)

7-23 Morphology of *Thecamonas* sp. contaminant in CCAP cryptomonad culture AY050179 (unpublished)

Chapter One

General introduction

To summarize the prospect for biological exploration, Earth is biologically still a mostly unexplored planet. The opportunities for basic research, which promise us great scientific and practical rewards, seem limitless. Their pursuit will help make the next hundred years the Century of the Environment.

- Edward O. Wilson (2001, *xiv*)

Chapter summary

Most species on Earth remain uncharacterized, especially in the microbial biosphere, where theoretical disagreements and methodological difficulties have led to microorganisms being particularly underrepresented in global biodiversity counts. However, it is established that unicellular eukaryotes within Kingdom Protozoa (protists) are cosmopolitan, resilient, and play vital ecological roles as predators of bacteria, grazers of phytoplankton, and prey to metazooplankton. The phylogenetic relationship between major protozoan groups remains unresolved, in part because protists are difficult to work with in the laboratory and even frequently observed lineages, therefore, remain enigmatic. This is the case of Apusozoa, a group of commonly observed, free-living heterotrophic flagellates of uncertain taxonomic position, under-sampled biodiversity, and unknown ecology. This work describes a collection of exploratory experiments seeking to better understand Apusozoa, chronicling a combination of traditional culturing and modern molecular methods that offers unprecedented insight into these potentially important, yet little-known, microorganisms.

Global biodiversity and Apusozoa

An important gap in our fundamental knowledge of Earth is that the total diversity of life remains unknown. In the past half century, the global conservation effort has established the concept of biodiversity and championed the need to better understand the planet's component species, in part to give context to extinction rates that are 100 to 1,000 times higher than the natural background rate (Pimm et al. 1995).

However, the number of catalogued species represents only a fraction of the total found in nature. A recent study suggests that 86% of species remain uncharacterized, although in microorganisms, such as prokaryotes and unicellular eukaryotes, where universal criteria for a species concept remain elusive, this proportion is thought to be even greater (Table 1) (Mora et al. 2011). Cataloguing all life, estimated at 8.7 million species (±1.3 million SE), is therefore a near-impossible task, despite increased public awareness, unprecedented funding opportunities, and advances in molecular techniques.

Kingdom Protozoa in the microscopic biosphere

Microorganisms, including prokaryotic Eubacteria and Archaebacteria, as well as unicellular eukaryote members of kingdom Protozoa (protists), are particularly underrepresented in biodiversity counts even though they play a central role within global biogeochemical interactions (Jürgens and Matz 2002; Kuikman et al. 1990; Thingstad and Lignell 1997). Soil microbes drive heterotrophic respiration, the oxidization of plant-derived organic matter and one of the major pathways by which carbon dioxide is released to the atmosphere (Falkowski et al. 2000). Soil-

dwelling microorganisms also mediate pathways critical to the health of most ecosystems, including nitrification, where ammonium, a product of anaerobic and aerobic decomposition, is oxidized into nitrate via nitrite (Cebron et al. 2003; Daims et al. 2001; Hendricks 1996; Neubacher et al. 2008).

The microscopic biosphere thus plays a vital part in linking the abiotic and biotic elements within most healthy ecosystems and protozoa serve many important ecological roles, including as bacterivores, herbivores, and prey.

Firstly, protist predation on bacteria or other protists represents a significant source of microbial mortality, with the relatively larger protozoa regulating the structure, productivity, and biomass of bacterial assemblages (Azam et al. 1983; Caron 2000; Christaki et al. 1999; Fenchel 1984; Güde 1989; Jürgens and Matz 2002; Lavrentyev et al. 1997; Neubacher et al. 2008; Pace et al. 1990; Pomeroy 1974; Sherr and Sherr 2002; Sieburth 1978; Simek et al. 1999; Simon et al. 1998; Strom 2000). The resilience and high metabolic rate of protists enable them to remain competitive even in densely populated microbial environments (Fenchel 1987; Sherr and Sherr 1994) or in extreme conditions, like at the freezing poles (Buck et al. 2000; Laybourn-Parry et al. 2000; Palmisano and Garrison 1993; Scott et al. 2001; Sherr and Sherr 2002), in hypersaline salterns (Pedrós-Alió et al. 2000), and in the deep sea (Buck et al. 2000; Turley and Carstens 1991).

Secondly, protist grazing of phytoplankton critically influences the structure and function of euphotic zone communities within the water column (Jacobson and Anderson 1996).

Finally, as prey, heterotrophic protists represent a significant food source for metazooplankton, thereby contributing to the regeneration of nutrients in aquatic habitats (Calbet and Landry 1999; Jeong et al. 1999; Nagata 2000).

Molecular methods and the eukaryotic tree of life

Despite their resilience in the natural environment, most protozoan strains are difficult to study in laboratory conditions and molecular biology provides a way to bypass the biases of culturing (Cavalier-Smith 2007; Dyer and Obar 1994; Vickerman 1998).

However, obtaining protist monocultures remains essential for the description of new species, enhancing the quality of microscopic observations, and facilitating sequencing of taxonomically informative genes. New protozoan species, isolated from environmental material, are important because they improve taxonomic resolution within known groups and bolster statistical support to the currently unresolved eukaryotic tree of life (Burki et al. 2008). A robustly supported eukaryotic phylogeny is a vital stepping-stone to research into the structure and function of eukaryotic communities, including their origins, adaptation mechanisms, life-history traits, and the evolution and persistence of vital processes (Lane and Archibald 2008). To this end, molecular biology has transformed our understanding of the evolutionary relationships between species, presenting powerful new tools to complement the traditional methods of morphological and biochemical inference (Keeling et al. 2005).

Molecular methods have already drastically altered our view of higher-level diversity, showing that unicellular eukaryotes are polyphyletic, scattered across the tree of life (Cavalier-Smith and Chao 2003; Nikolaev et al. 2004).

Most eukaryotic species are thought to belong to five or six major assemblages known as 'supergroups' (Figure 1) (Baldauf et al. 2000; Cavalier-Smith 2003; Cavalier-Smith 2003b; Richards and Bass 2005; Simpson and Roger 2002; Simpson and Roger 2004). The supergroups include the opisthokonts and Amoebozoa, known together as the unikonts (Cavalier-Smith 2002), as well as Plantae (or Archaeplastida), Excavata, Chromalveolata, and Rhizaria (Burki et al. 2008; Lane and Archibald 2008).

However, these hypothetical groupings are controversial and the precise order of evolutionary divergence between major eukaryotic clades remains unknown, not least because two phyla remain outside the current synthesis: Heliozoa (Cavalier-Smith and Chao 2003) and the recently discovered Apusozoa (Cavalier-Smith 2002, 2003, 2007; Cavalier-Smith and Chao 2003), the latter of which is the focus of this project.

Apusozoa: eukaryotic 'orphans'

Apusozoa is a recently described phylum of heterotrophic gliding zooflagellates. Cells resembling Apusozoa are frequently observed in mixed environmental samples (Lee and Patterson 2002; Nikolaev 2006; Patterson and Lee 2000) though, prior to this study, remarkably little work has focused specifically on the diversity within each apusozoan order or putatively related lineages, such as *Micronuclearia* (Mikrjukov and Mylnikov 2001) and *Collodictyon* (Brugerolle et al. 2002), the

amoeboflagellate formerly misidentified as *'Mastigamoeba' invertens* (Stiller et al. 1998; Stiller and Hall 1999) and now redescribed as *Breviata anathema* (Walker et al. 2006).

Until recently, Apusozoa comprised two main orders. The first, Apusomonadida (apusomonads) (Cavalier-Smith and Chao 2003; Karpov and Mylnikov 1989), recently underwent an important taxonomic revision and now comprises genera *Thecamonas*, *Manchomonas*, *Multimonas*, *Podomonas*, and the soil-dwelling *Apusomonas* (Cavalier-Smith and Chao 2010).

The second traditionally recognized apusozoan order is Planomonadida (planomonads), gliding flagellates whose cell characteristically nods as it glides along the substratum upon its posterior cilium. The taxonomy of planomonads has been the subject of several recent publications, including a major revision by Cavalier-Smith et al. (2008), based on the interpretation of Savile Kent's (1880-82) initial description of a flagellate that was observed, described, and named *Ancyromonas sigmoides* (Figure 2).

A third apusozoan order, Mantamonadida (mantamonads), was recently isolated from a sample of marine sediment and described for the first time by Glücksman et al. (2011, Chapter 2). Although two mantamonad 18S rDNA types are known, the type species, *Mantamonas plastica*, remains the only characterized isolate.

Aims and chapter content

The initial aim of this project was to investigate, for the first time, the diversity and ecology of Apusozoa. The chance discovery of *Mantamonas plastica*,

documented in the published article in **Chapter 2**, extends the analysis by demonstrating the extent by which the broader diversity of Apusozoa is vastly under-sampled. Focusing on the described order Planomonadida, the manuscript in **Chapter 3** emphasizes the value of traditional culturing methods, revealing new genera and many novel species in a thoroughly revised planomonad taxonomy based on morphology, 18S rDNA sequences, and ITS2 rDNA secondary structure.

The rDNA sequences obtained from culturing were subsequently used to design PCR probes specific to the two known mantamonad ribotypes and several apusomonad and planomonad lineages. In experiments outlined in **Chapters 4 and 5**, these primers were applied to a range of environmental DNA (eDNA), detecting many new putatively apusozoan lineages and providing unprecedented insight into their ubiquitous ecological distribution.

Chapter 4 also describes a new method of mining existing metagenomic and metatranscriptomic environmental sequence databases for previously unidentified putatively apusozoan lineages and, using metadata accompanying each retrieved sequence, develops a widely applicable framework for investigating the relationship between sequencing methodology and detected diversity.

Chapter 6 offers a potential future perspective in the study of Apusozoa by describing a microcosm predation experiment combining Cercozoa, another recently characterized group of free-living flagellates, with mixed bacterial assemblages. This study demonstrates how the monocultures and genetic data provided by explorative taxonomy and diversity studies, like those presented in **Chapters 2-5**, serve as stepping-stones to more complex ecological experiments.

A general discussion, closing remarks, and future perspectives are provided in **Chapter 7**.

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Table

Table 1. Number currently catalogued species by major group (modified from Mora et al. 2011).

	Earth	Ocean	Total	% Grand Total
Eukaryotes				
Animalia	953,434	171,082	1,124,516	78.2
Chromista	13,033	4,859	17,892	1.2
Fungi	43,271	1,097	44,368	3.1
Plantae	215,644	8,600	224,244	15.6
Protozoa[1]	8,118	8,118	16,236	1.1
Total	1,233,500	193,756	1,427,256	
Prokaryotes[2]				
Archaea	502	1	503	0
Bacteria	10,358	652	11,010	0.8
Total	10,860	653	11,513	0.8
Grand Total	*1,244,360*	*194,409*	*1,438,769*	

Percentages rounded to 3 significant digits.

[1] The protozoan database used here combines various elements of classification schemes from different ages. These figures should therefore be revisited once a stable global protozoan catalogue has been put in place.

[2] It is difficult to compare the prokaryotes with eukaryotes in this manner because different taxonomic communities, in particular the bacteriologists compared with most other taxonomists, use different criteria to define individual species. It is likely, however, that both prokaryotes and unicellular eukaryotes are vastly underrepresented here.

Figure legends

Figure 1: Eukaryotes and the tree of life. *Top*: Generalised trees of life (viruses excluded) showing two alternative views on the relationship of major lineages. These are known as the, a) Archaea tree, and, b) Eocyte tree. Figures copied from Tree of Life Web Project (1997). *Bottom*: A hypothetical tree of eukaryotes showing five 'supergroups' based on molecular, biochemical, and morphological data. 'Orphan' genera, such as Apusozoa, are not convincingly associated with any of these five supergroups and are thus omitted. From Keeling et al. (2005).

Figure 2: Scanned drawings of *Ancyromonas sigmoides* by Kent (1880-82). From Cavalier-Smith et al. (2008).

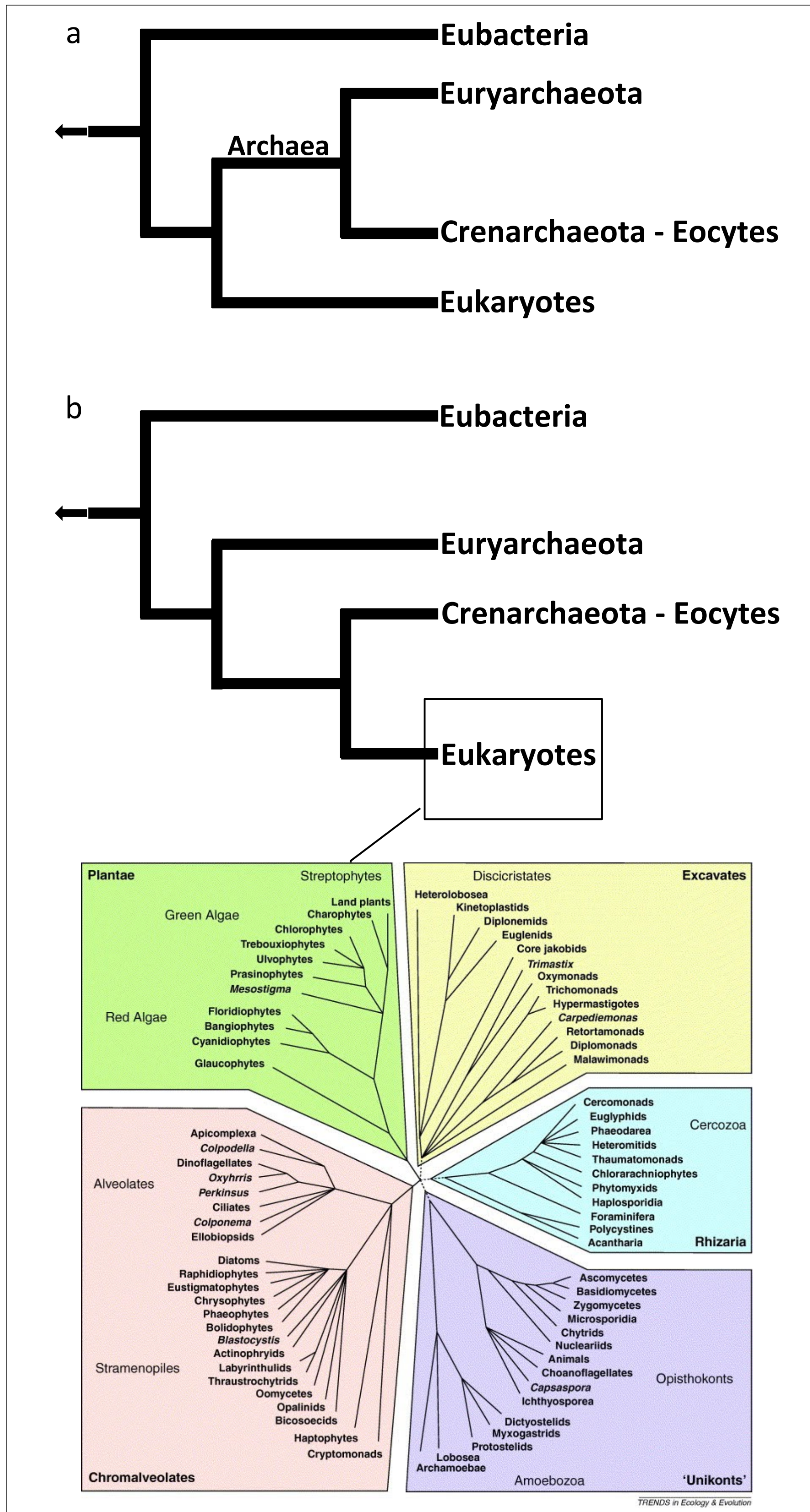

Figure 1

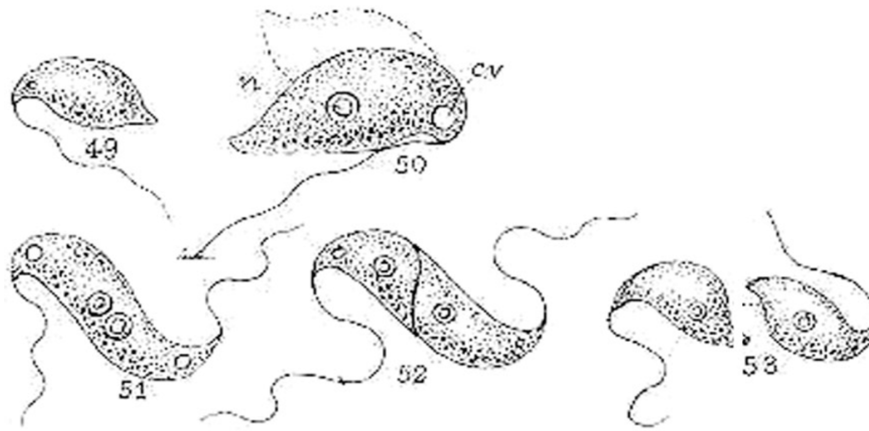

Figure 2

Chapter Two

The novel marine gliding zooflagellate genus *Mantamonas* (Mantamoandida ord. n.: Apusozoa)

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ORIGINAL PAPER

The Novel Marine Gliding Zooflagellate Genus *Mantamonas* (Mantamonadida ord. n.: Apusozoa)

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Mantamonas **is a novel genus of marine gliding zooflagellates probably related to apusomonad and planomonad Apusozoa. Using phase and differential interference contrast microscopy we describe the type species *Mantamonas plastica* sp. n. from coastal sediment in Cumbria, England. Cells are ~5 μm long, ~5 μm wide, asymmetric, flattened, biciliate, and somewhat plastic. The posterior cilium, on which they glide smoothly over the substratum, is long and highly acronematic. The much thinner, shorter, and almost immobile anterior cilium points forward to the cell's left. These morphological and behavioural traits suggest that *Mantamonas* is a member of the protozoan phylum Apusozoa. Analyses of 18S and 28S rRNA gene sequences of *Mantamonas plastica* and a second genetically very different marine species from coastal sediment in Tanzania show *Mantamonas* as a robustly monophyletic clade, that is very divergent from all other eukaryotes. 18S rRNA trees mostly place *Mantamonas* within unikonts (opisthokonts, Apusozoa, and Amoebozoa) but its precise position varies with phylogenetic algorithm and/or taxon and nucleotide position sampling; it may group equally weakly as sister to Planomonadida, Apusomonadida or *Breviata*. On 28S rRNA and joint 18/28S rRNA phylogenies (including 11 other newly obtained apusozoan/amoebozoan 28S rRNA sequences) it consistently strongly groups with Apusomonadida (Apusozoa).**
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Key words: Apusomonadida; Apusozoa; *Mantamonas*; Planomonadida; 18S rRNA phylogeny; 28S rRNA phylogeny.

Introduction

Free-living gliding zooflagellates are major constituents of marine, freshwater, and soil environments, yet the full extent of their diversity remains unknown (Foissner 2008; Patterson and Simpson 1996; Šlapeta et al. 2005). Most genera of gliding zooflagellates belong to the phylum Cercozoa (Cavalier-Smith and Chao 2003a), the focus of much recent work (Bass and Cavalier-Smith 2004; Bass et al. 2009a,b; Cavalier-Smith et al. 2008a, 2009; Chantangsi et al. 2008; Hoppenrath and Leander 2006; Howe et al. 2009), though gliding zooflagellates are also found in Euglenozoa, Heterokonta, and especially Apusozoa, where they are the predominant and probably ancestral phenotype.

Apusozoa (Cavalier-Smith 2002; Cavalier-Smith and Chao 2003b, 2010) is a small protozoan phylum primarily comprising biciliate gliding zooflagellates classified into two orders: Apusomonadida (Karpov

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and Mylnikov 1989) and Planomonadida (Cavalier-Smith et al. 2008b). The order Planomonadida (planomonads), until recently referred to as '*Ancyromonas*' (Hänel 1979; Patterson and Zölfell 1991), comprises seven named species of *Planomonas* belonging to one freshwater and two marine clades; elsewhere – Glücksman et al. (unpublished) - we describe ten additional planomonad species and revise planomonad/ancyromonad taxonomy in the light of this greatly expanded diversity and of Heiss et al. (2010), which appeared after final revision of the present paper. All planomonads are bacterivorous, biciliate, dorso-ventrally flattened, and use their usually acronematic posterior cilium to glide on solid substrata. The order Apusomonadida (apusomonads) comprises six genera, most with ventral grooves from which pseudopodia emerge for feeding on bacteria: *Amastigomonas* and *Apusomonas* from soil or freshwater; the marine *Podomonas, Manchomonas* and *Multimonas*; and the largely marine *Thecamonas* (Alexeieff 1924; Cavalier-Smith and Chao 2010; Larsen and Patterson 1990; Vickerman et al. 1974). Like the more rigid and non-pseudopodial planomonads, apusomonads are biciliate bacterivores, gliding on surfaces by means of their typically strongly acronematic posterior cilium. Both groups of Apusozoa have a distinctive dorsal pellicle with sub-membrane dense layer(s), a ventral groove without pellicular thickening, and acronematic anterior cilium, but differ in mitochondrial ultrastructure and in the presence of extrusomes only in planomonads. The little-known, non-flagellate freshwater *Micronuclearia* has been placed in phylum Apusozoa as a third order (Micronucleariida) because its pellicle structure and flat mitochondrial cristae resemble those of planomonads (Cavalier-Smith et al. 2008b); it lacks extrusomes and centrioles and has a ventral cavity that emits extremely slender pseudopodia as in apusomonads (Mikrjukov and Mylnikov 2001).

All three apusozoan orders branch so deeply on single-gene trees that they are hard to place, making their relationships to each other and to other eukaryotes controversial (Cavalier-Smith and Chao 2003b, 2010; Cavalier-Smith et al. 2008b); for apusomonads at least, an ancient relationship with opisthokonts is likely (Cavalier-Smith and Chao 1995; Kim et al. 2006). A fourth order of even less clear phylogenetic position, Diphylleida, comprising three genera of non-gliding zooflagellates with a ventral groove but no dorsal pellicular thickening (Brugerolle et al. 2002), was once tentatively placed in Apusozoa (Cavalier-Smith 2003a,b); it was later excluded (Cavalier-Smith et al. 2008b) and suggested to be closer to excavates like *Malawimonas* (Cavalier-Smith and Chao 2010).

We have now isolated from marine sediments two distinct strains of a previously unknown free-living gliding zooflagellate morphotype that is flattened and has a very thin anterior cilium, like *Planomonas*, but a more plastic body. We name them *Mantamonas* and describe by light microscopy the strain that remains alive in culture as the type species, *Mantamonas plastica*. We sequenced the 18S and 28S rRNA genes of both *Mantamonas* strains and a new *Planomonas* isolate and also 28S rRNA of 10 other Apusozoa and Amoebozoa in order to establish the evolutionary position of *Mantamonas* within eukaryotes and to obtain better two-gene trees for Apusozoa and Amoebozoa than previously available (Moreira et al. 2007). Both the 18S and 28S rRNA gene phylogenies show that our isolates represent two divergent species of a morphologically distinct lineage (mantamonads) for which we establish a new family and order within Apusozoa. Our morphological and behavioural observations support the genetic evidence that *Mantamonas* is related to the two known orders of apusozoan gliding zooflagellates.

Results and Discussion

Light Microscopy

The morphology of *Mantamonas plastica* is highly distinctive (Fig. 1). Cells are strongly flattened, somewhat plastic, and consistently asymmetric, with a clearly visible posterior cilium, on which they glide, and less conspicuous (distinctly thinner) anterior cilium. They are typically ~2 μm thick, ~5 μm long and ~5 μm wide, but vary distinctly in size and shape according to bacterial density and growth phase. In about a third of the cells the nucleus is conspicuous as a spherical body in the right anterior quadrant of the cell immediately to the right of the proximal end of the posterior cilium. Thinner cells have a right anterior hump just above the nucleus, likely caused by its presence.

Mantamonas is characterized by a blunt projection on the left-hand side of the cell when viewed dorsally. This projection is invariably present, though it is proportionately longer and narrower on smaller cells. Pseudopodia stick to the substratum on either or both of the left or right posterior immediately adjacent to the gliding cilium. They are sometimes drawn out by the forward progres-

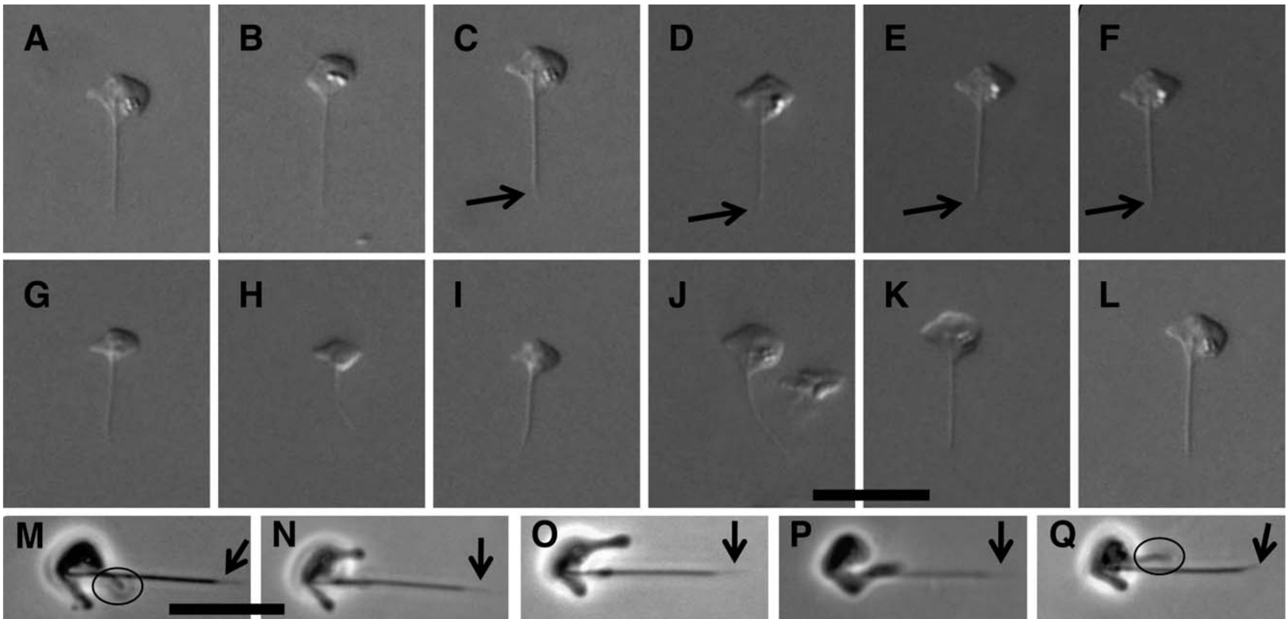

Figure 1. General morphology of living *Mantamonas plastica* type strain. Differential interference contrast (**A-L**) and phase contrast (**M-Q**) light micrographs. The cells shown exhibit characteristic morphological features of *Mantamonas*, including cell shape plasticity and extension of pseudopods (M-Q), blunt projection on the left-hand side of the cell, barely visible anterior cilium (A–C), thicker and often acronematic posterior cilium (C-F; M-Q), and sometimes visible spherical nucleus to the upper right of the posterior ciliary base (A, C, G, J). The highly refractile granules (B-F; J-L) are probably ingested bacteria. **A-L** Twelve different cells; **M-Q** Five different cells, flipped vertically to facilitate comparison with DIC pictures. Arrows indicate acroneme. Ovals encircle pseudopod. Scale bars 10 μm.

sion of the cell, being released before being quickly retracted back into the cell.

The anterior cilium (Fig. 2), emanating from just above the substratum (roughly 0.5 μm above the plane of the posterior cilium), is $\sim$3 μm in length and does not beat as in most biciliate eukaryotes, being almost rigid through most of its length. It is almost parallel to the substratum and is held forwards and to the left at an angle of $\sim$40–50 degrees to the longitudinal axis as the cell glides forward. The distal third of the anterior cilium may tilt slightly upwards and vibrates just perceptibly with extremely small amplitude. It is exceptionally thin as in most *Planomonas* species, as if virtually its whole length (except its extreme base as it enters the cell) is an acroneme. The posterior cilium is up to 10 μm in length and considerably thicker than the anterior one, being the normal thickness for cilia. It is variably acronematic; when visible (usually) the acroneme is never longer than 2 μm.

Cells glide smoothly and steadily along the surface of the substratum at about one cell body length per second (Fig. 3). Turning, almost always to the right, is effected by flexing the posterior cilium near the cell body, the bend being propagated distally as the cell glides forwards. *Mantamonas plastica* is a voracious bacterivore, gliding over bacterial cells, stopping, and ingesting them using broad pseudopods located mostly on the left side and a little on the posterior side of cells. Bacteria, ingested at up to three cells per minute, accumulate in the rear half of the cell but sometimes also stick to the posterior cilium and can move up and down it by the ciliary gliding motility. When stuck on the ventral side of *Mantamonas* cells, they can be propelled rapidly forward along the cilium by the return current of the ciliary gliding machinery; they often stick to the dorsal surface of the cell and can be actively propelled backwards, and can remain at the rear end of the cell without apparently being ingested.

Mantamonas plastica exhibits a regular pattern of growth, remaining sparse for up to 144 hours after introduction to a new dish. They flourish quickly afterwards, peaking in density at about 216 hours. Populations rapidly crash and die around three weeks after inocculation without forming cysts. Occasional cultures (about one in twenty) remain sparsely populated with slow moving cells for up to six months after introduction. We did not observe cell division.

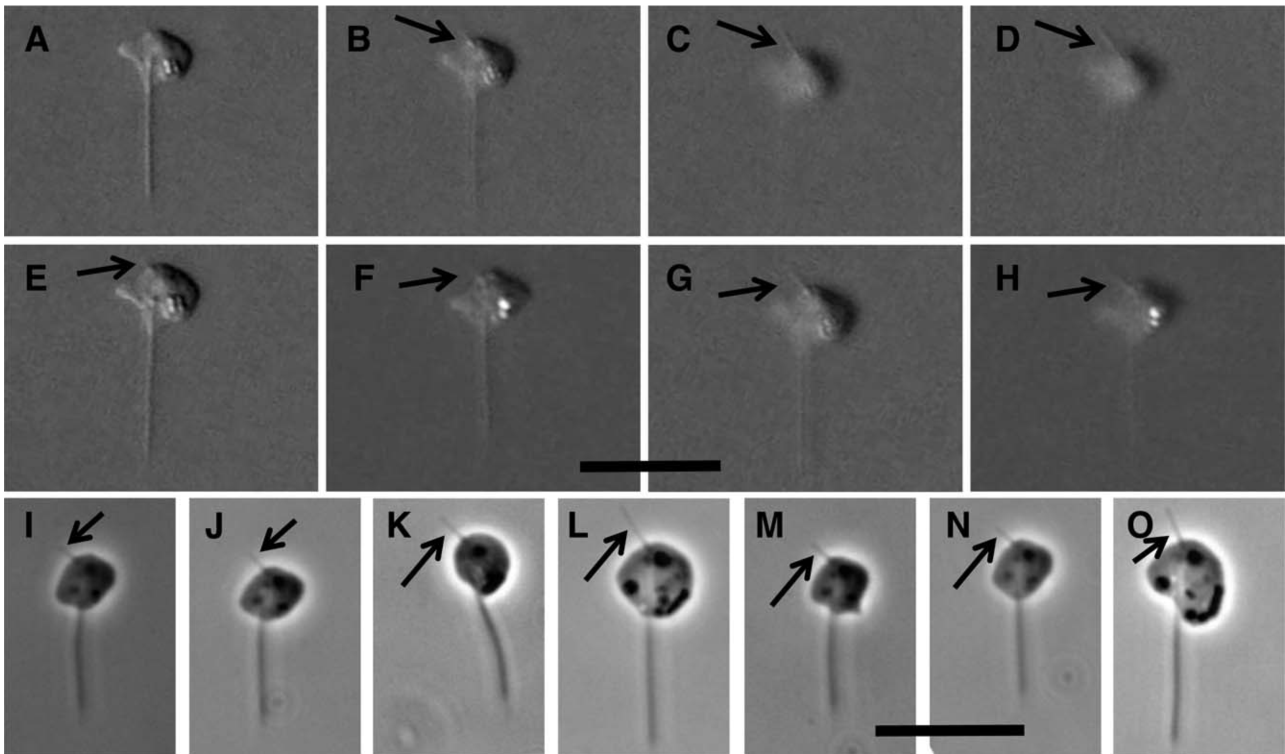

Figure 2. Anterior cilium of living *Mantamonas plastica* type strain. Differential interference contrast (**A-H**) and phase contrast (**I-O**) micrographs. Whereas the posterior cilium is conspicuous, the anterior cilium can only be seen by careful observation. **A-D** One cell at different focal positions. **E-H** Another cell at different focal positions (DIC). **I-O** Seven different cells. Arrows indicate anterior cilium. Scale bars 10 µm.

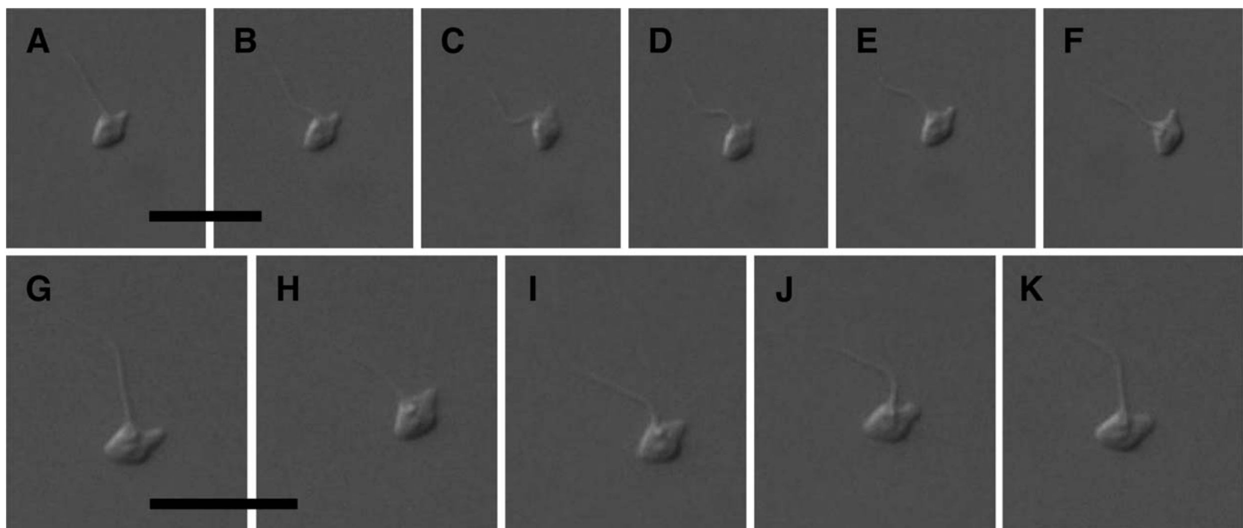

Figure 3. Locomotion of living *Mantamonas plastica* type strain. Differential interference contrast light micrographs. Turning is effected by flexing the posterior cilium initially near to the cell body. Turns more often to the right as in **G-K**. **A-F** Serial consecutive frames of a cell during gliding, over a 1 s period; **G-K** Serial non-consecutive frames of a cell during gliding, over a 1.5 s period (nucleus also visible in cell anterior). Scale bars 10 µm.

Sequence Phylogeny

We sequenced 18S and 28S rRNA genes from both isolates of *Mantamonas*, which proved to be very distinct genetically. In order to obtain concatenated 18S/28S trees with better taxon sampling of Apusozoa and key outgroups than any previously published ones we also sequenced 18S and 28S rDNA from a new isolate of *Planomonas* (3b) from the same locality (Cumbria, England) and 28S rDNA from 10 other species: two apusomonads (*Manchomonas bermudensis*; *Thecamonas trahens*); *Micronuclearia podoventralis*; *Breviata anathema*; and six Amoebozoa (*Phalansterium solitarium* and "*filosum*"; *Vannella septentrionalis*; "*Vermamoeba*" *vermiformis; Rhizamoeba saxonica* and *Acanthamoeba castellani*). When constructing trees, we compared three alignment masks of differing lengths to determine if greater or fewer positions in the dataset significantly affected the placement *Mantamonas* or the confidence values generated. For both 18S rRNA and 28S rRNA single-gene trees, the position of *Mantamonas* was identical for each mask; even the bootstrap support for it was almost the same. As the topology of both the 18S and 28S trees was also essentially the same for the liberal, medium and conservative alignments for each molecule (see Methods), though the bootstrap support for generally accepted relationships were frequently marginally greater the more positions were included, we carried out the two-gene analysis only with the most inclusive alignments (4014 nucleotide positions, in contrast to only 2574 in Moreira et al. 2007 or 3247 in Marande et al. 2009). None of the sequence trees can be correctly rooted, as the sequences for excavates, chosen as the outgroup for reasons explained by Cavalier-Smith (2010a), never all grouped together, Loukozoa and Metamonadida being wrongly separated into conflicting places on different trees; therefore they were arbitrarily rooted on Loukozoa.

The two distinct *Mantamonas* ribotypes make up a robustly monophyletic clade in all our analyses despite differing so substantially from each other that they must be separate species and mutually more distant than are many genera. Both 28S rRNA (Fig. 4) and concatenated 18S/28S rRNA (Fig. 5) gene analyses show Mantamonadida as sister to Apusomonadida with strong support (1 Bayesian posterior probability (PP)/100% bootstrap support (BS) ML and 1 PP/98% ML BS respectively); that topology was also strongly supported for a separate PhyML analysis using an ultra-stringent mask including only 997 nucleotides for 28S rRNA and 1227 nucleotides for 18S rRNA (selected by default setting of G-blocks). On the concatenated tree, the Mantamonadida/Apusomonadida clade is sister to Amoebozoa (with moderate Bayesian but negligible ML support), but only to some Amoebozoa with 28S rRNA alone; both the 28S rRNA and the concatenated analyses showed Planomonadida as sister to opisthokonts with high (Bayes) or negligible (ML) support (this position for *Planomonas* was previously found with concatenated rRNA trees: Moreira et al. 2007). With the taxa used for the concatenated tree, Mantamonadida is weakly sister to Planomonadida on the 18S rRNA maximum likelihood tree (23% BS; Fig. 6) and on the ultra-stringent PhyML tree (27% support), weakly sister to Apusomonadida on parsimony trees, and sister to an almost certainly artefactual clade comprising Planomonadida, Heterokonta, and Cercozoa on the Bayesian tree (0.27 PP). Distance methods for 18S rRNA placed *Mantamonas* as sister to *Breviata* (BioNJ 20% BS) (as did PhyML with the same mask) or clustered it with *Vannella* and Apusomonadida (minimum evolution).

To rule out the possibility that *Mantamonas* is more closely related to taxa for which 28S rRNA sequences are unavailable, e.g. the diphylleid, telonemid, or malawimonad zooflagellates, we also ran 18S rRNA trees including them and a greater diversity of other eukaryotes. The 121-taxon Bayesian tree in the electronic supplementary material shows that *Mantamonas* does not group with any of these other lineages, being sister to Planomonadida with weakish support (40% ML BS; 0.89 PP). On this tree Apusozoa are not holophyletic, since Apusomonadida is sister to opisthokonts whereas the Planomonadida/Mantamonadida clade is sister to Apusomonadida/opisthokonts plus Amoebozoa (0.98 PP and negligible 15% ML support). By running other trees with a still greater variety of models, algorithms, taxon samples, and masks, we concluded that the position of *Mantamonas* on 18S rRNA trees is not stable and is never significantly supported statistically.

Thus the two most robust trees (Figs 4 and 5) agree that *Mantamonas* and the two previously established gliding zooflagellate orders of Apusozoa, plus Amoebozoa, are probably the most closely related protozoa to opisthokonts, and the poorly resolved and mutually mildly conflicting 18S rRNA trees (e.g. Fig. 6 and others not shown) are consistent with this. However the mutual relationships among *Mantamonas*, *Planomonas*, Apusomonadida, opisthokonts, and Amoebozoa are less clear. The fourth, non-flagellate putatively

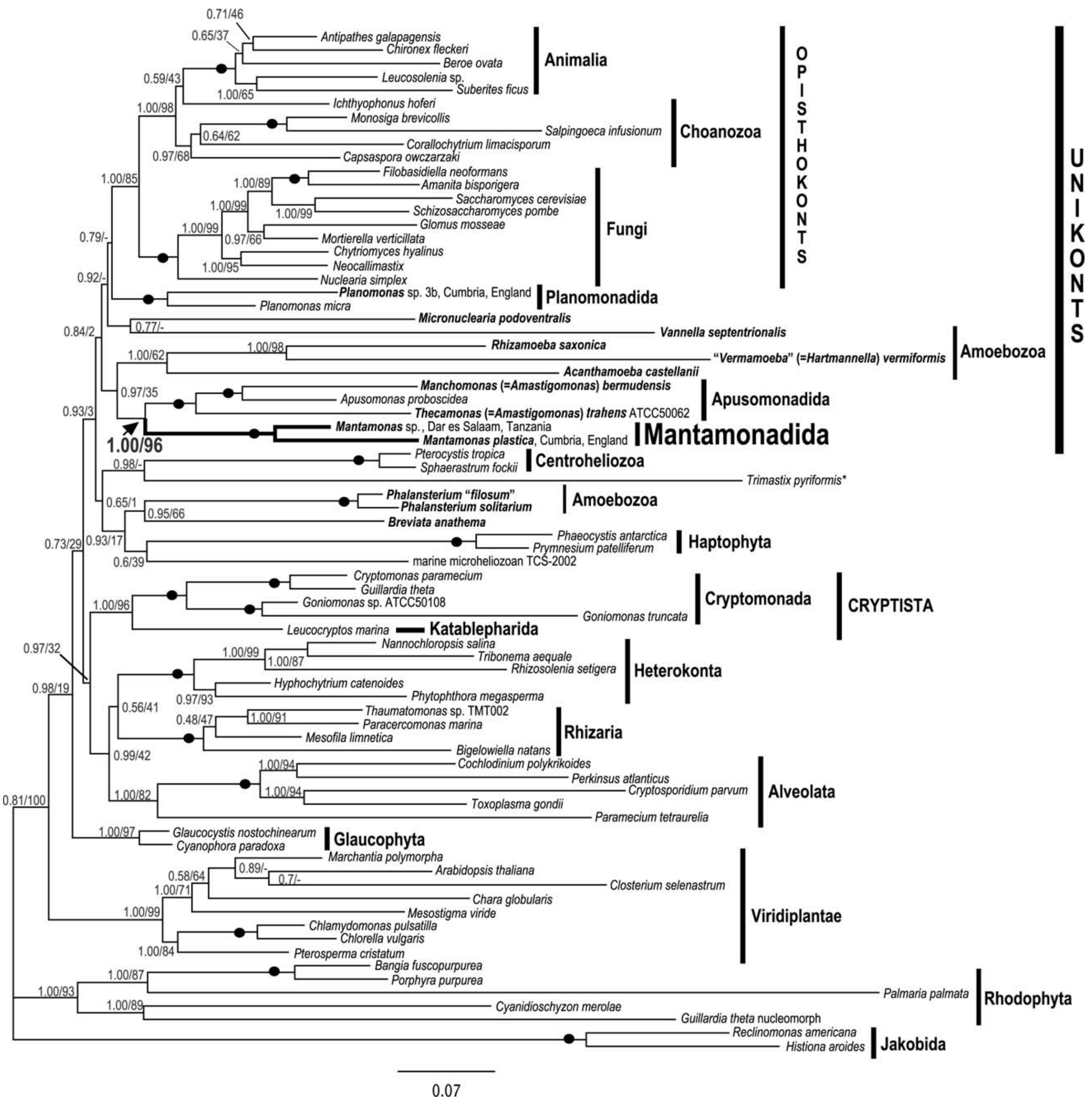

Figure 4. Position of Mantamonadida on 28S-rRNA gene phylogeny. Bayesian (GTR + gamma + I + covarion model) phylogeny of 76 28S rRNA sequences using 2454 nucleotide positions. Support values are Bayesian posterior probability (left) and bootstrap support (right) inferred using RAxML (GTRMIX + gamma + I), 100 pseudoreplicates. Dashes indicate nodes not observed by ML, black circles indicate 100% support by both methods. The scale bar indicates 7% substitutions per nucleotide site. New sequences are shown in bold. Asterisk indicates incomplete sequence**.** In all figures new generic and species names to be published by Smirnov et al. (2010 submitted) are placed in inverted commas: "*Vermamoeba*" for *Hartmannella vermiformis* and species *Phalansterium "filosum"*. That paper is their primary publication and their inclusion in the present paper (should it be published earlier) has no nomenclatural significance.

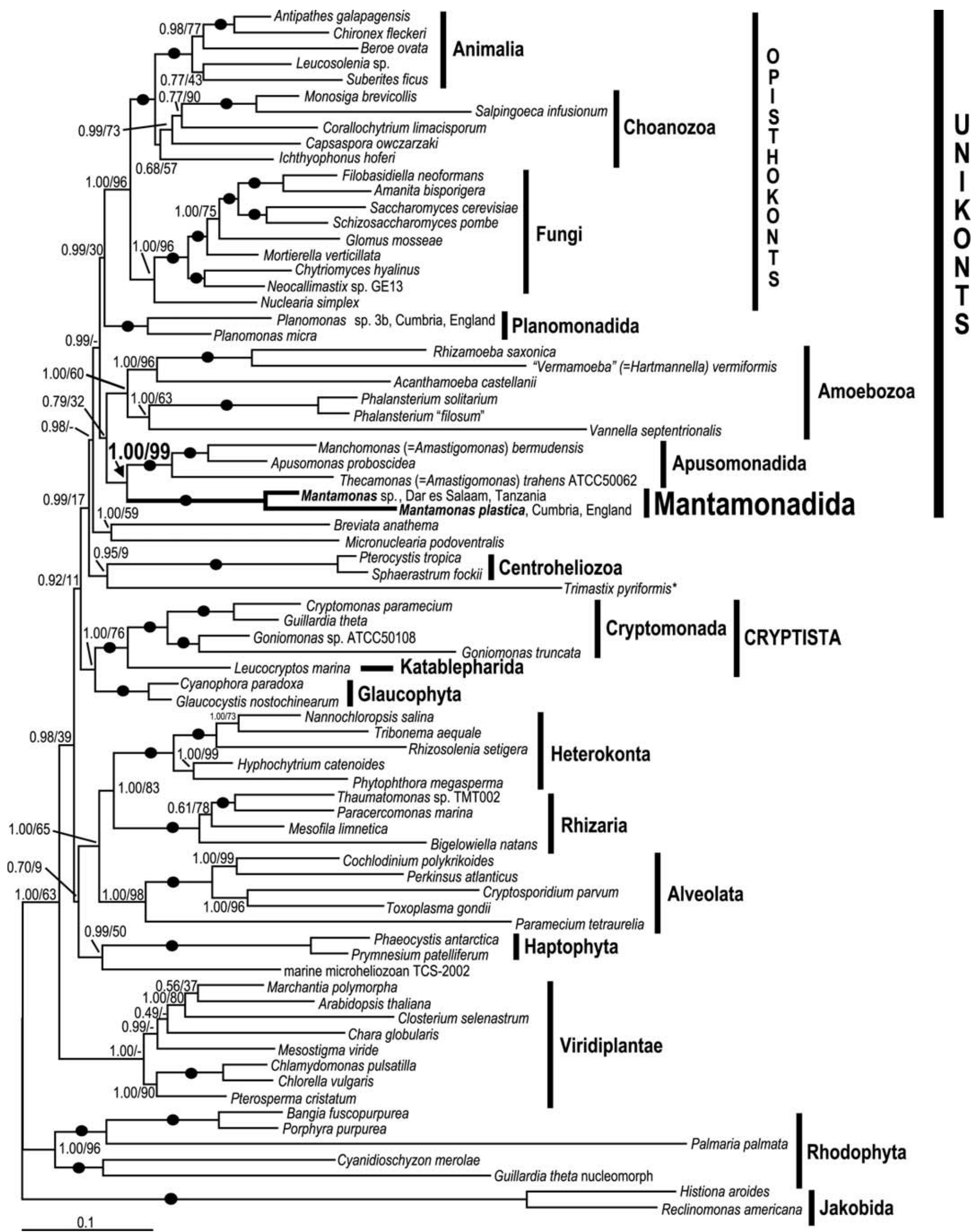

Figure 5. Position of Mantamonadida on 18S/28S-rRNA concatenated two-gene phylogeny. Bayesian (GTR and gamma plus covarion model) phylogeny of 76 18S and 28S rRNA sequences using 4014 nucleotide positions. Support values are Bayesian posterior probability (left), and bootstrap support (right) inferred using RAxML (GTRMIX + gamma + I), 100 pseudoreplicates. Dashes indicate nodes not observed by ML, black circles indicate 100% support by both methods. The scale bar indicates 10% substitutions per nucleotide site. New mantamonad sequences are shown in bold. Asterisk indicates incomplete 28S sequence.

apusozoan group *Micronuclearia* never grouped with *Mantamonas* or any other of these unikont groups; its position being even more inconsistent among trees. With 18S rRNA *Micronuclearia* was weakly sister to *Breviata* (Bayes) or to no particular group (ML) on the 76 taxon trees (Fig. 6). On the 121 taxon tree (Supplementary Fig.) it was sister to *Diphylleia rotans* (Bayesian tree), a non-gliding

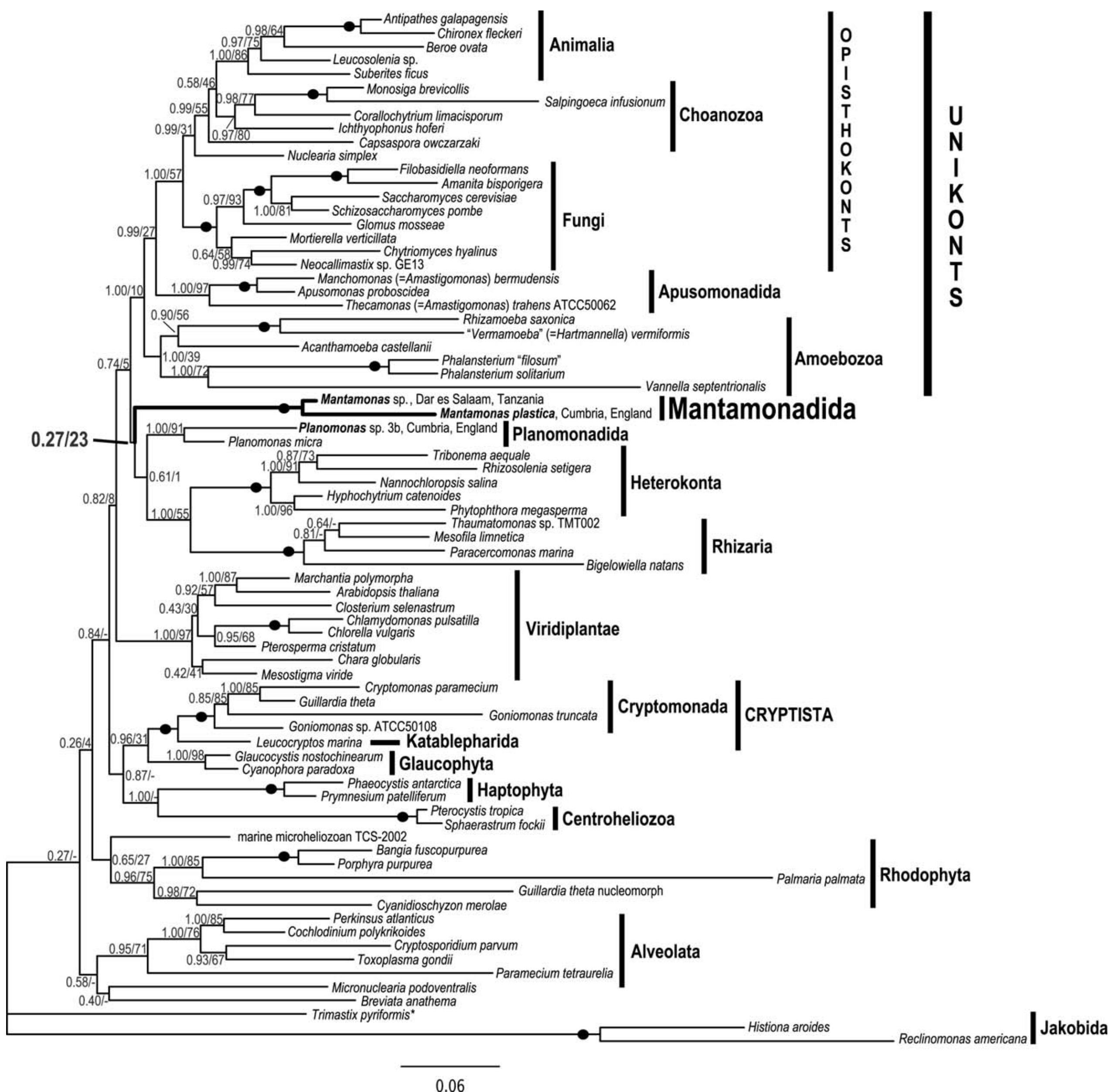

Figure 6. Maximum likelihood (RaxML) 18S rRNA gene phylogeny for 76 eukaryotes. Support values are Bayesian posterior probability (left), and bootstrap support (left) inferred using RAxML (GTRMIX + gamma + I), 100 pseudoreplicates. Dashes indicate nodes not observed by ML, black circles indicate 100% support by both methods. The scale bar indicates 6% substitutions per nucleotide site. New sequences are shown in bold.

zooflagellate of unclear evolutionary affinities (Brugerolle et al. 2002; Cavalier-Smith 2003a), or alveolates (ML). With 28S rRNA *Micronuclearia* was weakly sister to the amoebozoan *Vannella* (Bayesian), which was itself incorrectly placed, or to *Phalansterium* and the enigmatic anaerobic amoeboflagellate *Breviata* (ML). On the concatenated tree it was weakly sister to *Breviata* (Fig. 5). Thus our two-gene trees do not clarify whether *Nuclearia* is truly an apusozoan, or the position of *Breviata*; no tree showed *Breviata* as sister to Amoebozoa as on a multiprotein tree (Minge et al. 2009) or to Apusomonadida as Walker et al. (2006) suggested.

Other inconsistencies between the 18S and 28S rRNA trees not germane to the present paper emphasize that neither molecule alone adequately resolves the deepest eukaryotic branches.

Nonetheless, in comparison with a previous study (Moreira et al. 2007) the exclusion of long-branch Amoebozoa, Rhizaria, and Excavata, and the inclusion of a greater fraction of the molecule and many more Apusozoa, as well as new short-branch amoebozoan sequences, has considerably improved the 28S rDNA tree, though it still has problems in resolution as Amoebozoa are not all together. No longer is there a grouping of apusomonads and Rhizaria (probably artefactual) or an almost certainly artefactual grouping of Amoebozoa with red algae and excavates. Both single-gene trees and the concatenated tree now recover with reasonable support the relationship between heterokonts, alveolates and Rhizaria seen on all recent multiprotein trees (Burki et al. 2007, 2008, 2009; Liu et al. 2009; Yoon et al. 2008), as with weaker support did the concatenated rRNA gene tree of Marande et al. (2009). Our two-gene tree has the best taxon sampling of the major eukaryote lineages of any 18S/28S rRNA tree to date. It shows *Breviata*/*Micronuclearia* as sister to unikonts, not deep in the tree or disrupting established amoebozoan relationships as in the single-gene trees. The two-gene tree is superior to either single-gene tree in many places where support values are higher, judged by its congruence with other evidence, but it still disagrees with multigene trees in a few respects, notably the position of cryptists (for which 18S rRNA and 28S rRNA trees are discordant) and placement of jakobids within Plantae instead of with the other excavate *Trimastix* (a long-branch problem). Apart from the failure to recover the monophyly of excavates, probably because we could sample them only sparsely as we chose to exclude the longest branches, the most striking difference from Moreira et al. (2007) is that apusomonads no longer show any relationship with Rhizaria, but group within the unikonts together with *Mantamonas*, indicating that this feature of that previous two-gene tree was an artifact of inadequate taxon sampling, an inconsistency our new sequences rectify. The concatenated rRNA tree of Marande et al. (2009) also was not in total agreement with multiprotein trees, but unlike ours did recover the monophyly of Plantae and of Apusozoa and placed Apusozoa (*Planomonas micra* and *Apusomonas* only) as sister to opisthokonts, nowhere near Rhizaria; but apusozoan holophyly on that tree could be mainly because it omitted Amoebozoa, *Micronuclearia*, and *Trimastix*, and also differed from ours in other ways (e.g. omission of *Breviata* and inclusion of Euglenozoa but fewer nucleotides). Though the backbone in this part of the tree is well supported in the Bayesian analysis, ML is sometimes discordant and bootstrap support is weak.

Taxonomy

Mantamonas Cavalier-Smith and Glücksman gen. n. **Diagnosis:** gliding marine biciliate protozoa; cell shape strongly flattened, plastic, asymmetric, with characteristic projection on the left side of the cell (viewed dorsally); pseudopodia used for feeding; smooth gliding locomotion by means of the posterior cilium, held straight without undulation during forward movement, turning by a single asymmetric flexing close to the cell body, normally to the right; anterior cilium extremely thin and hard to see by light microscopy, held forwards and to the left of the longitudinal axis, almost immobile; posterior cilium conspicuous, held almost straight backwards, usually acronematic; bacterivorous; non cyst-forming. **Etymology:** *Manta*- from manta ray (*Manta birostris*), as the overall shape of the cell, its trailing cilium, and steady movement is reminiscent of a swimming ray; Gk *monas* unit. **Type species:** *Mantamonas plastica* Glücksman and Cavalier-Smith sp. n. **Diagnosis:** cell size ~2 μm thick, 3–7 μm long, 3–7 μm wide; projection to the left side of the cell proportionately longer and narrower on smaller cells; pseudopodia mostly on posterior end, stick to substratum and are drawn out by the forward progression of the cell before being released and retracted; anterior cilium up to ~3 μm long, held forwards and to the left ~40–50 degrees to the longitudinal axis, does not beat except for slight terminal vibration; posterior cilium, ~10 μm long, conspicuous and sometimes acronematic; nucleus visible in about a third of cells as a spherical body in the right anterior quadrant of the cell immediately to the right of the proximal end of the posterior cilium; bacterivorous; non cyst-forming. **Type culture**: CCAP 1946/1; type illustrations: Figures 1 and 2; type sequences: GenBank accession numbers GU001154 (18S rRNA) and GU001155 (28S rRNA); type locality: littoral marine sediment, Walney Island, Burrow-in-Furness, Cumbria, England, November 2007; collector and isolator: David Bass. **Etymology:** *plastica* emphasizes the plasticity of the cell.

New higher taxa: Family Mantamonadidae Cavalier-Smith, type genus *Mantamonas*, and order Mantamonadida Cavalier-Smith. **Diagnoses**: both as for the genus *Mantamonas* above. We do not assign Mantamonadida to either existing apusozoan class (Thecomonadea containing only Apusomonadida; Hilomonadea containing Planomonadida and Micronucleariida: Cavalier-Smith et al. 2008b) since electron microscopy is needed to establish whether *Mantamonas* belongs to Thecomonadea, as the 28S rRNA trees suggest is likely, or is ultrastructurally more like *Planomonas* or even has a novel ultrastructural morphotype meriting a third class.

Implications for Protist Evolution

Our microscopic observations and phylogenetic analyses indicate that *Mantamonas* is a distinctive new deep-branching lineage of gliding flagellates with several morphological features in common with Planomonadida and Apusomonadida, and which probably belongs to the unikont part of the eukaryotic tree - unikonts were defined by Stechmann and Cavalier-Smith (2003a,b) but have now been modified by the inclusion of Apusozoa (Cavalier-Smith

2010a; Cavalier-Smith and Chao 2010). Some gene trees long suggested that Apusomonadida might actually be sister to opisthokonts (Cavalier-Smith and Chao 1995; Cavalier-Smith and Chao 2010; Kim et al. 2006) and the same has been suggested for *Planomonas* (Atkins et al. 2000; Moreira et al. 2007). The fact that on almost all our many trees *Mantamonas*, Planomonadida, and Apusomonadida group more closely with opisthokonts plus Amoebozoa than they do with bikont eukaryotes, makes it likely that all these three groups of Apusozoa are now correctly placed in the unikonts. As Cavalier-Smith (2010a) explains in detail, the presence in an apusomonad, Multimonas (then called Amastigomonas) *marina,* of the dihydrofolate-thymidine-synthetase gene fusion that was formerly regarded as a marker for bikonts (Stechmann and Cavalier-Smith 2002, 2003b) probably means that Amoebozoa and opisthokonts independently reversed the fusion, which must have occurred earlier in eukaryote evolution than was once thought; thus its absence is not a unifying feature of unikonts. The inclusion of Apusozoa means that despite their name unikonts were ancestrally biciliate: the common ancestor of all unikonts probably actually had the same pattern of ciliary transformation (anterior cilium younger; posterior cilium older) as bikonts (Cavalier-Smith 2010a) - ciliary transformation in the amoebozoan *Physarum* that once suggested otherwise was previously misinterpreted (see Roger and Simpson 2009). Thus only certain myosin characters (Richards and Cavalier-Smith 2005) remain as unikont synapomorphies. The discovery of *Mantamonas* adds yet another clearly biciliate group to the unikonts.

Despite the absence of ultrastructural information, the strongly acronematic character of both cilia and apparently exclusively gliding lifestyle (no swimming was ever observed) together point to *Mantamonas* being more closely related to Planomonadida and Apusomonadida than to any other Protozoa, as no others share both characters so strongly. Some morphological characters (dorso-ventral flattening, extremely thin anterior cilium, absence of ciliary sleeve) are more similar to *Planomonas*, but others (pseudopodia, albeit of different form; body flexibility) are more characteristic of Apusomonadida. As *Mantamonas* has a unique combination of characters that distinguish it clearly from both other gliding apusozoan orders, and as our molecular analyses strongly indicate that *Mantamonas* is not closely related to either, we established the new order Mantamonadida for this unique morphotype. We suggest that the last common ancestor of Mantamonadida, Apusomonadida and Planomonadida was probably an obligatorily gliding biciliate with highly acronematic anterior and posterior cilia, and that Apusozoa, including all three orders, is probably monophyletic in the proper historical sense of that word (Cavalier-Smith 2010b), i.e. these characters probably did not arise independently from non-gliding ancestors.

But it remains to be determined whether Apusozoa are paraphyletic or holophyletic, an uncertainty arising because on single-gene trees planomonads and apusomonads sometimes group together (Cavalier-Smith and Chao 2003b) but often do not (Cavalier-Smith et al. 2008b; Cavalier-Smith and Chao 2010). On this point our sequence trees are indecisive, even though our concatenated tree is probably substantially more reliable for the relationships of Apusozoa than a previous one (Moreira et al. 2007). Marande et al. (2009) also demonstrate the superiority of concatenated 18S/28S trees over those for either gene alone, and recovered a well-supported apusozoan clade comprising *Planomonas* and *Apusomonas* that was sister to opisthokonts with moderate support. Though 28S rRNA and concatenated trees both suggest with relatively high support that mantamonads are sisters to apusomonads, 18S rRNA trees are collectively unresolving for the position of *Mantamonas*. The use of the PhyloBayes algorithm with the CAT model for heterogeneous evolution does not help in the inherent low resolution of 18S rRNA for most deep branches, for the positions it found for apusomonads (sister to opisthokonts) and planomonads (sister to Apusomonadida/opisthokonts) both remained weakly supported (Cavalier-Smith and Chao 2010). In some respects the 18S rRNA tree is inferior topologically to the 28S tree, e.g. in placing *Nuclearia* and Glaucophyta with the wrong sister group (as judged by trees using over 100 protein sequences, e.g. Burki et al. 2009; Liu et al. 2009) with quite strong support. If the 28S rRNA tree is also more reliable for *Mantamonas*, then it may really be sister to Apusomonadida not Planomonadida. We have initiated EST sequencing projects for *Mantamonas* and several planomonads and apusomonads, as sequence data may be needed from scores or even hundreds of genes for a firmer answer.

Functional and Ecological Implications

Mantamonas cells catch the eye in particulate environmental samples because of their smooth, regular, gliding effected by their posterior cilium. This mode of continual cell movement is compara-

ble to that of apusomonads, which glide smoothly along the substratum to which the cells adhere strongly by their ventral surface. Planomonads also glide on surfaces using their posterior cilium but exhibit a conspicuous, vigorous nodding motion characteristic of all members of the group. This planomonad nodding is absent in *Mantamonas*, but its characteristic turning to the right is produced by an asymmetric flexing close to the cell body; this single asymmetric flexing is very similar to the periodic flexing at this position that causes the nodding motion of planomonads. Thus the posterior gliding cilium of planomonads and mantamonads may have a fundamentally similar underlying structure that promotes asymmetric flexing close to the cell body. The essential difference may lie in the mantamonad posterior cilium adhering to the substratum throughout its length, whereas in planomonads the proximal region is free, allowing the cell body to nod. This contrast may stem from the greater plasticity of the ventral side of the cell body of *Mantamonas* allowing more complete ciliary contact with the substratum.

Another distinctive morphological characteristic of *Mantamonas* is its anterior cilium, so extremely thin and acronematic throughout its whole length that it is difficult to see by light microscopy; in this it resembles most *Planomonas* species (Cavalier-Smith et al. 2008b) more closely than apusomonads (though a few apusomonad species also have a largely acronematic anterior cilium: Cavalier-Smith and Chao 2010). Unlike in most protists, this cilium scarcely moves and is held forward motionless except for low amplitude vibrations of its tip as the cell glides, possibly functioning in a sensory capacity. Apusomonads, including *Apusomonas*, mostly have a far more conspicuous anterior cilium, often visible as extending through a thicker sleeve ('proboscis') and beating asymmetrically from right to left (Cavalier-Smith and Chao 2010); no ciliary sleeve was apparent in *Mantamonas*; in this respect they are more like planomonads which are also similarly dorsally flattened. However, in planomonads the slender anterior cilium beats stiffly from right to left as the cell nods vigorously by flexing its posterior gliding cilium during locomotion. The anterior cilium points forward and slightly to the left in all three groups.

The cell shape of *Mantamonas* is asymmetric because of its distinctive protrusion on the left side of the cell. This feature varies in size and shape between cells and according to the movement of the individual cell, though it is invariably present and easy to distinguish under the light microscope. No other known group has precisely this characteristic; the gliding biciliate marine heterokont *Caecitellus* has a less pronounced left bulge associated with a cytopharynx (Hausmann et al. 2006), but is readily distinguished by the active oar-like movement of its longer and thicker anterior cilium and its more rounded, somewhat triangular and less plastic cell shape, including the complete absence of posterior pseudopodial prolongations. Apart from its characteristic protrusion, *Mantamonas* cell shape is somewhat plastic, a trait shared by many apusomonads, which emit long branching pseudopods from their ventral groove. Both planomonads and apusomonads have a proteinaceous, dense pellicle layer under their dorsal plasma membrane reinforcing the shape of their cell. The greater plasticity of *Mantamonas* at its rear end and on its left side suggests that if it has a dorsal pellicular layer, like all previously known Apusozoa, this may not cover the whole of its dorsal surface; it might be concentrated especially in the right anterior quadrant of the cell which appears to be the most rigid as we never saw pseudopods or ingestion of bacteria there. Electron microscopy is needed to see if *Mantamonas* has a pellicle layer like other Apusozoa, and, if it does, whether it is double as in Apusomonadida or single as in Hilomonadea, but perhaps because of their greater plasticity we have so far been unable to achieve satisfactory fixation.

As a fourth order within Apusozoa, *Mantamonas* is an important new finding among the still substantially uncharted free-living gliding zooflagellates. As we also saw *Mantamonas* in cultures from Mexican and Welsh littoral samples, we suspect that the genus is probably cosmopolitan, though it may be restricted to marine environments - unlike planomonads and apusomonads; to establish its species richness and whether individual species also are cosmopolitan or geographically restricted, and to elucidate their ecology, numerous isolates must be cultured and genetically characterized. The other cultures unfortunately died before we could take pictures or extract DNA. However they were morphologically similar to *Mantamonas plastica*. As the two known sequences, though highly divergent, were likewise from morphologically rather similar strains, we suspect that it may not prove possible to identify *Mantamonas* species solely by morphology. That they have been overlooked previously and related sequences have not yet been found in environmental samples (Richards and Bass 2005) suggests that they may be relatively rare in most habitats compared with planomonads and apusomonads, both of which are known from environmental DNA analyses and are amongst the most frequently reported zooflagellates in micro-

scopic surveys (Patterson and Lee 2000). Possibly the best way of establishing their diversity will be to use the strategy of clade-specific primers to make gene libraries from environmental DNA (Bass and Cavalier-Smith 2004; Lara et al. 2009), which we are now implementing on the basis of our new sequences.

Methods

Isolation and culture: *Mantamonas plastica* was found by DB in November 2007 in an enriched marine sediment sample from Walney Island, Burrow-in-Furness, Cumbria, England. It was isolated by serial dilution into 96-well microtitre plates containing 250 μl aliquots of artificial seawater (CCAP recipe). After isolation it was introduced into 90 mm plates containing artificial seawater and one boiled wheat grain as food for the endogenous bacteria it eats. It was maintained in this medium at 18 °C and subcultured every 2 weeks and has been placed in the UK Culture Collection of Algae and Protozoa, Oban as strain CCAP 1946/1. A second *Mantamonas* strain (*Mantamonas* sp.) was isolated from muddy sand collected in January 2008 by TCS from a beach in Dar es Salaam, Tanzania. Though DNA was extracted from a clonal culture purified by EG, it died before we could photograph it. It was morphologically similar to *M. plastica*. Third and fourth strains were briefly observed in 96-well microtitre plates holding littoral marine samples from (a) the Yucatan peninsula, Mexico and (b) the northern coast of Wales, but died before they could be isolated. A new *Planomonas* strain (3b), closely related to the three South Altantic environmental DNA sequences that are sister to the tight 3-sequence clade that includes *Planomonas mylnikovi* (Cavalier-Smith et al. 2008b), was isolated from the same locality as *Mantamonas plastica* (Cumbria, UK) and will be described as a new species later.

Light microscopy: Differential interference contrast (DIC) pictures of living *Mantamonas* growing in a glass-based dish were taken on a Nikon Eclipse 80i microscope with x60 NA 1.0W dipping water immersion lens and recorded on a Sony HD HDR-HC3E digital video camera set to maximum optical zoom and frames captured by software Final Cut Express HD. For the DIC pictures, we designate the broad surface pressed against the glass bottom of the dish as ventral, whereas the dorsal surface faces away from the substratum during gliding. Micrographs captured from videos using water immersion dipping objectives and DIC microscopy show the organism from above (its dorsal side). Phase contrast pictures of living *Mantamonas* cells (Figs 1, M-Q; 2, I-O) were taken using a Zeiss Axiovert 200 M microscope equipped with a X100 immersion objective and an Axiocam HR digital camera. To facilitate comparison with DIC pictures, phase contrast micrographs of cells gliding on the underside of coverslips (thus viewed from the ventral surface) were flipped so that the characteristic protrusion from the cell body is on the cell's left.

Gene sequencing: DNA was extracted from uniprotist clonal cultures using the UltraClean soil DNA isolation kit (MoBio Laboratories Inc.). 18S rRNA gene sequences were amplified by PCR with primers SA1 (5'-CCTGGTTGATCCTGCCAGTAG-3') and SB1 (5'-GATCCTTCYGCAGGTTCACCT-3'). Sequencing primers were SB1 and internal primers S6R (5'-ACCGC-GGSTGCTGGCACCAGACTT-3') and Pre3NDF (5'-CAGC-AGGCGCGCAAATTACCC-3'). 28S rRNA gene sequences were amplified by PCR with eukaryotic and *Mantamonas*-specific primers. Initially we used general eukaryote primers 2F (5'-ACSCRYYGRRYTTAAGCA T-3') and 3180R (5'-GGG TAAAACTAACCTGTCTCACGACGGTC-3') to generate a ~2.8 kb primary amplicon for the Tanzanian species. Primers for *M. plastica* (Cumbria strain) were a *Mantamonas*-specific primer bass1380f (5'-ATTCCGATCACGAACGAC-3') and 3180R to generate a ~3 kb primary amplicon. These long fragments were then used as templates for nested PCR to amplify four overlapping internal fragments spanning the entire length of the initial amplicon generated by 2F and 3180R primers in conjunction with other eukaryote-specific internal primers so as to generate enough template for direct sequencing. Internal primers used were B20F (5'-ACCGATAGCRRABAAGTASHRYGA-3'), 12R (5'-CTATCCTGAGRGAAAATTCG-3'), 9F (5'-CCGTCTYGAAAC-ACGGAC-3'), 5R (5'-GTTACACAYTCCTTAGCGG-3'), 12F (5'-CGAARTTTCYCTCAGGATAG-3') and G19R (5'-GGGTGAA-CAATCCAACACTTKRNGAAT-3'). These primers were also used for sequencing along with the following: CDr (5'-GACTCCTTGGTCCGTGTTTCAAGA-3'), 5F (5'-CCGCTAA-GGAGTGTGTAA-3'), 19R (5'-CYRDBTTGCCGACTTCCCT-3') (EAS). *Breviata anathema* DNA (strain ATCC 50338) was kindly supplied by Jeffrey Silberman and *Acanthamoeba castellanii* DNA by Michael Gray. The other 9 amoebozoan and apusozoan DNAs had been extracted for 18S rRNA sequencing from cultures held in our laboratory as described previously (Smirnov et al. 2007, 2008, 2010; Cavalier-Smith et al. 2004, 2008b; Cavalier-Smith and Chao 2003b). Their 28S rRNA was amplified with various combinations of eukaryotic and specific primers and sequenced with appropriate internal primers using standard methods for ABI automated sequencing (CB and EC). For most sequences we amplified the 3' part of the 18S rRNA and the complete ITS1, 5.8S rRNA and ITS2 at the same time, so that they are included in the sequences submitted to GenBank. We also sequenced the 28S rRNA gene of *Apusomonas proboscidea* and *Planomonas micra* more fully than before. GenBank accession numbers: GU001154 to GU001170.

Phylogenetic analysis: 18S rRNA gene fragments of 1779 nt (*Mantamonas plastica*) and 1685 nt (Tanzania strain) were edited and concatenated using CodonCode Aligner 3.03 (http://www.codoncode.com/aligner/). Using MacGDE (http://www.msu.edu/~lintone/macgde) and BioEdit v7.0.9.0 (Hall, 1999), the nearly complete sequences were added to a pre-existing alignment of hundreds of diverse eukaryotes, from which we selected a representative subset of 121 taxa. Sequence alignment masks were selected by eye; the most liberal mask (1560 positions), included as many well-aligned positions as possible. Two stricter masks excluded the most rapidly evolving regions: medium (1470 positions), and conservative (1380 positions) and constructed trees for each for comparison.

28S rRNA gene sequences were edited and concatenated in the same way, yielding fragments of 3156 nt (*Mantamonas plastica*) and 2802 nt (Tanzania strain), and subsequently added to a pre-existing alignment of 74 representative eukaryotic 28S rRNA gene sequences, selected to match the taxonomic groups used in the 18S rRNA analysis. We chose by eye three different sequence masks for analysis: liberal (2454 positions), medium (2372 positions), and conservative (2270 positions), and constructed trees for each for comparison. To reduce long-branch artifacts, which can be particularly severe for Amoebozoa, retarian Rhizaria, and Excavata, which contain many long-branch taxa, we mostly included only the shortest branch representatives of each group; the amoebozoan species chosen for 28S rRNA sequencing were selected because of their substantially shorter branches on 18S rRNA trees than those included previously (Moreira et al. 2007); as expected, their branches are

also much shorter on 28S rRNA trees. By excluding numerous long-branch taxa and manually aligning each sequence carefully we were able to include many more nucleotide positions than in the earlier analysis (Moreira et al. 2007). A concatenated 18S/28S rRNA alignment was created; combining the liberal 18S rRNA sequence mask and the three different 28S rRNA masks, yielded three different sequence masks for analysis: liberal (4014 positions), medium (3932 positions), and conservative (3830 positions).

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian methods, with distance and parsimony also used just for 18S rRNA. We used RAxML-V (Stamatakis et al. 2005) for maximum likelihood analyses and for obtaining bootstrap values, running each of the masked alignments using the GTRMIXI model of sequence evolution (g=8); 100 runs used parsimony starting trees and 100 runs used randomly generated starting trees, and the bootstrap analyses were plotted onto the overall most likely tree. Bootstrap values were calculated using the default maximum parsimony start trees. We used MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) on the University of Oslo's Bioportal (https://www.bioportal.uio.no) for Bayesian analyses, running each of the masked alignments with 2 independent chains running for 5×10^6 generations (a burn-in of 2×10^6 was used) using the GTR + gamma (8 rate categories) + I + covarion model and sampling frequency 0.01. PAUP (Swofford 1999) was used for parsimony (heuristic searches with TBR) and distance trees the GTR + gamma + I method (parameters calculated by ModelTest: Posada and Crandall 1998) were used for BioNJ and minimum evolution trees. Neighbor joining distance trees were also calculated by Phylip using the F84 model and gamma distribution of rates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2010.06.004.

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Chapter Three

Phylogeny and evolution of Planomonadida (Apusozoa): nine new species and new genera *Fabomonas* and *Nutomomas*

Manuscript

Chapter notes

This chapter will shortly be submitted for publication. It is presented here already formatted for submission to *Protist*. Supplementary videos and alignments omitted. The planomonad taxonomy presented here is used in chapters 4 and 5.

Phylogeny and evolution of Planomonadida (Apusozoa): nine new species and new genera *Fabomonas* and *Nutomonas*

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Planomonads are widespread gliding zooflagellates from marine and freshwater sediments, but only seven *Planomonas* species are known. We cultured 13 new strains; morphology and 18S and ITS2 rDNA sequences show that 11 represent nine new species described here. Two belonging to existing species have minor ITS2 sequence differences from type strains. *Fabomonas tropica* sp. n. differs in shape and is genetically very distant from previously known planomonads, yet is ultrastructurally similar to them. ITS2 secondary structure is clade-specific, differing most sharply in the main freshwater clade from all marine species, being exceptionally short compared with earlier-diverging marine clades. Anterior cilium morphology maps simply onto the rDNA tree: one large clade (*N. mylnikovi* comb. n., known freshwater planomonads, plus new species *N. indica*, *atlantica*, *kenti, lacustris,* and very different *longa*), with a uniformly thin, entirely acronematic anterior cilium, constitutes the new family Nutomonadidae. The revised Planomonadidae (*Fabomonas*, *P. micra*, and new species *P. elongata*, *bulbosa*, and *brevis*) have a more conspicuous emergent basal region of the anterior cilium of normal thickness. *Nutomonas lacustris* differs from *N. howeae* and *N. limna* (new combinations, formerly *Planomonas*) mainly by ITS2 compensatory and/or hemi-compensatory mutations. The first soil planomonad (new *Nutomonas limna* subspecies) was isolated.

Key words: 18S rRNA phylogeny; *Ancyromonas*; *Fabomonas*; ITS2 secondary structure; *Nutomonas*; *Planomonas*

Running title: New genera and species of planomonads

Introduction

Bacterivorous protists are ecologically influential components of microbial communities (Glücksman et al. 2010; Jürgens 1994; Murase and Frenzel 2008; Neubacher et al. 2008) yet the true extent of their presumed high genetic diversity remains unknown and most species are probably still undescribed. In the past decade, many previously unclassified free-living

zooflagellates have been placed within either Cercozoa (Bass and Cavalier-Smith 2004; Cavalier-Smith et al. 2008; Cavalier-Smith and Chao 2003ab; Howe et al. 2009) or Apusozoa (Cavalier-Smith 1993, 2002, 2003a, 2003b; Cavalier-Smith and Chao 2003, 2009). Apusozoa are characterized by a distinctive dorsal pellicle and comprise three zooflagellate orders, Apusomonadida (Karpov and Mylnikov 1989; Cavalier-Smith and Chao 2010), Planomonadida (planomonads) (Cavalier-Smith et al. 2008) – the focus of this paper, and Mantamonadida (Glücksman et al. 2011), plus the non-flagellate filose Micronucleariida (non-flagellates).

Planomonads have a distinctive morphology, exhibiting a highly (often largely) acronematic anterior cilium emerging through an apical ciliary tunnel from a rounded, strongly flattened, largely rigid cell. The cell glides over surfaces on its often acronematic, posterior cilium, conspicuously nodding its cell body by kinking the cilium as it progresses. Bacteria are ingested in the ventral pellicle-free groove near where the posterior cilium emerges. Ultrastructurally, a single dense pellicle layer underlies the dorsal cell surface membrane, kinetocyst-like extrusomes are present in the rostrum, and mitochondrial cristae are flat. Planomonads are commonly found in surveys, largely of marine environments (Al-Qassab et al. 2002; Larsen and Patterson 1990; Lee and Patterson 2002; Patterson and Lee 2000), since Hänel (1979) first described freshwater examples. Cavalier-Smith et al. (2008) established the order Planomonadida after concluding that for three decades almost all recorded planomonads had been misidentified as *Ancyromonas sigmoides*, a small marine flagellate discovered and named by Saville Kent (1880-82) (Atkins et al. 2000; Hänel 1979; Lee and Patterson 2000; Patterson and Simpson 1996; Patterson and Zölffel 1991; Tong 1997; Tong et al. 1998; Vørs 1992, 1993). Cavalier-Smith et al. (2008) revised the group's taxonomy, basing it for the first time primarily on genetically and morphologically characterized clonal cultures, and described four new species: the seven then-known *Planomonas* species formed the family Planomonadidae and order Planomonadida. However, Heiss et al. (2010) have since argued that one of the new species, *Planomonas mylnikovi*, is a junior synonym of *Ancyromonas sigmoides* Saville Kent and that no serious

misidentification was involved earlier. We do not agree and consider this synonymy to be mistaken; our discussion explains why we do not accept their neotypification of *Ancyromonas sigmoides* by the *N. mylnikovi* type strain or renaming other *Planomonas* species.

To extend the revision of Cavalier-Smith et al. (2008), we isolated planomonads from three continents, studying cultures by differential interference contrast (DIC) and phase contrast light microscopy, and in one case transmission electron microscopy (TEM). We sequenced 18S and ITS rDNA and, for strains with identical 18S rRNA sequences, folded and analyzed the secondary structure of the internal transcribed spacer 2 (ITS2) rRNA in order to use the presence or absence of compensatory base changes (CBCs) as species demarcation criteria according to principles of Coleman (2003, 2009). By comparing ITS2 primary and secondary structures in the five major planomonad lineages now identified we show that three of them underwent large divergent departures from the probable ancestral state by extensive deletions or insertions. We describe 9 new species and the first planomonad clonally cultured from soil, which we make a new subspecies. By more than doubling the number of known species, we detected a striking dichotomy in how anterior ciliary structure maps onto the tree, and used this to divide planomonads into two morphologically and still more genetically distinct families: Nutomonadidae and Planomonadidae.

Results

We sequenced 18S rRNA genes for new planomonad isolates from 13 localities; nine from marine or brackish sediments, three from freshwater lake sediments, and one from soil. The nine new species include two lineages that are radically different from all others (Fig. 1): a deep-branching new freshwater species, *Nutomonas longa*, and a deep-branching new marine species, *Fabomonas tropica*. Bootstrap support for the position of *N. longa* is robust whereas that for *F. tropica* is weak. Uncertainty in the phylogenetic position of *F. tropica* is also apparent in its

sensitivity to taxon sampling; it is either the deepest-branching planomonad clade on its own (Suppl. Fig. S1) or sister to *Planomonas* (Fig. 1).

Morphologically, the most interesting novel conclusion from increasing known planomonads from seven to 16 species is that the structure of the anterior cilium maps onto the molecular tree in a phylogenetically consistent way. In the putative clade comprising *F. tropica* and *P. micra* (Fig. 1), the part of the anterior cilium that emerges from the ciliary tunnel is of normal thickness at its base and acronematic only in its distal portion. By contrast, in the entire clade comprising the original freshwater clade, *N. longa*, and the *N. mylnikovi* clade the anterior cilium is acronematic throughout its length, making it uniformly thin and much harder to see than in *F. tropica* and the *P. micra* clade. Because this consistent difference in anterior cilium maps so reliably onto the molecular tree and reflects a very ancient divergence within planomonads, we have chosen to use it to subdivide planomonads into two families. Family Planomonadidae is now restricted to those that, like the *Planomonas* type species, *P. micra*, have an anterior cilium with a thick base. The new family Nutomonadidae and its sole genus *Nutomonas* are introduced for all planomonads with a uniformly acronematic anterior cilium. This dichotomy reflects both the deep early genetic divergence between these two groups and their contrasting morphology. Given the also deep divergence within Planomonadidae, we subdivide them into two genera of contrasting body shapes, which correspond to the two clades that are sometimes sisters (Fig. 1) and sometimes not (Fig. S1).

New and Revised Families and Genera

Nutomonadidae Cavalier-Smith fam. n. **Diagnosis:** Planomonadida with ventral groove and flattened body like Planomonadidae, but with anterior cilium barely visible, acronematic throughout its emergent length; unlike in *Fabomonas* and in *Planomonas* as emended below, even the base of the light microscopically visible emergent part of the anterior cilium is extremely thin. G present at positions 19 and 79 in 5.8S rDNA, unlike in Planomonadidae (Table 3). **Type genus:** *Nutomonas* Cavalier-Smith.

Nutomonas Cavalier-Smith gen. n. **Diagnosis:** Anterior cilium barely visible, mostly acronematic; cells range from almost isodiametric to elongated ovals; ventral indentation on cell's left where posterior cilium emerges; comprises marine and freshwater species. **Type species:** ***Nutomonas howeae*** Cavalier-Smith comb. n. **Basionym:** *Planomonas howeae* Cavalier-Smith in Cavalier-Smith et al. (2008 p. 549). Synonym *Ancyromonas howeae* Heiss et al., 2010. **Etymology:** *Nuto* L. I nod; *monas* Gk unit. We also place three other existing species in *Nutomonas*: ***Nutomonas limna*** Cavalier-Smith comb. n. **Basionym:** *Planomonas limna* Cavalier-Smith in Cavalier-Smith et al. (2008 p. 550). Synonym *Ancyromonas limna* Heiss et al., 2010. ***Nutomonas mylnikovi*** Cavalier-Smith comb. n. **Basionym:** *Planomonas mylnikovi* Cavalier-Smith in Cavalier-Smith et al. (2008 p. 549). Contrary to Heiss et al. (2010), we do not accept *Ancyromonas sigmoides* Saville Kent, 1882 as the same species as *N. mylnikovi*. ***Nutomonas sinistra*** Cavalier-Smith comb. n. **Basionym:** *Ancyromonas sinistra* Al-Qassab et al., 2002 p. 138. Synonym *Planomonas sinistra* Cavalier-Smith, 2008.

Planomonadidae Cavalier-Smith, 2008 em. **Emended Diagnosis:** add to the original diagnosis (Cavalier-Smith et al. 2008 p. 548): basal part of anterior cilium conspicuous and of normal thickness (like the posterior cilium), extremely slender acroneme restricted to distal portion; 5.8S rDNA with 6 unique signatures (Table 3).

Fabomonas Cavalier-Smith gen. n. **Diagnosis:** bean-shaped cell (this elongated shape and its divergent 18S rDNA sequence distinguish it from *Planomonas* as here emended); anterior cilium obvious and acronematic; posterior cilium elongated by acroneme, up to doubling its total length; marine. ITS2 much shorter (291-293 nt) than in *Planomonas* (395-427 nt) 5.8S rDNA from *Planomonas* by having 3 unique sequence signatures and lacking the 2 *Planomonas* signatures (Table 3). Type and sole species: *Fabomonas tropica* Glücksman and Cavalier-Smith. **Etymology:** L. *faba* because bean-shaped.

Planomonas Cavalier-Smith, 2008 emended by excluding *Nutomonas*. **Emended Diagnosis:** add to the original diagnosis (Cavalier-Smith et al. 2008 p. 547): basal part of anterior cilium conspicuous, of normal thickness (like posterior cilium), extremely slender acroneme restricted to distal portion; 5.8S rDNA has two unique sequence signatures (Table 3); ITS2 structures usually with four helices (helix 1-III and a single helix distal to helix III). **Type species:** *P. micra* Cavalier-Smith, 2008. Other existing species: *Planomonas cephalopora* (Larsen and Patterson) Cavalier-Smith, 2008 and *Planomonas melba* (Patterson and Simpson, 1996).

Morphology of *Fabomonas tropica*

Fabomonas tropica was found only in marine sediment; no cysts or contractile vacuoles were seen. Figure 2 shows living *F. tropica* cells during active gliding. They are bean-shaped, 3-5 µm

long, 1.5-2.5 µm wide, and approximately 1 µm thick, though strain il19 (Red Sea) is slightly less elongated (Fig. 2S-X). Like all planomonads, *F. tropica* is biciliate and glides on its longer posterior cilium. Cells lie to its right, nodding at 2-4 cycles/second; larger cells do so faster. They change direction by a kink in the proximal fifth of the posterior cilium, turning almost exclusively to the right. Video clips of this and some other new strains are included as electronic supplementary material.

The slender anterior cilium (Fig. 2A) is about half as long as the posterior cilium, emerging from a gentle depression at the leading end of the cell. It flickers rapidly at 3-4 cycles per second with amplitude of 90-200º to the left of the cell. Its shape resembles most closely that of *Planomonas* but is less visibly acronematic, its thick base occupying a third to half its length, making it distinctly more obvious than in any *Nutomonas*. As in *P. micra*, the posterior cilium is almost always visibly acronematic, emerging from an oblique groove along the cell's ventral side (about one third of the way from its apex) and trailing with close adhesion to the substratum and without beating during straight gliding. The acroneme varies in length between 5% and 30% (Fig. 2W and H respectively) of the length of the thicker segment of the posterior cilium, appearing marginally longer in the type strain (nyk4) and strain mex1. It is most obvious in phase contrast (Fig. 2Y-AA).

A granule-bearing rounded rostrum (Fig. 2J) is at the anterior end of the cell between the ventral groove and cell apex. It is shorter and less obvious than in *P. micra*. We did not observe feeding but assume it feeds on bacteria. Probable digestive vacuoles (Fig. 2L, R, U) are in the middle or rear of the cell, and were also seen with bacteria inside them by electron microscopy (Fig. 4); these are less prominent on strain nyk4. Generally more resilient than other planomonads under laboratory conditions, the first isolate (nyk4) survived five years in a tube of marine sediment before being discovered. Cell division was not studied.

Because some trees (e.g. Fig. S1) suggested that *F. tropica* might not branch within other planomonads, we examined the type strain by TEM to check that it was ultrastructurally related

and found it has the same ultrastructure as other planomonads (Figs 3-4). A single dense pellicular layer is clearly visible beneath the dorsal plasma membrane; it is absent from the ventral groove. Oval extrusomes are clearly visible docked in the rostral rim. The mitochondrion is situated in the cell's dorsal side and has flat, radially arranged, discoid cristae. Thus, basically the same ultrastructural phenotype is found across the whole planomonad tree.

Fabomonas tropica Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Body bean-shaped, 3-5μm long, 1.5-2.5μm wide (length/width ratio ~2.0), ~1μm thick; anterior cilium obvious, acronematic (less visibly than in *P. micra*), but of normal thickness in its basal third or half, flickers rapidly; ventral groove shallower and less obvious than *P. micra*; rostrum extrusome-bearing, shorter than *P. micra*; nucleus and digestive vacuoles visible through cell surface membrane; posterior cilium emerges from ventral groove, invariably visibly acronematic, trails during forward continuous gliding; during gliding cell nods at 2-4 cycles/second, larger cells faster; turns mostly to right; marine; no cysts. **Type culture:** CCAP 1968/4; ITS2 sequence of 291 nucleotides. Type illustrations: Figure 2A-L, Y-AA. Type sequences: 18S rRNA **XXXXX** and ITS/5.8S rRNA **XXXXX**. Type locality: seawater and sediment, Ankobra Beach, Ghana, 2002 (collector J. Saldarriaga); left in a collection tube until discovery in 2007. **Etymology:** *tropica* because so far found exclusively near or in the tropics. **Other strains*: Fabomonas tropica*** strain il19, CCAP 1958/8 (Fig. 2S-X); isolated from seawater and sediment from a beach in Eilat, Israel. Rounder cells than type strain; shorter, less obviously acronematic posterior cilium; ITS2 sequence (293 nt.) 2 nucleotides longer than type strain and some differences in sequence but with no CBCs or hemi-CBCs. Type sequences: 18S rRNA **XXXXX** and ITS/5.8S rRNA **XXXXX**. ***Fabomonas tropica*** strain mex1 (Fig. 2M-R); from seawater and sand at a beach on the Yucatan Peninsula, Mexico, 2008 (coll. TCS; now dead). More prominent refractile granules on cell surface membrane than type strain; ITS2 sequence (291 nt.) same length as type strain, fewer differences from it than has CCAP 1958/8, and no CBCs or hemi-CBCs.

Nutomonas longa morphology and behaviour

Nutomonas longa was isolated from only one freshwater location. Figure 5 shows the morphology of living cells during active gliding. The mostly obviously elongated cells are 3.5-5 μm long, 3-3.5 μm wide, and approximately 1μm thick. The ventral groove (Fig. 5A) is more obviously deeply indented, especially at its anterior end, than in other freshwater nutomonads

(Fig. 7A-F). The posterior end of the cell is bulbous (Fig. 5C). A rostrum with rounded apex (Fig. 5M) sits beside the base of the posterior cilium left of the cell's anterior end. The nucleus is in the middle of the cell (Fig. 5G). A contractile vacuole is in the narrow isthmus between the deeply incised anterior of the ventral groove and the convex right side of the cell (Fig. 5D,K) and discharges on the left just posterior to the posterior ciliary base; though usually somewhat more on the right side, in some cells it is on the left or may even apparently alternate between left and right. Other non-contractile vacuoles are sometimes visible in the cell's posterior lobe (Fig. 5S).

The usually acronematic posterior cilium, on which the cell glides by adhering to the substratum throughout its length, emanates from the anterior end of the ventral groove. It is unusually variable in length, typically approximately double the cell length, and often appears longer still because of the acroneme, which sometimes even doubles its overall length. Under differential interference contrast, the acroneme may fade in and out of view during gliding (Fig. 5N-S). Cell nodding during gliding (Fig. 5N-S) is slower and within a narrower range than in other planomonads. Turns are mostly to the right, occasionally to the left. The anterior cilium, emerging (Fig. 5B, K), is evenly thin (unlike in *Planomonas* and *Fabomonas*), and flicks rapidly over a narrow range (~40º). It is difficult to see in living cultures but noticeable on micrographs grabbed from videos. We did not observe feeding, but assume it is bacterivorous; like other freshwater planomonads, it grows more slowly and less densely in culture than marine planomonads.

Nutomonas longa sp. n. Cavalier-Smith and Glücksman. **Diagnosis:** Body ellipsoidal, bulbous posterior end, 3.5-5 µm long, 3-3.5 µm wide (length/width ratio: ~1.31), ~1 µm thick; anterior cilium 3 µm but hard to see as extremely thin and of even thinness, flicks rapidly over narrow range (~40°) from pointing straight forwards to pointing partially to left; posterior cilium approximately double body length, variably acronematic, acroneme sometimes invisible; ventral groove more obvious and deeply penetrating than in *N. howeae*, triangular and deeply incised at anterior end and covered dorsally by a very thin flap; rostrum immediately anterior to base of posterior cilium is pushed to and fro by its ciliary kinking, rounded apex with about two small refractile granules; nucleus further back than in other planomonads, in mid cell;

anterior contractile vacuole close to insertion of posterior cilium on either right (usually) or left of cell; locomotion by continuous gliding on posterior cilium, cell nodding less rapid (2-2.5 nodding cycles/s) than other planomonads by kinking posterior cilium to right very close to cell, less often bending it to left slightly more distally; cells vibrate less than most planomonads during nodding; turns mostly to right; exclusively freshwater; no cysts. **Type culture:** CCAP 1958/5. Type illustrations: Fig. 5. Type sequences: 18S rRNA **XXXXX** and ITS/5.8S rRNA **XXXXX**. **Type locality:** water and sediment, Boiling Springs Lake (freshwater), North Carolina, United States (EAS 2008). **Etymology:** *longa* because cell slightly elongated.

Divergent ITS2 secondary structures in Planomonadida

We sequenced the rDNA ITS1, 5.8S and ITS2 of each species, and found marked differences in ITS2 length (Table 2) and inferred secondary structures (Fig. 6). *Fabomonas tropica* ITS2, with 291-3 nucleotides, most resembles that of other unikonts in length and structure, having three easily recognizable major helices, helix I-III (Fig. 6A) plus minor helices in the helix IV region. Helices I-III seem to be homologous with those of opisthokonts (Coleman, 2007), so the *Fabomonas* structure may be regarded as close to the ancestral type for planomonads, which is more consistent with its earlier branching than the aberrant freshwater clade, whose 18S rDNA, 5.8SrDNA and ITS sequences have all diverged much more from those of the earlier branching marine clades. Compared with *Fabomonas*, the other two marine clades have greatly lengthened their ITS2 by multiple insertions, giving lengths of 394-426 nucleotides in *Planomonas* and slightly less (331-395) in the *Nutomonas* subgenus *Kentomonas*, unsurprisingly yielding somewhat different secondary structures with much elongated helix III (Fig. 6 B, C). In the sequence alignment of ITS2 (Supplementary Material), reasonably good alignment is possible throughout each genus but only crudely if at all between genera.

The clearly derived freshwater nutomonad clade has drastically shortened its ITS2 (185-217 nucleotides) (Fig. 6D-E); some deletions seem to have been independent in *N. longa* and the major freshwater clade. *Nutomonas longa* (subgenus *Incisomonas*), with 217 nucleotides, has the least altered secondary structure in which the standard three helices are readily apparent and the

helix II side branches, and extra small 3' end helices seen in *F. tropica* are absent (Fig. 6D). The *N. howeae/limna* clade (subgenus *Nutomonas*) has the most highly modified secondary structures. Modification is greater in the shortest sequences (*N. lacustris* (Fig. 6E) and its sister *N. howeae*, which have undergone additional deletions in four regions) than in longer ones, making structural comparison with other eukaryotes difficult. Despite considerable divergence between the freshwater and marine nutomonads, the alignment reveals that some ITS2 shortening took place in regions corresponding to all three helices prior to the last common ancestor of the two freshwater subgenera (*Incisomonas* and *Nutomonas*). As helix I and II are generally shorter than III, this makes this region very short in subgenus *Nutomonas*, especially in the shortest ones where helix I and helix II merge into a single helix (Fig. 6D, E).

Figures S2-S4 (Supplementary Material) show the best ITS2 folds for all other new strains isolated here as well as for all new species and strains described by Cavalier-Smith et al. (2008). There is some variation within major clades but generally markedly less than between them. Secondary structure of *N. howeae* (Fig. S4) was identical to that of its sister species *N. lacustris* except for slight differences in length of unpaired regions. In the new soil planomonad, *N. limna* ssp. *terrestris*, the second lowest free energy structure was essentially the same as in its sister *N. limna*, and thus seems more likely to be the biological structure than the theoretically lowest free energy structure, in which helix III is rearranged to show two extra lateral helices (Fig. S4A). Interpreting the folds of the two marine clades with extra long ITS2 is less straightforward and mfold yielded more different folds of only somewhat differing free energy than for the freshwater clade where there were only two (or in *N. howeae* only one) very similar ones for each species.

Marine nutomonads all had broadly similar folds with an extra helix IIA (Fig. 6C) in addition to helices I-III, but *N. mylnikovi*'s very long ITS2 (386 nt) was distinctly aberrant with extra branches near the base of helix III and as many as 13 different folds of varying stability, differing especially in the arrangement at the base of helices I, II, and IIA and at the distal end of

helix III. Helix IIA lacks homologues in the other clades and is a synapomorphy for this clade. Of the two sisters, *N. indica* and *N. atlantica*, *N. indica* has the shorter ITS2 with only three different but closely related possible folds; in addition to that shown, the slightly less stable versions had a less branched end to helix III – of these the more stable alterative also differed from Figure 6C by a rearrangement of the pairing at the base of helices II and IIA, yielding short helices between the 5' and 3' regions and below the base of helices II and IIA. The longer *atlantica* ITS2 had a very similar folded structure with distinct helix I, II and IIA and free energy (dG) of -75; however the theoretically optimal fold structure (dG -70.4) showed a rearranged helix I/II region with a long basal helix bifurcating to give two long terminal helices (both shown in Fig. S3B). Evolutionary conservation arguments suggest that the former is more likely in vivo. In the optimal *N. kenti* and *N. mylnikovi* folds, the pairing arrangements below the base of helices I, II and IIA differ even more (Fig. S3).

In *Planomonas*, *P. elongata* and *micra* have the shortest ITS2; that of *P. elongata* is most similar to *F. tropica*, but with a single helix IV and a much longer helix III with somewhat different sidearms. That of the type strain (ATCC502670) of *P. micra* is similar overall to *P. elongata* but with proportionately much shorter helix II and IV in its lowest free energy fold (dG -151.41); this shortness of helix II is suspicious given the close similarity of primary structure in this region and the fact that the second lowest free energy version of this strain (dG -152.46 only slightly less stable) has a long helix II with strong sequence identity to that of *P. elongata* (both shown in Fig. S2), making it evolutionarily more plausible. The difficulty of predicting folds in this region for *P. micra* is emphasized by comparison with the other two strains of this species and the generation by mfold of 11-13 different structures for each strain, some differing only very slightly in free energy. In the South African strain the lowest (dG -146.88) and second lowest free energy (dG -149.53) predicted structures have helix II unfolded and paired instead with a region proximal to the 3' end of the molecule; a *P. elongata*-like helix II (but with a side arm) was seen only in a higher energy structure (dG -152.17). In the Millport strain also the lowest free energy

version (dG -138.31) was idiosyncratic, but a higher energy version (dG -148.27) was quite similar to that of the type strain and *P. elongata* (Fig S2). The optimal fold for *P. bulbosa* was much more like that of *P. elongata* despite numerous insertions. But *P. brevis* has far more indels compared with the other *Planomonas* species, making its secondary structure substantially different: an extra helix is between II and III and the complete helix III/IV region is rearranged into two very long helices, with the distal helix somewhat longer.

Too much should not be made of the differences in folding amongst closely related sequences as in *Planomonas*, since folding is only of heuristic value and cannot necessarily be expected to reconstruct reliably the actual in vivo structure. That is because in vivo folding is likely to be progressive from 5' to 3' end during transcription whilst it is attached upstream to 5.8S RNA (not simultaneously across the in silico excised molecule as was done with mfold), and actual free-energies and helix stability would also be modulated in the cell nucleus in unpredictable ways by the attachment of proteins (and other RNAs); moreover the current version of mfold allows RNA modeling only at 37C, whereas natural planomonad habitats have generally much lower but widely differing temperatures, ranging from the mostly frozen Lake Baikal at about 4C to tropical beaches and Boiling Springs Lake, (we tested the folding of the corresponding DNA sequences at different temperatures and found marked differences in predicted secondary structure for the longer sequences, which are clearly harder to model predictably than the shortened ones). To compare the compensatory base changes for discriminating between closely related species we used the most stable structures found by mfold, except for *N. limna* ssp. *terrestris* CCAP1958/10 where we chose a higher free energy structure, whose shape conformed more closely to that of the rest of the clade (Fig. S4).

In 5.8S RNA, which is normally a very conservative molecule, we also noted marked differences within the freshwater clade between *N. longa* (with exceptionally divergent 5.8S rRNA) and all others, and between all these and the marine clades, indicating that as for 18S rRNA their 5.8S rDNA has been more prone to rapid spurts of evolution than the marine clades,

though such spurts have clearly occurred in the stem of every major lineage. These evolutionary spurts in sequence structure have yielded a pattern of sequence signatures unique to the families, genera, and subgenera described here (as well as for the freshwater clade) that are easily identified (Table 3). They independently corroborate the new supra-specific taxa erected here on the basis of morphology and 18S rDNA trees.

Because of the three radically different ITS2 patterns in *Nutomonas*, and distinctive morphology and locomotory behaviour of *N. longa*, we establish three new subgenera:

Subgenus *Nutomonas* Cavalier-Smith. **Diagnosis:** freshwater, with anterior contractile vacuole; very short ITS2 (under 220 nucleotides: range 185-204), with partial or complete merger of helix I and II; 5.8S rDNA with 11 unique sequence signatures (Table 3); cells slightly elongated with a length/width ratio ranging from 1.26-1.67; nucleus anterior.

Subgenus *Incisomonas* Cavalier-Smith. **Diagnosis:** freshwater, with anterior contractile vacuole; ventral groove triangular, deeply incised and with dorsal flap; Cells slightly elongated; nucleus median. Type species *Nutomonas longa*. **Etymology:** *incisum* L. cut into, because of deeply incised ventral groove; plus *monas* Gk unit.

Subgenus *Kentomonas* Cavalier-Smith. **Diagnosis:** marine, no contractile vacuole; long ITS2 (331-395 nucleotides) with a novel helix IIA in addition to helices I, II and III (Fig 6C) that is absent in all other planomonads; 5.8S rDNA has 6 unique sequence signatures (Table 3); cell shape closer to isodiametric than in other two subgenera; nucleus anterior. Rostrum slightly less obvious than in subgenus *Nutomonas* and markedly less obvious than in *Incisomonas*. Type species *Nutomonas kenti*. **Etymology:** After William Saville Kent and type species.

Nutomonas lacustris a new sibling species

As Figure 1 shows, freshwater *Nutomonas*, except *N. longa*, are extremely closely related. The new lacustrine species from the Sea of Galilee, *N. lacustris*, is very closely related to the existing species *N. limna* and *howeae* (both formerly *Planomonas*). Genetically (ITS2), it is distinctly more like *N. howeae* (see alignment in Supplementary Material) and morphologically marginally more like *N. limna*. Despite minor morphological differences amongst these three species, which constitute subgenus *Nutomonas*, they are safely distinguishable only by their different ITS2 sequences (Table 1).

Nutomonas lacustris (Fig. 7C-F) closely resembles *N. howeae* and *N. limna* in shape and ciliary proportions but is easily distinguished from *N. longa* by cell shape and locomotory behaviour (in particular, by more vigorously nodding). *Nutomonas lacustris* is on average marginally narrower with an often slightly longer and more often acronematic posterior flagellum and more conspicuous transverse groove than *N. limna* CCAP1958/10, but the range of variation in these properties overlaps: both have a thin anterior cilium, best visible by phase contrast (Fig. 7A-F, Y-Z), emanating from a slight anterior depression. The thicker posterior cilium, more visibly acronematic in *N. lacustris* (Fig. 7C, E), emerges from an oblique groove, shallower than in *N. longa*, on the left ventral side of the cell. The rostrum (Fig. 7C, F, Y-Z) bearing small inconspicuous granules (Fig. 7C,D) is immediately anterior to the groove; it is shorter relative to cell size and less pointed than in *Fabomonas* (Fig. 2) and *Planomonas* (Fig. 7Q-X). As in all freshwater planomonads of subgenus *Nutomonas*, a contractile vacuole is in the cell's anterior right quadrant (Fig. 7A, C, F). Gliding on their posterior cilium, *N. limna* CCAP1958/10 (4-5 nodding cycles/s) and *N. lacustris* (3-4 cycles/s) nod more rapidly than *N. longa*. *Nutomonas limna* CCAP1958/10 is the first planomonad isolated from soil.

Nutomonas lacustris Glücksman and Cavalier-Smith sp. n. **Diagnosis:** identical 18S rDNA and near identical morphology to *N. limna* type and *N. limna* CCAP1958/10; cells 4.2-4.8 X 2.5-2.9 μm (length/width ratio: ~1.67); posterior cilium (~8 μm) typically longer than in *N. howeae* and proportionally slightly longer than in *N. limna*; during gliding nods at 3-4 cycles/s; anterior cilium twice as long as in *N. mylnikovi*, beats through 90º from anterior to left. ITS2 (185 nt.) substantially shorter than in marine planomonads; with only 152 nt. identical to *N. limna* type strain 120 (differ by 1 CBC and 3 HBCs), 149 nt identical to *N. limna* CCAP1958/10 (differ by 1 CBC and 6 HBCs), 168 nt. identical to *N. howeae* strain 124 (differ by 1 HBC). **Type illustrations:** Fig. 7C-F Type sequences: 18S rRNA and ITS/5.8S rRNA **XXXXX**. Type locality: water and sediment from Sea of Galilee, Israel, 2008. Culture now dead. **Etymology:** *lacustris* because isolated from a lake.

A soil planomonad

A new genetically distinct strain of *Nutomonas limna* (staf1; CCAP 1958/10) was isolated from soil from the Old Course, St Andrews, Scotland, 2008, the first soil planomonad to be brought into uniprotist culture and have its rDNA sequenced. It has identical 18S rDNA to *N. limna* and *N. lacustris*. It has near identical morphology to *N. howeae* type strain, but more commonly visible snout (Fig. 7A-B, Y-Z); cells are ~4.4 X 3.5 μm (length/width ratio: ~1.26, indistinguishable from *N. howeae*); often somewhat shorter posterior cilium with less obvious acroneme than *N. lacustris*. Its ITS2 (216 nt.) is most similar to that of the *N. limna* type strain (which has 219 nt.) but differs by 20 nucleotide substitutions, 12 indels of from one to 4 nucleotides, and one ambiguity in *N. limna* 120. We conservatively do not make it a new species as no CBCs or h-CBCs were found, but the relevance of this is questionable as its ITS2 apparently most stable secondary structure differs from that of the three freshwater strains of subgenus *Nutomonas* (Fig. S4A); we therefore recognize it as a distinct terrestrial subspecies:

N. limna* ssp. *terrestris Cavalier-Smith and Glücksman subsp. n. **Diagnosis:** distinguished from nominal subsp. *limna* by three differences in 5.8S rRNA sequence and more substantially in ITS sequences, and in the theoretically most stable folding pattern of helix III of ITS2 deduced secondary structure having two long helical sidearms (Fig. S4A); type strain CCAP 1958/10; type ITS/5.8S rDNA sequence GenBank XXXX.

New marine *Nutomonas* species

The three new marine species, *Nutomonas indica* (Fig. 7G-I, AA-AB), *N. atlantica* (Fig. 7J-L, AC-AD), and *N. kenti* (Fig. 7M-N), are morphologically similar to *N. mylnikovi*. One of them (*N. atlantica*), which forms a robust and more distant subclade with *N. indica* (Fig. 1), is slightly

more morphologically different from *N. mylnikovi*, which forms an also robust subclade with *kenti* and five environmental sequences. Sequence differences are substantially greater among all four species now recognized in the broad *mylnikovi* clade than amongst the more uniform three species of the *limna* clade, all being easily distinguished by 18S rRNA sequencing alone. In all three new species, cells are more rounded and the rostrum less prominent than in Planomonadidae. Their invariably present anterior cilium is slender, nearly entirely acronematic and thus difficult to discern using x60 water immersion DIC lens; it emerges from a dimple next to a slight apical projection on the anterior right end of the cell and flickers rapidly. Difficult to observe even in phase contrast, we saw it more frequently than did Cavalier-Smith et al. (2008) in *N. mylnikovi* – even for *N. mylnikovi* whose anterior cilium is sometimes reduced to a stub (Heiss et al. 2010) reexamination of the type strain revealed the anterior cilium somewhat more often than in the original study (Cavalier-Smith et al. (2008), suggesting that its degree of development may vary somewhat, as Mylnikov's scanning electron micrographs in Cavalier-Smith et al. (2008) also showed.

Nutomonas atlantica (Fig. 7J-L) has a marginally shorter posterior cilium than *N. mylnikovi* and the other new species; it is less visibly acronematic than in *N. mylnikovi*, but the acroneme is longer and easier to distinguish than in closely related *N. indica* (Fig. 7G-I). The posterior cilium emerges from a shallower and less obvious groove than in Planomonadidae. The cell surface is smoother and more rounded compared to Planomonadidae; vacuoles and other organelles are rarely visible. Cells glide on the posterior cilium and nod as *N. mylnikovi*, but raise their right side off the substratum and roll up to 90° on their axis. Nodding is more rapid than in other species. Turns, effected by a kink in the proximal third of the posterior cilium, are equally frequent in both directions.

Nutomonas atlantica Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Smaller and chubbier than *N. mylnikovi* or *indica* **(**3.5-3.8 X 2.5-3.20 μm, length/width ratio: ~1.28); 18S, 5.8S and ITS rRNA sequences distinct from both. ITS2 395 nt. long. Posterior cilium as short as or shorter than in *N. indica* (5.7-7.3 μm), unlike *indica* and like

mylnikovi usually extended by a short acroneme; cell body nods during gliding at 5-7 cycles/s; anterior cilium extremely thin and short (probably <1 μm), visible only with difficulty. **Type culture:** CCAP 1958/9. Type illustrations: Fig. 7J-L. Type sequences: 18S rRNA **XXXXX** and ITS/5.8S rRNA **XXXXX**. Type locality: seawater and sediment from beach near Peniche, Portugal, 2008 (EG). **Etymology:** *atlantica* because isolated from the Atlantic shore.

Nutomonas indica Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Cell shape and size **(**4.1-5.7 X 3.2-4.4 μm, length/width ratio: ~1.29) indistinguishable from *N. mylnikovi* and *N. atlantica*; posterior cilium differs from *N. mylnikovi* by usually not being visibly acronematic and often relatively shorter relative to body (5.7-9.5 μm); emergent part of anterior cilium entirely acronematic, exceedingly thin and hard to see (2 μm or shorter); notably distinct 18S and ITS rDNA sequences, branching with *N. atlantica*, not *N. mylnikovi* and *kenti*, on 18S rRNA tree. 5.8S rRNA most similar to that of *N. kenti*, differing by 1 nt; ITS2 sequences 335 nt. long. **Type culture:** CCAP 1958/6. Type illustrations: Fig. 7G-I, AA-AB. Type sequences: 18S rRNA **XXXXX**; ITS/5.8S rRNA **XXXXX**. Type locality: seawater and sediment from muddy sandy beach, Dar es Salaam, Tanzania, 2007 (TCS). Etymology: *indica* because isolated from Indian Ocean.

Nutomonas kenti Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Body shape, size **(**3.5-4.1 X 2.5-3.5 μm, length/width ratio: ~1.27) and variability closest to *N. atlantica*; posterior cilium 5-11 μm plus obvious acroneme; 18S, 5.8S and ITS rRNA sequences distinctive. ITS2 331 nt. long. **Type culture**: CCAP XXXX; this is *Planomonas* sp. strain 3b, whose 18S, ITS, 5.8S and 28S rDNA were sequenced by Glücksman et al. (2010b). **Type illustrations:** Fig. 7M-N. Type 18S ITS/5.8S and 28S rRNA sequences: GenBank GU001166. Type locality: seawater and beach sediment, Cumbria, England, 2007. Strain 11b also from Cumbria (2007) had identical 18S rDNA and ITS. **Etymology:** *kenti* after William Saville Kent (who Heiss et al. 2010 think first saw a nutomonad).

Three new *Planomonas* species

All new *Planomonas* species are broadly similar to *P. micra* but distinguishable by small morphological differences, slightly different 18S rRNA, and more substantially different ITS2 sequences. The *P. micra* strain from South Africa (saf, Fig. 7O-P) had a somewhat shorter

posterior cilium than the *P. micra* type strain but was morphologically effectively indistinguishable; it had identical 18S rDNA and slightly different ITS2. For comparison we sequenced ITS of the Millport strain of *P. micra* (Cavalier-Smith et al. 2008); its ITS2 is slightly more similar to saf than to the *micra* type strain. Even though Millport ITS2 differs from the type by 20 nucleotides, including one hemi-CBC, we adhere to the earlier decision not to make it a separate species (Cavalier-Smith et al. 2008) as the new South African strain is approximately intermediate and equidistant in ITS2 sequence from it and the type.

New marine species *Planomonas elongata* (Fig. 7U-V), *P. bulbosa* (Fig. 7W-X), and *P. brevis* (Fig. 7Q-R), as well as South African *Planomonas micra* most resemble the *P. micra* type (ATCC502670) with which they cluster on Figure 1. They have an obviously acronematic anterior cilium; a much longer, barely acronematic posterior cilium; a long, granule-bearing rostrum; and pronounced ventral groove. Though all four isolates are nearly indistinguishable microscopically, *Planomonas micra* saf has a marginally longer anterior cilium relative to body length and its posterior cilium is more obviously acronematic than the three new species. They all have a broadly similar, asymmetrical, cell shape. The three new species each differ slightly from *P. micra* in length-to-breadth ratio, *P. elongata* being relatively most elongated. The nucleus and other organelles are frequently visible. Cells glide continuously on their posterior cilium and nod at 3.5 cycles/s. Turns are mostly to the right. *Planomonas* strains are the least resilient Planomonadida under our culturing conditions; three died before submission to CCAP.

Planomonas bulbosa Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Near identical shape to *P. micra* type though slightly larger (5 X 4, not 4 X 3 μm, length/width ratio: ~1.29), slightly more bulbous rostrum and non-acronematic, marginally shorter posterior cilium (8-9 μm); anterior cilium ~4 μm, its proximal half of normal thickness, distal half (or slightly more) a thin acroneme. 18S rDNA differs from *micra* by 4 nucleotides (ignoring the C not G at *micra* position 1276 that is probably a PCR/sequencing error, and the ambiguity at *micra* nt 1337); ITS2 sequence 426 nt. long. Culture dead; isolated from brackish river water, it grew in 100% artificial seawater. Type illustrations: Figure 7W-X. Type sequences: 18S rRNA **XXXXX**; ITS/5.8S rRNA **XXXXX**. Type locality: brackish sediment, Cape Fear

River, Wilmington, North Carolina, United States, 2008 (EAS). **Etymology:** *bulbosa* because of bulbous rostrum.

Planomonas elongata Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Cell shape usually proportionally distinctly more elongated than *P. micra*, *P. bulbosa*, *P. cephalopora* and *P. melba* (4-6.3 X 3.7-4.μm, length/width ratio: ~1.34). Non-acronematic posterior cilium 11-13 μm, longer than *P. micra*, shorter than *P. cephalopora*; anterior cilium 2.5-3.0 μm, proximal half normal thickness, distal half acronematic. 18S rDNA differs from *P. micra* by 1 nucleotide (ignoring the two putative errors in *P. micra* mentioned under *P. bulbosa*; at position 707 there may also be heterogeneity among copies with either a C or the G found in *P. micra* and other *Planomonas*). ITS2 394 nt. long. Culture dead. **Type illustrations:** Figure 7T-V. Type sequences: 18S rRNA **XXXXX**; ITS/5.8S rRNA **XXXXX**. Type locality: seawater and sediment from beach, Bodrum peninsula, Turkey, 2008. **Etymology:** *elongata* because cell body relatively more elongated than in other *Planomonas* (and posterior cilium longer than in *P. micra*).

Planomonas brevis Glücksman and Cavalier-Smith sp. n. **Diagnosis:** Essentially same size and shape as *P. bulbosa* **(**5-5.4 X 4 μm, length/width ratio: ~1.3); posterior cilium intermediate in length between *P. bulbosa* and *elongata* (10-12 μm), non acronematic or with short acroneme; anterior cilium with very short (0.67 μm), thick emergent base and acroneme varying from almost invisible to 4 μm. 18S rDNA unique, differs from *micra* by 26 nt. (2.4%), ITS2 411 nt. Long, substantially different from *P. micra*. Culture now dead. **Type illustrations:** Fig. 7Q-S. Type sequences: 18S rRNA **XXXXX**; ITS/5.8S rRNA **XXXXX**. Type locality: seawater and sediment, Paralimni, Cyprus, 2008. Etymology: *brevis* L. short, because of shortness of thick basal region of anterior cilium.

In addition to these new species a genetically novel new strain of *Planomonas micra* (saf: CCAP 1958/7) with identical 18S rDNA sequence to the type strain was isolated from seawater and sediment at Cape Town, South Africa in 2008. Its ITS2 (396 nt. long), differs from the *P. micra* type (399 nt.) at 12 positions (97.5% identity) but as it exhibits no CBCs or hemi-CBCs. and its morphology (Fig. 7O-P) is indistinguishable from the type strain we consider it to be the same species.

Discussion

Of the 13 new planomonad strains, only two belong to known species: *P. micra* and *N. limna*. Nine of the other 11 are new species, seven relatively closely related to existing ones and two (*F. tropica* and *N. longa*) differing strongly from known species both genetically and morphologically, revealing two new and unsuspected deep-branching clades. Thus, there are now five major planomonad clades, grouped here into three genera according to their cell shape and anterior ciliary morphology. Each clade corresponds to a genus or subgenus in the revised taxonomy.

Quantum evolution in ITS2 structure

A striking discovery is that the length and secondary structure of ITS2 differs radically in freshwater and marine planomonads; it is much shorter and sometimes has fewer helices in the highly divergent freshwater compared with all marine clades. These major changes in secondary structure imply a temporary destabilization of the pre-rRNA processing machinery, probably at the time of the transition to freshwater and a temporally associated reduction in stabilizing selection on 18S rRNA structure that putatively caused its subsequent accelerated evolution. The structure of ITS in the early diverging new marine clade *Fabomonas* is basically like that in other unikonts such as fungi, whose secondary structure it clearly resembles more than those of plants and chromists, which tend to have more helices (Coleman 2007). The other two marine clades have both increased the length of their ITS2 compared with *Fabomonas* by multiple insertions; in *Planomonas*, whose ITS2 is alignable in many more places with *Fabomonas* than are those *Nutomonas* there appear to have been at least 12 insertions of often sizeable blocks and there is usually a single long helix in the distal region where helix IV is found in green algae (Coleman 2007). ITS sequence comparisons between the two families are more difficult, but both the topology of the 18S rDNA tree in Figure 1 and our inability to align substantial regions between

families and the distribution of short patches that are apparently alignable are consist with a combination of extreme divergence from a common ancestral sequence at least some independent insertions in *Planomonas* and the marine nutomonad subgenus *Kentomonas* are responsible for the divergent sequences and the rather dissimilar secondary structures of the two long-ITS2 marine clades. Helix IIA is a synapomorphy for *Kentomonas* and was probably a unique insertion prior to its last common ancestor.

To varying degrees ITS2 has a different secondary structure in each of the three genera and in all four of the major clades on the 18S rRNA tree; these differences in molecular morphology are more striking than the differences in cell morphology. Loss or gain of helices or rearranged helices causing substantial differences in secondary structure (as in subgenus *Nutomonas* or *Planomonas brevis*) are known in some other lineages of advanced eukaryotes (Coleman 2007). They are particularly unsurprising within the planomonad clade, which both 18S sequence trees and our unpublished multiprotein trees (Snell et al. in prep) suggest are probably somewhat older than the entire animal kingdom (i.e. over 600 My) even though it is only ranked as an order. Several groups of eukaryotes appear even to have lost helix III (e.g. Heterolobosea like *Naegleria* De Jonckeere 2004) which is almost present (though in *Planomonas brevis* rearranged) in planomonads. Even greater truncation of ITS2 than seen here for the main freshwater *Nutomonas* clade evolved in the parasitic microsporidia and metamonad flagellates, which independently eliminated the pre-rRNA processing step that ancestrally generated 5.8S rRNA and truncated both ITS, e.g. the metamonad *Giardia* ITS2 has only 53 nucleotides and a radically simplified secondary structure (Edlind et al. 1990). The changes we found amongst the major planomonad lineages, though less radical than these, are quite striking, and strongly reinforce earlier arguments against past lumping of almost all planomonads as a single species (i.e. *Ancyromonas sigmoides*: Cavalier-Smith et al. 2008).

Anterior cilium diversity

The flicking anterolateral motion of the extremely thin anterior cilium of *Nutomonas* seems unlikely to help locomotion; we suggest it has a sensory function in detecting prey and triggers cessation of gliding and attempted ingestion when it encounters bacteria attached to or just above the substratum. In most species its length is about right in conjunction with the amplitude of nodding to detect any bacteria by touch that lie within a path about one planomonad cell body wide. The greater length of the thick basal portion of the anterior cilium in Planomonadidae compared with Nutomonadidae probably allows it to beat with greater amplitude (most marked in *Fabomonas*) thus detecting bacteria over a broader swath, but at a cost of more energy consumption for its motion and biogenesis.

Thus, our striking finding of the grouping as a robustly supported clade of all strains with a reduced anterior cilium that is entirely acronematic in its emergent part (family Nutomonadidae; genus *Nutomonas*) might reflect a basic adaptive and functional difference between Nutomonadidae and Planomonadidae. The nutomonads *N. limna* and *howeae* were discovered in the highly oligotrophic Lake Baikal where bacteria are extremely sparse (Cavalier-Smith et al. 2008) compared with the marine habitats from which most Planomonadidae have been isolated. We are investigating a broad range of habitats by environmental DNA sequencing to clarify such potential environmental preferences amongst planomonad genotypes and to test whether those now apparently restricted to the marine or freshwater clades sometimes occur in the other habitat.

Major new planomonad clades

As the totally acronematic state is absent in the nearest outgroups (i.e. other unikonts, e.g. Apusomonadida, *Breviata*, core Amoebozoa, Choanozoa), it is a derived state for nutomonads alone that has probably been stable in evolution for several hundred million years. The better-developed anterior cilium of Planomonadidae, with its conspicuous thick base, must be the ancestral state for planomonads, irrespective of whether Planomonadidae is paraphyletic, as

suggested by the supplementary figure, or holophyletic, as suggested by Figure 1. Multigene trees are needed to decide between these alternatives; our own preliminary ones based on scores of genes strongly support the holophyly of both Planomonadidae and Nutomonadidae (Snell et al. in prep). In other respects, the overall phylogeny of planomonads is consistently supported by all methods and taxon samples.

It is increasingly clear that the deepest branching clades are essentially marine and that the large freshwater *Nutomonas* subclade, now augmented by the highly divergent *N. longa*, is derived secondarily from marine ancestors and is consistently sister to the marine *Kentomonas* clade. The long branches of the freshwater clade show that rRNA evolutionary rates permanently increased roughly threefold around the time of that transition, which also happened during the analogous single marine to freshwater transition in goniomonads (von der Heyden et al. 2004). Our TEM examination of *Fabomonas tropica* shows that the basic ultrastructural features of planomonads (a single dense layered dorsal pellicle, kinetocysts, and flat mitochondrial cristae) occur in all three marine clades, thus confirming their ultrastructural conservatism.

The discovery of *N. longa* and *F. tropica* shows that the round, almost isodiametric body form of all previously described planomonads, is not universal. *F. tropica*, in particular, is relatively more elongated than any other known species. This higher length-to-breadth ratio (seen to a more variable or lesser extent in *N. longa*) is of special interest as it is shared also by *Ancyromonas sigmoides*, which Heiss et al. (2010) recently argued is a senior synonym for *N.* (then *P.*) *mylnikovi*. The greater length-to-breadth ratio of *A. sigmoides* compared with *N. mylnikovi*, which was too lightly dismissed by Heiss et al. (2010), is one of several reasons given below why it was premature to try to equate *A. sigmoides* with any previously known planomonads and why we cannot accept the synonymy they propose.

Species diversity amongst planomonads

Despite the marked genetic divergence of all five clades, morphological differences among the three previously known clades are very slight and appear not to correlate with phylogenetic position. Except for the systematic difference in anterior ciliary structure between the two families, sequences are a better guide than morphology to planomonad phylogenetic relationships and usually essential for species demarcation. Our discovery of *Nutomonas atlantica* and *indica* indicates that the marine clade containing *N. mylnikovi* (subgenus *Kentomonas*) is substantially more diverse than was previously thought. Our description of *N. kenti* provides a close cultured relative to three South Atlantic environmental DNA sequences that differ from it by about five 18S rDNA nucleotides and are probably another closely related species (or two, if the few differences of one of them are genuine). From its morphology, we expect *N. sinistra* also to belong to this marine *Nutomonas* clade. Likewise, the discovery of three marine relatives of *P. micra* substantially expands the diversity of that clade; given the structure of their anterior cilia and cell shape, we suggest that *P. cephalopora* and *melba* also will be found to belong in this *Planomonas* clade when cultured and sequenced. The fact that over three quarters of our new isolates are novel species means that several times more planomonad species must exist than the 16 now described.

To avoid describing every genetically distinct clone as a separate species we adopted the principle that if two clones have identical 18S rDNA sequence and almost identical morphology we should not make them separate species unless their ITS2 sequences differ in at least one compensatory base change (or hemi-CBC), which are clear indicators that the differences are selectively significant. Thus, we treat all three *F. tropica* clones as the same species despite clear genetic differences. For each strain, we sequenced ITS from six or more separate plasmid clones and found either no heterogeneity or that it was at a much lower level than that between different *F. tropica* or *P. micra* isolates (and never involved CBCs or HBCs or altered pairing properties as judged from the inferred secondary structures).

Without knowing whether planomonads are sexual or clonal in population structure, we cannot say whether thus defined entities such as *P. micra* and *F. tropica* are biological species or just assemblages of closely related clones. Nonetheless, this species demarcation criterion is a usefully objective way of limiting the otherwise endless proliferation of names that increased genetic discrimination could lead to without a deliberate check of some sort. It also has the advantage that closely related planomonad species defined by it will differ genetically to a comparable degree to closely related species in those numerous groups where genetic isolation among close relative biological species evolves at a similar rate to ITS2 CBCs (Coleman 2009). We recommend a similar policy in other protozoan groups where (as usually) sexuality and breeding patterns are unknown, rather than establishing new species on the basis solely of small non-compensatory differences in ITS (sometime single nucleotide differences have been used: De Jonckheere 2004, 2007; such small differences are important for understanding population differentiation, but routinely recognizing them all by species names would be oversplitting).

We observed and cultured samples from a wide array of geographical locations across five continents. Only rarely did we fail to observe at least one living planomonad cell in a mixed culture from a particular location, showing that the group is globally ubiquitous in freshwater and marine environments. Samples appearing to lack planomonads were usually taken during winter (for example, from sediment in a semi-frozen pond in Sweden), where observed overall protozoan diversity was visibly lower than in summertime samples from the same location. Combining present and past evidence (Cavalier-Smith et al. 2008), *P. micra* strains have now been cultivated at least three times independently from the American and European sides of the North Atlantic and from the seashore in South Africa; further sampling is needed to decide whether their small differences in ITS2 (at about 7 positions) are just individual clone differences or differences among geographic races; either way these similar genotypes must be common and widespread in temperate waters. *Fabomonas tropica* was found three times, so it is widespread in the tropics or near-tropics, but in each case the ITS was slightly different showing similar genetic

variation to *P. micra* (variants at 8 positions). Whether the greater genetic similarity of the two Atlantic strains of *F. tropica* (from Mexico and Ghana) compared with that from the more distant (as sea currents flow) Red Sea strain reflects a systematic geographic genetic differentiation or is just a chance result of few samples can only be determined by more sampling. The differences between the soil and freshwater subspecies of *N. limna* are more substantial.

Planomonad nomenclature

We used the marked ITS2 secondary structure differences we discovered to subdivide *Nutomonas* into three subgenera corresponding to the three lineages on the rRNA tree (Fig. 1). We decided against making them full genera, as we prefer to make genera more easily distinguishable by light microscopy, as are all three now recognized. Although *N. longa* is unambiguously separable by its cell shape and locomotory behaviour from the other two *Nutomonas* clades, subgenera *Nutomonas* and *Kentomonas* cannot be distinguished solely by light microcopy, except for the absence of contractile vacuoles in *Kentomonas*, a distinction that might break down if freshwater *Kentomonas* or marine members of subgenus *Nutomonas* were ever discovered. Heiss et al. (2010) noted that these two clades and *Planomonas* sensu stricto are all genetically so divergent that making them separate genera would be reasonable.

It would be nomenclaturally desirable to raise the nutomonad subgenera to full genera if it were ever established (by the discovery and genetic characterization of a novel strain that is genuinely morphologically indistinguishable from *A. sigmoides*) that this species belongs in the *Kentomonas* clade, as Heiss et al. (2010) argued is likely. Our discovery of *N. longa* and *F. tropica* increases the likelihood that a marine member of *Kentomonas* clade with a more elongated body form than any known species will also one day be discovered. Such an organism would be a much better candidate for equating with *A. sigmoides* than *N. mylnikovi,* that should only be done if the majority of its cells have a protuberance at the non-ciliary end giving it a sigmoid shape as in *A. sigmoides*. Already the discovery of *N. kenti*, *indica* and *atlantica* provides

three more species, none of which is less like *A. sigmoides* than is *N. mylnikovi*. One of them (*N. indica*), when grown in 25%, 50% or 75% seawater, in which it thrives, does display ciliary 'anchoring' behaviour similar to that observed by Heiss et al. (2010) for *N. mylnikovi*. By contrast, freshwater nutomonads, which grow exclusively in 100% freshwater, and *Fabomonas* and *Planomonas* sensu stricto, never exhibited such anchoring behaviour in our cultures. We made the freshwater *N. howeae* the type for the genus and subgenus *Nutomonas*, so that if it were ever demonstrated that *A. sigmoides* does belong in the *Kentomonas* clade, as Heiss et al. (2010) proposed, the name *Ancyromonas* could be applied to it alone; the names *Nutomonas* and *Incisomonas* could both be retained, and only *Kentomonas* would become a junior synonym of *Ancyromonas*.

However, it is also possible that Saville Kent's *A. sigmoides* was either (a) a nutomonad belonging to a different (possibly still unknown) subclade from *Kentomonas* or (b) a cercozoan or an anoecid heterokont (see below). Given these uncertainties and the rate of new discoveries, the ideal of nomenclatural stability was not well served by applying the name *Ancyromonas* to a previously known planomonad. Because Heiss et al. (2010) devoted a whole paper to a contrary view we explain below why we disagree. Their arguments that Saville Kent's *A. sigmoides* may have been a mixture of some kind of planomonad and an unknown protist capable of multiple fission are much stronger (but still far from being compelling) than their claim that it was specifically *N. mylnikovi*, which we consider almost certainly incorrect (as TCS told AS before they published it). We focus primarily on the latter as it caused (in our view regrettable) nomenclatural conflict.

Nutomonas (=*Planomonas*) *mylnikovi* is not *Ancyromonas sigmoides* Saville Kent

Heiss et al. (2010) exaggerate the similarities between these two taxa in several ways. We shall not reply to every detail of their arguments, but emphasize key reasons why we strongly disagree with synonymizing *Planomonas* (now *Nutomonas*) *mylnikovi* and *Ancyromonas sigmoides*. They

misinterpret three important characters that are clearly different:

When discussing shape, their assertion of 'a close similarity, with some discrepancies' attempts to discount Saville Kent's statement that *Ancyromonas* is 'about three times longer than broad' (his figures showed it only about 2.38 times as long as broad; as *Nutomonas mylnikovi* is normally 4-5 by 3-4 μm, its length-to-breadth ratio is about 1.3, not 3 – a large discrepancy); he also said it was 'ovate or elongate', the word elongate being ignored by Heiss et al. (2010). Heiss et al. mentioned that Saville Kent did not draw his own plates, but if that were the cause of the ratio being lower in the plate it is not reasonable to suppose that the artist exaggerated the length/breadth ratio. Quite the reverse: given Saville Kent's use of '1:3 ratio' and 'elongate', it is more likely that the artist slightly underrepresented the length/breadth ratio and/or that the text figure of 1:3 was rounded up from something between 2.5 and 3 (Saville Kent never used fractions in such comparisons). The mismatch between his text and figures is thus much more trivial than Heiss et al. imply, objectively less than the substantially larger mismatch between the range of 2.38-3 for *Ancyromonas* and the 1.3 ratio for *N. mylnikovi*. It gives no reason to de-emphasize Saville Kent's clear statements about the substantial degree of elongation. *Nutomonas mylnikovi* and all other *Nutomonas* and all currently known planomonads (Table 2) are indeed distinctly less elongated than was *Ancyromonas*. Heiss et al. suggest that Saville Kent's 3:1 ratio refers not to length/breadth but to length/thickness of a hypothetically flattened cell, which seems unlikely as this is a less frequent view than the face-on one apparently depicted in Saville Kent's figures. Hänel (1979), who in our view was the first to depict planomonads unambiguously, as well as all subsequent authors prior to Cavalier-Smith et al. (2008), drew them only in surface view.

Secondly, the detailed shape of *N. mylnikovi* and all other *Nutomonas* in face view is very different from Saville Kent's figures of *Ancyromonas*, as Cavalier-Smith et al. (2008) correctly emphasized. Heiss et al. unreasonably downplay the sigmoidal nature of *Ancyromonas* that gave it its name. All our and all their micrographs contradict *N. mylnikovi* being sigmoid; their Fig. 6

does not show the same shape as Saville Kent's drawings. Heiss et al. (2010) overemphasize that they saw one (we suspect moribund, damaged or dying) cell with a pointed posterior. Even this abnormal cell cannot justify the claim that the shape is 'closely' similar, as it did not have a recurved posterior as Saville Kent said was usual: 'posterior one [i.e. extremity] sometimes rounded, but more often slightly recurved in an opposite direction'; speculating that *Ancyromonas sigmoides* was not actually sigmoid, but that Saville Kent may have been misled about its shape by viewing malformed cells or ones seen from the wrong angle (Heiss et al. 2010) is a totally unconvincing excuse for ignoring the very aspect of cell shape that his species name emphasized. This speculative dismissal as an artifact of the key character distinguishing Saville Kent's flagellate from other species invalidates the claimed neotypification of *Ancyromonas sigmoides* by *N. mylnikovi*. Cavalier-Smith et al. (2008), when saying 'completely different shape' had in mind the cell outline not the flatness; 'completely' rather than 'very' as above somewhat overstated the difference, as Heiss et al (2010) remarked, but the shape really is sufficiently different (based on both text and figures) to make it **not** consistent enough with that of *N. mylnikovi* to satisfy the requirements for neotypification of clause 5.3.5 of the International Code of Zoological Nomenclature (ICZN). If detailed cell shape is rejected as a differentiating character, all that is left is cell size, the presence of but one cilium, and its length and beat pattern: too little positive data to justify neotypification. It was also unwise to use the type strain of the well-characterized species *Planomonas mylnikovi* to neotypify an old name for a flagellate that does not correspond precisely in cell shape to it and where other experts disagree with the identity claimed (contrary to recommendation 75B).

Heiss et al.'s (2010) discussion of Saville Kent with respect to 'anchoring and gliding' muddles the two and fails to convince us that *Ancyromonas* was a glider. Saville Kent stated that the flagellum is 'adhesive or anchorate *at its distal extremity*' [our italics], that it is 'free-swimming or anchorate at will', and that 'free-swimming animalcules' have the flagellum trailing at the rear 'slowly undulating throughout its length'. We have never observed any planomonad

swimming freely; neither did Heiss et al., nor has any other paper on the group. As far as we know, they progress only by gliding on surfaces. However, as a distinction between gliding on surfaces and true free-swimming in the media had not then been made, Saville Kent **might** simply have been contrasting translational movement of some sort with stationary fixation to the substratum; analogously, his generic description of *Petalomonas* and *Cercomonas* stated that they are 'entirely free swimming', though we now know that all of them glide and almost none swim freely (Bass et al. 2009). Thus we agree with Heiss et al. (2010) up to a point: using a realistic historical perspective, we cannot exclude the possibility that his *Ancyromonas* moved by gliding not swimming, even though ciliary gliding on surfaces was not actually mentioned. In describing *Cercomonas crassicauda*, however, Saville Kent did contrast creeping [i.e. gliding] with free swimming, but the emphasis there may have been of the role of the cytoplasmic tail rather than the non-vibratile posterior cilium that was not then distinguished from it.

Conversely, we cannot rule out the still reasonable possibility that *Ancyromonas* was a genuine swimmer not a glider: interpretation by Heiss et al. of Saville Kent's comparison with *Heteromita* and *Anisonema* as evidence for gliding is overconfident and probably wrong. He did not, as they wrongly assert, 'equate the movement of his specimens' to *Heteromita* and *Anisonema*. What Saville Kent actually wrote was that 'the single trailing filament combines the **functions** of both the trailing and vibratile flagella of *Heteromita* and *Anisonema*', which most likely means two things. First that the posterior flagellum **simultaneously** trails and undulates during locomotion, unlike the other two genera, as he made clear in the passage on free swimming, which speaks of the flagellum 'undulating throughout its length, and accomplishing by its vibrations the advancement made' by 'free-swimming animalcules'. We have not observed *N. mylnikovi* or other planomonads gliding like that with as much short period undulation as in his figure 49 of a 'free-swimming cell'. There is no evidence that Saville Kent's *Heteromita* species even belonged to the same genus or phylum, let alone that his three *Heteromita* species that anchored by their posterior ciliary tips also glided; Saville Kent's *Heteromita* might not be

related to *Neoheteromita globosa* and other glissomonads that do glide (Howe et al. 2009). Secondly, the **function** of the posterior cilium that led him to select '*Heteromita*' and *Anisonema* for comparison with *Ancyromonas* was almost certainly **tip anchorage** not gliding; in contrasting the two flagella of Anisonemidae it **was** anchorage, not movement by gliding or any other propulsive mechanism, that he explicitly attributed to the posterior one. Moreover, his *Anisonema* was heterogeneous, including not only euglenoids that glide but also flagellates like *Bodo ludibundus* that do not glide but attach by the extreme tip of their posterior cilium like *Ancyromonas*. Thus the assumption by Heiss et al. that he had in mind gliding is anachronistic and unwarranted speculation; he made no comparison with the gliding (but not anchoring) uniciliate *Petalomonas* whose anterior gliding cilium (as he specified) is rigid through most of its length. Saville Kent compared the flagellum to an undulating membrane, but specifically said that the gliding posterior flagellum of *Anisonema* is 'non-vibratile' whereas he repeatedly called that of *Ancyromonas* 'vibratile'; it is therefore beyond question that he did not consider them to cause locomotion in precisely the same way. Heiss et al.'s statement that 'anchoring sensu Saville Kent and gliding as currently understood were clearly not mutually exclusive behaviours to him' seems wrong because (a) he **did** contrast 'free swimming' with 'adherent' or 'anchorate'; (b) it is unreasonable to guess what he thought about 'a concept of gliding as currently understood' as he had no such concept; nothing can sensibly be said about what Saville Kent believed about gliding versus anchoring. If as we suspect Heiss et al. simply meant that his term 'free-swimming' **sometimes** included gliding, we agree, but they present no evidence that it did so in the case of *Ancyromonas*.

Saville Kent's text twice stated that anchoring was at 'its distal extremity', so it was pedantically misleading to say that 'Saville Kent's text does not specify that the anchoring is only at the tip' (Heiss et al. 2010); his words 'at the distal extremity' do specify the tip; his figure specifies anchorage **only** at the tip; his figure legend for a third time specified 'fixed by distal **termination** of the single flagellum' stresses that anchoring was terminal not lateral, making this

a third mismatch between *A. sigmoides* and *N. mylnikovi*. Behaviour like that shown in S4 of Heiss et al. probably does not correspond to what Saville Kent depicted or described and is therefore irrelevant to the argument. The cell shown in S3, however, is anchored very close to the tip, but as tip anchorage of a posterior flagellum is known in other groups it does not narrow the possibilities to *N. mylnikovi* where it has not been shown to be the usual mode of anchorage. Until we discovered *F. tropica* we had not seen multiple undulations on a planomonad gliding cilium as depicted in Figure 2 (Y, Z, AA). In this respect, *F. tropica* is more like *Ancyromonas* than is *N. mylnikovi*. In body proportions its elongate shape is also more similar, but it is not sigmoid or pointed, nor does it show the kind of 'anchoring' behaviour that Heiss et al. noted for *N. mylnikovi*, and we have seen in *N. indica* (which is as like – or as unlike – *Ancyromonas* as is *mylnikovi*). Saville Kent's figure 50 showed the flagellum undulating through its whole length while attached at its extreme tip; we have not seen such extreme tip attachment in planomonads.

The most significant novelty in Heiss et al. (2010) is their useful observations of division. Their figure 8, which corresponds to a late stage of division not observed by Cavalier-Smith et al. (2008), superficially resembles figure 52 of Saville Kent (1882), but is apparently not precisely the same, as in figure 8 the left margins of the cells face each other, whereas in Saville Kent the right margins are facing; more thorough study is needed to see if that is a consistent difference. Heiss et al. speculate that Saville Kent did not observe the same stages of division as Cavalier-Smith et al. (2008). But Saville Kent did clearly describe early stages of division in words flatly contradicted by the observations of Cavalier-Smith et al. (2008), writing that division 'takes place obliquely the **first indication** … being a lengthening out of the body, accompanied by the greater prolongation of the more or less pointed posterior extremity until it attains a curvature …'. Thus, the argument that *Ancyromonas* did not divide in the same way as planomonads (Cavalier-Smith et al. 2008) was correct, even though Heiss et el. (2010) showed that its terminal phase may be similar.

We agree with Heiss et al. (2010) that Saville Kent's description of multiple fission probably

related to a different organism than the one he figured (one candidate would be a colpodellid, its posteriorly flagellate phase being easily overlookable in an *Ancyromonas* bloom). Saville Kent may have made other errors, notably concerning the contractile vacuole which he believed to be present in every protozoan (and wrongly thought absent in green algae), and we agree that we need to make some allowance for the optical and conceptual limitations of his day, but if we ignore or discount too much of what he reported (as do Heiss et al. 2010), we would depart so far from his stated observations as to destroy any merit in retaining his name. Its cell shape is objective evidence that *Ancyromonas sigmoides* was not the same species as *N. mylnikovi;* its mode of movement, anchoring, and division are arguably also somewhat different or at least not convincingly demonstrated to be identical. Just as important in relation to the claimed species identity is our present demonstration that some previously undiscovered planomonads resemble *A. sigmoides* in length/breadth ratio **more** than does *N. mylnikovi*, albeit not yet perfectly, though all are distinguishable from *Ancyromonas* in cell posterior shape as well as in mode of movement.

Cavalier-Smith et al. (2008) were consistent in accepting that Hänel (1979) observed planomonads but denying that Saville Kent did so, because Hänel's freshwater cells are totally indistinguishable from those of *N. limna* and *howeae* (also freshwater), whereas marine *A. sigmoides* is very dissimilar from them; Hänel's drawings unambiguously portray the correct rostral shape and the emarginated character of the ventral groove of subgenus *Nutomonas* (the latter not characteristic of subgenus *Kentomonas*) as well the anterior dimple from which the anterior cilium arises, and even depict a likely contractile vacuole in precisely the correct position for subgenus *Nutomonas*, whereas Saville Kent's drawings and text evince no trace whatever of a ventral groove or anterior dimple. Cavalier-Smith et al. (2008) emphasized the overall positive evidence of morphology with respect to such similarities and differences in shape and grooviness in making their contrasting judgment concerning the two authors, and did not rely solely on whether or not flatness was explicitly mentioned or denied, a point excessively emphasized by

Heiss et al. (2010). Hänel's drawings confirm in every single detail that he saw a member of subgenus *Nutomonas*, as Cavalier-Smith et al. (2008) argued, so we may reasonably infer that Hänel's cells were flat. By contrast, Saville Kent's drawings and text are inconsistent with the claim that he saw *N. mylnikovi* or any known member of subgenus *Kentomonas* or any well-characterized planomonad; therefore assuming that *A. sigmoides* was also flat is more hazardous given the three discrepancies stressed above. Heiss et al. (2010) strongly criticized Cavalier-Smith et al. (2008) for assuming that *Ancyromonas* was not flat on the grounds that Saville Kent did not assert that it was not flat. But that criticism is unfair because Saville Kent described scores of protists that are known not to be flat without explicitly calling any 'not flat'; but in every diagnosis of the gliding euglenoid *Petalomonas* he did specify that they are flat and used 'depressed' in the generic diagnosis; it is surely significant that he did not use any of these words for *Ancyromonas*, despite calling even *Entosiphon* (a markedly less flat gliding euglenoid) 'depressed' and 'compressed'. We therefore reasonably assumed that it was more likely than not that *Ancyromonas* was no flatter than the generality of small flagellates; indeed he called it ovate (egg-shaped) or gibbous, which means convex, protuberant or swollen on one side or humpbacked); naturalists used gibbous as a contrast to flat. Heiss et al.'s (2010) pure speculation that it was flat ignores these considerations and is less likely than our assumption that it was not. Despite their criticisms of the precise words of Cavalier-Smith et al. (2008) that are tangential to their central arguments, it remains likely that *Ancyromonas* lacked this key and extremely obvious characteristic of all planomonads, even though we cannot prove this.

Heiss et al. (2010) inaccurately outlined the history of doubts over the identity of *Ancyromonas*: saying that Larsen and Patterson (1990) 'questioned whether' Hänel's and Saville Kent's organisms were the 'same' is wrong. Larsen and Patterson (1990, p. 912) seem to **accept** (not question) that Hänel was describing the same organism as Saville Kent; their only questioning concerning *Ancyromonas* was the statement 'it is not clear if Hänel (1979) was observing the same organism as Klebs (1982)' – i.e. not a comparison with Saville Kent's marine

strain but with Klebs's also freshwater *Phyllomonas contorta*, which Lemmerman (1914) transferred in *Ancyromonas* without mentioning *A. sigmoides*. Nor when describing *Planomonas cephalopora* as *Bodo cephaloporus* did Larsen and Patterson (1990, p. 9) consider the possibility that *P. cephalopora* might be 'the same organism' as *A. sigmoides* (the only doubt expressed was whether it was actually a *Bodo*), which they did not even mention. Later Patterson and Zölffel (1991) unequivocally supported Hänel's interpretation, wrongly treated *B. cephaloporus* as synonym of *A. sigmoides*, and (rightly we think) removed *Phyllomonas contorta* again from *Ancyromonas*, contradicting Larsen and Patterson (1990, p. 912) who favoured 'allowing the concept of *Ancyromonas* developed by Lemmerman and Hänel to stand'.

In claiming *N. mylnikovi* to be a synonym for *A. sigmoides*, Heiss et al (2010) tacitly accept our view that Hänel's equation of freshwater *Nutomonas* and *A. sigmoides* was mistaken and therefore that there was indeed formerly an excessively liberal interpretation of the concept of *A. sigmoides*. Yet, despite their now accepting that there really are many more hard-to-distinguish planomonad species than was long recognized, more than a tinge of that excessive liberality remains in Heiss et al. (2010), most manifest in their mistaken *mylnikovi*/*sigmoides* species synonymy and astounding statement that 'depending on one's species concept, *A. cephalopora* could be synonymous with *A. sigmoides*, or perhaps *A. micra*'. We have shown here that *N. mylnikovi*, which they equate with *A. sigmoides*, should not be in the same genus or even family as *P. micra*, as they differ in anterior ciliary structure that is remarkably conserved within the two primary clades on the planomonad tree, which are as genetically divergent as the radiate animal phyla (see Fig. S1). Even in the light of the trees of Cavalier-Smith et al. (2008), and now still more so, a species concept that could allow one to consider equating *P. cephalopora* (which ciliary structure unambiguously places it in Planomonadidae not Nutomonadidae) with **either** *N. mylnikovi* **or** *P. micra* is too lax by orders of magnitude. They also failed to emphasize that, well before Cavalier-Smith et al. (2008), Ekelund and Patterson (1997) stated that Hänel's species 'is probably not the original concept of Saville Kent' - apparently the first forthright criticism of

twentieth century identifications of Saville Kent's *A. sigmoides*. Ekelund and Patterson (1997) considered these organisms to be different, though nobody took that view to its proper nomenclatural conclusion before Cavalier-Smith et al. (2008). Until then all planomonads other than *P. melba* (Patterson and Simpson 1996) and the distinctive snoutless *P. sinistra* - not found since its original description (Al-Qassab et al. 2002), were wrongly lumped as one species: *A. sigmoides*. Recognition now of 16, of which 13 from five highly divergent lineages are genetically characterized, gives a much more realistic picture of their biodiversity - though still far from complete, it no longer allows such lax species concepts as formerly prevailed.

What was *Ancyromonas sigmoides* Saville Kent?

If *A. sigmoides* was a gliding flagellate (Heiss et al. 2010), we concede that it is somewhat more likely to have been a member of Planomonadida than of any other currently well-characterized group. But since new planomonads are continually being discovered it was premature to try to make a final judgement by considering only planomonads known in 2008. Our unpublished environmental DNA sequencing reveals two other novel deep nutomonad lineages (Glücksman et al. in prep.), both from marine and freshwater samples; either might have the sigmoid shape and recurved posterior plus correct size that could potentially identify it as *A. sigmoides* - neither (unlike *N. mylnikovi*) is in the *Kentomonas* clade; or both could prove irrelevant to its identity.

Moreover, other gliding lineages are being discovered within Apusozoa (Glücksman et al. 2010), and within the cercozoan subphylum Filosa, which appear to have been ancestrally gliding zooflagellates with two cilia (Howe et al. 2011a). Several lineages of Cercozoa have independently lost the anterior cilium (e.g. *Clautriavia* within Imbricatea, *Metopion* within Metromonadea, *Allantion* within Glissomonadida); there are still numerous marine environmental DNA clades with no cultured representatives of known morphology; and novel gliding phenotypes are being described at a high rate (Howe et al. 2011a,b). Therefore the possibility exists that *A. sigmoides* belongs to one of these not to Planomonadida. Attachment by the distal

portion of the gliding flagellum (anchoring behaviour) is known for *Metromonas* and for the oscillating glissomonad *Mollimonas vickermani*; some glissomonads have a rostrum (e.g. *Bodomorpha*) and many nod while gliding (Howe et al. 2011b), making them fairly easy to confuse with planomonads without critical study. An eminent flagellate specialist of long experience gave us cultures he identified as *Ancyromonas* sp. which turned out on sequencing to be novel nodding glissomonads. Though most glissomonads are freshwater, at least one is marine (Howe et al. 2011b); recently we had in temporary crude culture another nodding, gliding marine flagellate seemingly not a planomonad, which might have been a glissomonad or related to *Ancyromonas* (or both), but we were unable to isolate it for further study. The frequency with which we encounter such novel flagellates shows that we know too little to say whether the right combination of characters to identify *A. sigmoides* will first turn up in a planomonad, a cercozoan, or something else.

One clue that *A. sigmoides* may have been a nodding glider, whether planomonad, cercozoan or a more novel group, is that Saville Kent said 'progression was in a straight line, accompanied by an oscillating motion, the single flagellum trailing in the rear'. 'Nodding' motility does not unambiguously make it a glider, but combined with straight progression makes it more plausible than any consideration mentioned by Heiss et al. There is no other evidence that it may have glided and none that it was flat. It might instead have been a non-gliding, non-flattened flagellate that can alternate between genuine swimming in the medium or anchorage by a posterior-directed cilium, as Cavalier-Smith et al. (2008) reasonably assumed most likely. Heiss et al. (2010) tried to deduce or guess what species Saville Kent saw and what confusions he may have made. But why restrict such speculative comparison to planomonads just because of their historical misidentification as *A. sigmoides* by Hänel (1979)? Can a better or as good a case be made for other groups?

One possibility is an anoecid heterokont (Cavalier-Smith and Chao 2006), perhaps of a somewhat different phenotype from any species known in culture. Anoecids like *Cafeteria* and

Pseudobodo are very common in marine habitats and their normal mode of feeding is by attaching to the substratum by the extreme tip of the posterior cilium and undulating the anterior one; they also swim with a trailing cilium. Thus they both anchor to substrata by a ciliary tip and swim, exactly as *Ancyromonas* was said to, unlike planomonads; when swimming the anterior cilium of many heterokonts undulates so rapidly that it can be almost invisible by phase contrast or DIC. Though without modern optics Saville Kent might have overlooked it, it is perhaps more likely that some undescribed anoecids have lost the anterior flagellum. The anterior flagellum of *Pseudobodo minima* (Ruinen 1938), a putative anoecid, is very short and easy to overlook or may even be absent in some cells. In cell size many anoecids overlap with *A. sigmoides*. In shape they are more similar to *A. sigmoides* than are most planomonads; in particular *Pseudobodo minima*, *Anoeca atlantica, Rictus* and *Cafeteria* normally have a pointed posterior (Cavalier-Smith and Chao 2006; Yubuki et al. 2010). Possibly Saville Kent saw an anoecid with strongly reduced or absent anterior cilium.

No known anoecid corresponds exactly with *A. sigmoides* but they have considerable diversity in ciliary patterns. Anoecids are very poorly understood, with new genera such as *Anoeca*, *Rictus* (which feeds anchored to substrata and swims with long undulating posterior cilium), *Nanos* and *Filos* (Cavalier-Smith and Chao 2006; Kim et al. 2010; Yubuki et al. 2010), only recently discovered, and the number of undescribed lineages from environmental DNA surveys is great in this part of the heterokont tree (Massana et al. 2006; Park et al. 2010). The possibility of the eventual discovery of a new anoecid species corresponding precisely to *A. sigmoides* cannot therefore be excluded. Except for *Caecitellus*, which could not be confused with *Ancyromonas*, all anoecids are non-gliders. Perhaps more far fetched, he might even have seen one more like *Cafeteria*, which when feeding bends its long anterior flagellum backwards undulating it asymmetrically all the while (Larsen and Patterson, 1990), the angle of the undulating flagellum varying among species (Cavalier-Smith and Chao 2006). But if the attached posterior cilium was short or buried in bacterial debris, he might have mistakenly have thought

that the cell was attached by the tip of its anterior undulating cilium. The anoecid idea is offered as a possibility not a probability simply to emphasize that our knowledge is still too incomplete to decide the issue. It is not obviously less likely than some past speculations about what Saville Kent may or may not have seen that allowed too much lumping and too little discrimination amongst closely similar organisms.

Much more work on planomonads, Cercozoa, and anoecids may be needed before *A. sigmoide*s can be safely reidentified – if ever. Had we accepted neotypification of *A. sigmoides* by *N. mylnikovi* despite strong counterarguments, the possibility of applying the name to the real *Ancyromonas sigmoides* when it is eventually rediscovered (whether a planomonad, cercozoan, anoecid or member of another group) would have been lost.

Conclusions. Our present work raises the total number of planomonad species from 7 to 16, increases the number of genera and families, and clarifies their relationships. Even if the species synonymy of Heiss et al. (2010) were one day to be accepted, *Planomonas* would not be a junior synonym of *Ancyromonas*; though *Kentomonas* or *Nutomonas* would be, depending on whether or not one chose to elevate the *Kentomonas* clade from subgenus to genus. However, it is highly premature to identify *A. sigmoides* with confidence or rename any planomonad family or genus, especially as our environmental DNA studies (shortly to be submitted elsewhere) reveal still more lineages. Should *Ancyromonas* eventually turn out to belong in Nutomonadidae, as Heiss et al. (2010) thought most likely, but which is only one of several reasonable possibilities, Nutomonadidae would necessarily become a junior synonym of Ancyromonadidae. But even then the order Planomonadida could and should be retained in preference to Ancyromonadida because it is descriptively more apposite for all its currently unambiguously established members.

Methods

Isolation and culture: Strains were found by EG between November 2007 and September 2009 in marine sediment samples from ten locations, freshwater samples from two, and in a soil

sample (Table 2). All were isolated by several rounds of serial dilution into 96-well microtitre plates containing 250 µl aliquots of either artificial seawater (CCAP recipe) or Volvic mineral water. After isolation, strains were introduced into 90 mm plates containing artificial seawater (marine) or Volvic (freshwater/soil) with one boiled wheat grain serving as food for the endogenous bacteria. They were maintained in this medium at 18-22C, subcultured every four weeks, and placed in the UK Culture Collection of Algae and Protozoa (CCAP), Oban.

Microscopy: DIC pictures of living strains growing in a glass-based dish were taken on a Nikon Eclipse 80i microscope with x60 NA 1.0W dipping water immersion lens and recorded on a Sony HD HDR-HC3E digital video camera set to maximum optical zoom and frames captured by software Final Cut Express HD. For the DIC pictures, we designate the surface pressed against the glass bottom of the dish (and posterior flagellum) as ventral, whereas the dorsal surface faces away from the substratum during gliding. Micrographs captured from videos using water immersion dipping objectives and DIC microscopy show the organism from above. Cell size was measured on at least ten different cells of each strain, chosen by eye as generally representative of the species based on observation of at least five living cultures.

Phase contrast pictures of living cells were taken using a Zeiss Axiovert 200M microscope equipped with a X100 immersion objective and an Axiocam HR digital camera. To facilitate comparison with DIC pictures, phase contrast micrographs of cells gliding on the underside of cover slips (thus seen from below) were flipped horizontally. Specimens were prepared for TEM, sectioned, and observed as in Heiss et al. (2010).

Electron microscopy: Cell cultures were pelleted at 3,000 *g* for 40 min (20°C) in a microcentrifuge tube and then fixed on ice in a cocktail of 2.5% (v/v) glutaraldehyde and 1.0% (w/v) OsO_4 in 100 mM cacodylate buffer (pH 7.4) with 10% (w/v) sucrose. Cells were then rinsed 3X in cacodylate buffer and twice with water before being injected into 2% (w/v) melted agarose and allowed to set. Agarose blocks were dehydrated in a graded series of ethanol and subsequently embedded in Spurr's resin and allowed to polymerize at 65°C overnight. Serial

sections of 70 nm were cut with a diamond knife on a Leica UC6 ultramicrotome (Leica, Knowlhill, Wetzlar, Germany) and were subsequently mounted on slot grids with pioloform resin (SPI, as in Rowley and Moran, 1975), stained with saturated (2% w/v) uranyl acetate in 50% ethanol for 10 minutes, and counterstained with Reynold's lead citrate (Reynolds 1963) for 5 minutes. Sections were observed using a Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR, Philips) and photographed with a 1-megapixel digital camera (Soft Imaging System: MegaView 2).

Gene sequencing: DNA was extracted from uniprotist clonal cultures using the UltraClean soil DNA isolation kit (MoBio Laboratories Inc.). 18S rRNA gene sequences were amplified by PCR with primers SA1 (5'-CCTGGTTGATCCTGCCAGTAG-3', forward primer, located at the 5'-end of the 18S rDNA) and SB1 (5'-GATCCTTCYGCAGGTTCACCT-3', reverse primer, located at the 3'-end of the 18S rDNA). PCR reactions were run under the following conditions: initial denaturation at 95 ˚C for 5 min, 35 cycles (denaturation at 95 ˚C for 40 s, annealing at 58 ˚C for 40 s, extension at 72 ˚C for 2 min 20 sec), and final extension at 72 ˚C for 5 min. Sequencing primers were SB1 and internal primers S6R (5'-ACCGCGGSTGCTGGCACCAGACTT-3', reverse primer, located in the C4 region of the 18S rDNA) and Pre3NDF (5'-CAGCAGGCGCGCAAATTACCC-3', forward primer, located in the C3 region of the 18S rDNA).

ITS rRNA was amplified under the conditions listed above using forward primer Pre3NDF and reverse primer B10r (5'-CCT CCG CTT ABT DAT ATG CTT-3', located at 5'-end of 28S rDNA). rDNA clone libraries were constructed using the TOPO TA Cloning Kit (Invitrogen, as described in Bass and Cavalier-Smith, 2004). White colonies were individually picked using sterile pipette tips and dropped into 25 μl PCR reactions. Colony PCR reactions were run with the Pre3NDF/B10r primer set and under the conditions described above. Colony PCR products were cleaned using the PEG protocol described elsewhere (Howe et al. 2009). Sequencing primers were PreBf (5'-GTA GGT GAA CCT GCA GAA GGA TC-3') and B10r. GenBank accession

numbers for all new sequences are XXXX [to be inserted on acceptance of MS after all corrections are finalized].

Phylogenetic analysis: 18S rRNA gene fragments of 1683-1787 nt. were edited and concatenated using CodonCode Aligner 3.03 (http://www.codoncode.com/aligner/) and Geneious Pro 4.7.6 (http://www.geneious.com/). Using MacGDE (http://www.msu.edu/~lintone/macgde) and BioEdit v7.0.9.0 (Hall 1999), for the Apusozoa-only tree (Fig. 1), we selected 34 sequences representing the three main apusozoan clades and selected 1702 unambiguously aligned positions for phylogenetic analysis. By excluding non-Apusozoa and manually aligning each sequence carefully we could include more nucleotide positions than in the unikont-wide analysis (Fig. S1: 1613) and in previously published 18S rRNA trees (Moreira et al. 2007; Glücksman et al. 2011). Phylogenetic trees were reconstructed by maximum likelihood (ML) and Bayesian methods. ML used RAxML-V (Stamatakis et al. 2005) with the GTRMIXI model (g=8); 100 runs used maximum parsimony starting trees and 100 runs used randomly generated starting trees; bootstrap analyses used parsimony start trees and 500 pseudoreplicates (100 pseudoreplicates for the tree in Supplementary Figure 1); results were plotted onto the overall most likely tree. We used MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) on the University of Oslo's Bioportal (https://www.bioportal.uio.no) for Bayesian analyses, with 2 independent chains running for 7×10^6 generations (burn-in of 2×10^6) using the GTR + gamma (8 rate categories) + I + covarion model and sampling frequency 0.01.

ITS2 sequence folding: The boundaries of ITS1, ITS2, and 5.8S rRNA for each sequenced clone were determined and each aligned independently using the online MAFFT aligner (http://mafft.cbrc.jp/alignment/server/cgi-bin/mafft4.cgi; Q-INS-i model). ITS2 sequences were delimited and cropped using an online ITS2-annotation tool (Eddy, 1998) (http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?annotator) with the following parameters: Model – eukaryotes; Maximum E-values – E<1.0; Minimum size of ITS2 – 0nt, except for *N. longa* for which the corresponding region was selected from the alignment - as the

program wrongly included non-homologous regions of its rather divergent 5.8S and 28S rRNA. Annotated ITS2 sequences were aligned separately using the online MAFFT aligner with considerable manual improvements. ITS2 sequences were folded using the RNA folding program of mfold (web version, v4.6, http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) under default settings (37ºC, 1 M NaCl). Visual representations of each fold were generated in PDF with "p-num" structure annotations. Because 37ºC is not physiological for Planomonadida, for comparisons within the *N. limna*/*howeae*-clade we also used mfold older version v2.3 at temperatures 10C and 4C. Temperature often greatly affected the structures found.

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Table 1. Number of identical sites between species of subgenus *Nutomonas*

	***Nutomonas howeae* strain124**	***Nutomonas limna* type strain 120**	***Nutomonas limna* CCAP 1958/10**	***Nutomonas lacustris***
***Nutomonas howeae* strain 124 (191)**	-	146	146	168
***Nutomonas limna* type strain 120 (219)**	146	-	181	152
***Nutomonas limna* CCAP 1958/10 (212)**	146	181	-	149
***Nutomonas lacustris* (185)**	168	152	149	-

ITS2 length in nucleotides for each strain is in brackets.

Table 2. Newly isolated strains.

							GenBank		
Species	**Code**	**Salinity tolerated in culture**	**Location**	**Location medium**	**Length/ width ratio**	**ITS2 rDNA length**	**18S rDNA**	**ITS2 rDNA**	**Culture collection**
Nutomonas longa	ncfw	100% freshwater only	Boiling Springs, North Carolina, USA	sediment	~1.31	204			CCAP 1958/5
Nutomonas limna	staf1	100% freshwater only	Old Course, St Andrews, Scotland	soil	~1.26	215			CCAP 1958/10
Nutomonas lacustris	il18	freshwater	Sea of Galilee, Israel	sediment	~1.67	185			dead
Nutomonas indica	af	25-100% marine	Dar es Salaam, Tanzania	Muddy beach/sand	~1.29	336			CCAP 1958/6
Nutomonas atlantica	pen1	marine	Peniche, Portugal	sediment	~1.28	395			CCAP 1958/9
Nutomonas kenti	edm	marine	Burrow-in-Furness, Cumbria, England	sediment	~1.27	331			CCAP XXXX
Planomonas elongata	turk	marine	Bodrum peninsula, Turkey	sediment	~1.34	394			dead
Planomonas bulbosa	ncm	marine/brackish	Cape Fear River, Wilmington, North Carolina	sediment	~1.29	426			dead
Planomonas brevis	cy	marine	Malama Beach, Paralimni, Cyprus	sediment	~1.30	411			dead
Fabomonas tropica type	nyk4	25-100% marine	Ankobra Beach, Ghana	sediment	~2.00	291			CCAP 1958/4
Fabomonas tropica	mex1	marine	Cancun, Mexico	sediment	~2.00	291			dead
Fabomonas tropica	il19	marine	Eilat, Israel (Red Sea)	sediment	~2.00	293			CCAP 1958/8
Planomonas micra	saf	25-100% marine	Cape Town, South Africa	seawater	see type	396			CCAP 1958/7

Table 3. Clade-specific 5.8S rDNA sequence signatures

Clade	Position	Signature
Planomonadidae	55-62	**T**GCG**TAC**
	84	A
	122	**G**G**G**
	126	**A**
	128-130	CC**C**
	151	**T**
Planomonas	79-81	**C**GC
	110	**T**
Fabomonas	6	**C**
	79-83	**A**ATT**G**
	139	**T**
Nutomonadidae	19	**G**
	79	**G**
Subgenus *Kentomonas*	1-2	T**C**
	35	**T**
	55	**C**
	131	**G**
	146	**C**
	151	**C**
Subgenus *Nutomonas*	4-5	**TA**TTT**C**T
	26-31	**C**T**T**T**GA**
	44-48	**C**CG**TG**
	55-56	**AA**
	63	**T**
	82-86	**C**CCG**A**
	110	**A**
	120-122	G**A**T
	130-132	TA**T**C
	145-146	AA
	151-153	GG**A**
Subgenera *Nutomonas* plus *Incisomonas* (freshwater nutomonads)	28-31	**T**T**GA**
	51	**A**
	97	**G**
	100	**G**
	122	**T**
	131	**A**
	148	**C**

Nucleotide positions are numbered as in *Fabomonas tropica* which has the most conservative 5.8S rDNA with fewest deletions; the corresponding positions in other species can be determined from the alignment in the supplementary material. Those in bold are different in all other groups of Planomonadida.

Figures

Figure 1. 18S rRNA gene phylogeny of Planomonadida and other Apusozoa. Bayesian tree (GTR + gamma + I + covarion model) of 34 sequences using 1702 nucleotide positions. Support values are Bayesian posterior probability (left) and RAxML bootstraps (right). Black blobs indicate 100% support by both methods. Scale bar indicates 10% substitutions per nucleotide site. Names and branches of new species are shown in bold.

Figure 2. Morphology of *Fabomonas tropica*. Differential interference contrast (**A-X**) and phase contrast (**Y-AA**) light micrographs of different living *Fabomonas tropica* cells from Ghana (A-L, Y-AA; type strain nyk4), Mexico (M-R), and Israel (Red Sea; S-X). **A-X** 24 different cells; **Y-AA** Three different cells of type strain nyk4. Black arrows indicate anterior cilium. White arrows indicate acroneme. Scale bars 10 µm.

Figure 3. Transmission electron micrograph of *Fabomonas tropica* type strain nyk4. Section showing characteristic planomonad features, including a mitochondrion (**m)** with flat cristae and a dense dorsal single submembrane pellicular layer (**pl**), as well as three underlying singlet microtubules and a long posterior cilium (**pc**). X 30,000.

Figure 4. Transmission electron micrograph of *Fabomonas tropica* type strain nyk4. Section through cytoplasm showing the mitochondria (**m**), extrusomes (**e**), dorsal submembrane pellicular layer (**pl)**, nucleus (**n**), food vacuole containing a bacterial cell (**f**), and posterior cilium (**pc**) lying within the ventral groove (**v)** lined by a surface membrane devoid of the dense layer. X 30,000.

Figure 5. Differential interference contrast light micrographs of living *Nutomonas longa*. A-M Thirteen different cells; **N-S** Serial non-consecutive frames of a cell during gliding over a 1.5 s period, showing nodding movement and contraction of posterior cilium acroneme. Black arrows indicate anterior cilium. White arrows indicate acroneme. Rostrum (r) in M. Contractile vacuole (v) in D. Scale bars 10 µm.

Figure 6. Ribosomal RNA Internal Transcribed Spacer 2 (ITS2) secondary structures of selected new strains from each major planomonad clade. All are the lowest free energy structures produced with the default settings of the RNA-folding algorithm of mfold v. 4.6. **A-C** Representatives of the divergent marine clades**: A** *Fabomonas tropica* type strain nyk4 (291 nt.).

B *Planomonas elongata* (394 nt.). **C** *Nutomonas indica* (336 nt.). **D-F** Representatives of the freshwater clades differing in ITS2 length**:** **D** *Nutomonas longa* (204 nt.). **E** *Nutomonas limna* (217 nt.). **F** *Nutomonas lacustris* (185 nt.). For reference, lowest free energy (and a few theoretically suboptimal but evolutionarily more plausible structures) for all others of the 18 sequenced strains are in the electronic supplementary material (Figs S2-4).

Figure 7. Morphological diversity amongst nine novel planomonad isolates. Differential interference contrast (**A-N**) and phase contrast (**Y-AD**) light micrographs of living *Nutomonas* cells: soil *N. limna* CCAP 1958/10 (A-B, Y-Z) freshwater *N. lacustris* (C-F), and marine *N. indica* (G-I, AA-AB), *N. atlantica* (J-L), and *N. kenti* (M-N, AC-AD). Differential interference contrast (**O-X**) of living *Planomonas* cells belonging to marine species *Planomonas brevis* (O-P), *P. micra* strain saf (Q-S), *P. elongata* (T-V), *P. bulbosa* (W-X). **A-AD** 30 different cells. Contractile vacuoles (**v**) in A and C. Scale bars 10 µm.

Supplementary material

Supplementary Figure S1. 18S rRNA gene phylogeny of Planomonadida and other unikonts. Bayesian phylogeny (GTR + gamma + I + covarion model) of 54 18S rRNA sequences using 1613 nucleotide positions. Support values are Bayesian posterior probability (left) and bootstrap values (right) inferred using RAxML (GTRMIX + gamma + I), 100 pseudoreplicates. Black blobs indicate 100% support by both methods. Scale bar indicates 10% substitutions per nucleotide site. Names and branches of new species are shown in bold.

Supplementary Figure S2. Optimal folds for all 3 *Planomonas* species (marine). The lowest free energy structure from Mfold except where otherwise stated:
A. *Planomonas micra* ATCC502670. (a) Lowest dG -151.41; (b) dG -152.46. B. *Planomonas micra* saf. (a) lowest dG -146.88; (b) dG -152.17. C. *Planomonas micra* Millport. (a) Lowest dG -138.31; (b) dG -148.27. D. *Planomonas bulbosa.* E. *Planomonas brevis.*

Supplementary Figure S3. Optimal folds for 3 *Nutomonas* species of subgenus *Kentomonas* (marine). A. *Nutomonas kenti.* B. *Nutomonas atlantica.* We show both the lowest free energy fold (a) dG -70.4, and a theoretically less optimal fold, which more resembles that of its sister *N. indica* and thus seems more likely to be the biologically meaningful one (b) dG -75. C. *Nutomonas mylnikovi.*

Supplementary Figure S4. Optimal folds for 2 *Nutomonas* species of subgenus *Nutomonas*.
A. *Nutomonas limna* ssp. *terrestris.* For this subspecies we include both folds found as the slightly higher free energy one more resembles the lowest free energy one of other species. B. *Nutomonas howeae* type strain 124.

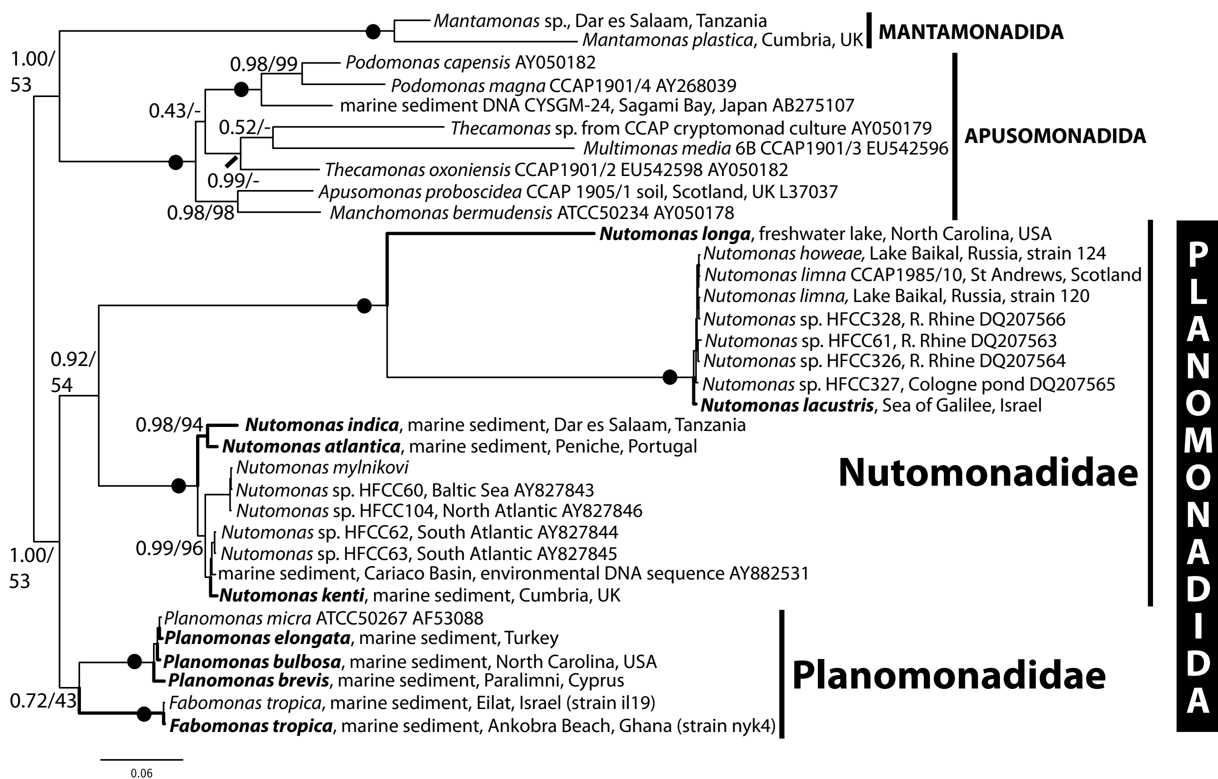


Figure 1

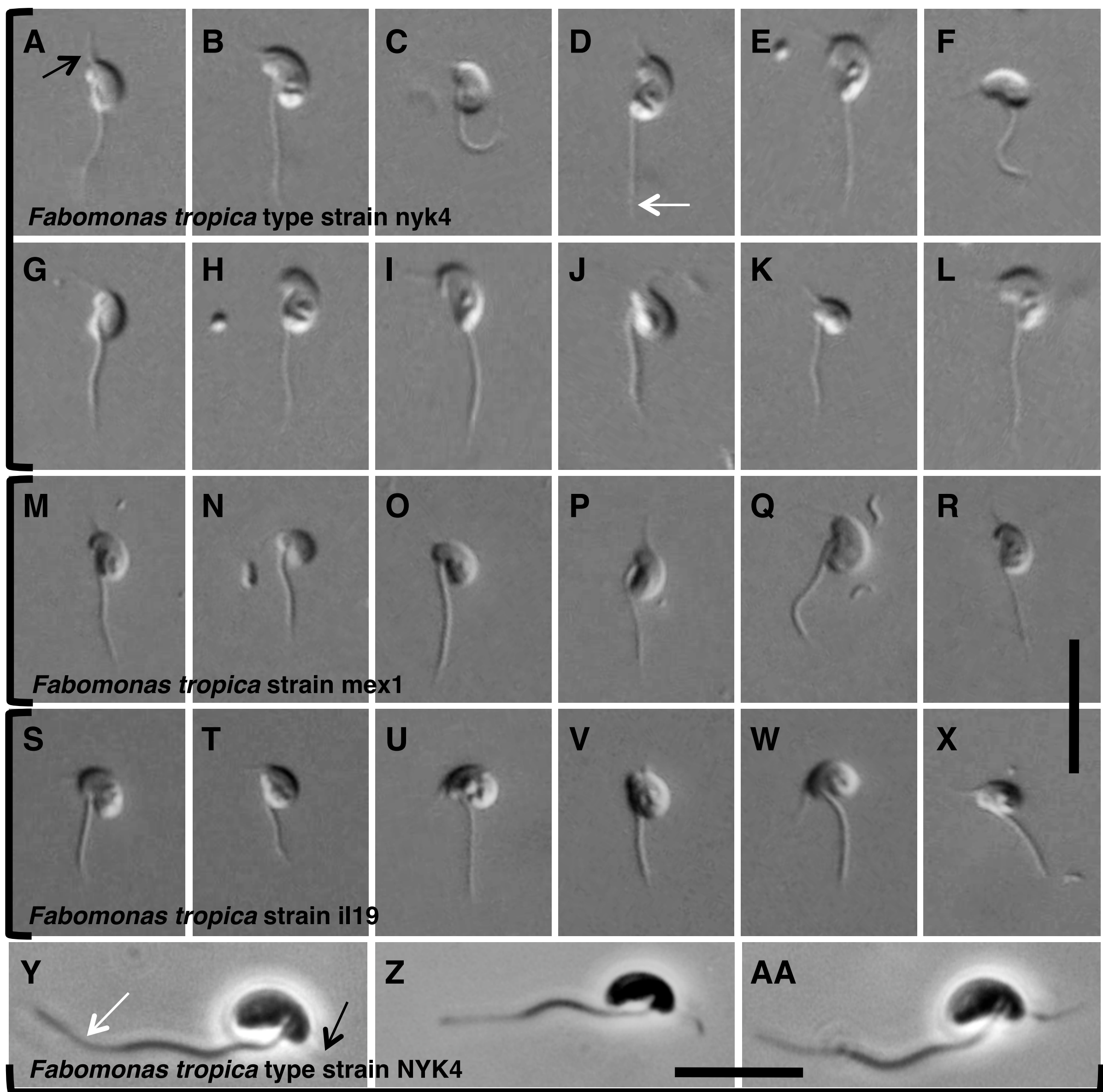
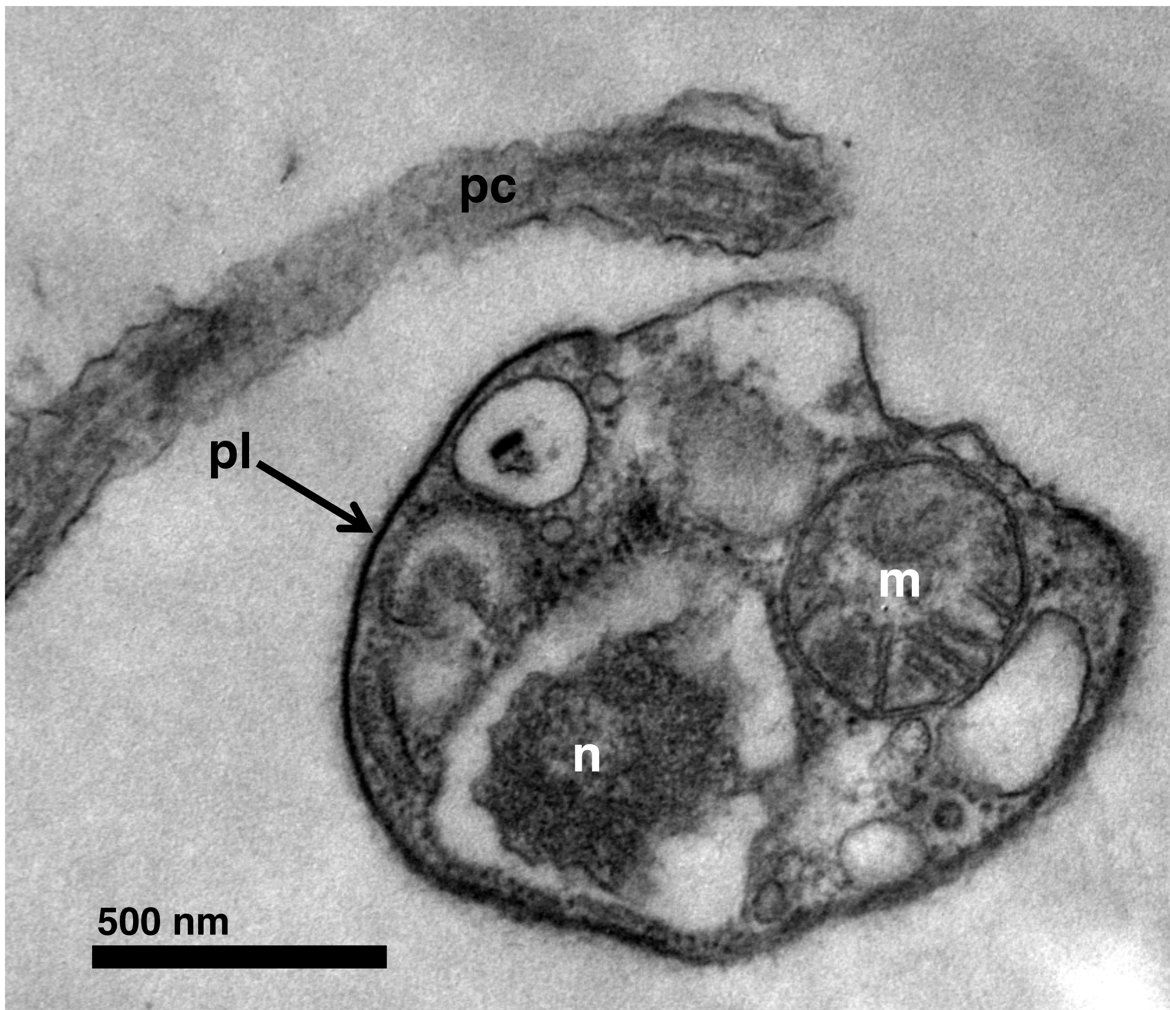


Figure 2



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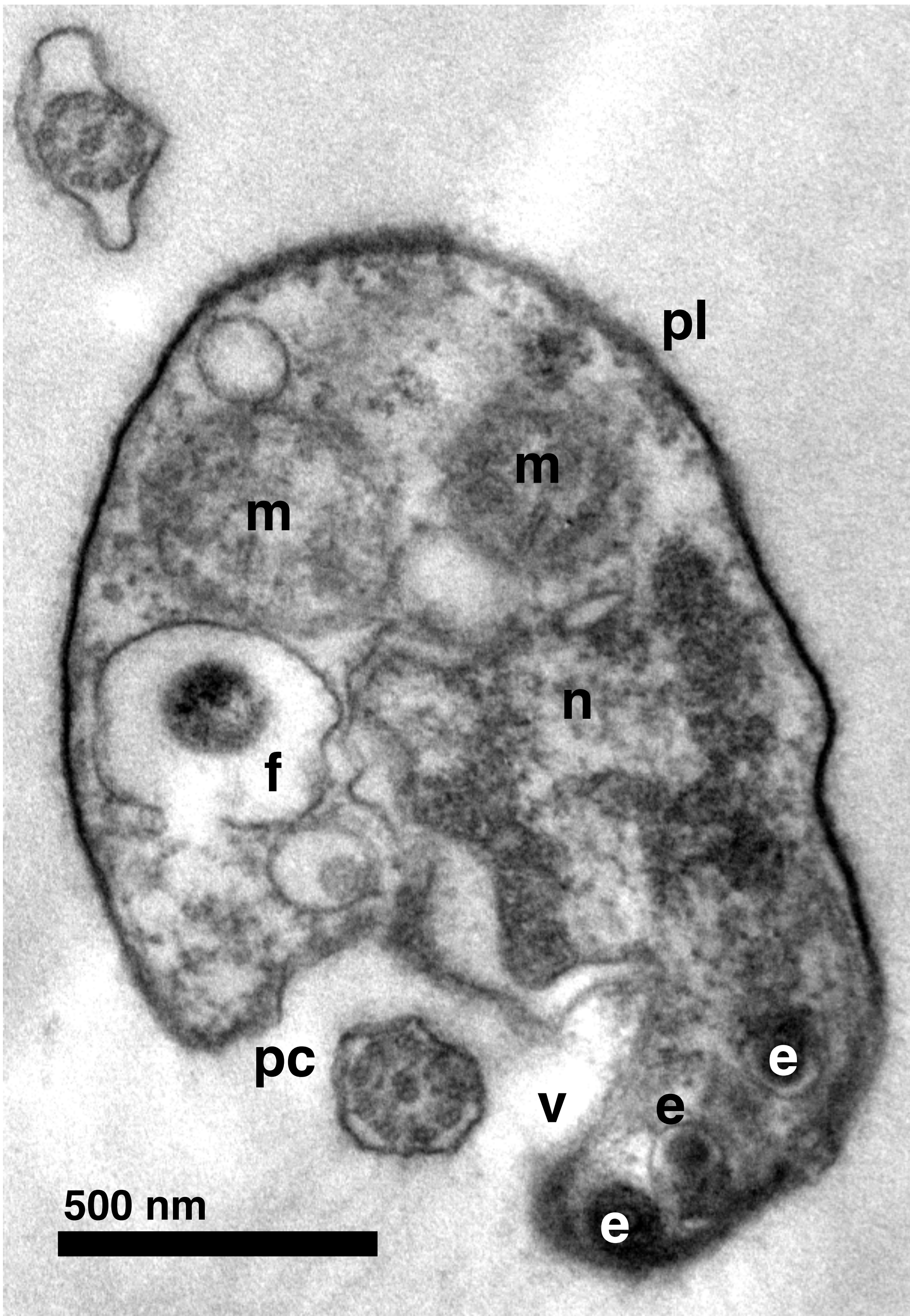

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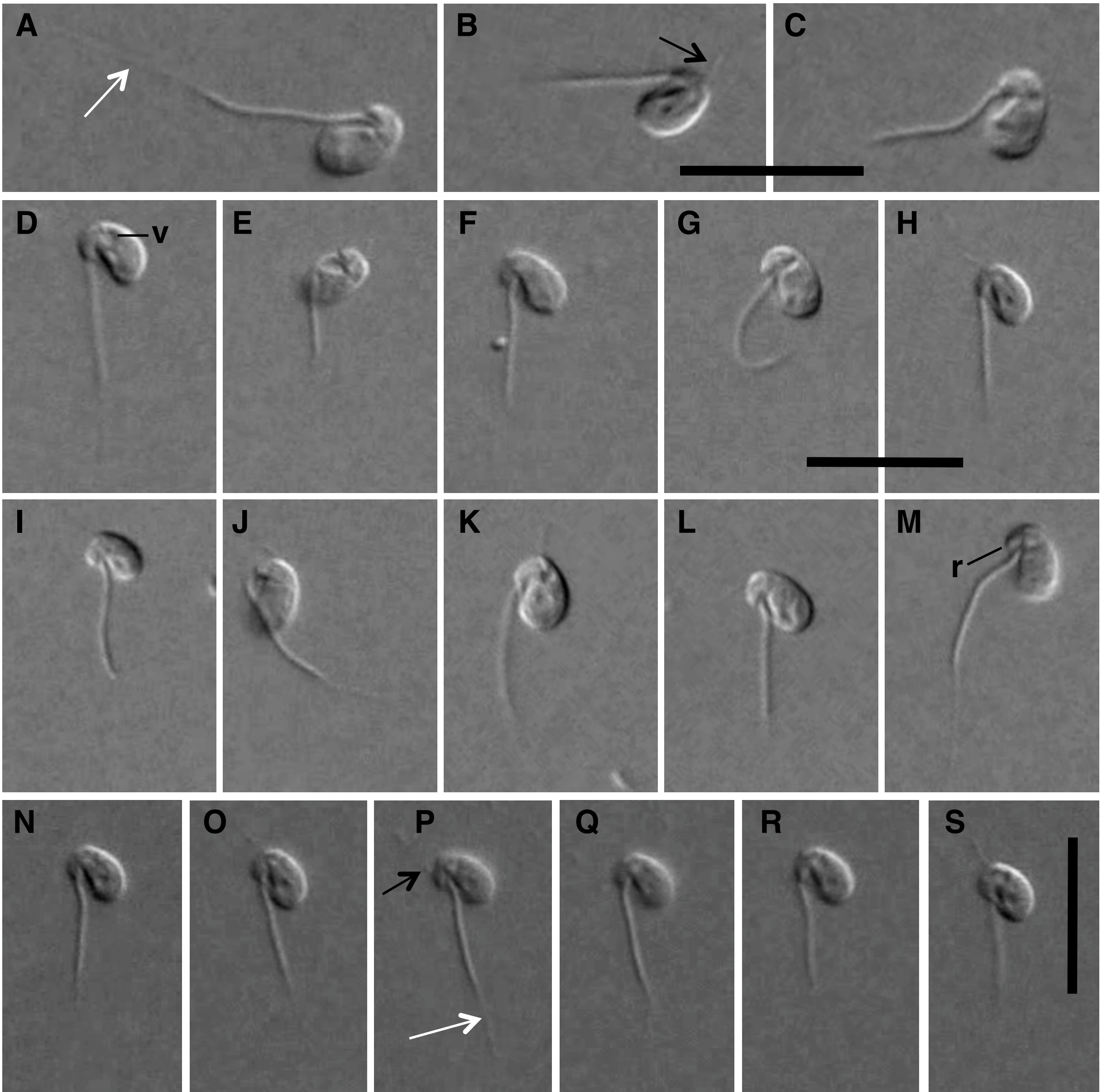

Figure 5

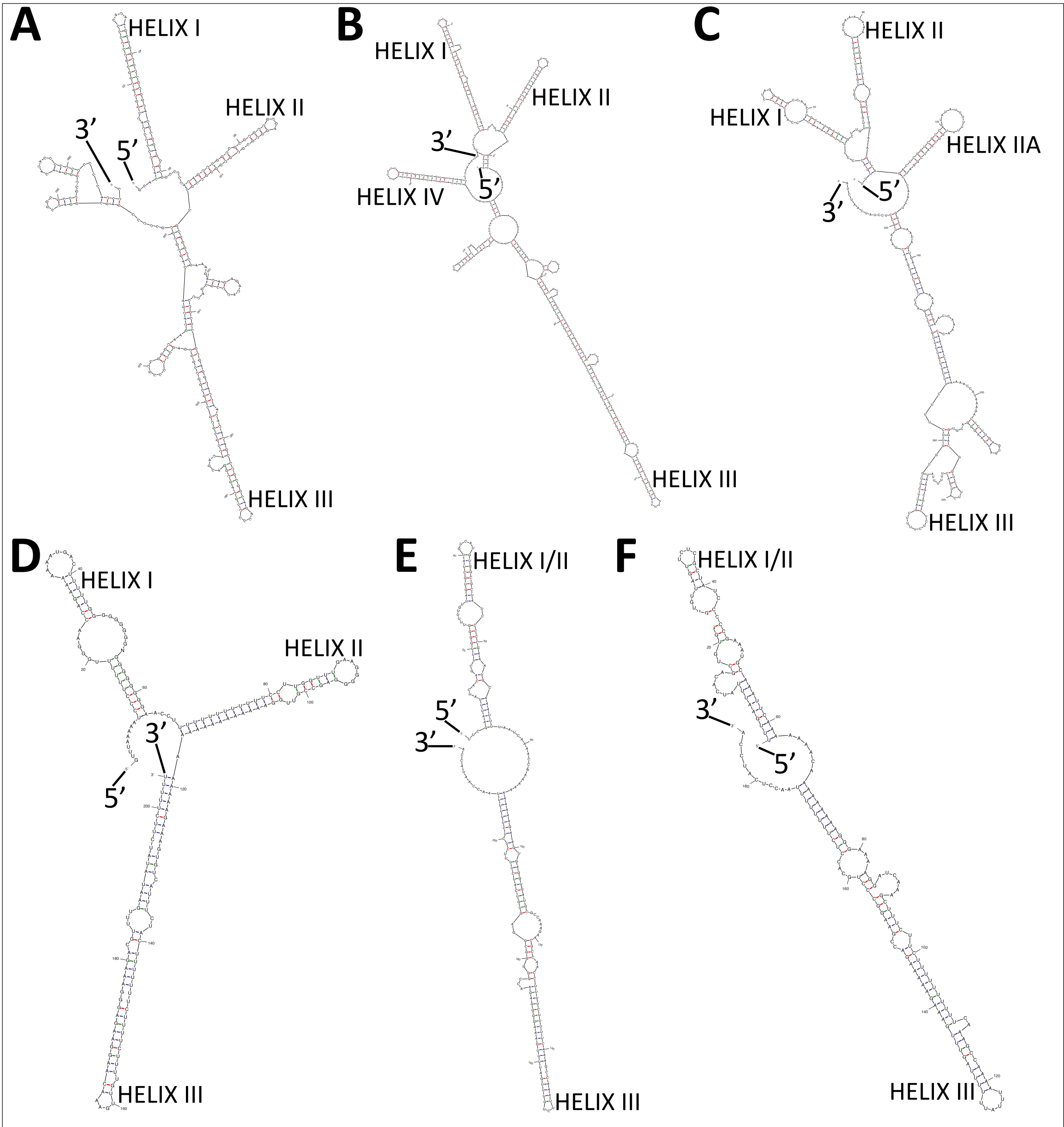

Figure 6

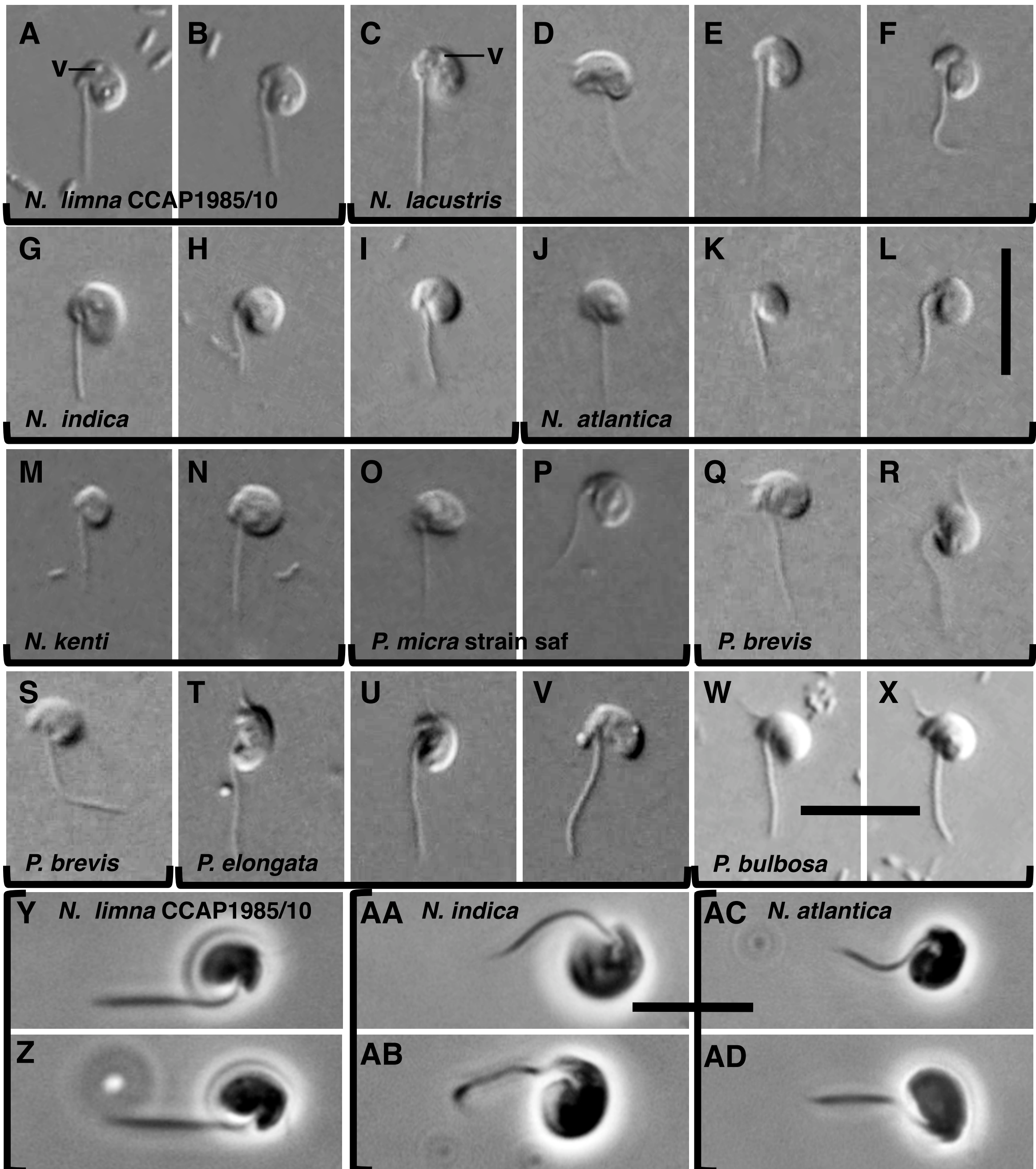

Figure 7

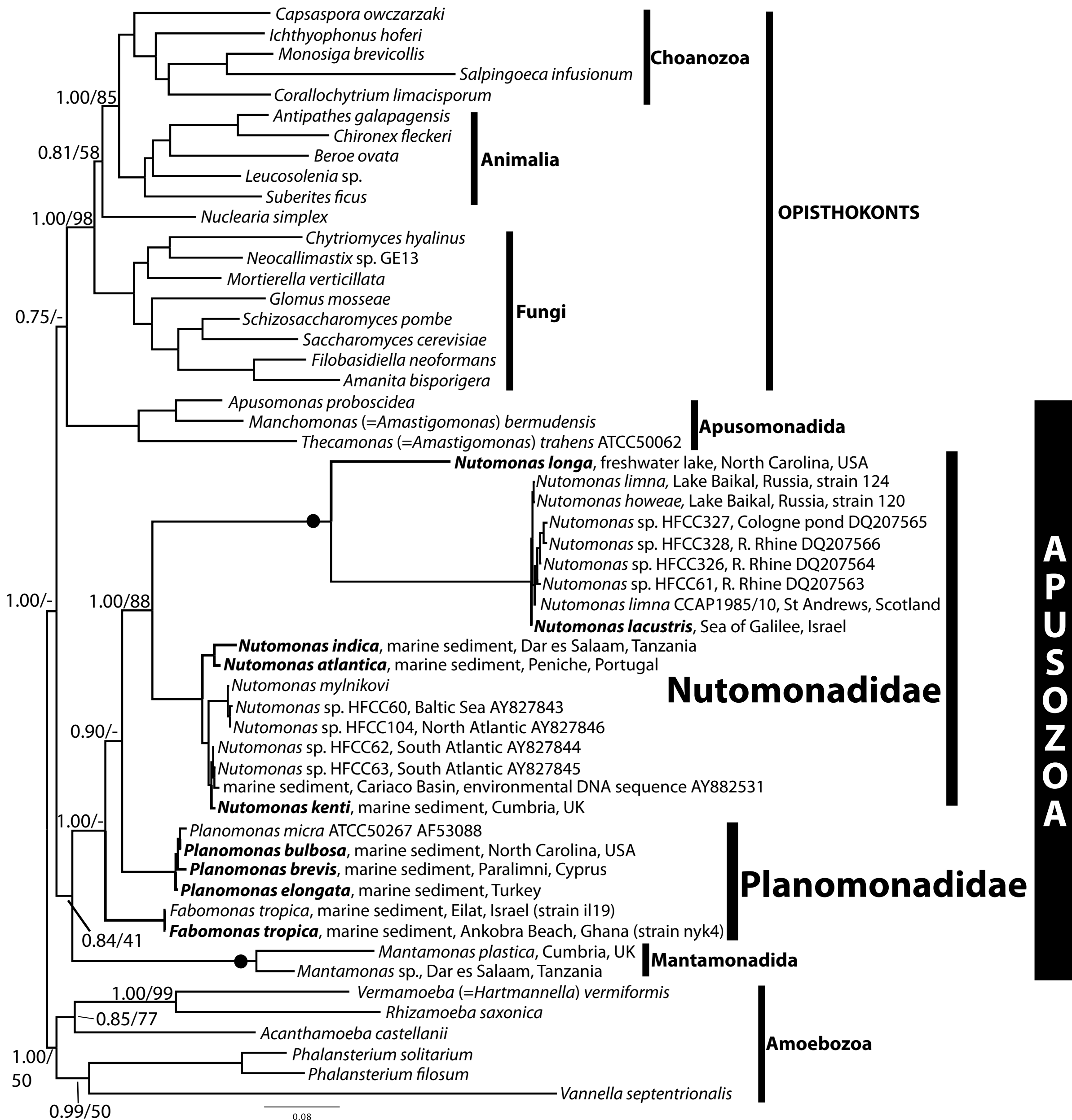

Figure S1

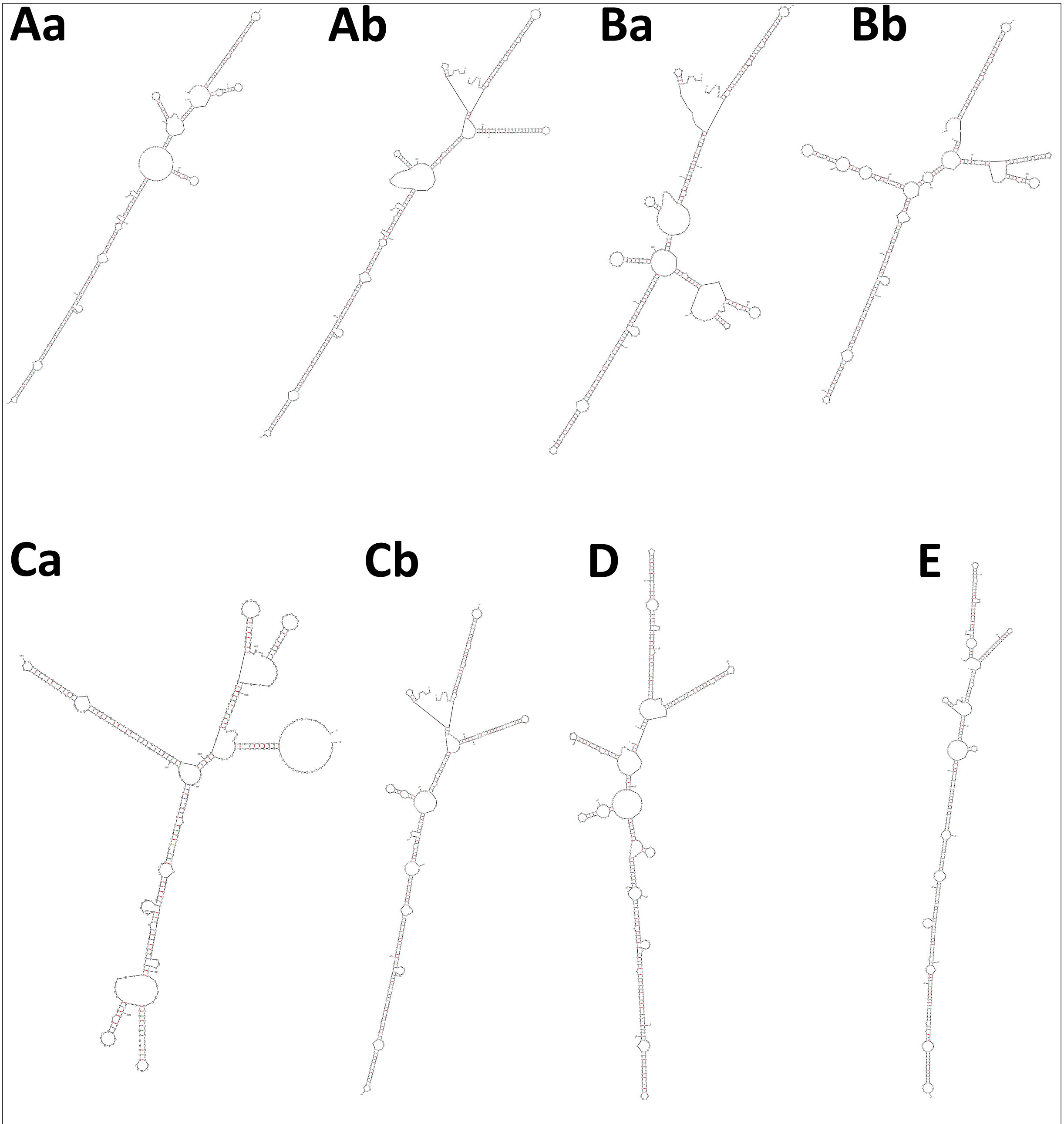

Figure S2

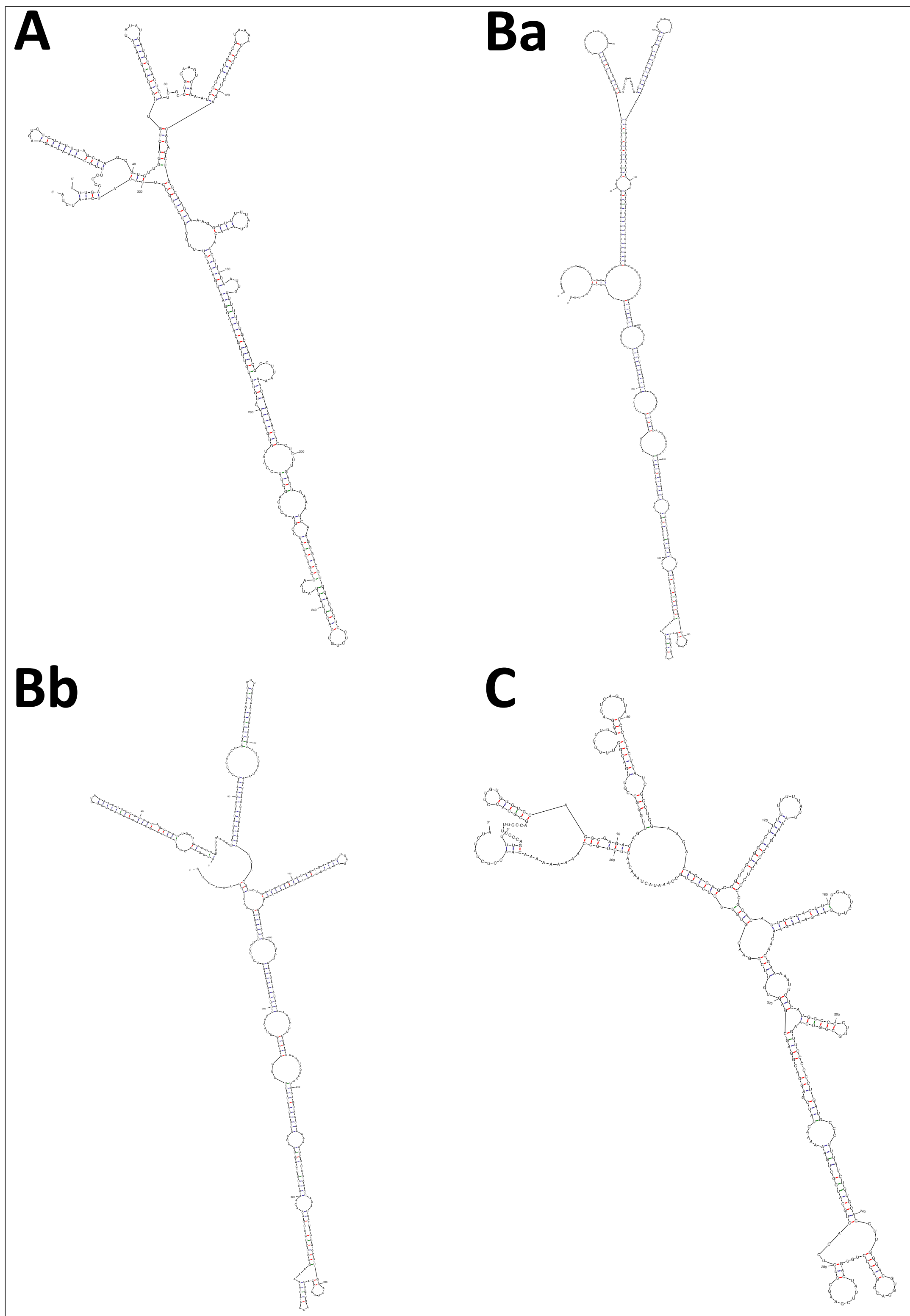

Figure S3

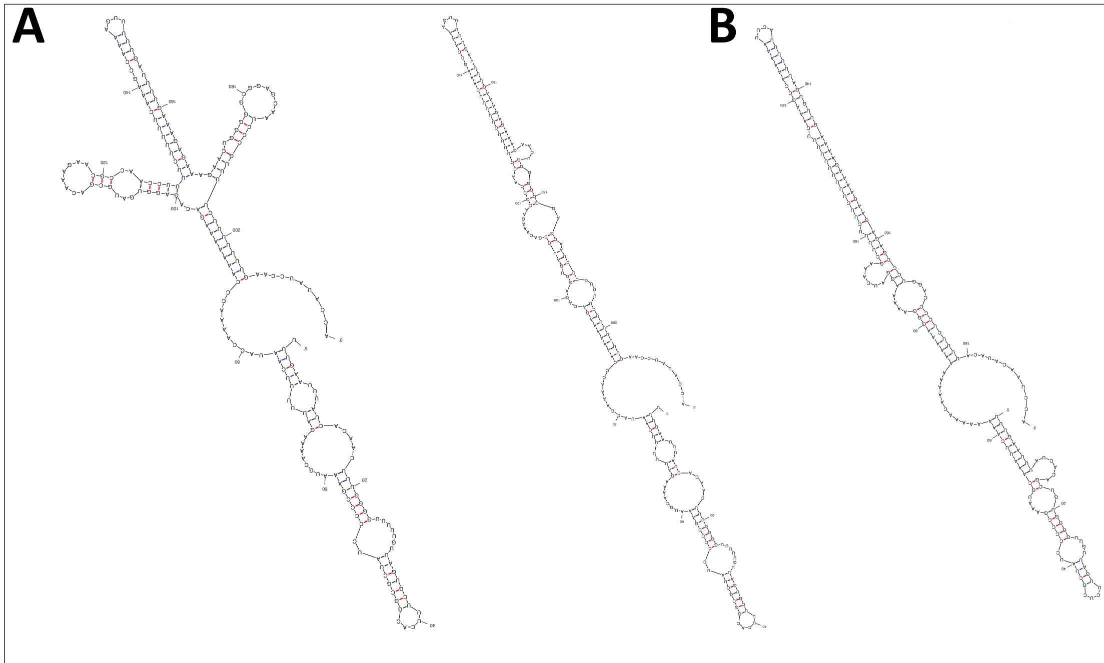

Figure S4

Chapter Four

A PCR-based environmental survey and mining of publically available metagenomic databases to explore the diversity and ecology of Mantamonadida (Protozoa: Apusozoa)

Manuscript

Chapter notes

This chapter will shortly be submitted for publication as a manuscript co-authored by David Bass and Tom Cavalier-Smith. Formatting and content have been adjusted specifically for this thesis, including with the addition of chapter citations. For consistency, this chapter adopts the revised nomenclature of Planomonadida, presented in Chapter 3, even though this system has not yet been formally accepted.

Chapter abstract

Mantamonadida is a recently discovered order of gliding zooflagellates related to either Planomonadida or Apusomonadida (Apusozoa). To investigate the diversity and ecology of mantamonads, we designed group-specific PCR primers and applied them to environmental DNA extracted from a range of habitats. We show that type species *Mantamonas plastica* 18S rDNA amplified in samples from marine, freshwater, and soil. Three new ITS1 rDNA-types were also detected. To search for additional diversity and to investigate the relationship between detected diversity and sequencing methods and technologies, we mined publically available databases of PCR-generated and PCR-independent metagenomic and metatranscriptomic sequences using known apusozoan sequences as seeds. We found 31 directly sequenced genomic and transcriptomic environmental reads that were identical to the known mantamonad 18S rDNA types, all obtained from marine samples. Our relatively conservative approach also found three new planomonad lineages, including the first ever putatively freshwater fabomonads. The remaining planomonad sequences belong to known major clades and were mostly detected by direct transcriptomic sequencing. We also present five new apusomonad lineages and identify sequences belonging to known clades, detected by direct transcriptomic sequencing and targeted PCR-based approaches. Detection rates differed by clade, suggesting some lineages may be more common in the natural environment. Based on this survey, direct transcriptomic sequencing appears to be the most effective method of detecting rare cells in mixed microbial assemblages.

Keywords: Apusomonadida, Apusozoa, CAMERA Portal 2.0, Environmental survey, Mantamonadida, Metagenomic, Metatranscriptomic, Planomonadida

Introduction

Free-living protists in the nanoplankton size class (2-20 μm) numerically dominate microbial eukaryotic assemblages and are responsible for a variety of vital trophic processes within environmental food webs (Foissner 1999; Glücksman et al. 2010; Lim 1996; Moore and de Ruiter 1991; Puitika et al. 2007). Phylum Apusozoa (Cavalier-Smith 2002) comprises small gliding heterotrophic zooflagellates that are commonly observed in environmental samples. However, their diversity, ecology, and phylogenetic relationship with other eukaryotes, are incompletely understood (Lee and Patterson 2002; Patterson and Lee 2000).

Mantamonadida

Apusozoa was traditionally thought to comprise two orders, Apusomonadida (Cavalier-Smith and Chao 2003, 2010; Karpov and Mylnikov 1989) and Planomonadida (Cavalier-Smith et al. 2008; Chapter 3; Heiss et al. 2009; Heiss et al. 2011), but we recently identified a third, Mantamonadida, taken from marine sediment in Cumbria, UK (Glücksman et al. 2011 - Chapter 2).

Only one mantamonad species has been described, *Mantamonas plastica*, and it remains the only strain currently alive in culture. Another unique 18S rDNA type (*Mantamonas* sp. AF21 GU001156.1) was isolated from marine sediment from Dar Es Salaam, Tanzania, but the culture died before pictures could be taken. Since then, we have seen mantamonad-like cells on several occasions in mixed marine environmental cultures, but all of them died during the isolation process. Therefore, although potentially ecologically important, little is known about their

diversity and ecology, partly because they are difficult to work with in the laboratory.

Culture-independent molecular techniques

In recent years, the introduction of culture-independent molecular techniques, which bypass some of the selective biases inherent in cultivation or microscopy methods, has revealed a far greater microbial diversity than suggested by traditional morphological investigations (Dawson and Pace 2002; Edgcomb et al. 2002; Richards and Bass 2005; Zettler et al. 2002). Group-specific PCR primers have the potential to exclude unrelated taxa from an analysis, making it possible to target individual eukaryotic lineages within mixed samples of environmental DNA (eDNA) (e.g. Bass and Cavalier-Smith 2004; David et al. 1996; Habura et al. 2004; Howe et al. 2009; Lin et al. 2006; von der Heyden and Cavalier-Smith 2005). To that end, we used the only two existing 18S rDNA types of mantamonads to design group-specific PCR primers, applying them to eDNAs sampled from a range of locations and habitat types.

Apusozoa in metagenomic and metatranscriptomic sequence databases

We also developed a replicable method of using apusozoan 18S rDNA signatures as seeds to mine publically available databases containing PCR-based and amplification-independent, metagenomic and metatranscriptomic sequences, obtained by either Sanger or 454 sequencing technologies. These distinctions are important because, though all of them bypass the biases of culturing, each method differentially detects microbial diversity.

For example, metatranscriptomics, based on sequencing of microbial community total RNA or poly-A-selected messenger RNA (mRNA; see below), differs from metagenomics, which is based on sequencing total DNA, because it detects *in situ* gene expression patterns within microbial assemblages at a specific time and place rather than entire genomes from microbial communities as a single unit (Bailly et al. 2007; Moran 2009). Transcriptomic sequencing also avoids non-coding DNA and introns so gives proportionally greater coverage of expressed genes. One would expect it to be easier to detect low abundance eukaryotes in metatranscriptomic studies due to a number of features inherent of mRNA that make it possible to increase the relative count of expressed reads from rare organisms. For example, it is possible to separate out eukaryotic from prokaryotic mRNA by targeting the polyadelynated tail on the 3'-end of molecules, mostly unique to eukaryotes. Moreover, since only a small fraction of an individual genome is expressed as mRNA and multiple copies are produced, transcripts from rare cells may be highly abundant in community mRNA, yet undetectable in genomic DNA from the same sample.

Likewise, both metagenomic and metatranscriptomic sequencing can be carried out with or without an amplification (PCR) step. Amplification, using molecular probes, offers directed detection of conserved target genes. Yet, such an approach amplifies different organisms differentially, and thus will detect rare species only if they amplify readily (see Chapter 7). PCR-based approaches also target relatively few genes at a time are only applicable to genes that are known, currently just a fraction of the total genetic makeup of microbial communities. These biases are bypassed in direct sequencing studies, where total community DNA or RNA is

randomly sequenced and where the representation of template molecules is not skewed, as is the case with PCR-based approaches.

Different sequencing technologies may also influence the detected outcome of diversity surveys. Although both next-generation 454 Pyrosequencing and Sanger dideoxy sequencing provide unbiased methods of sequencing large numbers of reads from environmental samples, Sanger sequencing relies on a cloning step, which is known to be associated with biases against less abundant taxa (Claesson et al. 2009). Furthermore, for a lower price, next-generation sequencing methods are able to generate far more environmental reads than Sanger, and are therefore more likely to provide a comprehensive survey of sample communities.

Study aims

Given the elusive nature of mantamonads within environmental cultures, the overall aims of this chapter were to, a) explore the diversity and ecology of mantamonads using PCR-independent methods, b) mine metagenomic and metatranscriptomic environmental sequence databases for mantamonad, planomonad, and apusomonad sequences, and, c) relate the relative abundance and diversity of retrieved apusozoan reads to sequencing technologies used, and, more specifically, target discrepancies between metagenomic and metatranscriptomic reads, sequenced using either PCR-based or amplification-independent methods.

Methods

DNA extraction

Environmental samples were collected in sterile Eppendorf or Falcon tubes and DNA was extracted as soon as possible after collection, following the Maximum Yield Protocol of the UltraClean Soil DNA Isolation Kit (MoBio Laboratories), using c. 0.5 g of soil/sediment. Environmental DNA samples were extracted as stated in Table 1 of Chapter 5.

PCR reactions

Primers were designed to be specific to both known 18S rDNA types of *Mantamonas*. A nested PCR strategy was employed because it was impossible to visualise amplicons on agarose gels after an initial round of PCR reactions. We amplified a portion of the 18S and the entire ITS1 rDNA, using two *Mantamonas*-specific 18S rDNA forward primers, located approximately 300 and 1350 nucleotides from the 5'-end of the molecule, and paired them with two universal eukaryote reverse primers, located near the 5'-end of the 5.8S rDNA gene (Table 1).

In the initial PCR reaction, eDNA was diluted 1/10 with de-ionized water to reduce the effect of PCR inhibitors, and then amplified using two semi-nested primer sets. Each round of PCR included at least one positive control (gDNA extracted from a known culture of the primer target) and a negative control (reagents with molecular grade water added in place of DNA). PCR reactions were run under the following conditions: initial denaturation at 95 °C for 5 min, 35

cycles (denaturation at 95 °C for 40 s, annealing at 58 °C for 40 s, extension at 72 °C for 2 min 20 sec), and final extension at 72 °C for 5 min.

PCR products from the initial reaction were diluted 1/100 in preparation for the nested reaction and subsequently run under the same conditions as the initial reaction, with the exception of a higher annealing temperature (65°C). Full PCR products (25 μl) were run on 1% agarose gels and visible bands of the appropriate lengths were excised and cleaned following the protocol of the GFX PCR DNA Gel Band Purification Kit (GE Healthcare).

Cloning, sequencing, and editing

rDNA clone libraries were constructed using the TOPO TA Cloning Kit (Invitrogen, as described in Bass and Cavalier-Smith, 2004). White colonies were individually picked using sterile pipette tips and dropped into 25 μl PCR reactions. Colony PCR reactions were run with the same primer set and under the same conditions as nested PCR reactions. Colony PCR products were cleaned using the PEG protocol described elsewhere (Howe et al. 2009). Sequencing reactions were carried out using primers from the nested PCR, then run using dye terminators and separated on an automated ABI-377 sequencer.

Sequences were edited and concatenated in Geneious Pro 5.0+ (Drummond et al. 2010), where aligning by eye, Neighbour-Joining (Jukes-Cantor), and PhyML trees (substitution model: JC69) confirmed that the 5'-end of all the new sequences (located on the 18S rDNA) was identical to *M. plastica*. We then cut out ITS1 rDNA sequences (3'-flank of the amplicon) and aligned them, using a small

portion of the 3'-flank of the 18S rDNA, with a *M. plastica* ITS1 rDNA sequence obtained by direct sequencing of a strain in culture.

The resulting sequences were aligned first by MAFFT (Q-ins-i model) and then refined by eye using MacGDE (http://macgde.bio.cmich.edu/). Using the remaining alignment of approximately 123 nt, we grouped the *M. plastica* ITS1 rDNA sequences into three distinct lineages by eye.

Metagenomic and metatranscriptomic sequence database search on CAMERA 2.0 Portal

To mine publically available environmental sequence databases for apusozoan sequence signatures, we seeded searches on the CAMERA 2.0 Portal (Sun et al. 2010) with 18S rDNA sequences of 20 known representative mantamonads, planomonads, and apusomonads (for detailed descriptions of the datasets, see Supplementary Table 1). The seeds included eight planomonad species (*Fabomonas tropica*; *Nutomonas longa; N. howeae* strain124; *N. indica; N. atlantica; N. mylnikovi; N. kenti*; and *Planomonas micra* AF174363), and ten apusomonad species (*Thecamonas* sp. Bamfield; *Apusomonas proboscidea* L37037; *Manchomonas bermudensis* AY050178; *Podomonas capensis* AY050182; *Podomonas magna* AY268039; *Thecamonas* sp. AY050179; *Thecamonas oxoniensis* EU542598; *Thecamonas trahens* AY050180; *Multimonas media* 6B EU542596; and environmental sequence CYSGM-25 AB275107).

To avoid length-based search biases, full or near-full 18S rDNA seed sequences were quartered in length, making, from the 5'-end of *M. plastica* GU001154.1: Section 1 (nts. 0-430), Section 2 (431-774), Section 3 (775-1254), and Section 4 (1255-1802).

Separate blastn searches were carried out across three CAMERA databases as well as the NCBI (env_nt) environmental sample database (Table 2). All queries were carried out using the following parameters: *evalue* exponent=1, filter low-complexity seq=T, lower case filtering=false. Once searches were complete, returned hits ([4 x 18S rDNA quarter-length sequences] x 20 strains x 50 hits per seed = 4,000 hits per database) were downloaded as fasta and metadata spreadsheet files.

Returned hits were merged into three independent fasta files according to their database of origin and then imported to Geneious 5.4.3, where identical names were eliminated to facilitate the next step. Each alignment was then exported as a fasta file and fed into the *mothur* bioinformatics package (Schloss et al. 2009), where duplicate sequences were eliminated using the 'unique.seqs' function, as described in the online manual (http://www.mothur.org/wiki/Unique.seqs).

In Geneious 5.4.3, we created a 'custom blast' template sequence database comprising an 18S rDNA alignment of 79 representative eukaryotes (identical to that used for 18S rDNA phylogenies in Chapter 2, with the addition of *Fabomonas tropica*). We subsequently ran custom blast queries (hits= 1, *blastn* algorithm; though identical results were achieved with *megablast*) with each of the three fasta files against the template sequence database, retrieving the environmental sequences that matched to an existing apusozoan sequence as its first hit. Rows containing these sequences were highlighted and isolated on the CAMERA output metadata spreadsheet. To it were added the name of each metagenomic study or, if from NCBI, information about the source publication.

All the retrieved sequences that matched mantamonads belonged to one of two 18S rDNA ribotypes (Table 3).

Cluster analyses

For planomonads and apusomonads, in order to improve the resolution of our analysis and minimise the risk of leaving out relevant sequences, we divided the metadata spreadsheets by order, according to the custom blast labels. Cluster analyses were carried out using default settings on the *cd-hit-est* (command line: -d 0 –n 10 –l 11 –r 1 –p 1 –g 1 –G 0 –c 0.95 –aS 0.8) application run remotely on the CAMERA 2.0 Portal interface. The cluster cut-off point was thus a conservative 95% similarity (see Methods, Chapter 5, for more details).

In order to take into account the possibility of sequencing errors or chimaeras, only cluster representatives of lineages found independently in more than one study were used for our phylogenies. These were added to the alignment of 79 representative eukaryotes (same as the custom blast template) in Geneious 5.4.3 and aligned using both the inbuilt MAFFT (algorithm FFT-NS-1, scoring matrix=200PAM/k=2) and ClustalW alignment functions. The alignments were run remotely on the Green Button Supercomputer, New Zealand (http://geneious.greenbutton.net/), accessed via Geneious.

As an additional refinement stage, hypervariable alignment regions were masked out in MacGDE. Subsequently, Maximum-Likelihood (PhyML) and quick Neighbour-Joining trees were run within Geneious 5.4.3 (substitution model: JC69; Optimisation: tree/length), and all sequences grouping with either of the two orders Planomonadida and Apusomonadida were extracted and added to

order-specific alignments used in previous work (Cavalier-Smith and Chao 2010 for Apusomonadida, Chapter 3 for Planomonadida).

Phylogenetic analyses

After being aligned and further refined by eye, hypervariable regions of the alignments were masked, unmasked regions were removed, and phylogenies reconstructed using Maximum-Likelihood (RAxML) and Bayesian methods.

We used RAxML-V (Stamatakis et al. 2005) for the final Maximum-Likelihood analyses and for obtaining bootstrap values, running the masked alignment using the GTRMIXI model of sequence evolution (g=8); 100 runs used parsimony starting trees and 100 runs used randomly generated starting trees, and the bootstrap analyses were plotted onto the overall most likely tree. Bootstrap values were calculated using the default maximum parsimony start trees. We used MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) on the Cyberinfrastructure for Phylogenetic Research (CIPRES www.phylo.org/sub_sections/portal/) Portal for the Bayesian analysis, running the masked alignment with 4 independent chains running for 7 x 10^6 generations (a burn-in of 2 x 10^6 generations was used) using the GTR + gamma (8 rate categories) + I + covarion model and sampling frequency 0.01.

Results

Mantamonas-specific PCR

We generated mantamonad amplicons from 29 (36.7%) of 79 samples from marine (7/24, 29.1%), freshwater (17/40, 42.5%), and soil (5/15, 33.3%). Although our primers were designed to detect 18S rDNA sequence signatures of both known mantamonad 18S rDNA-types, the sequences we obtained from our clone libraries were identical only to *Mantamonas plastica* in their 18S rDNA (approx. 450 nucleotides in length) and differed from the *M. plastica* type strain in their ITS1 rDNA (133-141 nt. in length).

Mantamonas diversity and ecological distribution

The tree in Figure 1 shows new mantamonad environmental ITS1 rDNA lineages with the *M. plastica* type strain, labelled according to source eDNA and habitat type.

Our search for metagenomic and metatranscriptomic mantamonad sequences in online databases (within CAMERA) identified 31 partial 18S rDNA sequences (on average 174 nt. long), from two different sequencing studies, identical either to *M. plastica* or to *Mantamonas* sp. AF21 (Table 3).

The retrieved sequences were found in two sampling locations of the total nine (Supplementary Table 1) included in the databases we searched, both from marine habitats. Both *Mantamonas* sp. AF21 sequences were from the South Pacific Ocean, Antarctica Aquatic Microbial Metagenome project (genomic DNA, no amplification step, Sanger sequencing). 29 *M. plastica* sequences were from the

Gulf of Mexico, Dauphin Island Cubitainer Experiment (DICE, cDNA from extracted RNA, no amplification step, pyrosequencing).

Planomonads in PCR-based and PCR-independent metagenomic/metatranscriptomic datasets

Our database search also uncovered many other environmental sequences with likely affinities to known Apusozoa (Table 4).

192 environmental sequences from CAMERA initially grouped with planomonads during the custom blast. Using the *cd-hit-est* algorithm, these sequences were subsequently grouped alongside known species into 61 clusters, of which 16 were detected in more than one study. A further five lineage representative sequences were either too short or too divergent and were subsequently removed from the analysis. The tree in Figure 2 shows the remaining three new lineages that were detected in more than one study alongside known species and the number of studies in which they were detected.

The cluster analysis grouped previously published planomonad species into six clusters (Figure 2), of which five were detected in the databases: two were found exclusively by direct sequencing (*P. micra*-clade and *F. tropica*), one was found exclusively by PCR-based sequencing (*N. limna*-clade), and two were found by both (*N. indica*, *N. mylnikovi*-clade). Table 5 shows the number of individual retrieved sequences that grouped within each cluster containing a previously described species.

All three new lineages form a clade with *F. tropica*. The *N. mylnikovi*-clade (except divergent *N. indica*) was independently detected most frequently (five times, three direct and two PCR-based).

Apusomonads in PCR-based and PCR-independent metagenomic/metatranscriptomic datasets

158 environmental sequences from CAMERA initially grouped with apusomonads, grouping into 40 different clusters, of which ten were detected in more than one study, including five new lineages that grouped within apusomonads on our final phylogenies after too short or divergent sequences were removed. The tree in Figure 3 shows the five new lineages that were detected in more than one study alongside known apusomonads.

The cluster analysis grouped previously published apusomonad species into 13 clusters (Table 5, Figure 3), of which six were detected in the databases: one was detected exclusively by direct sequencing (*Thecamonas* sp. Bamfield), four exclusively by PCR-based sequencing (environmental clones EF023187, AB275107, and AF530544, and *M. bermudensis*), and two by both methods (*Thecamonas trahens*-clade and *Apusomonas*-clade).

New lineage A1 was detected by targeted PCR, and groups, with A27, with environmental lineage EF023187 with strong support. New lineages A25, A30, and A31 group with *P. magna* with moderate support (Figure 3).

Discussion

With so little known about Mantamonadida, the three newly sequenced *M. plastica* ITS1 rDNA types and 31 retrieved transcriptomic sequences are valuable discoveries that make up, along with the paper presented in Chapter 2, the only ever work dedicated to mantamonads.

Mantamonad diversity

Our explorative PCR-based survey suggests *M. plastica* is ubiquitous across habitats, with mantamonad-specific primers amplifying genetic material in over half the environmental samples tested. In Figure 1, the three new ITS-types are shown having been amplified from the following habitat types: Environmental Lineage 1 - soil and freshwater; Env. Lin. 2 – exclusively marine; Env. Lin 3 – exclusively freshwater. Together with our observations of monocultures thriving in 50% mineral water (50% artificial seawater) media, these results indicate that mantamonads occur in non-marine environments. However, no mantamonad sequences were retrieved from freshwater metagenomic and metatranscriptomic databases online and mantamonads have never been observed in non-marine mixed environmental samples. Therefore, until living cells are observed either in culture or using PCR-independent visualization techniques, such as fluorescence in-situ hybridization (FISH), the ability for mantamonads to survive in non-marine environments is impossible to confirm.

Taken together, these results also suggest that the overall genetic diversity of mantamonads may be relatively low compared with planomonad and apusomonad Apusozoa, and other recently studied free-living flagellate groups, such as Cercozoa (Howe et al. 2009; 2011a; 2011b). This hypothesis is reinforced by the discovery of just 31 metagenomic and metatranscriptomic sequences obtained from PCR-independent studies, compared with 192 planomonad and 158 apusomonad reads (Table 4), all identical to the two previously published 18S rDNA types. Of these, 29 retrieved transcriptomic sequences were identical to *M. plastica*, suggesting, together with its exclusive presence within our PCR survey, it

may be more common, or live at higher densities, than *Mantamonas* sp. AF21 in natural environments.

Importantly, our finding of relatively fewer mantamonad sequences compared with other apusozoan orders, coupled with the relatively higher detection rates of *M. plastica* over *M.* sp. AF21, are particularly important in the context of metagenomic and metatranscriptomic sequence data, because these sequences were generated without the biases inherent in the PCR amplification process, well-documented to impede studies of relative abundance (see General Discussion in Chapter 7). However, only further sequencing could confirm discrepancies in relative abundance between apusozoan orders, as large-scale sequencing requires a sizeable number of reads to be representative.

Strain AF21, on the other hand, has only ever been isolated once, from marine material taken from Tanzania, and its culture died before it could be morphologically characterized. Thus, although identical to the known sequence, the two AF21 reads we retrieved from the databases, both from the South Pacific, serve to verify the original sequence reads obtained from the dead culture and confirm that AF21 may also be ubiquitous, in relatively lower densities than the type species, across marine environments.

The three new *M. plastica* ITS1 rDNA types represent the only new diversity we found during our study, and suggest that future environmental surveys of mantamonads may be more effective if based on the hypervariable ITS region, which can measure diversity at a higher resolution. However, only further sequencing and secondary structure analysis (as demonstrated in Chapter 3 with planomonad ITS2 rDNA) could test whether, based on established methodology

(Coleman 2009), the observed variation could be considered true speciation or is the product of intra-genomic differences within the same 18S rDNA species.

Given time constraints, we were not able to fully test different PCR protocols for their effect on detected mantamonad 18S rDNA diversity. However, in order to further investigate the 18S rDNA diversity, future work could employ several methods known to increase the detection of diversity by PCR, for example, by using more general primers, lower annealing temperatures, decreasing the number of cycles in each reaction, and changing the primer target sequence.

Unlike the other two major apusozoan orders (Chapter 5), mantamonads were difficult to work with even after nested PCR reactions produced visible amplicons. These were cut and cleaned, but cloning and sequencing was often unsuccessful: a phenomenon that never occurred during our work with planomonad-specific and apusomonad-specific primers using identical methods. Coupled with the retrieval of exclusively transcriptomic mantamonad sequences from public databases, these results, as well as the complete lack of mantamonad-like sequences in the historical literature, suggest that mantamonads may be inherently difficult to detect using PCR-based approaches. More generally, this finding is important because it suggests that direct sequencing of transcriptomic material is a more effective method for detecting rare species in environmental surveys compared with metagenomic, amplification-dependent, studies.

In the case of mantamonads, transcriptomic sequencing may be instrumental in detecting further genetic diversity and could in future serve to verify the habitat data suggested by our PCR-based survey, where cysts or extracellular DNA may have caused false positives.

Apusozoan diversity

We expanded our search of previously published environmental sequence databases to examine diversity within planomonads and apusomonads, and to relate detected diversity patterns to the methods used in the sequencing process. Overall, our customized search method revealed three distinctly new planomonad and five new apusomonad lineages that were detected independently by two or more studies. In planomonads (Figure 2), the novel lineages form a clade with *Fabomonas tropica*, an important discovery because no sister lineages are previously described for this species, characterized for the first time in Chapter 3. By adding context to the *Fabomonas* clade, these new lineages will enable the identification of clade-specific sequence signatures and therefore facilitate targeted sequencing approaches in future diversity studies.

The new *Fabomonas*-like lineages are also important from an ecological perspective because two of them were found in surveys of both freshwater and marine habitats, thus making them the first known lineages detected in freshwater habitats but not grouping on the putatively freshwater *N. limna*/*N. longa* clade. This result is vital in the context of the PCR-based environmental survey (Chapter 5) because it reinforces our findings that apusozoan habitat constraints are overestimated by observation of living cells in culture.

In apusomonads (Figure 3), new lineages group with the environmental sequence EF023187 and with *Podomonas magna*, both with moderate support. Although novel environmental lineage A1 was detected in soil, like lineage EF023187, its long-branching sister, A27, was detected in a marine habitat and therefore represents the only known putatively marine-dwelling lineage within the

Apusomonas clade. As with the new planomonad lineages described here, this finding demonstrates that lineages within the same clade are detected in multiple habitat types. However, since *Apusomonas* is known to encyst, and has even been observed emerging from 3,000-year-old ice cores (informal account by D. Tikhonenkov), only further work could confirm the extent of its ecological tolerance.

In both orders, the most species-rich marine clusters were also the ones independently detected in the largest number of studies: *N. mylnikovi*-clade (Figure 2) in planomonads (5 studies) and the *Thecamonas trahens*-clade (Figure 3) in apusomonads (5 studies). These clusters were detected both by direct sequencing of transcriptomic and amplification-dependent sequencing of metagenomic genetic material. Taken together with culture-based observations (Cavalier-Smith and Chao 2010; Chapter 3), these results suggest that apusozoan clades are differentially detected, and perhaps differentially abundant, in the natural environment, either as a result of higher ecological tolerance or because they exist at higher densities. This finding represents the first ever examination of relative diversity within the apusozoan orders.

Methodological remarks

As with mantamonads, overall detection rates of planomonad and apusomonad clades were higher in studies relying on direct sequencing of transcriptomic material. This result is noteworthy when compared to direct sequencing of metagenomic material, which detected only two planomonad and no apusomonad lineages. Although this observation can partly be attributed to the slight bias in

favour of transcriptomic (six) compared to metagenomic (five) studies within the databases, the discrepancy is sufficiently large to suggest that amplification-independent, RNA-based, studies may be more effective at detecting rare species within microbial assemblages.

Furthermore, despite relatively high detection rates using targeted PCR and cloning methods, only one of the eight new apusozoan lineages was detected exclusively by targeted PCR, compared with five by amplification-independent transcriptomic methods. This indicates that, whereas targeted PCR-based approaches successfully detect rare groups, they are not as effective as direct sequencing at detecting novel lineages, perhaps because of primer-based restrictions.

For this pilot study, we employed conservative parameters throughout our customized environmental sequence database search. For the sake of consistency, all our cluster analyses were carried out with a 95% sequence identity threshold (*cd-hit-est* command line: -c 0.95, sequences have to differ by ≥5% to form a new cluster), meaning that new clusters were less likely to be designated than with commonly applied cluster thresholds of 97-98%. Although this high cluster threshold likely reduced the amount of perceived diversity, the resulting clusters largely support the topology of described clades based on morphological and genetic traits. We also maintained a conservative methodology when determining which lineages to include in our phylogenetic analyses, only keeping those that were independently detected in two or more studies. This process collapsed the majority of planomonad (45 of 61) and apusomonad (30 of 40) lineages, recovered from the metagenomic and metatranscriptomic datasets, into previously known

genotype boundaries. Finally, after adding remaining lineages to their respective alignments, we excluded any new sequences that were too divergent and/or short to align properly.

Future perspectives

Together, our conservative sequence filtering methodology meant that, after the relatively conservative clustering analysis, planomonads were reduced from 61 original clusters to three new lineages and apusomonads from 40 to five new lineages. Given more time, in place of the custom blast algorithm, it would be valuable to use a linear time phylogenetic placement application such as *pplacer* (Matsen et al. 2010) to place all the unique retrieved sequences onto a representative eukaryotic reference tree. This would more accurately determine the likely identity of each sequence prior to the cluster analysis. The same application could then be used after a less conservative cluster analysis, to place each cluster onto order-specific reference trees, thus minimizing the number of excluded sequences.

Finally, a phylogenetic placement application could drastically expand on this study by combining, through a linear time alignment, all described species with all relevant database sequences, as well as all the new lineages we detected in the PCR-based environmental survey described in Chapter 5. Apart from presenting many new putatively planomonad and apusomonad lineages obtained from online databases in a broader context, such a complete phylogeny could offer a unique perspective on the detection patterns of each lineage according to sequencing method and study of origin.

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Table 1. Details on PCR primers used.

PCR round	C°	Amp.	FORWARD PRIMER Name	Position (nt. from 5'-end, *M. plastica*)	Sequence (5' to 3')	Spec.	REVERSE PRIMER Name	Pos.	Sequence (5' to 3')	Spec.
Initial	58	~1750	Mta4f	18S (302)	TTTCTGCCCTATCACTCTATTGTTG	Mantam.	MEuk5.8Sr1	5.8S	GCAAAGGGGTCAAATGACGCTCAG	Euk.
Nested	65	~700	Mta1356f	18S (1356)	TTAGAGGGACTAGCTGYATCCAGCAG	Mantam.	MEuk5.8Sr2	5.8S	GTGCGTTCAAARATTTGATGATTCACGTT	Euk.

Table 2. Summary of the four mined databases on CAMERA 2.0 Portal, including method by which they were obtained.

Name	Description	Bases	Sequences
All NCBI Environmental Samples (ENV_NT)	All NCBI Environmental Samples. This is a mirror data set of NCBI "Environmental nucleotide sequences" BLAST database (ENV_NT). It has entries from the NCBI Genbank division ENV and UNA. *Database updated on 2011-05-14 from NCBI Refseq release 47 and Genbank release 183*	7,562,313,920	18,402,261
CAMERA's Non-Identical Nucleotide Sequences	Non-redundant nucleotide database that includes but expands upon nrnt *Database updated on 2011-05-14 from NCBI Refseq release 47 and Genbank release 183*	244,705,868,858	77,616,618
All Metagenomic 454 Reads	Complete set of metagenomic sequencing reads obtained by 454 sequencing	52,358,203,154	182,688,787
All Metagenomic Sanger Reads	Complete set of metagenomic Sanger reads in CAMERA (excludes 454 reads)	15,557,009,999	15,068,493

Table 3. Retrieved mantamonad sequences from the Camera Portal 2.0.

Identical to	Length	Sample ID	Subject Name	Sampling Location	Citation
M. plastica	169	CAM_S_A001	CAM_READ_0081859241	Dauphin Island Cubitainer Experiment (DICE), Gulf of Mexico, AL, USA	Howard et al. 2011
"	171	CAM_S_A001	CAM_READ_0081881727	"	"
"	172	CAM_S_A001	CAM_READ_0081893129	"	"
"	171	CAM_S_A001	CAM_READ_0081893345	"	"
"	172	CAM_S_A001	CAM_READ_0081896133	"	"
"	173	CAM_S_A001	CAM_READ_0081914789	"	"
"	172	CAM_S_A001	CAM_READ_0081920415	"	"
"	169	CAM_S_A001	CAM_READ_0081934239	"	"
"	170	CAM_S_A001	CAM_READ_0081939003	"	"
"	163	CAM_S_A001	CAM_READ_0081940589	"	"
"	172	CAM_S_A001	CAM_READ_0081968617	"	"
"	169	CAM_S_A001	CAM_READ_0081992433	"	"
"	169	CAM_S_A001	CAM_READ_0082030713	"	"
"	169	CAM_S_A001	CAM_READ_0082065913	"	"
"	172	CAM_S_A001	CAM_READ_0082069557	"	"
"	172	CAM_S_A001	CAM_READ_0082165303	"	"
"	170	CAM_S_A001	CAM_READ_0082172805	"	"
"	170	CAM_S_A001	CAM_READ_0082191431	"	"
"	160	CAM_S_A002	CAM_READ_0082353709	"	"
"	163	CAM_S_A002	CAM_READ_0082448903	"	"
"	160	CAM_S_A002	CAM_READ_0082504235	"	"
"	163	CAM_S_A002	CAM_READ_0082544499	"	"
"	163	CAM_S_A002	CAM_READ_0082568893	"	"
"	160	CAM_S_A002	CAM_READ_0082572567	"	"
"	161	CAM_S_A002	CAM_READ_0082646557	"	"
"	157	CAM_S_A002	CAM_READ_0082648717	"	"
"	172	CAM_S_A002	CAM_READ_0082693343	"	"
"	169	CAM_S_A002	CAM_READ_0082695627	"	"
"	161	CAM_S_A002	CAM_READ_0082724595	"	"
M. sp. AF21	270	CAM_SMPL_TA_34915	CAM_READ_0088082879	Antarctica Aquatic Microbial Metagenome, South Pacific Subtropical Gyre	Hoffman et al. (unpublished)
"	381	CAM_SMPL_SRA022156	CAM_READ_0126364577	"	"

Table 4. Summary of CAMERA Portal 2.0 database searches, seeded with quarter-length 18S rDNA sequences of 20 known Apusozoa, and subsequent cluster analyses. Counts of unique sequences are out of the 4,000 total returned hits from each database. NCBI figures are combined hits from the NCBI_n and env_nt (environmental) databases. Results from our custom blasts, where all unique retrieved sequences were compared to a representative alignment of eukaryotes, are grouped by order as Mantamonadida (M), Planomonadida (P), or Apusomonadida (A). Parentheses indicate cluster groupings reinforced by two or more studies. Sequences from known strains are excluded from cluster figures.

		Custom Blast results			
Database	Total Size	Unique	M	P	A
NCBI	76,951,238	2,402	2	105	59
MetSanger	15,068,493	328	0	6	3
Met454	182,688,787	2,120	34	81	96
Clusters			2 (1)	61 (16)	40 (10)

Table 5. Number of environmental sequences retrieved from the CAMERA Portal 2.0 database searches by lineage cluster. The number of sequences excludes those from published species.

Order	Lineage cluster	Seqs.
Planomonadida	All marine *Nutomonas*, except *N. indica*	20
	N. indica	9
	All *Planomonas*	4
	All freshwater *Nutomonas,* except *N. longa*	2
	All *Fabomonas*	2
	Nutomonas longa	0
Apusomonadida	All *Thecamonas trahens*	56
	All *Apusomonas proboscidea*	5
	soil DNA clone Amb_18S_480 EF023187	4
	Podomonas capensis AY050182	2
	environmental sequence AB275107	2
	Thecamonas sp. Bamfield	1
	Manchomonas bermudensis AY050178	1
	environmental sequence AT-50 AF530544	1
	All *Multimonas media*	0
	environmental sequence AT-41 AF530526	0
	Thecamonas sp. AY050179	0
	Thecamonas oxoniensis EU542598	0
	Podomonas magna AY268039	0

Supplementary Table 1. Description of metagenomic databases on Camera Portal 2.0.

Code	Name	Description
CAM_P_0000545	Guaymas Basin deep-sea Metagenome	Metagenomic and metatranscriptomic sequencing (454-Titanium) of deep-sea hydrothermal plumes and background seawater of Guaymas Basin and Carmen Basin in the Gulf of California
CAM_PROJ_AmazonRiverPlume	Microbial community gene expression across a productivity gradient of the Amazon River plume	Metagenomic and metatranscriptomic samples were collected during an oceanographic research cruise on the R/V Seward Johnson across the Amazon River plume to the eastern Equatorial Atlantic Ocean
CAM_PROJ_CAM_P0000101	Polyamine Metatranscriptomic Study	Experimental metatranscriptomic approach to identify and compare the taxonomic composition and functional genes of bacterioplankton that are involved in utilizing putrescine and spermidine in a Southeast US coastal marine system.
CAM_PROJ_PacificOcean	Influence of nitrogen-fixation on microbial community gene expression in the oligotrophic Southwest Pacific Ocean	Metatranscriptomic samples were collected along a North-South transect in the western South Pacific Ocean, to investigate the relationship between nitrogen-fixing populations and microbial community gene expression in the nitrogen-limited oligotrophic water.
CAM_PROJ_AntarcticaAquatic	Antarctica Aquatic Microbial Metagenom	The metagenome data includes sequencing results from two lakes in the Vestfold Hills region of East Antarctica and ocean samples in the vicinity of Casey Research Station. All samples were collected using size fraction filtering methods that were used on the the Venter Institute"s Global Ocean Sampling Expedition. DNA was isolated from the 0.1 to 0.8 microns size fraction.
CAM_PROJ_GOS	Global Ocean Sampling Expedition	These studies have produced the largest catalogue of genes to date from thousands of new species, with no apparent slowing of the rate of discovery (i.e., attaining saturation of data).
CAM_PROJ_GeneExpression	Surface Water Marine Microbial Community Gene Expression	Surface water microorganisms were collected on two research cruises in the equatorial North Atlantic ocean and South Pacific Subtropical gyre to understand variability in gene expression and gene complement in the oligotrophic ocean.
CAM_PROJ_SapeloIsland	Sapelo Island Bacterioplankton Metagenome	Metagenomic DNA of active cells were captured and pyrosequenced to examine whether individual taxa specialize on particular components of the marine DOC pool.
CAM_PROJ_DICE	Dauphin Island Cubitainer Experiment (DICE)	Two replicate cubitainers were amended with nutrients (N and P) to stimulate phytoplankton bloom, while two untreated cubitainers served as controls. The bacterial community metatranscriptome (0.22 - 5um size fraction) was sequenced from nutrient amended microcosms and untreated controls on day 5, while the phytoplankton bloom had reached its peak.

Taken from database descriptions at http://camera.calit2.net/datasets/

Figure legends

Figure 1. New *Mantamonas plastica* ITS1 rDNA lineages. Neighbour-Joining (NJ) tree of representative sequences from three new lineages of *Mantamonas plastica* obtained from clone libraries across a range of environments. Two of the new lineages were found in several separate libraries. In the table on the left, black boxes next to eDNA sample names indicate that bands were visible after semi-nested PCR was carried out using primers specific to both known mantamonad 18S rDNA types. Stars, circles, and ovals relate lineages to specific eDNA samples.

Figure 2: 18S rDNA gene phylogeny of Planomonadida and other Apusozoa. 18S rDNA gene phylogeny of Planomonadida and other Apusozoa. Maximum-Likelihood tree (RAxML, GTRMIXI model, g=8, best of 100 parsimony starting trees) of 38 sequences using 1643 nucleotide positions. Support values are RAxML boostraps (left) and Bayesian posterior probability (right). Black blobs indicate 100% support by both methods. Scale bar indicates 10% substitutions per nucleotide side. Names and branches of newly placed lineages are shown in bold. Table on left lists the origin of all sequences from each clade, by the number of independent studies featured in the CAMERA Portal 2.0 databases and the method by which they were generated. For either genomic DNA (DNA) or complementary DNA from RNA (transcriptomic, cDNA), sequences were generated either directly (D) or through an amplification step (A). The sequencing method is listed as either pyrosequencing (P) or Sanger (S). Sequences generated by targeted PCR and cloning (T) are also listed. Only sequences found in more

than one CAMERA database are included here. The habitat of origin of described species and environmental lineages retrieved from sequence databases is shown at the branch tips as either marine (black hexagon) or freshwater (star). Both a star and a hexagon signify the lineage was detected in both marine and freshwater habitats.

Figure 3: 18S rDNA gene phylogeny of Apusomonadida and other Apusozoa. Maximum-Likelihood tree (RAxML, GTRMIXI model, g=8, best of 100 parsimony starting trees) of 36 sequences using 1596 nucleotide positions. Support values are RAxML boostraps (left) and Bayesian posterior probability (right). Black blobs indicate 100% support by both methods. Scale bar indicates 10% substitutions per nucleotide side. Names and branches of newly placed lineages are shown in bold. Table on left lists the origin of all sequences from each clade, by the number of independent studies featured in the CAMERA Portal 2.0 databases and the method by which they were generated. For either genomic DNA (DNA) or complementary DNA from RNA (transcriptomic, cDNA), sequences were generated either directly (D) or through an amplification step (A). The sequencing method is listed as either pyrosequencing (P) or Sanger (S). Sequences generated by targeted PCR and cloning (T) are also listed. Only sequences found in more than one CAMERA database are included here. The habitat of origin of described species and environmental lineages retrieved from sequence databases is shown at the branch tips as either marine (black hexagon) or freshwater (star). Both a star and a hexagon signify the lineage was detected in both marine and freshwater habitats.

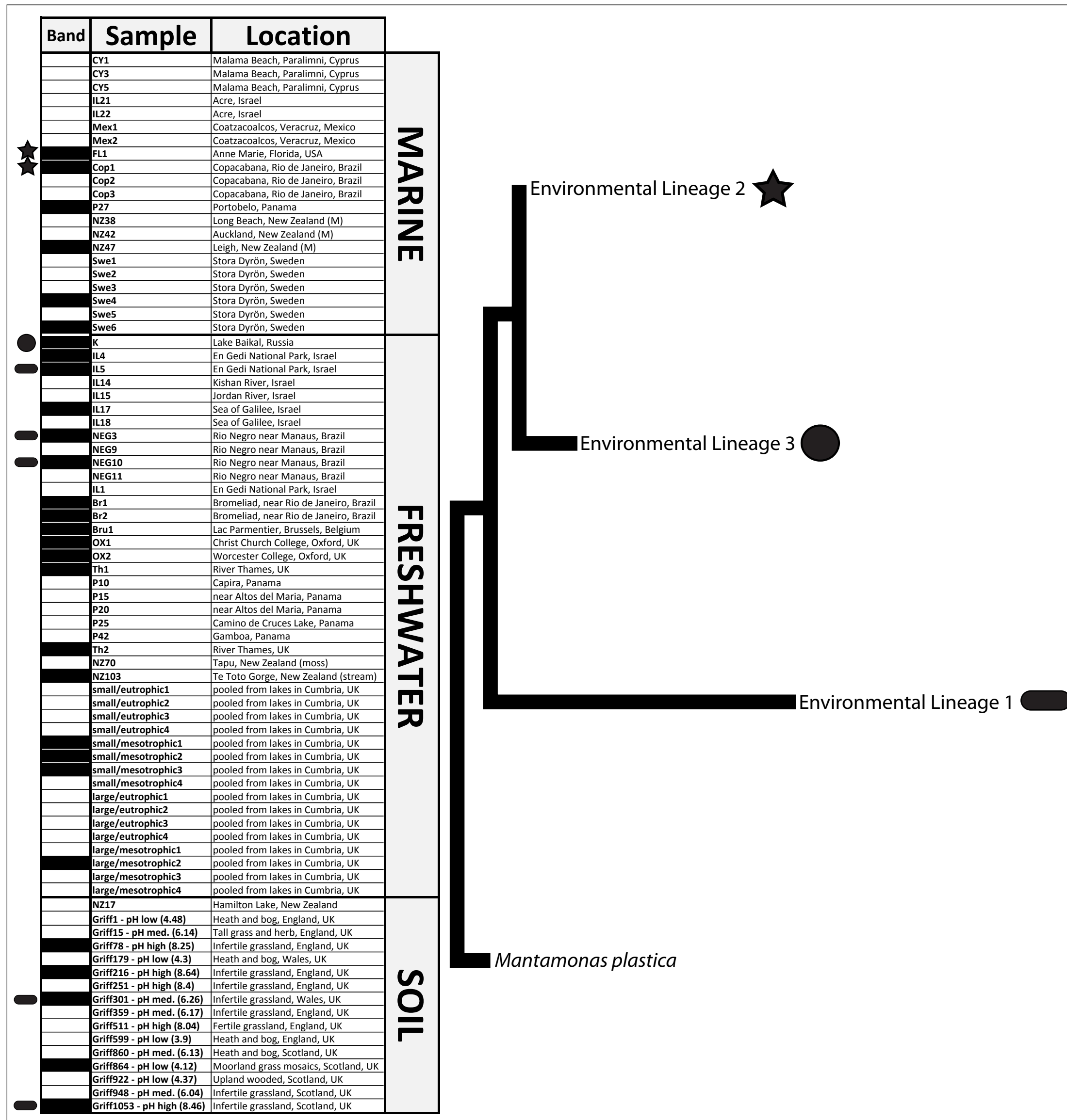


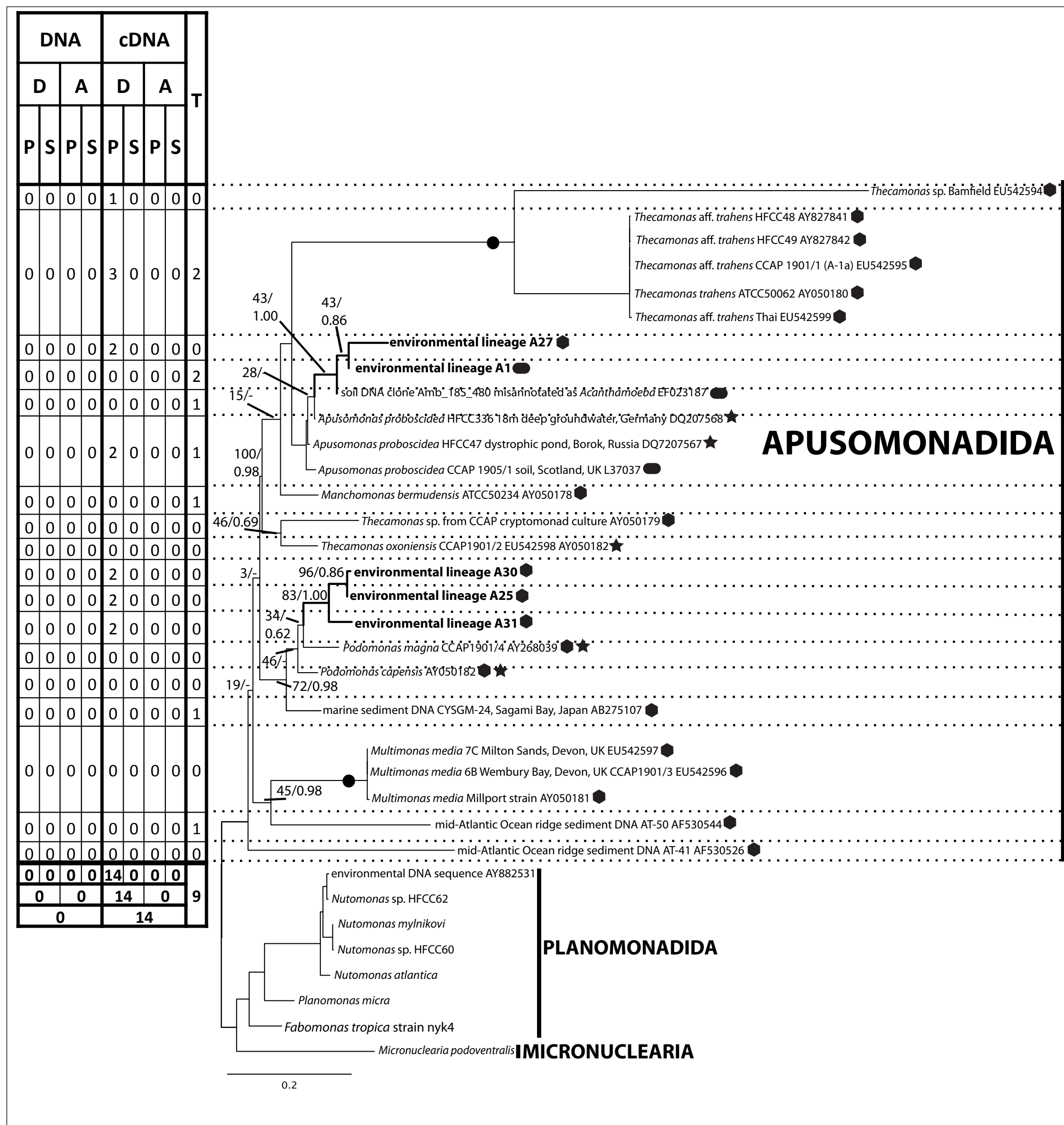
Figure 1

DNA				cDNA				T
D		A		D		A		
P	S	P	S	P	S	P	S	
0	0	0	0	1	0	0	0	0
0	1	0	0	0	0	0	0	0
1	0	0	0	3	0	0	0	0
1	0	0	0	1	0	0	0	2
0	0	0	0	1	0	0	0	1
0	0	0	0	1	0	0	0	0
0	0	0	0	2	0	0	0	0
0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0
0	0	0	0	3	0	0	0	2
0	0	0	0	1	0	0	0	1
0	1	0	0	1	0	0	0	0
1		0		1		0		
1				1				
2	0	0	0	12	0	0	0	7
2		0		12		0		
2				12				

Mantamonadida
Planomonadida

Mantamonas plastica, Cumbria, UK
Mantamonas sp. AF21, Dar es Salaam, Tanzania
MANTAMONADIDA
37/0.82
Thecamonas sp. from CCAP cryptomonad culture AY050179
Multimonas media 6B CCAP1901/3 EU542596
Podomonas capensis AY050182
APUSOMONADIDA
Apusomonas proboscidea CCAP 1905/1 soil, Scotland, UK L37037
94/1.00
Manchomonas bermudensis ATCC50234 AY050178
Thecamonas oxoniensis CCAP1901/2 EU542598
Podomonas magna CCAP1901/4 AY268039
marine sediment DNA CYSGM-24, Sagami Bay, Japan AB275107
Planomonas micra ATCC50267 AF53088
Planomonas micra AF174363
Planomonas bulbosa, marine sediment, North Carolina, USA
Planomonas brevis, marine sediment, Paralimni, Cyprus
Planomonadidae
Planomonas elongata, marine sediment, Turkey
24/-
65/0.77
environmental lineage P22
87/0.94
environmental lineage P18
Fabomonas tropica, marine sediment, Ankobra Beach, Ghana (strain nyk4)
40/-
environmental lineage P55
Nutomonas sp. HFCC61, R. Rhine DQ207563
Nutomonas sp. HFCC326, R. Rhine DQ207564
37/0.82
Nutomonas limna CCAP1985/10, St Andrews, Scotland
Nutomonas sp. HFCC328, R. Rhine DQ207566
Nutomonas howeae, Lake Baikal, Russia, strain 120
Nutomonas sp. HFCC327, Cologne pond DQ207565
Nutomonas limna, Lake Baikal, Russia, strain 124
Nutomonas lacustris, Sea of Galilee, Israel
Nutomonas longa, freshwater lake, North Carolina, USA
Nutomonas sp. HFCC62, South Atlantic AY827844
Nutomonas sp. HFCC63, South Atlantic AY827845
46/0.99
marine sediment, Cariaco Basin, environmental DNA sequence AY882531
Nutomonadidae
86/0.99
Nutomonas kenti, marine sediment, Cumbria, UK
Nutomonas sp. HFCC104, North Atlantic AY827846
Nutomonas sp. HFCC60, Baltic Sea AY827843
Nutomonas mylnikovi
0.07
Nutomonas atlantica, marine sediment, Peniche, Portugal
Nutomonas indica, marine sediment, Dar es Salaam, Tanzania
PLANOMONADIDA

Figure 2



D P (DNA)	D S (DNA)	A P (DNA)	A S (DNA)	D P (cDNA)	D S (cDNA)	A P (cDNA)	A S (cDNA)	T
0	0	0	0	1	0	0	0	0
0	0	0	0	3	0	0	0	2
0	0	0	0	2	0	0	0	0
0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	1
0	0	0	0	2	0	0	0	1
0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	2	0	0	0	0
0	0	0	0	2	0	0	0	0
0	0	0	0	2	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0
0	**0**	**0**	**0**	**14**	**0**	**0**	**0**	
0		0		14		0		9
0				14				

Figure 3

Chapter Five

A PCR-based environmental survey of free-living flagellates Apusomonadida and Planomonadida (Protozoa: Apusozoa)

Manuscript

Chapter notes

This chapter will shortly be submitted for publication as a manuscript co-authored by David Bass and Tom Cavalier-Smith. Formatting and content have been adjusted specifically for this thesis, including with the addition of chapter citations. For consistency, this chapter adopts the revised nomenclature of Planomonadida, presented in Chapter 3, even though this system has not yet been formally accepted.

Chapter abstract

Apusozoa is a phylum of free-living zooflagellates. Apusomonadida and Planomonadida, the two main apusozoan orders, are frequently observed by light microscopy of freshwater, marine, and soil samples, yet little is known about their diversity and ecology. Using sequences of known strains, we designed lineage-specific molecular probes and used them to construct environmental rDNA libraries from a wide range of habitats. We detected two new 18S rDNA lineages of putatively freshwater Nutomonadida (*N. limna*-clade, Planomonadida) in samples from freshwater, soil, and marine habitats. Using primers targeting ITS1 rDNA, we demonstrate a similar ecological tolerance in the putatively marine Nutomonadida (*N. mylnikovi*-clade) and compare the environmental lineages to ITS1-types of described species. Finally, we detected new lineages within order Apusomonadida using primers specific to soil-dwelling *Apusomonas* and putatively marine Thecamonadida, again suggesting considerable ecological tolerance and providing early evidence for lineage-specific differentiation according to environmental variables like pH or nutrient levels. We conclude that Apusomonadida and Planomonadida are more diverse and ecologically tolerant than previously thought based on culturing alone.

Keywords: Apusomonadida, Apusozoa, environmental survey, Mantamonadida, PCR, Planomonadida

Introduction

Apusozoa (Cavalier-Smith 2002) are gliding heterotrophic zooflagellates found in freshwater, soil, and marine environments. Though they are commonly observed in mixed microbial assemblages (Lee and Patterson 2002; Patterson and Lee 2000), very little is known about their diversity and ecology. Recently, traditional culturing methods, electron microscopy, and 18S rDNA gene phylogenies, have expanded our knowledge of the two most described apusozoan orders, Apusomonadida (apusomonads) (Cavalier-Smith and Chao 2003, 2010; Karpov and Mylnikov 1989) and Planomonadida (planomonads) (Cavalier-Smith et al. 2008; Chapter 3; Heiss et al. 2009, 2011). A third order, Mantamonadida (mantamonads) (Chapter 4, Glücksman et al. 2011 in Chapter 2), was described only recently, illustrating the paucity of knowledge around a group whose true diversity and position on the eukaryotic tree remain unknown.

Previous studies show that the majority of protozoan diversity is lost in culturing experiments because most species do not grow under standard culturing conditions (Boenigk et al. 2006). To that end, in the wake of culture-independent surveys of the free-living Cercozoa (Bass and Cavalier-Smith 2004; Howe et al. 2009, 2011a, 2011b), we designed PCR primers specific to apusozoan lineages and applied them to environmental DNA (eDNA) extracted from a range of habitat types.

Apart from to provide a culture-independent perspective of apusozoan genetic diversity, the aims of this study were to investigate several different habitat types

and to test whether, a) the diversity found in nature matched patterns suggested by culturing, and, b) certain lineages and clades are restricted to certain habitats.

In Chapter 4, we show that the type species of Mantamonadida, *Mantamonas plastica*, is detected across several habitat types, yet its 18S rDNA diversity appears to be restricted to the two ribotypes described from culturing. Here we show that planomonads and apusomonads are also detected across different habitats but, unlike mantamonads, they are more diverse than discerned from culturing alone.

Methods

DNA extractions and PCR amplification

Environmental samples were collected in sterile Eppendorf or Falcon tubes and eDNA was extracted as soon as possible after collection, following the Maximum Yield Protocol of the UltraClean Soil DNA Isolation Kit (MoBio Laboratories), or alternative methods summarized in Table 1.

Since we were unable to visualize amplicons by gel electrophoresis after initial PCR reactions, we used a nested PCR strategy for all our experiments. The primers used in our environmental survey were designed as outlined in Table 2.

To create our template ITS1 rDNA alignment of putatively marine nutomonads (*N. mylnikovi*-clade), we obtained sequences from described species by PCR amplification of genomic DNA (gDNA) with primers Pre3NDF (5'-CAGCAGGCGCGCAAATTACCC-3', forward primer, located in the C3 region of the 18S rDNA) and reverse primer B10r (5'-

CCTCCGCTTABTDATATGCTT-3’, located at the 5’-end of 28S rDNA. PCR products were cloned as above and eight colonies were individually picked for sequencing using sterile pipette tips. Sequencing primers were preBf (5’-GTAGGTGAACCTGCAGAAGGATC-3’, forward primer, located in the C10 region of 18S rDNA) and B10r (5’-CCTCCGCTTABTDATATGCTT-3’, reverse primer, located at 5’-end of 28S rDNA).

In the environmental survey, eDNA was diluted 1/10 with de-ionized water prior to the initial PCR reaction, to reduce the effect of inhibitors (see Chapter 7 – General Discussion) and then amplified by PCR using two specific or semi-specific primer sets. Each round of PCR included at least one positive control (gDNA extracted from a known culture of the primer target) and a negative control (reagents with molecular grade water added in place of DNA). Reactions were run under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles (denaturation at 95 °C for 40 s, annealing at X °C [Table 2] for 40 s, extension at 72 °C for 2 min 20 sec), and final extension at 72 °C for 5 min. Products from the initial reaction were diluted 1/100 and transferred to a fresh mix of PCR reagents, run under the same conditions as the initial reaction with the exception of a higher annealing temperature. Nested PCR products were run on a 1% agarose gel and visible bands of the appropriate lengths were excised and cleaned following the protocol of the GFX PCR DNA Gel Band Purification Kit (GE Healthcare).

Cloning, sequencing, and editing

Clone libraries were constructed using the TOPO TA Cloning Kit (Invitrogen, as described in Bass and Cavalier-Smith 2004). White colonies (eight initially, but up to 24 depending on detected diversity) were individually picked using sterile pipette tips and dropped into 25 µl PCR reactions. Colony PCR reactions were run with the same primer set and under the same conditions as the nested PCR described above. Colony PCR products were cleaned using the PEG protocol described elsewhere (Howe et al. 2009). Sequencing reactions were carried out in both directions using primers from the nested PCR reaction, then run using dye terminators and separated on an automated ABI-377 sequencer.

Sequences were edited and concatenated in Geneious Pro 5.0+ (Drummond et al. 2010). From the two apusomonad-specific (*Thecamonas* and *Apusomonas*) primer set amplifications, the resulting 18S rDNA sequences were merged into one alignment file and were then run through cluster analyses with sequences of known species. Cluster analyses were carried out using the *cd-hit-est* application, run remotely on the CAMERA 2.0 Portal (Sun et al. 2010) using default settings (command line: -d 0 –n 10 –l 11 –r 1 –p 1 –g 1 –G 0 –c 0.95 –aS 0.8). Important for the purpose of this analysis, the sequence identity threshold (-c 0.95) represents the global sequence identity and is used as a cut-off for determining clade clusters (see Li et al. 2010 for full *cd-hit-est* documentation).

Following the cluster analysis, the longest sequence (the *cd-hit-est* 'representative sequence') from each determined cluster that did not contain a known species was extracted and added to apusomonad and planomonad alignments featuring known species.

Phylogenetic analysis

We used RAxML-V (Stamatakis et al. 2005) for Maximum-Likelihood analyses and for obtaining bootstrap values, running masked alignments using the GTRMIXI model of sequence evolution (g=8). 100 runs using parsimony and 100 runs using randomly generated starting trees, as well as the bootstrap values, were plotted onto the overall most likely tree. Bootstrap values were calculated using the default maximum-parsimony start trees.

We used MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) remotely run on the Cyberinfrastructure for Phylogenetic Research Portal (CIPRES www.phylo.org/sub_sections/portal/) for the Bayesian analysis, running the masked alignment with four independent chains for 7 x 10^6 generations (burn-in of 2 x 10^6 generations) using the GTR + gamma (8 rate categories) + I + covarion model and sampling frequency 0.01. Hypervariable ITS1 rDNA region phylogeny of marine nutomonads was calculated with a Neighbour-Joining tree (model: Jukes-Cantor) within Geneious 5.4.4.

Results

Freshwater planomonads

For planomonads, our primers were based on 18S rDNA sequence signatures of all known putatively soil-dwelling and freshwater species (family Nutomonadidae), including the most lineage-rich clade comprising *N. limna*, *N. howeae*, and *N. lacustris* and related sequences from GenBank as well as the unique long-branching lineage *N. longa*. Amplicons were visible in 38/77 (49.4%) of nested PCRs from

marine, freshwater, soil, and across an estuarine gradient. We made 27 clone libraries and sequenced 8-24 colonies per library, depending on detected diversity. New lineages EP47 and EP43 were found in 3 (11.1%) and 7 (25.9%) of the 27 libraries respectively. 17/27 (63%) libraries contain sequences that cluster with *N. limna* at the 95% sequence identity threshold established by *cd-hit-est*. No environmental sequences cluster with *N. longa* (Figure 1).

A single eDNA sample, extracted from ~0.5 g of sediment or 1-2 L of lake water, occasionally contained multiple detected lineages. One library (cop2) contained all three detected lineages, whereas five samples contained two lineages: the *N. limna*-cluster and either EP43 (il17, il21, Grif864) or EP47 (Grif216, Grif599). All three lineages were detected in marine, freshwater, and soil habitats. However, the *N. limna*-cluster was detected in the widest range of habitats, including at all three soil pH levels, in a freshwater planktonic sample, and nearly every freshwater (4/6) and in all marine (4/4) samples. Novel lineage EP43 was found in soil, freshwater, and marine environments, including in two different eutrophic freshwater planktonic samples (but was absent in mesotrophic samples).

Marine planomonads

In order to investigate diversity and ecological preference at a higher phylogenetic resolution, we also obtained environmental ITS1 rDNA sequences for the putatively marine nutomonad clade that includes described *N. mylnikovi* (black box 'N' in Fig. 1). Amplicons were visible in 23/96 (24%) samples and clone libraries were created for 20 of them. Fig. 2 shows an ITS1 rDNA phylogeny of putatively marine nutomonads obtained from environmental clone libraries alongside

sequences generated from cloned monocultures. The new environmental ITS1 rDNA sequences group differentially with the four described *Nutomonas* lineages: *N. kenti*, *N. mylnikovi*, *N. atlantica*, and *N. indica*.

N. indica was detected in soil, freshwater, and marine habitats, and was the only putatively marine planomonad detected in freshwater planktonic habitats. Seven different ITS-types were detected from eDNA extracted in the Mediterranean Sea, of which the most lineage-rich sample contained three distinct lineages (il22). Four libraries (EstA, Grif251, IL22++, P27) contained two lineages.

Surprisingly, amplified ITS1 rDNA sequences of *N. mylnikovi* and *N. kenti* each group across multiple clusters and, in the case of *N. mylnikovi*, across two different clades.

Apusomonads

We obtained environmental 18S rDNA sequences of Apusomonadida using primers specific to putatively marine thecamonads and the soil-dwelling *Apusomonas* clade. Only two clusters were detected independently by both primer sets (the *Thecamonas trahens* clade and EA28). Fig. 3 shows a phylogeny of new environmental lineages with described apusomonads.

Whereas many of the environmental sequences clustered with known species *Thecamonas trahens*, *Thecamonas* sp. Bamfield, *Apusomonas*, and *Podomonas capensis*, five lineages were both new and verifiable by detection in multiple libraries.

Two environmental lineages grouping near marine *T. trahens* were also found in soil habitats, including novel lineage EA25, which was also found in two

freshwater and two marine libraries. The *T. trahens* cluster itself was widely detected in all three major habitat types.

Other new environmental lineages were detected in multiple samples and across habitat types: notably, EA30 (7 libraries; soil and marine), EA31 (6 libraries; soil, freshwater, marine), and EA25 (6 libraries; soil, freshwater, marine). Several samples contained multiple genotypes, including three libraries containing four (Plank1, Grif864, PanM) and six libraries containing three lineages (Grif359, Grif78, MexFlaM, NZ47, NzM, P27).

Finally, phylogenetic and cluster analyses reveal that most of the clonal sequences amplified with *Apusomonas*-specific primers group outside *Apusomonas* with low support.

Discussion

Some of the apusozoan diversity patterns demonstrated by our environmental survey are commensurate with culturing observations. For example, culturing suggests that the putatively freshwater nutomonad clade comprises a species-rich *N. limna*-cluster coupled with a single divergent lineage *N. longa*. This taxonomic divide is reinforced by our environmental survey, which reveals little intermediate 18S rDNA diversity (two lineages) between the two known clades. As observed from culturing, our survey shows that lineages belonging to the *N. limna*-cluster are far more common and widespread in a range of habitat types compared to any other putatively freshwater planomonad lineages. Meanwhile, *N. longa*, only ever

isolated once in culture, was never detected in our survey and therefore likely represents a rare strain with a unique habitat preference (Fig. 1).

The two novel lineages EP47 and EP43 shown in Fig. 1, both detected in multiple samples, are an important contribution to the putatively freshwater clade because these two new environmental lineages may represent morphological intermediates between *N. longa*, morphologically distinct from all other known species (described for the first time in Chapter 3), and the *N. limna*-cluster. In future, the morphology of these lineages could be confirmed by further culturing or by culture-independent techniques such as fluorescence in-situ hybridization (FISH) which, combined with flow cytometry, may also be useful in identifying and counting otherwise elusive *N. longa* cells in mixed environmental samples (see Chapter 7 – General Discussion).

We used a different set of primers, specific to the hypervariable ITS1 rDNA sequence signatures, to target putatively marine nutomonads and compare these environmental sequences to described species *N. indica*, *N. atlantica*, *N. kenti*, and *N. mylnikovi* (Fig. 2) With the exception of one new clade, environmental lineages were interspersed between sequences from described species in our phylogeny. Since ITS1 rDNA is subject to considerable intra-genomic variation, we were not able to discern how many new species were represented by our environmental sequences but, judging by eye, it is likely that at least lineages ENL3, ENL15/16, ENL13, and ENL8 are divergent enough to represent new species. In future, a secondary structure analysis, similar to the one presented in Chapter 3, could provide a useful framework for the delineation of species on this phylogeny.

With primer sets specific to the soil-dwelling *Apusomonas* and putatively marine thecamonads, we found 14 new environmental lineages within Apusomonadida (Fig. 3). Likely due to the shorter environmental sequence reads, support for the overall topology presented in Fig. 3 was weak. However, it is probable that some of the sequences detected with *Apusomonas*-specific primers group outside the target clade, most of them near *Podomonas capensis;* an important finding because it shows our primers were not always specific to their target. In future, designing primers specific to the flanks of these new environmental lineages, to sequence the remaining portions of their 18S rDNA, could strengthen the phylogenetic support for this tree.

Only two clusters were detected independently by both primer sets and none of the environmental lineages were similar to the sequences retrieved during our search through publically available metagenomic databases (described at length in Chapter 4; in this chapter, these sequences are labelled on Figs. 1 and 3 as 'environmental lineages A*x* or P*x*'), highlighting the importance of using a variety of methods when discerning diversity based on culture-independent techniques. Future experiments could test for the influence of methodological variables, such as PCR annealing temperature, to illustrate further whether perceived diversity differs according to the methodology used.

Major habitat types and other ecological variables

For this exploratory study, we classify sampling habitat type as being one of three possibilities: marine, freshwater (aquatic), or soil. In our culturing experiments, we

commonly grow monocultures in either artificial seawater (marine) or Volvic mineral water (freshwater/soil).

The culturing record suggests apusozoan strains are restricted to certain habitats. However, our environmental survey shows that some lineages and clusters of >95% similar sequences were detected in multiple habitat types; for example, the putatively marine clade comprising *Thecamonas trahens* was also found in freshwater and soil samples. This result was consistent across all three orders, including the putatively marine mantamonads, and may be indicative that apusozoan species are ecologically more tolerant than observed using traditional methods. For raw data on detected clades across each individual environmental sample, see Supplementary Figure 1.

Whereas many clades were found in several habitat types, some appeared restricted or biased towards the habitats in which they are known to grow in culture. For example, the only detected environmental lineage that groups with *Apusomonas* was found in soil or, more striking even, a majority (12 of 16) of detected *N. mylnikovi*-cluster lineages were amplified from marine samples. Moreover, the two new apusomonad environmental lineages, EA31 and EA30, found in marine and soil habitats, group closely with *Podomonas capensis* AY050182 (marine estuary mouth, Western Cap, South Africa) and *Podomonas magna* AY268039 (White Sea, 12 ppm salinity), both isolated from brackish environments.

Lineages demonstrated potentially differential rates of habitat tolerance. For example, *N. indica*, which was detected in all three habitat types, is perhaps more ecologically tolerant than environmental lineage ENL0, found independently in

two different soil marine samples but not in any other habitat type. Similarly, apusomonad lineage EA28 was found twice independently in marine samples but not in soil or freshwater. Other putatively marine nutomonad environmental clades, one comprising ENL15 and ENL16 (differing from each other genetically by 14/~170 nucleotide positions), found in the mid-range salinity level of the brackish estuarine sample, or another comprising ENL8 and ENL13 (12/~169 positions difference), may also represent ecologically specialized single lineages if intra-clade differences are attributed to intracellular sequence variation. Although these results suggest some lineages may be restricted to certain habitats, only further sampling and sequencing could confirm these ecological patterns.

To precede our survey, we previously investigated habitat tolerance by running acclimatization experiments on monocultures of known planomonad species (Chapter 3, Table 2). Although putatively freshwater nutomonads, such as *N. limna* and *N. longa*, only grew in 100% mineral water media, putatively marine nutomonads, planomonads, as well as fabomonads, were all able to grow, and even thrive, in brackish conditions containing as little as 25% artificial seawater (mixed with Volvic).

Our salinity gradient experiments show that some apusozoan species, although they prefer certain environmental conditions, could be capable of surviving in multiple habitat types. Therefore, it is possible that, over time, and perhaps by only a minority of cells, they can transition from marine to freshwater environments. On the other hand, it is also possible that the genetic material detected in our experiments comes from extracellular DNA or cysts, although encysting has not been observed in most of these species. In such a situation, our

nested PCR protocol would likely be powerful enough to detect the sequence signature of even a single cell in ~0.5 g of environmental material. To avoid these biases, future work could focus exclusively on active cells by way of targeted experiments using RNA extractions from the environment. Further salinity gradient experiments, examining apusomonad monocultures, would also provide important context for the interpretation of environmental results.

Some environmental samples were included in our study to systematically test for the effects of environmental variables such as pH or nutrient levels, revealing some interesting patterns when compared with the genetic data. For example, the putatively marine nutomonads, which our survey suggested prefer marine habitats, were only detected in medium (once) and high (twice) pH soil samples, indicating a possible preference to pH levels above 6.04. Likewise, apusomonad environmental lineages EA25 and EA31 amplified more readily in the highest and lowest pH categories respectively. In contrast, the freshwater nutomonad clade containing *N. limna* was detected in all three pH categories; a particularly interesting result given only one species within this clade has previously been isolated from soil. In future, the relationship between perceived diversity and variables, such as pH, could be more directly examined by experimentally varying culturing media.

Another important finding reported here is the detection of gliding Apusozoa in planktonic lake samples, indicating that, barring the detection of cysts or extracellular DNA, currents in aquatic environments could disperse cells either freely or upon debris in the water column.

Methodological remarks and future directions

Apart from the under-sampling bias inherent in PCR-based environmental surveys, our results may also be influenced by the PCR protocol itself; therefore, the relative rates of lineage detection reported here likely do not reflect lineage proportional abundance in nature. Previous work suggests cell density and size, eDNA inhibitor levels, primer bias, and the relative proportion of eukaryotic and prokaryotic DNA in each sample, could all influence relative detection rates of different lineages (Bass and Cavalier-Smith 2004; Cavalier-Smith 2005; Farris and Olson 2007; Hansen et al. 1998; Potvin and Lovejoy 2009).

Moreover, we relied on nested PCR reactions to obtain visible amplicons. Though powerful enough to detect a single cell in DNA from a mixed assemblage, nested PCR additionally influences perceived diversity, accumulating biases produced throughout the initial amplification step. The lack of visible amplicons after one PCR round contrasts sharply with recent work on the abundant Cercozoa (Bass et al. 2009; Howe et al. 2009), where clone libraries containing up to 27 unique lineages were generated from a single eDNA sample. This suggests that apusozoan cells, though found ubiquitously, are likely present at relatively low densities in the environment, perhaps occasionally experiencing blooms, as has sometimes been observed in mixed environmental cultures.

Although true apusozoan diversity will likely remain difficult to measure, this study shows that culture-independent methods provide important data to complement traditional culturing methods. Grounded in these exploratory findings, future work could feature more ecologically targeted PCR and next-

generation sequencing experiments to further improve our understanding of Apusozoa and other elusive free-living gliding flagellates.

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Tables

Table 1. Environmental DNA sampling and extraction protocol. Names refer to: EG - Edvard Glücksman, DB - David Bass. "Samples amplified" refers to the number of visible bands after nested PCR reactions. Primer pair targets listed as: (1) putatively marine thecamonads (18S rDNA), (2) *Apusomonas* (18S), (3) putatively marine nutomonads (ITS1), (4) putatively freshwater nutomonads (18S).

Name	Description	Extraction method	Sampling sites	Total eDNAs	Samples amplified			
					1	2	3	4
Marine general	Sediment and filtered seawater	~0.5 g of soil or sediment. Maximum Yield protocol of the UltraClean soil DNA Isolation Kit (MoBio Laboratories) (EG, DB)	Mediterranean Sea, Atlantic Ocean, Pacific Ocean	18	3	12	5	13
Freshwater general	Sediment and filtered water	~0.5 g of soil or sediment. Maximum Yield protocol of the UltraClean soil DNA Isolation Kit (MoBio Laboratories) (EG, DB)	River Jordan, Sea of Galilee, Israel; Rio Negro, Brazil; Oxford, Thames, UK; Panama; France	28	-	-	-	6
Lake planktonic	Filtered from water column	Pre-filtration: 1-2 L lake water through 10, 5, 2 µm pore size, 47 mm polycarbonate filters (Millipore) and then onto a 0.2 µm pore size filter. The 0.2 µm polycarbonate filter captured the nanoplanktonic size fraction; the 2, 5, and 10 µm filters were combined as picoplanktonic fraction and above. The filters were then cut, folded, and placed in the bead-beating tubes of the UltraClean Soil DNA Isolation kit. (DB)	DNA was pooled from 16 lakes in Cumbria, UK	16	2	13	4	7
Soil	Soil	Griffiths et al. 2011	15 representative locations across Britain, as part of the Countryside Survey. Samples taken at 3 pH ranges (low – 3.9-4.48; medium – 6.04-6.27; high – 8.04-8.64)	15	3	12	2	12
Estuarine	Sediment	Smith et al. 2007	Colen Estuary, Essex, United Kingdom (3 sites)	47	-	-	11	31

Table 2. Primers used and PCR conditions.

PCR Round	Annealing temp. (C°)	FORWARD PRIMER				REVERSE PRIMER			
		Name	Location (nt. from 5′-end)	Sequence (5′ to 3′)	Specificity	Name	Location	Sequence	Specificity
Initial	58	Th479f	18S (479)	GCGCGCAAATTACCCAATGGCG	*Thecamonas*	SB1n	18S (end)	GATCCTTCYGCAGGTTCACCT	Eukaryote
Nested	65	Th507f	18S (507)	CGAGGTAGTGAMGAACAATAACGATGG	*Thecamonas*	Th1451r	18S (1451)	AACTTCCTCTGGTTAMAACAC	*Thecamonas*
Initial	58	Pre3NDF	18S	CAGCAGGCGCGCAAATTA CC	Eukaryote	Apu1570r	18S (1570)	ACTAGGAATTCCTCGTTKAAGATC CA	*Apusomonas*
Nested	65	Apu789f	18S (789)	GCCTTGAATACATTAGCATGGAATAACG	*Apusomonas*	Apu1401r	18S (1401)	CTCAAACTTCCTTYGGTTAAAGCA CGC	*Apusomonas*
Initial	68.5	Myl1479f	18S (1479)	GCGCGCTACACTGAAAGCGT	*N. mylnikovi*-clade	Myl5.8Sr1	5.8S	GCAAAGGGGTCAAATGACGCTCAG	*N. mylnikovi*-clade
Nested	67.5	Myl1530f	18S (1530)	GTCTGGGTAATCTTTTGAAACGCT	*N. mylnikovi*-clade	Myl5.8Sr2	5.8S	GTGCGTTCAAARATTTGATGATTCACGTT	*N. mylnikovi*-clade
Initial	67.5	Plfw137f	18S (137)	CTAGAGCTAATACATGCGTCAATG	*N. limna*-clade	Plfw1218r	18S (1218)	ACGGCCATGCACCACYAACCATCA	*N. limna*-clade
Nested	68	Plfw192f	18S (192)	CAAAACCAATGCGKGGCAACMCG	*N. limna*-clade	Plfw1054r	18S (1054)	CATACTCCCCCCGGAACCCAAAAT	*N. limna*-clade

Figure legends

Figure 1: 18S rDNA gene phylogeny of Planomonadida, including new lineages found with primers specific to all putatively freshwater nutomonads. Black rectangle (F) indicates nested PCR primer target. Names and branches of new lineages are shown in bold. The heat map (left) indicates number of libraries in which each lineage was obtained, grouped by environmental variable. Maximum-Likelihood tree (RAxML, GTRMIXI model, g=8, best of 100 parsimony starting trees) of 39 sequences using 1680 nucleotide positions. Support values are RAxML boostraps (left) and Bayesian posterior probability (right). Black blobs indicate 100% support by both methods. Scale bar indicates 10% substitutions per nucleotide side. Soil pH levels are: low – 3.9-4.48; medium – 6.04-6.27; high – 8.04-8.64. Freshwater biogeographic sites are: F1 – Israel; F2 –

Brazil; F3 – Oxford, United Kingdom; F4 – Mexico; F5 – Panama; F6 – France. Marine biogeographic sites are: M1 – Mediterranean, comprising Israel and Cyprus; M2 – Mexico and Florida; M3 – Brazil, Atlantic Ocean; M4 – New Zealand. Freshwater planktonic sites divided by filter size at each of two trophic levels: M – Mesotrophic; E – Eutrophic. Black rectangle (N) indicates nested PCR target for marine nutomonad-specific nested primer set featured in Figure 2. The habitat of origin of described species is shown at the branch tips as either marine (black hexagon) or freshwater (star).

Figure 2: ITS1 rDNA gene phylogeny of the putatively marine nutomonad clade, including new lineages. Heat map (left) indicates the number of libraries from which each lineage was isolated and corresponding environmental variables. Maximum-Likelihood tree (PHYML, substitution model = JC69) of 17 sequences using 284 nucleotide positions. Scale bar indicates 10% substitutions per nucleotide side. Soil pH levels: low – 3.9-4.48; medium – 6.04-6.27; high – 8.04-8.64. Freshwater planktonic mesotrophic (M) and eutrophic (E) samples, according to two filter sizes. Marine biogeographic sites: M1 – Mediterranean, comprising Israel and Cyprus; M2 – Mexico and Florida; M3 – Brazil, Atlantic Ocean; M4 – New Zealand; M5 – Panama. Estuarine (Estu.) salinity gradient levels: B – Brightlingsea Reach (high); A – Alresford (medium); H – The Hythe (low). Primer specificity indicated in black rectangle (N) in Figure X2.

Figure 3: 18S rDNA gene phylogeny of Apusomonadida, including new lineages. Black rectangles indicate nested PCR primer targets, marine

thecamonads (T) and *Apusomonas* (A). Names and branches of new lineages are shown in bold. The heat map (left) indicates number of libraries in which each lineage was obtained, grouped according to environmental variables. Maximum-Likelihood tree (RAxML, GTRMIXI model, g=8, best of 100 parsimony starting trees) of 51 sequences using 1484 nucleotide positions. Support values are RAxML boostraps (left) and Bayesian posterior probability (right). Black blobs indicate 100% support by both methods. Scale bar indicates 10% substitutions per nucleotide side. Soil pH levels: low – 3.9-4.48; medium – 6.04-6.27; high – 8.04-8.64. Freshwater planktonic mesotrophic (M) and eutrophic (E) samples, divided by two filter sizes. Marine biogeographic sites: M1 – Mediterranean, comprising Israel and Cyprus; M2 – Mexico and Florida; M3 – Brazil, Atlantic Ocean; M4 – New Zealand; M5 – Panama. Columns on the far right indicate primer set used to obtain each library. The habitat of origin of described species is shown at the branch tips as either marine (black hexagon), freshwater (star), or brackish (both).

Supplementary Figure 1: Heat map showing PCR amplifications by environmental DNA sample and group-specific primer pair. Black boxes indicate presence of visible amplicon. Grey boxes indicate no attempted amplification.

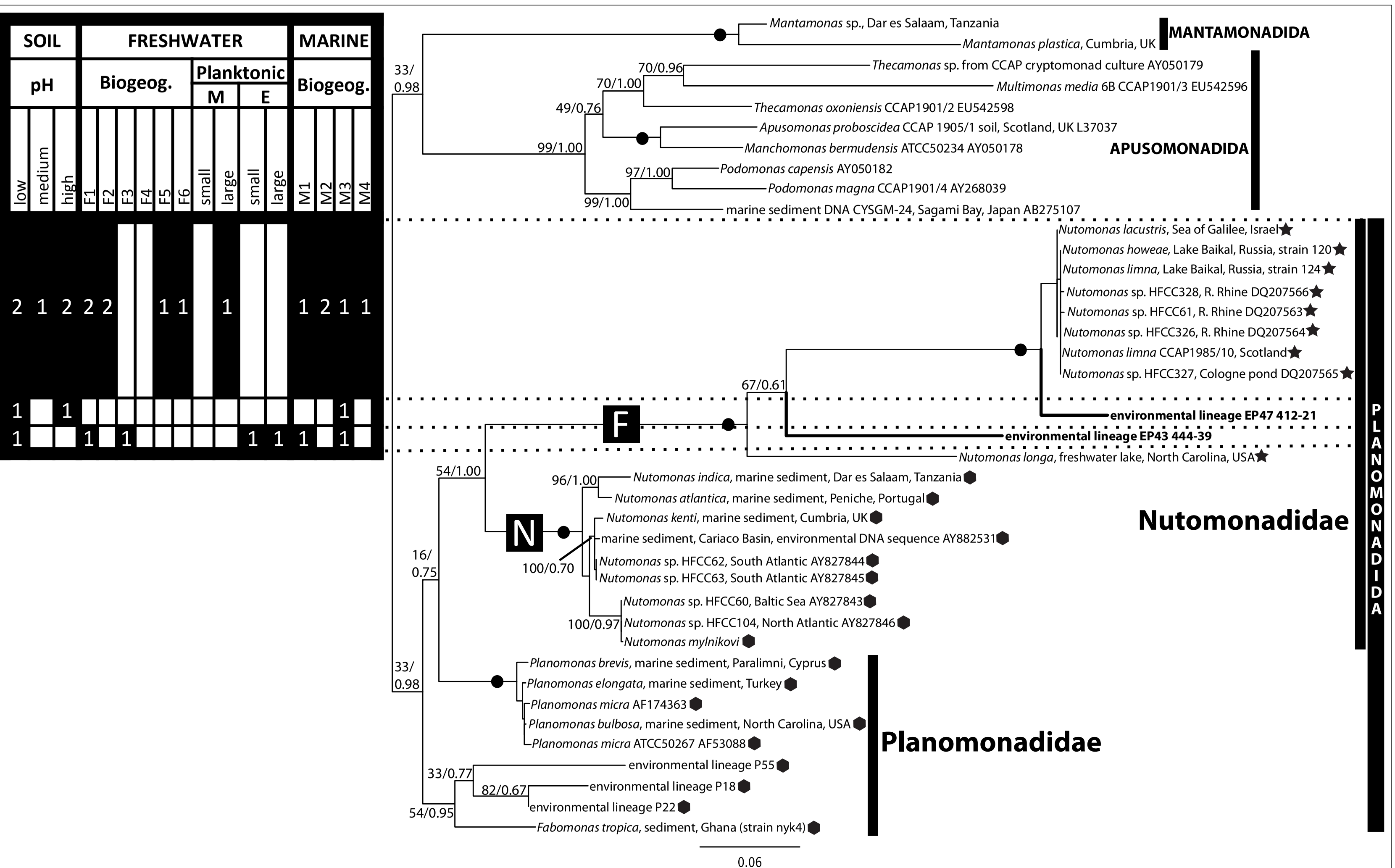

Figure 1

	SOIL			FRESHWATER				MARINE					ESTU.		
	pH			Plankt.				Biogeography							
				M		E									
	low	medium	high	small	large	small	large	M1	M2	M3	M4	M5	B	A	H
Nutomonas sp. 3b GU001166								1							
Nutomonas kenti, marine sediment, Cumbria, UK, clone 203-2								1							
environmental lineage ENL6 486-27			1					1							
Nutomonas kenti, marine sediment, Cumbria, UK, clone 293-10															
Nutomonas kenti, marine sediment, Cumbria, UK, clone 293-12															
Nutomonas atlantica, marine sediment, Peniche, Portugal, clone 293-7															
environmental lineage ENL3 486-37								1							
environmental lineage ENL7 475-9											1				
environmental lineage ENL16 486-1														1	
environmental lineage ENL15 486-4														1	
environmental lineage ENL0 475-2								1		1					
Nutomonas mylnikovi B70 EU349250, clone 04															
Nutomonas mylnikovi, clone 292-53															
Nutomonas indica, marine sediment, Dar es Salaam, Tanzania, clone 292-72		1	1		1			1			1		1		1
Nutomonas mylnikovi, clone 292-73								1							
environmental lineage ENL13 486-74												1			
environmental lineage ENL8 486-80												1			

Nutomonadidae

Figure 2

TOTAL			Soil pH			FW Plankt. M		FW Plankt. E		Marine Biogeog.					Primer target	
soil	freshwater	marine	low	medium	high	small	large	small	large	M1	M2	M3	M4	M5	Thecamonads	Apusomonads
		1										1			0	1
2	1	1	1		1			1		1					3	1
1	0	1			1					1					2	0
0	0	2											1	1	1	1
0	0	1											1		1	0
2	2	2			2			1	1				1	1	6	0
1	0	0		1											0	1
2	0	0		1	1										0	2
0	0	1								1					0	1
0	1	0						1							0	1
1	0	0		1											0	1
0	0	1												1	0	1
6	0	1	2	2	2									1	0	7
0	1	1					1							1	0	2
1	0	0			1										0	1
3	1	2	2	1			1						1	1	0	6
1	0	0	1												0	1
0	1	0						1							0	1

Fabomonas tropica strain nyk4
Planomonas micra
Nutomonas atlantica
environmental DNA sequence AY882531
Nutomonas sp. HFCC62
Nutomonas sp. HFCC60
Nutomonas mylnikovi
Planomonadida
71/0.99
64/ 0.92
98/0.85
12/0.67
environmental lineage P8
mid-Atlantic Ocean ridge sediment DNA AT-41 AF530526
environmental lineage EA16 505-26
99/0.99
Thecamonas aff. *trahens* Thai EU542599
Thecamonas aff. *trahens* CCAP 1901/1 (A-1a) EU542595
Thecamonas trahens ATCC50062 AY050180
Thecamonas aff. *trahens* HFCC48 AY827841
Thecamonas aff. *trahens* HFCC49 AY827842
environmental lineage EA26 484-17
T
2/-
71/0.99
76/0.92
environmental lineage EA28 505-51
Thecamonas sp. Bamfield EU542594
environmental lineage EA25 484-25
8/-
Apusomonas proboscidea CCAP 1905/1 soil, Scotland, UK L37037
Apusomonas proboscidea HFCC47 dystrophic pond, Borok, Russia DQ7207567
Apusomonas proboscidea HFCC336 18m deep groundwater, Germany DQ207568
soil DNA clone Amb_18S_480 misannotated as *Acanthamoeba* EF023187
environmental lineage A27
environmental lineage A1
40/0.72
6/-
41/-
A
Manchomonas bermudensis ATCC50234 AY050178
34/0.67
Thecamonas sp. from CCAP cryptomonad culture AY050179
Thecamonas oxoniensis CCAP1901/2 EU542598 AY050182
Apusomonadida
11/-
mid-Atlantic Ocean ridge sediment DNA AT-50 AF530544
Multimonas media Millport strain AY050181
Multimonas media 7C Milton Sands, Devon, UK EU542597
Multimonas media 6B Wembury Bay, Devon, UK CCAP1901/3 EU542596
32/0.90
70/0.75
environmental lineage A0
1/-
marine sediment DNA CYSGM-24, Sagami Bay, Japan AB275107
Podomonas capensis AY050182
environmental lineage EA42 505-23
16/-
environmental lineage EA40 505-58
11/-
4/-
environmental lineage EA32 502-14
1/-
13/-
environmental lineage EA29 505-47
56/-
98/-
environmental lineage EA30 484-48
environmental lineage EA36 505-81
3/-
81/ 0.93
environmental lineage EA33 502-49
3/-
environmental lineage EA31 484-44
19/0.52
environmental lineage EA37 484-45
environmental lineage EA38 505-64
30/0.46
Podomonas magna CCAP1901/4 AY268039
31/-
environmental lineage A31
70/0.23
environmental lineage A30
94/0.83
environmental lineage A25
0.09

Figure 3

	Sample	Location	Thecamonads	*Apusomonas*	*N. mylnikovi*-clade	Freshwater nutomonads
MARINE	CY1	Malama Beach, Paralimni, Cyprus				
	CY3	Malama Beach, Paralimni, Cyprus				
	CY5	Malama Beach, Paralimni, Cyprus				
	IL21	Acre, Israel				
	IL22	Acre, Israel				
	Mex1	Coatzacoalcos, Veracruz, Mexico				
	Mex2	Coatzacoalcos, Veracruz, Mexico				
	FL1	Anne Marie, Florida, USA				
	Cop1	Copacabana, Rio de Janeiro, Brazil				
	Cop2	Copacabana, Rio de Janeiro, Brazil				
	Cop3	Copacabana, Rio de Janeiro, Brazil				
	P27	Portobelo, Panama				
	NZ38	Long Beach, New Zealand				
	NZ42	Auckland, New Zealand				
	NZ47	Leigh, New Zealand				
FRESHWATER	IL1	En Gedi National Park, Israel				
	IL4	En Gedi National Park, Israel				
	IL5	En Gedi National Park, Israel				
	IL14	Kishan River, Israel				
	IL15	Jordan River, Israel				
	IL17	Sea of Galilee, Israel				
	IL18	Sea of Galilee, Israel				
	NEG3	Rio Negro near Manaus, Brazil				
	NEG9	Rio Negro near Manaus, Brazil				
	NEG10	Rio Negro near Manaus, Brazil				
	NEG11	Rio Negro near Manaus, Brazil				
	K	Lake Baikal, Russia				
	Br1	Bromeliad, near Rio de Janeiro, Brazil				
	Br2	Bromeliad, near Rio de Janeiro, Brazil				
	Bru1	Lac Parmentier, Brussels, Belgium				
	OX1	Christ Church College, Oxford, UK				
	OX2	Worcester College, Oxford, UK				
	Th1	River Thames, UK				
	P10	Capira, Panama				
	P15	near Altos del Maria, Panama				
	P20	near Altos del Maria, Panama				
	P25	Camino de Cruces Lake, Panama				
	P42	Gamboa, Panama				
	Th2	River Thames, UK				
	F1	southeastern France				
	F6	southeastern France				
	AQ2	Aquarium				
	OX3	Christ Church College, Oxford, UK				
	NZ70	Tapu, New Zealand (moss)				
	NZ103	Te Toto Gorge, New Zealand				
	small/eutrophic1	pooled from lakes in Cumbria, UK				
	small/eutrophic2	pooled from lakes in Cumbria, UK				
	small/eutrophic3	pooled from lakes in Cumbria, UK				
	small/eutrophic4	pooled from lakes in Cumbria, UK				
	small/mesotrophic1	pooled from lakes in Cumbria, UK				
	small/mesotrophic2	pooled from lakes in Cumbria, UK				
	small/mesotrophic3	pooled from lakes in Cumbria, UK				
	small/mesotrophic4	pooled from lakes in Cumbria, UK				
	large/eutrophic1	pooled from lakes in Cumbria, UK				
	large/eutrophic2	pooled from lakes in Cumbria, UK				
	large/eutrophic3	pooled from lakes in Cumbria, UK				
	large/eutrophic4	pooled from lakes in Cumbria, UK				
	large/mesotrophic1	pooled from lakes in Cumbria, UK				
	large/mesotrophic2	pooled from lakes in Cumbria, UK				
	large/mesotrophic3	pooled from lakes in Cumbria, UK				
	large/mesotrophic4	pooled from lakes in Cumbria, UK				
SOIL	NZ17	Hamilton Lake, New Zealand				
	Griff1 - pH low (4.48)	Heath and bog, England, UK				
	Griff179 - pH low (4.3)	Heath and bog, Wales, UK				
	Griff599 - pH low (3.9)	Heath and bog, England, UK				
	Griff864 - pH low (4.12)	Moorland grass mosaics, Scotland, UK				
	Griff922 - pH low (4.37)	Upland wooded, Scotland, UK				
	Griff15 - pH med. (6.14)	Tall grass and herb, England, UK				
	Griff301 - pH med. (6.26)	Infertile grassland, Wales, UK				
	Griff359 - pH med. (6.17)	Infertile grassland, England, UK				
	Griff860 - pH med. (6.13)	Heath and bog, Scotland, UK				
	Griff948 - pH med. (6.04)	Infertile grassland, Scotland, UK				
	Griff78 - pH high (8.25)	Infertile grassland, England, UK				
	Griff216 - pH high (8.64)	Infertile grassland, England, UK				
	Griff251 - pH high (8.4)	Infertile grassland, England, UK				
	Griff511 - pH high (8.04)	Fertile grassland, England, UK				
	Griff1053 - pH high (8.46)	Infertile grassland, Scotland, UK				
ESTUARINE GRADIENT	A1	02/2005				
	A1	04/2005				
	A1	06/2006				
	A1	02/2006				
	A2	02/2005				
	A2	04/2005				
	A2	06/2006				
	A2	08/2006				
	A2	10/2006				
	A2	02/2006				
	A3	02/2005				
	A3	04/2005				
	A3	06/2006				
	A3	08/2006				
	A3	10/2006				
	A3	02/2006				
	B1	02/2005				
	B1	04/2005				
	B1	06/2006				
	B1	08/2006				
	B1	02/2006				
	B2	02/2005				
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	B2	02/2006				
	B3	02/2005				
	B3	04/2005				
	B3	06/2006				
	B3	08/2006				
	B3	02/2006				
	H1	02/2005				
	H1	04/2005				
	H1	06/2006				
	H1	08/2006				
	H1	02/2006				
	H2	02/2005				
	H2	04/2005				
	H2	06/2006				
	H2	08/2006				
	H2	02/2006				
	H3	02/2005				
	H3	04/2005				
	H3	06/2006				
	H3	08/2006				
	H3	10/2006				
	H3	02/2006				

Supplementary Figure 1

Chapter Six

Closely related protist strains have different grazing impacts on natural bacterial communities

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Closely related protist strains have different grazing impacts on natural bacterial communities

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Summary

Heterotrophic protists are abundant in most environments and exert a strong top-down control on bacterial communities. However, little is known about how selective most protists are with respect to their bacterial prey. We conducted feeding trials using cercomonad and glissomonad Cercozoa by assaying them on a standardized, diverse bacterial community washed from beech leaf litter. For each of the nine protist strains assayed here, we measured several phenotypic traits (cell volume, speed, plasticity and protist cell density) that we anticipated would be important for their feeding ecology. We also estimated the genetic relatedness of the strains based on the 18S rRNA gene. We found that the nine protist strains had significantly different impacts on both the abundance and the composition of the bacterial communities. Both the phylogenetic distance between protist strains and differences in protist strain traits were important in explaining variation in the bacterial communities. Of the morphological traits that we investigated, protist cell volume and morphological plasticity (the extent to which cells showed amoeboid cell shape flexibility) were most important in determining bacterial community composition. The results demonstrate that closely related and morphologically similar protist species can have different impacts on their prey base.

Introduction

Environmental rDNA cloning and culture-based studies have recently shown protozoan diversity to be many times higher than morphological studies alone would suggest (Bass and Cavalier-Smith, 2004; Bass *et al.*, 2009; Howe *et al.*, 2009). However, it is unclear whether this newly revealed molecular diversity is associated with correspondingly high levels of functional diversity. There is some evidence that even closely related, morphologically similar strains may differ in their ecological preferences and responses (Corno and Jürgens, 2008) but such studies remain in their infancy and it is not yet possible to exclude the possibility that most molecular differences simply reflect neutral sequence variation (Fenchel and Finlay, 2006).

Protist feeding preferences are of particular interest because protozoa and bacteria are key players in the microbial loop (Jürgens, 1994; Murase and Frenzel, 2008; Neubacher *et al.*, 2008). Bacteria are responsible for the trophic movement of a sizeable fraction of the organic carbon available on earth (Jürgens and Gude, 1994; Jürgens and Matz, 2002; Falkowski *et al.*, 2008). There is often strong top-down control of bacterial populations by predatory protozoa (Fuhrman *et al.*, 1992; Hahn and Hofle, 2001; Corno and Jürgens, 2008), so protozoa play a critical role in modulating ecosystem processes and in setting the amount of organic matter available to higher trophic levels (e.g. Zöllner *et al.*, 2009). Although trophic level interactions between protists and bacteria have been well documented, relatively little is known about how different protist strains influence the diversity and composition of bacterial communities. If protists differ in their feeding preferences, the large numbers of protist genotypes being discovered from gene libraries could have significant repercussions both for understanding bacterial community dynamics and for explaining variation in ecosystem processes.

Protistan grazing is known to have a number of important consequences for bacterial communities. Protists tend to graze a limited size range of bacterial cells, thus removing medium-sized bacterial cells and shifting the size structure of the bacterial community towards larger and smaller cell sizes (Hahn and Hofle, 2001; Pernthaler, 2005). Similarly, as a consequence of this selective grazing, protists can alter the behaviour of bacterial assemblages, favouring the development of filaments or clumps of bacterial cells that are resistant to grazers (Hahn and Hofle, 2001). Finally, several studies have shown that protist grazing not only favours particular

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morphological and behavioural traits, but that they also alter the composition of the bacterial community by favouring grazing resistant bacterial strains (Boenigk *et al*., 2004; Pernthaler, 2005), which are selected if the cost of grazing resistance is sufficiently small. It is therefore critical that experiments that look at the effect of protist grazing on bacterial communities do so under tightly controlled conditions.

Previous work has indicated that morphologically similar protist strains can differ in their feeding preferences (Weisse *et al*., 2001). However, molecular surveys have now revealed high levels of morphological conservation as well as convergent evolution in protist morphologies (Nikolaev *et al*., 2004; Hoef-Emden, 2007; Darling and Wade, 2008; Howe *et al*., 2009), so individual protist morphospecies may harbour a genetically diverse mixture of strains. Such high levels of genetic divergence can compromise studies seeking to identify a relationship between grazing preferences and degree of relatedness among protist strains. To avoid this potential problem, we conducted grazing experiments in microcosms using protist strains with well-described molecular phylogenies.

We were interested in the effect of bacterivory of different but evolutionarily related protist strains of the same morphological type on a diverse but standardized bacterial community. Many previous studies have focused on the protist feeding on a single bacterial strain, but this approach does not provide insight into the ability of protist strains to differentially modify diverse bacterial communities. This process is fundamental to understanding the functioning of ecosystems, but is currently poorly understood. This study offers preliminary insight into these effects by offering protists a choice of prey in an experimental environment that approximates their natural habitat.

We examined the effects of protist predation on a well-characterized, diverse bacterial community under controlled conditions. The nine protist species used in this study were from well-sampled groups of cercozoan gliding heterotrophic biflagellates: members of genera *Sandona* and *Allapsa* (Glissomonadida; Howe *et al*., 2009), and of *Cercomonas* and *Paracercomonas* (Cercomonadida; Karpov *et al*., 2006). Molecular phylogenies have recently been constructed for all of the strains. Cercomonads and glissomonads are together among the most abundant and widespread microbial bacterivores in soils and freshwater habitats (Foissner, 1991; Ekelund *et al*., 2001; Bass *et al*., 2007), yet very little is known about their feeding habits. Whereas one would expect to see grazing differences across the full range of protist morphotypes (e.g. amoebae, flagellates, heliozoa, swimming forms, gliding forms, etc.) it is less obvious whether more closely related protists previously lumped into a single functional group differ significantly in grazing behaviour. If such differences were seen we wanted to know whether they were most strongly associated with genetic distance between protist strains (as measured by the universally used phylogenetic marker gene 18S rRNA) or with phenotypic cellular traits such as size, morphological plasticity, speed of locomotion and relative growth rate.

We inoculated the protist strains as monocultures into identical bacterial communities derived from leaf litter. We examined the bacterial community structure following grazing to determine the extent of protist feeding preferences. We also examined the extent to which differences in feeding preference were associated with phylogenetic distance and with phenotypic trait differences between the protist species.

Results

The phylogenetic tree of the nine protist species (Fig. 1A) is concordant with recent phylogenies (Bass *et al*., 2009; Howe *et al*., 2009). The cluster diagrams showing composite strain trait and bacterial terminal restriction fragment polymorphism (tRFLP) profile similarities are shown in Fig. 1B and C respectively. Clearly, neither strain traits nor phylogeny is solely responsible for explaining the tRFLP banding patterns. For example, the two most closely related protozoan strains, *Cercomonas longicauda* and *C. paravarians*, generated very different tRFLP profiles occupying completely different clusters in the tRFLP cluster diagram. However, there was an overall positive correlation between the trait and phylogenetic distance matrices (Fig. 2A), between bacterial community (tRFLP profile) distance and phylogenetic distance (Fig. 2B), and between tRFLP profile and strain trait distance (Fig. 2C). However, the correlation between trait and phylogenetic distance levelled out and even declined beyond a moderate level of trait difference (Fig. 2A). A similar pattern was seen between tRFLP profile and trait distance (Fig. 2C). The no-protist controls produced closely similar tRFLP profiles at the end of the grazing period (not shown), showing that in the absence of protists the bacterial communities did not diverge from each other.

We tested for associations between the three distance matrices using Mantel and partial-Mantel tests (Table 1), where partial-Mantel tests controlled for the effect of traits (when testing the effect of phylogenetic distance) or for the effect of phylogenetic distance (when testing for the effect of traits). When we analysed the four traits separately, there were significant associations between grazed bacterial community (tRFLP) differences and cell volume, plasticity and speed when partial-Mantel tests controlled for phylogenetic distance (Table 1). When we did not account for phylogenetic distance, cell volume

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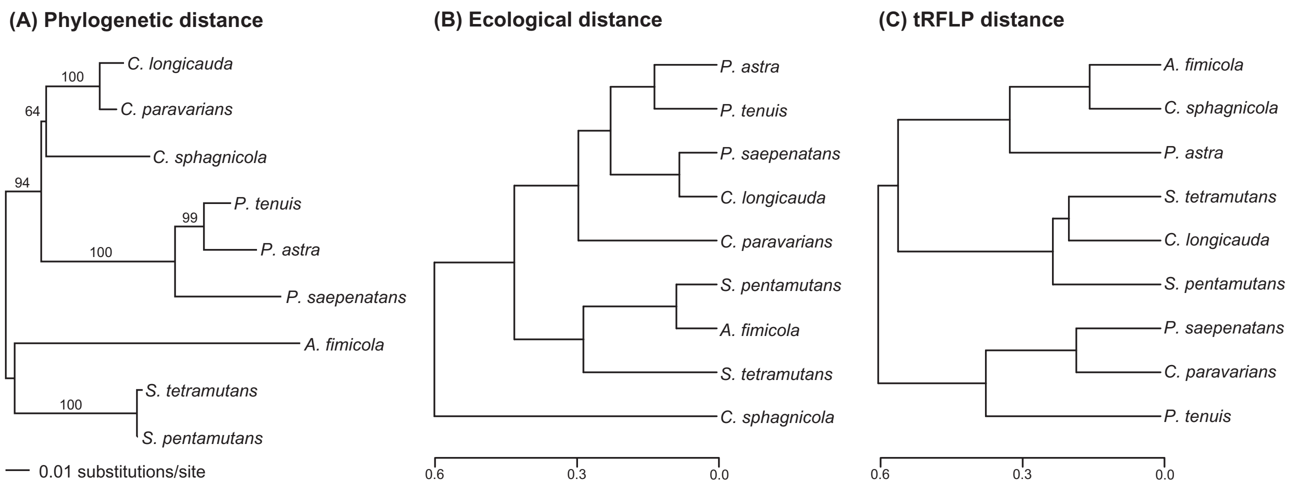

Fig. 1. Hierarchical clustering of the protozoan strains according to their (A) phylogenetic distance, (B) trait distance, (C) mean bacterial tRFLP distance. Phylogenetic distance shown as a distance tree using GTR+Γ+I model (see *Experimental procedures* for details). Mean tRFLP distance was calculated by first taking the mean relative abundance of each bacterial species and then creating a distance matrix from these mean values.

and plasticity showed highly significant associations with tRFLP differences, but cell speed did not. The bacterial communities obtained for each protist strain are shown in Fig. 3. The bacterial tRFLP profiles produced by each protozoan strain were repeatable with a few exceptions. On the whole, four restriction fragment polymorphisms (with lengths of 111, 251, 455 and 456 base pairs) were responsible for the main differences among protozoan strains.

Discussion

We have demonstrated that the effect of protist species on bacterial communities can depend on their morphological traits or on their phylogenetic history. Much of the previous work on protist predation of bacterial assemblages has been within the context of whether predation ('top-down control') or the availability of resources ('bottom-up control') regulates community prey biomass, structure and function (Corno and Jürgens, 2008; Murase and Frenzel, 2008; Bell *et al.*, 2010). Using well-characterized, clonally cultured protist strains, our study is among the first to examine the protest–bacteria interaction from a comparative-phylogenetic perspective. A robust 18S rDNA protist phylogeny enabled us to explain variation in the bacterial communities in terms of the phylogenetic relatedness of the protist strains that were grazing on them and the phenotypic traits of those strains. The results demonstrate a significant association between the composition of grazed (final) bacterial assemblages and the phylogeny of their protist predators. Phylogeny and morphological traits (cell size and morphological plasticity) both appear to influence the grazing behaviour of protists, lending support to the idea that feeding preference might play a role in the evolutionary divergence of eukaryotic organisms.

From the perspective of bacterial assemblage composition, these findings are consistent with past work (Billen *et al.*, 1990; Pace and Cole, 1994; Gasol *et al.*, 2002; Corno and Jürgens, 2008) by further highlighting the important effect of predation ('top-down' processes) in the regulation of natural ecosystems. We have shown that strain traits are also strongly associated with protist feeding behaviours. Differences in mode of protist movement (plasticity and speed) were significantly correlated with differences in bacterial grazing activity. Our observa-

Table 1. The Mantel statistic (r) was computed to test for a significant association between the distance matrix of tRFLP profiles and several predictor distance matrices. Partial Mantel statistics controlled for phylogeny (when strain traits were used as predictors) and vice versa.

Predictor	Mantel r	P	Partial-Mantel r	P
Phylogenetic distance	0.25	0.007	0.19	0.03
Trait distance	0.25	0.005	0.19	0.03
Volume	0.27	0.0003	0.19	0.007
Plasticity	0.30	0.0004	0.20	0.004
Speed	0.15	0.067	0.19	0.030
Growth rate	0.11	0.083	0.06	0.218

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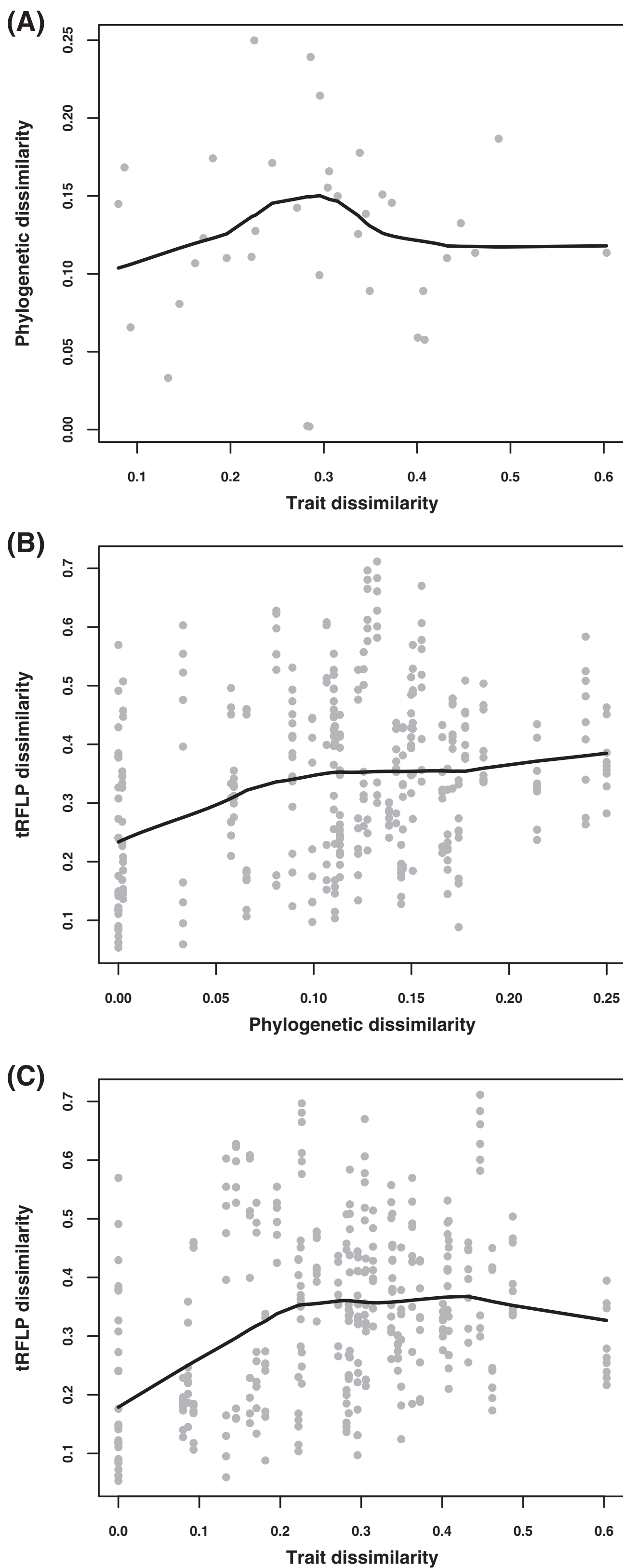

Fig. 2. Relationship between (A) phylogenetic distance and trait distance, (B) phylogenetic distance and tRFLP distance, (C) trait distance and tRFLP distance. Each data point is a single pairwise comparison. Fitted lines are lowess regressions, which fits a localized regression on a sliding window across the *x*-axis. The main objective of the lowess regression in this context is to document general trends in the data rather than to estimate their significance (significance tests of these relationships are estimated using Mantel tests, see Table 1).

tion that cell size is highly significant in explaining differences between grazing activity is concordant with the results of previous experiments using individual bacterial strains and artificial bacterial communities (Boenigk *et al*., 2004; Pfandl *et al*., 2004), and would appear plausible since the bacterial community comprises strains varying in size and therefore ease of uptake by protist predators. However, there were some interesting exceptions to the general patterns revealed by our data. For example, *Cercomonas sphagnicola* and *Allapsa fimicola* produced similar bacterial tRFLP profiles but are morphologically dissimilar and phylogenetically only distantly related. Conversely, morphologically similar strains, such as *C. longicauda* and *C. paravarians*, can exhibit more marked differences in grazing behaviour than their phenotypic differences might suggest. Further experiments are now required to determine how bacterial morphology, behaviour and phylogeny affect their likelihood of being grazed by particular protist morphotypes and taxonomic groups. Our results suggest that grazing preferences are both more specific and less predictable than assumptions based on simple morphological similarities might suggest. Therefore, the apparent bacterial omnivory of protozoan 'species' may conflate narrower and more specific preferences of individual protist lineages within taxonomically crude clusters of strains. Whether or not protist species are specialist or generalist predators will have important repercussions for how the ecosystem as a whole responds to perturbations. For example, increasing the supply of resources will tend to alter bacterial diversity if their protist predators are generalists but not if they are specialized on specific bacterial taxa (Bell *et al*., 2010).

The cercomonad and glissomonad protozoa used in these experiments are of particular interest because they have long been known to include the most abundant and widespread zooflagellates in soil. Like most protist groups that have been subjected to intensive molecular analysis they have recently been shown to be highly genetically diverse, far more so than their conserved and convergent morphologies suggest (Bass *et al*., 2007; 2009; Howe *et al*., 2009). We suggest that the cryptic diversity recently revealed by molecular studies hides a highly complex diversity of ecological function, and that individual protist strains can moderate their bacterial environment in dis-

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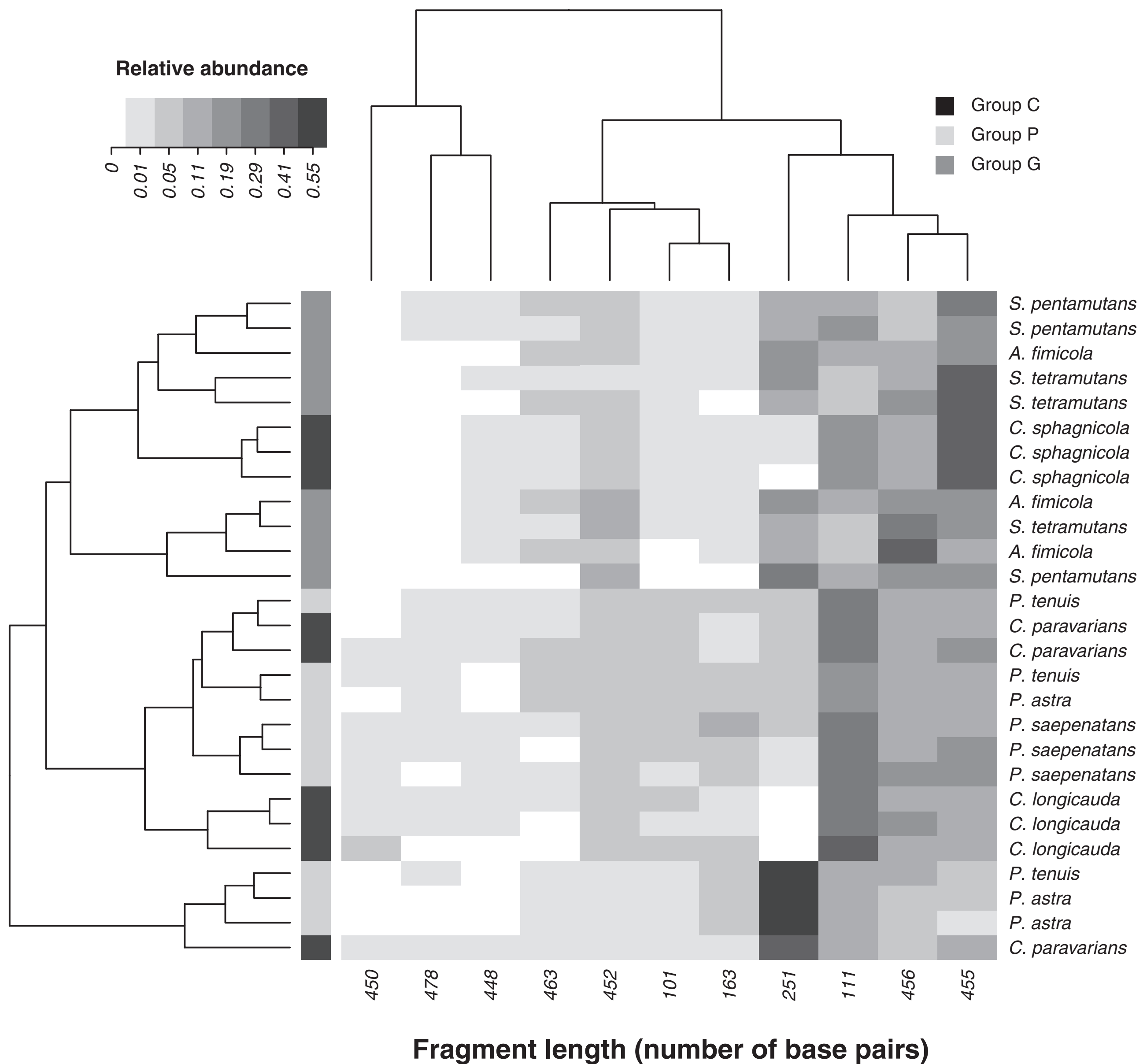

Fig. 3. Heatmap of relative abundance of each bacterial taxon (columns) in each microcosm (rows). The relative abundance of each taxon is along a greyscale from absent (black) to abundant (white). Microcosms are labelled with the protist species name (three replicates). Bacterial taxa are labelled by the size of the restriction fragment produced (in base pairs). Rows are hierarchically clustered according to the similarity in their species composition. Relative abundance values have been squared to emphasize differences.

tinct, if not always predictable ways. New insight into the significance and magnitude of these differences can be gained from comparative approaches such as the one we present here, and by utilizing more realistic, complex bacterial communities than are often employed in protist feeding experiments. More work is required to understand how grazing characteristics evolve among strains and to understand the implications of trophic mode on protist community structures and bacterial function.

Although 18S rDNA divergence itself is not directly linked to phenotypic traits of direct relevance to grazing behaviour, phylogenetic analyses using this gene produce evolutionary models strongly concordant with other phenotypic differences between sarcomonad genera and species (Karpov *et al*., 2006; Bass *et al*., 2009; Howe *et al*., 2009). Cell size and degree of amoeboid plasticity were strongly associated with feeding differences, but other phenotypic traits, possibly including physiological and biochemical traits (e.g. Roberts *et al*., 2006; Wootton *et al*., 2006), may also be important determinants of sarcomonad grazing differences. Future studies will investigate these possibilities.

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Bacteria play important functional roles, so alterations of the composition of bacterial community composition can have important impacts on ecosystem functioning (Bell *et al.*, 2005). The selective grazing activity of protists has the potential to significantly modify fundamental ecosystem processes such as nutrient cycling equilibria by exerting differential grazing pressures (Corno and Jürgens, 2008). For example, the contribution of protists to important ecological networks such as rhizosphere–bacteria–protist interactions has been shown to be complex (Griffiths *et al.*, 1999; Kreuzer *et al.*, 2006; Jousset *et al.*, 2008; Rosenberg *et al.*, 2009). More knowledge of the specificity of the feeding relationships between bacteria and protists is required to understand how these networks moderate plant development. Although microcosm experiments have been criticized for being inherently over-simplistic to properly explore fundamental questions of ecosystem ecology (Carpenter, 1996), our results support an opposing view (Jessup *et al.*, 2004): that replicated, reproducible experimental work is essential for isolating and examining ecological interactions under semi-natural conditions. The statistically significant associations identified here are based on nine protist strains. Further work will be required to document whether these patterns apply when additional protist strains and bacterial communities are assayed. However, we have shown that, under controlled conditions, closely related and morphologically similar protist species can have widely divergent effects on bacterial communities. We therefore anticipate that future research will reveal that much of the cryptic diversity within the protists will have significant functional consequences.

Experimental procedures

Protist collection, identification and description

We assayed 17 protist strains on a standardized bacterial community. Of these, eight were eliminated from the analysis because of insufficient growth under the experimental conditions (see above). The protist strains that were used were sarcomonad bacterivorous flagellates from the phylum Cercozoa, including three *Cercomonas* species (*C. longicauda*, *C. paravarians* and *C. sphagnicola*), three *Paracercomonas* species (*P. tenuis*, *P. astra*, *P. saepenatans*), and three glissomonads (order Glissomonadida: *A. fimicola*, *Sandona tetramutans*, *Sandona pentamutans*). Recent taxonomic treatments of these species can be found in Bass and colleagues (2009) and Howe and colleagues (2009), where availability of the strains is also given (see Table S1). A distance tree (100 replicates, random addition, minimum evolution) for the species used in the present study was generated in PAUP (Swofford, 2002) using a GTR+Γ+I nucleotide substitution model selected by MODELTEST (Posada and Crandall, 1998) (nine taxa, 1062 positions), which also generated the distance matrix used in the statistical analyses described above. The bootstrap values for Fig. 1A were generated in PAUP using the same model (1000 replicates).

We described a suite of traits for each protist strain: cell volume, speed, plasticity and protist cell density in the experimental wells during the course of the experiment. Speed, plasticity and cell volume were allocated an ordinal score based on observations of at least five individual cells (Table S1). Speed scores were assigned on the relative rate of movement of cells. Plasticity was a measure of the extent and speed of internal cytoplasmic movement and cell shape distortions. Cell volume was estimated assuming a generalized cone structure for each cell: $(1/3)\pi r^2 h$, where h = length of cell in μm. Cell density was taken as the average number of cells per field of view across the three replicate experimental microcosms counted at 24 h intervals after the point of inoculation for 5 days until the end of the inoculation period. Each of these traits was standardized by dividing by the maximum value to put each on a scale from zero to one. We were interested in both the effect of each of the traits individually and their overall effect on the bacterial communities. For each trait, the pairwise dissimilarity between each protist strain was simply the Euclidean distance between the standardized trait measurements. The total pairwise trait distance between each protist strain was the dissimilarity summed across the four traits.

Preparation of the protist cultures

Prior to the start of the experiment, protist cultures were maintained for prolonged periods on a freshwater medium (35 ml of Volvic water, with one sterile wheat grain that had been boiled in Volvic for 3 min). To ensure each strain was uncontaminated by any other eukaryote, we serially diluted each of the protist strains until a uniclonal protist culture was achieved (Bass *et al.*, 2009). Protist strains were then incubated at the experimental temperature (21°C) for 2 weeks prior to the beginning of the experiment. These cultures were inspected (200× magnification) every 2 days and pure cultures were transferred to fresh medium. This process was repeated until we were confident that the cultures were uncontaminated.

To ensure that the same bacterial community was present in each of the protist culture flasks prior to the start of the experiment, small volumes (*c.* 5 μl) of the protist cultures were inoculated into the bacterial community used for the experimental microcosms (see above), allowed to grow to moderate density, then repeatedly re-inoculated into the same (protist-free) bacterial community. In this manner, we diluted out the bacteria that were originally associated with each protist strain, resulting in protists that were growing in the standard bacterial community. On the day on which the experiment was initiated approximately 10^2 cells of each protist strain were inoculated into the experimental microcosm wells (three replicate wells per strain).

Bacterial communities and microcosm set-up

A diverse, natural bacterial community was obtained by incubating 1 g of unsterilized beech leaves in 40 ml of Volvic

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mineral water at 21°C for 3 days in closed microcosms. The resultant bacterial communities were filtered through a 1.2 μm cellulose acetate filter (Sartorius) to eliminate particulate matter and larger organisms. The cell density was then estimated using fluorescence activated cell sorting (FACS; see above), and bacterial cell density was adjusted to 10^5 cells ml^{-1}. A beech leaf medium was used to sustain the experimental bacterial communities. This was created by autoclaving 50 g of beech leaves in 500 ml of Volvic water, and this extract was diluted 16-fold in Volvic.

The microcosms consisted of 24-well plates each containing 2 ml of the diluted beech leaf medium and 100 μl of the bacterial community (i.e. 10^4 bacterial cells were inoculated into each well). The bacterial communities were allowed 1 week to develop prior to inoculation of the protist communities. Each protist strain was then inoculated into three microcosms. The protists were allowed to graze on the bacterial communities for 5 days, after which we assessed the bacterial community composition using tRFLP (see above). Only protist strains that reached an average density of > 100 cells per field of view (FOV) at 200× magnification were used for further analysis, ensuring that protist growth was sufficient to have a significant effect on the bacterial communities. Out of an initial 17 protist strains, eight did not grow sufficiently under the microcosm conditions to attain sufficient levels of abundance, and nine showed significant growth and so were analysed for the resulting bacterial communities.

Bacterial counts

Total bacterial counts at the end of the experiment were determined using FACS. A 50 μl sample from each microcosm was fixed with 1% paraformaldehyde, and stained using SYBR Green I, which fluoresces when bound to double-stranded DNA. Samples were passed through a Becton Dickinson FACScalibur flow cytometer using a timed aspiration (2 min). They were subsequently converted to absolute cell concentrations by measuring the volume aspirated during the 2 min analysis and comparing it with the weight of blank samples before and after aspiration (typical flow rates were 0.009 ml min^{-1}). Internal standards (10 μl of 0.45 μm Polysciences MultiSpec beads per 50 ml of solution) were added to all samples to account for any instrument drift. Bacterial counts estimated from the resulting side-scatter and green fluorescence cytograms using the WinMDI software package.

Bacterial community structure

We removed 1.5 ml from each microcosm and pelleted the cells by centrifuging at around 10 000 *g* for 10 min. The supernatant was subsequently discarded and the tubes stored at −20°C. We used the method of Bailey (1995) to extract the bacterial DNA. Briefly, the cell pellet was suspended in 500 μl of lysis buffer (10% SDS and 20 μg ml^{-1} Proteinase K) and incubated at 55°C for 30 min. The lysed cells were then amended with a solution containing 80 μl of 10% Cetyl trimethylammonium bromide solution (CTAB) and 100 μl of 5 M NaCl and incubated at 65°C for 10 min. DNA was extracted with 1:24 isoamyl alcohol : chloroform, and precipitated with isopropanol. Approximately 10 ng of the resultant DNA was subsequently used as template from which a 456-base-pair fragment of the bacterial 16S rRNA gene. D4-labelled primer 63f (Marchesi *et al.*, 1998) and unlabelled 519r (Lane, 1991) were used in PCR reactions containing 0.2 mM dNTPs, 1.25 U *Taq* DNA polymerase, 25 pmol of each primer, 5 μg of BSA and 5 μl of 10× PCR buffer in 50 μl volumes. Thermocycling conditions consisted of an initial denaturing step at 94°C for 90 s, 29 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for 90 s, and a final extension of 72°C for 5 min. Amplified DNA was purified in spin columns (GE Healthcare) according the manufacturer's instructions. The purified product was then cut with MspI restriction endonuclease (10 μl total reaction volume containing 10 U msp1 enzyme, 1 μl of 10× enzyme buffer, 1 μg of BSA and approximately 50 ng of PCR product) and incubated for 2 h at 37°C. MspI cleaves the DNA at a specific sequence (CCGG). The resulting profile of DNA fragments represents a 'fingerprint' of the taxonomic composition of the final bacterial communities. The tRFLP profiles were obtained on a Beckman CEQ2000 capillary sequencer and analysed using Genemarker (Softgenetics™) software.

Experiment design and analysis

The experiment consisted of testing each protozoan strain on the standard bacterial community. Each protozoan strain was assayed in triplicate. We were interested in explaining the variation in the tRFLP profiles in terms of the traits and phylogenetic information for each protozoan strain. To do this, we converted the tRFLP profiles into a matrix of pairwise tRFLP dissimilarities (Bray-Curtis dissimilarity). We predicted that the composition of the bacterial community was determined by the suite of traits and phylogenetic history of the protist strain. Protist strains that had different trait characteristics or that were only distantly related were predicted to result in different bacterial communities. Significant association between the trait and phylogenetic dissimilarity matrices and the tRFLP dissimilarity matrix was tested using Mantel and partial-Mantel tests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Protist traits used in the study.

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Chapter Seven

General discussion, conclusions, and future perspectives

Importance of this work

This work appears in the context of increased attention on species- and taxon-specific investigations of protozoa, conducted to further understand speciation, biodiversity, phylogenetic relationships, and species interactions within microbial food webs (Boenigk et al. 2005, Cleven and Weisse 2001; Kinner et al. 1998). Therefore, the results reported here are important because, as with recent work on Cercozoa (Howe et al. 2009, 2011a, 2011b), they offer substantial pioneering advances in the understanding of Apusozoa, free-living zooflagellates thought to inhabit most aquatic and soil microbial assemblages. Their presumed role as predators and prey within microbial assemblages, coupled with their under-sampled taxonomic diversity and environmental tolerance characterized here, suggest Apusozoa may play a fundamental role within ecological networks in natural ecosystems.

Methodological advances

On a broader level, these papers also boast a series of methodological advances that can be applied to other overlooked protozoan groups. For example, the gene alignments (available online) used in **Chapter 2** can be used as an initial template for studies of other under-studied organisms once isolated by intensive and selective culturing techniques. Furthermore, the phylogenetic reconstructions derived from those alignments, apart from providing a rationale for the inclusion of mantamonads within Apusozoa, served to demonstrate the important influence

of good taxon sampling, choice of target genes, alignment masks, and calculation algorithm, on the final topology of phylogenetic trees.

Chapter 3 also provides a methodological insight with potential future applications, whereby a published method of species delineation, using ITS2 rDNA secondary structure, is for the first time applied to planomonads, a group currently undergoing major taxonomic scrutiny. This technique provides an objective way of distinguishing between genetically and morphologically similar species and could in future be applied to other taxonomically enigmatic protozoan strains and, in particular, to superficially indistinguishable colourless flagellates. Other transferable methodological components of this study are the group-specific molecular probes, reported in **Chapter 4**; some of their potential applications in future studies are described later in this chapter.

Chapter 5 presents a new method of mining multiple databases of metagenomic environmental sequences by seeding searches with short apusozoan signature reads. Simply by varying the seed, this method could easily be applied to other protozoan groups and provides a unique way of characterizing the phylogenetic affiliation of otherwise unidentified environmental sequences.

Metadata attached to each sequence within the databases also provides valuable information for assessing diversity detected across different sequencing technologies, between genomic and transcriptomic studies, and between sequences obtained by PCR or amplification-independent means. According to the relatively conservative clustering and phylogenetic methodology employed in Chapter 5, amplification-independent, RNA-based, methodologies may be more effective at detecting rare lineages within microbial assemblages. Such systematic

application of metadata to investigate the relationship between genetic diversity and methodology is an important new framework presented in this thesis, with relevant applications to any future diversity studies focusing on low abundance microorganisms.

Finally, the experimental design of the published study in **Chapter 6** also has vast potential as a template for future investigations of predator-prey relationships between protozoa and bacteria, as long as the protozoan genetic characteristics are known. Planomonads, for example, would make an ideal future study group for microcosm grazing experiments, largely as a result of the taxonomic contributions presented here.

The discovery of Mantamonadida

I was unable to expand the study to focus on putatively apusozoan groups, such as *Discocelis*, *Micronuclearia* and *Breviata*, but the methodological framework provided here could be applied to these and other under-sampled taxa. To that end, the fortunate discovery of *Mantamonas plastica* exemplifies the extent to which higher-level apusozoan diversity is unknown, particularly given the sudden proliferation of mantamonad sightings after its initial isolation: the isolation of a second sequenced genotype followed by observations of mantamonad-like cells from marine isolates taken in the Atlantic (Mexico) and Pacific (Japan) oceans, as well as informal reports from a colleague that similar cells were also alive in a sample from the Pacific Ocean.

Though apparently far outnumbered by planomonad and apusomonad sequences, mantamonad metagenomic sequences belonging exclusively to both

known 18S rDNA types were found in online databases. Furthermore, the environmental survey detected *M. plastica* in freshwater and soil samples, and salinity gradient experiments reveal that the strain can thrive in brackish media. Together, these results suggest *M. plastica* and *Mantamonas* sp. Tanzania inhabit marine sediment in at least the Atlantic, Indian, and Pacific oceans, but may also inhabit non-marine environments: a remarkable find given the complete absence of this organism in the historical literature.

Additional molecular surveys, preferably of RNA, could more accurately characterize the ecological tolerance of mantamonads in the natural environment by targeting active gene expressions to confirm that cells are alive within positive samples and thus discounting the detection of extracellular DNA or cysts.

Apusomonadida and Planomonadida: an environmental perspective

I isolated two apusomonad strains, from marine sediment in the Atlantic Ocean (Portugal) and the Mediterranean Sea (Cyprus). The 18S rDNA of these isolates is nearly identical to the uncharacterized *Thecamonas* sp. (contaminant in cryptomonad AY050179) presented in Cavalier-Smith and Chao's (2010) recently revised apusomonad taxonomy. A follow-up paper is currently being prepared featuring a morphological description of this lineage (Figure 1), alongside an apusomonad multi-gene analysis that will include heat-shock protein *Hsp90* and cytochrome c oxidase subunit 1 (*cox1*) genes.

Thus, at present the genetic relationship between apusomonad genera remains unresolved; the topology of the most recent trees (Chapter 5, Figs. 1-3), which include environmental sequences, is weakly supported. However, sequence

signatures shared by multiple apusomonad lineages make it possible to detect deeper diversity in the environmental survey, and the idea that further apusomonad diversity exists is commensurate with preliminary data from a large-scale marine environmental study carried out using next-generation 454 sequencing (BioMarKs, see Future Perspectives below). Our study therefore shows evidence that apusomonads are far more diverse than we currently know from culturing alone and, moreover, adds important environmental context to new and existing lineages.

As mentioned in Chapter 4 (Discussion), a useful future direction for this project would be to combine apusozoan sequences from described species with the new environmental lineages presented in Chapter 5 as well as all the Apusozoa-like sequence reads retrieved from online databases (listed in Chapter 4, Table 4). Given the short length and low quality of some of these reads, this procedure would best be accomplished using a linear time phylogenetic placement application such as *pplacer* (Matsen et al. 2010). Such an alignment, its accompanying metadata, and resulting estimated phylogenetic relationships, would not only provide a compilation of known apusozoan diversity but could also provide unprecedented insight into the relationship between diversity detected and method used.

Lastly, this collection of papers presents an important contribution to the current taxonomic revision of planomonads. The paper featured in Chapter 3 is our answer to two rebuttals by Heiss et al. (2009, 2011), written in response to the initial Planomonadida paper written by Cavalier-Smith et al. (2008). Briefly, the discussion is grounded in an interpretation of Savile Kent's (1882-84) description

of *Ancyromonas sigmoides*, a free-living heterotrophic flagellate. Cavalier-Smith et al. claim that most published descriptions of *A. sigmoides* describe an uncharacterized organism other than the one Kent observed, but Heiss et al. disagree, citing similarities between Kent's drawings and planomonads.

In future, it is important that disagreements over basic taxonomy do not overshadow the importance of diversity and ecology studies: the work presented here is primarily based on the culturing and isolation of new strains and, regardless of the outcome of the taxonomic debate, presents morphologically and genetically distinct new species. Although it is difficult to find conclusive evidence in either direction, the work presented in Chapter 3 offers a reasonable argument in favour of further splitting the order into three genera based on combined morphological, molecular, and ultrastructural characteristics.

Methodological implications

The results presented here were made possible by an intensive and targeted culturing effort, whereby apusozoan strains were isolated from environmental samples using serially diluted sub-cultures. The resulting monocultures were the basis of behavioural observations and morphological diagnoses, provided the genetic material for 18S rDNA gene sequences, and were subsequently used to design group-specific molecular probes. Culture media was prepared by adding a single wheat grain to sterile artificial seawater (ASW) or mineral water (Volvic) and cultures were kept at room temperature and in natural light conditions. However, previous experiments have shown that a large proportion of flagellates can be lost

during the isolation process, particularly if medium acclimatization steps are lacking (Boenigk et al. 2005). In future, in hope of isolating more sensitive apusozoan species, mixed environmental cultures could be grown in several types of media and under differing conditions, including with acclimatization steps.

Microscopy studies

Living apusozoan cells from mixed environmental samples were isolated by several rounds of sub-culturing by serial dilution. Although monocultures of several species were isolated, serial dilution inherently relies on an element of chance and can be both ineffective and laborious. To avoid these biases, other isolation methods could be attempted in future; for example, the raw culture approach (Ekelund 1999; Vørs 1992), the none-flooded petri-dish method (Foissner 1987), or the cover slip method (Rasmussen et al. 2001).

To bypass culturing altogether, single-cell polymerase chain reaction (SC-PCR), has been shown to be effective in protist diversity studies, carried out either by placing a single cell in a PCR reaction tube and amplifying the gene of interest (Auinger et al. 2008), or by PCR amplification of RNA or DNA extracted from a single cell (see review by Lynn and Pinheiro 2009). However, this technique, which does not require the creation of monocultures, makes it more difficult to study morphology and impedes accurate taxonomic interpretation, and was therefore not a good option for this study due to its explorative nature.

I did not employ any systematic strategies for obtaining apusozoan monocultures from environmental material. Previous studies show that microorganisms generally exhibit high turnover rates in natural environments and

that microbial species composition, cell counts, biomass, and activity levels, can vary substantially according to short-term fluctuations in environmental variables, such as the availability of sunlight, temperature, and nutrient levels (Amblard et al. 1994; Berdjeb et al. 2011; Jugnia et al. 2000; Sime-Ngando et al. 2008; Sime-Ngando and Hartmann 1991; Winter et al. 2004). Moreover, other work has shown that the structure and dynamics of microorganisms may change over longer timeframes and, in particular, by season (Fonseca and Bicudo 2008, 2011). Therefore, it is likely that both short-term and long-term environmental factors influenced the apusozoan diversity we observed in cultured material and detected by PCR in environmental DNA (eDNA). Future work, linking abundance and diversity to the environment, best done by targeting RNA, and thereby examining *in situ* gene expression patterns at a particular time and place, could investigate how these biotic and abiotic environmental changes affect perceived diversity, including by screening samples taken from the same site at regular intervals over the course of several years.

With Aaron Heiss, I carried out Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) on *Fabomonas tropica* and *Mantamonas plastica* during a visit to the Alastair Simpson lab at Dalhousie University, Canada (June 2007). Whereas important ultrastructural traits common to all planomonads, such as flat mitochondrial cristae and a single-layered proteinaceous dorsal pellicle, were revealed in the *F. tropica* fix, we were unable to observe the ultrastructural features of *M. plastica* because cells ruptured during the fixation process. For practical reasons we were unable to perform a second fixation, though this would be taxonomically valuable because it could provide ultrastructural evidence linking

mantamonads to one of the two major apusozoan orders. Given the fragile nature of mantamonad cells, future EM fixes could be improved by using a higher density of cells, thus increasing the likelihood of finding cells intact for observation.

PCR amplification and clone libraries

During the environmental survey, phylogenetic analyses were run using sequences derived from cloned amplified eDNA, extracted from marine and freshwater sediment, filtered water, and soil. The results were therefore subject to the well-documented biases inherent to the PCR and cloning processes.

To that end, humic acids, complex polyphenols produced during the decomposition of organic matter and present in soil and water (Tsai and Olson 1992), represent important inhibitors of PCR and the effect of these can be lowered either by physically purifying or chemically enhancing the PCR reagent mix (Demke and Adams 1992; Kreader 1996). Therefore, the DNA template was purified by diluting eDNA by 1/10 before the initial PCR reaction and by further diluting the initial PCR reaction product by 1/100 prior to nested reactions. I also added bovine serum albumin (BSA), known to facilitate PCR by increasing DNA polymerase stability and reducing the loss or reagents through adsorption to tube walls (Nagai et al. 1998; Promega 2011), and dimethyl sulfoxide (DMSO), an organosulfur compound used in PCR to disrupt secondary structure formation in the DNA template by hydrogen bonding to the major and minor grooves of template DNA (Hardjasa et al. 2010; Simon et al. 2009).

Given more time, experiments could be carried out to investigate the effect of different stabilizing agents and alternating PCR protocols on perceived diversity of Apusozoa. Apart from the use of alternative reagents, such as thermostable, inhibitor-resistant DNA polymerase (Al-Soud and Rådström 2000; Al-Soud et al. 1998), organic solvents, and proteinase inhibitors (Chandler et al. 1998), it is possible that annealing temperatures also affect perceived diversity. Similarly, a standardized approach to extracting eDNA (material screened, elution concentration), here carried out by several individuals over an extended period, would eliminate biases created by the differential quality of the PCR template.

Using amplification-dependent techniques to assess diversity also has other limitations (Doherty et al. 2007). For example, 'universal' eukaryote primers have been shown not to amplify all eukaryotic targets equally in DNA extracted from mixed assemblages, leaving some groups overrepresented in clone libraries (Bass and Cavalier-Smith 2004; Potvin and Lovejoy 2009; Farris and Olson 2007; Hansen et al. 1998). The size of target cells, with larger cells often containing more DNA, may also influence relative amplification rates (Cavalier-Smith 2005), and could partly explain lower detection rates of the relatively smaller mantamonads in PCR-based environmental studies featured in the online databases (Chapter 5).

Finally, primers may differ in their effectiveness because of molecular secondary structure, thus differentially detecting diversity from the same sample (Doherty et al. 2007; Stoeck et al. 2006). I adjusted the PCR protocol to consider these potential biases, most markedly by designing and testing up to two sets of primers for each round of PCR reactions. Based on published observations that

perceived diversity is inversely related with amplicon size (Sogin 2006), and that shorter fragments are preferentially picked up during the plasmid ligation step in cloning (Huber et al. 2009), I also tried to amplify the shortest possible fragment containing enough variable nucleotides to enable our analysis.

Our apusozoan environmental sequences were from clone libraries of amplified eDNA using group-specific primers. However, several factors inherent of cloning are known to complicate diversity assessments. Firstly, chimeric sequences can be formed during PCR due to the recombination of partially homologous templates, either by template switching during a single PCR cycle or, in subsequent cycles, by truncated extension products annealing to partially homologous templates (Odelberg et al. 1995). According to published recommendations (Judo 1998), I reduced the chances of obtaining chimeric sequences by lengthening elongation times and minimizing the number of total PCR cycles.

Secondly, diversity assessments can be influenced by intra-individual RNA polymorphisms, or sequence variation in multi-copy genes such as the rDNA cistron (Dover et al. 1982). I took this into account in interpreting highly similar internal transcribed spacer (ITS) rDNA types, although secondary structure analysis, as carried out in the planomonad study, provides a quantitative level of measuring diversity. Alternative, cloning-independent, methods are presented in the Future Perspectives section of this chapter.

Species delineation and phylogenetic analysis

To describe differences between closely related planomonad strains, I sequenced and predicted, with Tom Cavalier-Smith, the secondary structure of the ITS2

rDNA within the nuclear ribosomal cistron. This molecule varies in sequence and secondary structure in a way that has been shown to correlate highly with taxonomic classification (Coleman 2009); the methodology, as applied to planomonads, is explained in detail in Chapter 3.

The hypervariable ITS1 rDNA gene, on the other hand, is less frequently used for phylogenetic purposes, although pioneering studies show that it may also be examined for high-resolution, species-level analyses (Hoshina 2010). To that end, extending the planomonad secondary structure analysis featured here to include ITS1 rDNA secondary structure folds would both contribute to a growing database of eukaryote-wide ITS1 rDNA secondary structures and verify species delineations indicated by the ITS2 rDNA folds.

Furthermore, as demonstrated with mantamonads, ITS1 rDNA can be additionally informative because it may be amplified alongside the 3'-end of the 18S rDNA molecule, important for the purposes of verifying the source of the read. Although secondary structure analysis is now a widely accepted method within protozoan systematics, interpretation of predicted folds remains a controversial and sometimes divisive subject area and, therefore, it is important to interpret results, as presented here, in the context of other morphological, ultrastructural, and genetic data.

Most of my phylogenetic trees were reconstructed based on 18S rDNA sequences. This is a commonly used marker because it is already well documented, relatively easy to amplify, and evolves at a rate that is appropriate with other characters of speciation (Amaral-Zettler et al. 2009; Brown et al. 2009; Diez et al. 2001; Heywood et al. 2011; Massana et al 2004; Moon-van der Staay et al. 2001;

(Pawlowski and Lecroq 2010). However, other molecular markers, most notably the mitochondrial protein-coding genes *cox1* and cytochrome *b* (*cytb*), have been proposed as potential alternative universal markers for the identification of protist species, although studies have so far mostly focused on red algae and ciliates (Lane 2009; Lynn and Strüder-Kypke 2006; Ogedengbe et al. 2011; Saunders 2005). The cytochrome-encoding genes may be advantageous for taxonomic purposes because they, a) are present in all eukaryotes and function homologously across most of them, enabling sequences to be effectively compared, b) are variable enough that they can be used to discriminate between closely related species, c) are easily amplified, particularly the *cox1* gene, using universal primers targeting conserved regions (Folmer et al. 1994), and, d) compared to ribosomal genes, are less likely to contain insertions, deletions, or other large-scale rearrangements, since mitochondria reproduce by binary fission and without sexual recombination (Chantangsi et al. 2007).

Time restrictions meant I was not able to obtain cytochrome-encoding genes for Apusozoa, although a project is currently underway to sequence these in apusomonads and contrast them with 18S rDNA sequences. A similar project for planomonads would be equally valuable, in particular to complement the taxonomic advances presented within these papers.

I used the character-based (tree-searching) maximum likelihood (ML) and Bayesian methods for the gene phylogeny reconstructions presented in figures. Character-based methods search for the most probable tree for a specific sequence set based on characters at each position of the sequence alignment and model of evolution (GTRMIXI model was used for ML trees). However, several

other methods exist for inferring evolutionary relatedness, including the character-based maximum parsimony (MP) and distance-based methods, such as neighbour-joining (NJ) trees (discussed at length in a review by Sleator 2011). To guard for the inherent biases of particular phylogenetic methods, I often reconstructed NJ trees and MP trees using two different applications (PhyML and RAxML) in preliminary analyses, to check alignment accuracy, and to get an exploratory idea of the species relationships within the alignment. For the eukaryote-wide study, featured in Chapter 2, I also ran analyses using alignments where portions of variable regions had been differentially removed (masked). The trees showed that the phylogenetic relationship between Mantamonadida and the two other apusozoan orders is consistent across liberal, normal, and conservative alignment masks. Future work could provide more intensive phylogenetic analysis of apusozoan diversity, using different computer packages and algorithms.

Future perspectives

Obtaining sequences by randomly picking clone colonies is relatively expensive and labour-intensive. Consequently, a number of alternative methods, free of a cloning step, have recently been developed and applied to microbial diversity studies. Some of these, like denaturing gradient gel electrophoresis (DGGE), thermal gradient gel electrophoresis (TGGE), or real-time quantitative PCR (RT-qPCR), rely on an initial PCR amplification step; others, like fluorescence in-situ hybridization (FISH), provide measures of abundance and diversity without

amplification, bypassing the biases of PCR. These techniques could all provide informative data for the further discovery of apusozoan diversity.

DGGE and TGGE are methods that rely on the separation and visualization of PCR-amplified genes to obtain profiles, or fingerprints, of microbial communities (Kowalchuk et al. 1997; Muyzer et al. 1993; Nübel et al. 1997). These techniques were originally applied to bacterial assemblages but are now also used to understand the diversity and abundance of free-living protozoa in natural environments (Jousset et al. 2010; Piquet et al. 2010; Rasmussen et al. 2001; Vaerewijck 2011). These techniques are particularly useful for monitoring changes in the composition of microbial communities over time; they could thus be applied to detect fluctuations in relative diversity between apusozoan strains over time at the same location. Furthermore, through the possibility of excising, re-amplifying, and sequencing specific DNA fragments taken from the gels, gene sequences that characterize abundance and diversity data can be obtained.

Another amplification-reliant method of microbial community analysis is real-time quantitative PCR (RT-qPCR), ideal for quantifying target lineage genetic material within an eDNA sample, as inferred from the fluorescent emissions of SYBR Green dye-stained amplicon present in a PCR reaction (Dorigo et al. 2005; Heid et al. 1996; Schmittgen 2001). Traditionally used in clinical trials, real-time PCR has only recently been applied to free living protozoa (Galluzzi et al. 2004) and could potentially be used to quantify apusozoan DNA or RNA in environmental samples using the primers presented here.

FISH, on the other hand, can be carried out without a PCR step. It works by the targeting of specific taxa within a mixed microbial community by fluorescent

rDNA probes designed to attach to group-specific sequence signatures. The fluorophore on the probe causes target genetic material to fluoresce after hybridization with the probes and can be subsequently viewed under an epifluorescent microscope or counted by flow cytometry (Simon et al. 1995). In recent years, FISH has been applied to several protist taxa (Borrelli et al. 2011; Fried et al. 2002; Lim et al. 1993, 1996; Medlin and Kooistra 2010; Rice et al. 1997a, 1997b). For smaller cells, such as mantamonads and planomonads, a variation of fish called tyramide amplification (TSA-FISH) could be used, producing a strengthened fluorescent signal (Lebaron et al. 1997; Schönuber et al. 1997).

Next-generation sequencing

Despite the range of available culture-dependent techniques, it is widely accepted that protist diversity may always be vastly underestimated when inferred by *in vitro* techniques. Recent work suggests around 90% of protozoan diversity is lost throughout the culturing process (Boenigk et al. 2006; Leander and Keeling 2003; Medinger et al. 2010; Slapeta et al. 2005). To that end, recent advances in high-throughput sequencing, or next-generation sequencing, produce affordable large-scale sequencing of genomic and environmental genetic material. Prominent sequencing technologies include Sanger capillary sequencing, Roche/454 GS FLX Titanium sequencing, and Illumina Genome Analyzer II/IIx (see Kircher and Kelso 2010 and Madis 2008 for reviews). It is likely that this rapid proliferation of new technologies, coupled with an increased focus on the species dynamics of microbial assemblages, will uncover a hidden depth of apusozoan diversity both

within and around currently characterized lineages. Preliminary data from the pan-European BioMarKs (Not et al. 2009) marine protist biodiversity survey, for example, using 454 sequencing of around 45 million DNA and cDNA sequences from three depths at nine coastal water sites, suggests that over a thousand sequence reads, representing dozens of new lineages, belong to Apusozoa.

Nevertheless, despite the impending torrent of useful environmental sequences, it remains important to frame these new molecular data within existing morphological and behavioural species characterizations. It is therefore clear that, although labour intensive, culturing methods, such as those providing the foundations for all the work featured in these papers, will continue to influence microbial diversity studies in the coming years and, perhaps even throughout this, the "Century of the Environment".

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Figure legend

Figure 1. Morphology of *Thecamonas* sp. contaminant in CCAP cryptomonad culture AY050179 (unpublished). Differential interference contrast light micrographs of different living cells. Scale bar 10 µl.

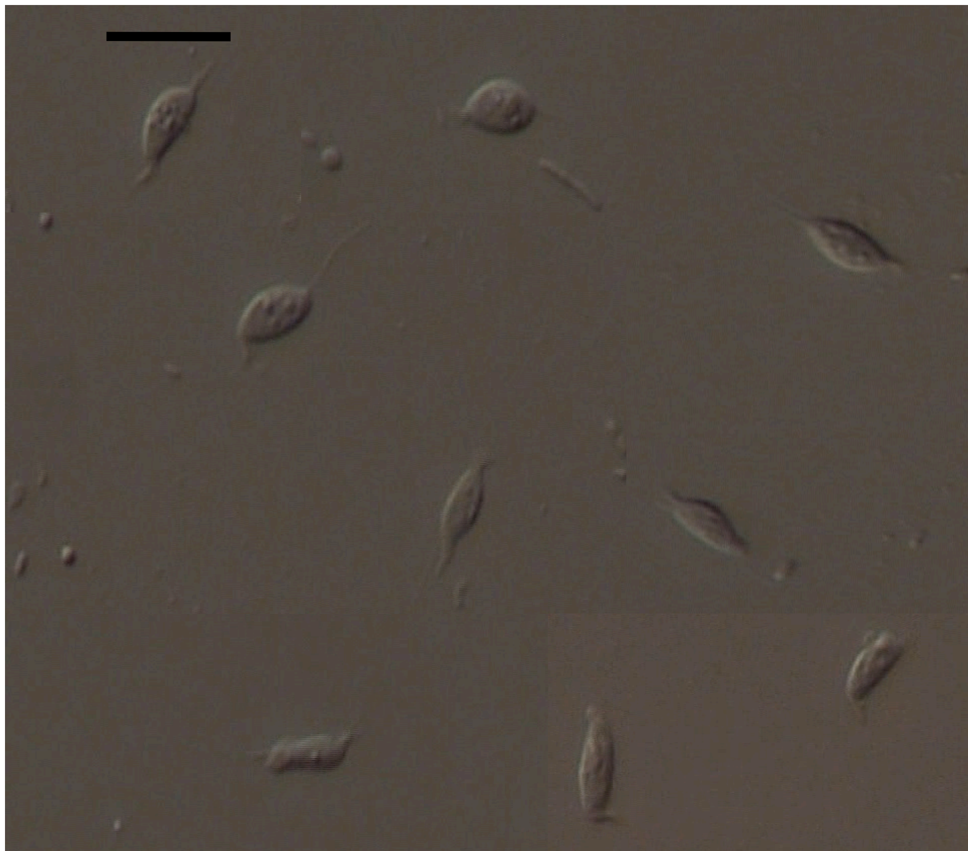

Figure 1

Chapter Eight

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Appendices

Appendix I: Posters and presentations

Appendix II: Parliamentary briefing - ***Biodiversity Offsetting***

Appendix I: Posters and presentations

<u>October 2011</u>
European Commission (DG Environment); Brussels, Belgium
Invited speaker, *Landscapes of the Future* seminar
Talk: Biodiversity offsetting from a UK perspective

<u>July 2011</u>
European Congress of Protistology (ECOP)
Berlin, Germany
Poster: Diversity and ecology of Apusozoa (Protozoa), a mysterious phylum of free-living zooflagellates
Travel Funding, Department of Zoology, University of Oxford

<u>July 2011</u>
International Society of Protistologists (ISOP) Annual Meeting
Seattle, Washington, USA
Talk: Diversity and ecology of Apusozoa (Protozoa), a mysterious phylum of free-living zooflagellates
ISOP Holz-Conner Travel Award and funding from Pembroke College, Oxford

<u>April 2011</u>
British Society of Protist Biology (BSPB) Annual Meeting
University of Southampton, UK
Talk: Exploring the diversity and ecology of Apusozoa
BSPB student travel funding

<u>March 2011</u>
BioMarKs Consortium Meeting
Barcelona, Spain
Talk: BioMarKs and Apusozoa

<u>January 2011</u>
Parliamentary Office of Science & Technology
Houses of Parliament, Westminster, UK
Principal organizer of a parliamentary seminar on *biodiversity offsetting*

<u>July 2010</u>
International Society of Protistology and British Society of Protist Biology
University of Kent, Canterbury
Talk: An exploration of the biodiversity of Apusozoa using traditional culturing and modern molecular methods
ISOP Holz-Conner Travel Award
*** Runner-up: Best Talk**

July 2010
International Society of Evolutionary Protistology (ISEP)
Kanazawa, Japan
Talk: An exploration of the biodiversity of Apusozoa using traditional culturing and modern molecular methods
Dean of Graduates Travel Award, Pembroke College, Oxford

December 2009
ESRC/NERC Knowledge Exchange Workshop
Living with Environmental Change
Botanical Gardens, Birmingham, England

December 2009
Young Systematists' Forum (YSF'09)
Natural History Museum, London, England
Talk: *Mantamonas* gen. n., a major new deep-branching eukaryotic lineage of marine gliding zooflagellates
*** 1st Prize: Best Talk**

August 2009
International Congress of Protistology (ICOP)
Armação dos Búzios, Rio de Janeiro, Brazil
Poster: *Mantamonas* gen. n., a major new deep-branching eukaryotic lineage of marine gliding zooflagellates
Dean of Graduates Travel Award, Pembroke College, Oxford
ISOP Holz-Conner Travel Award

April 2009
British Society for Protist Biology (BSPB) Annual Meeting
University of East Anglia, Norwich, England
Dean of Graduates Travel Award, Pembroke College, Oxford
Talk: Diversity of *Planomonas* (Apusozoa): an updated perspective

August 2008
Symposium of Biology Students in Europe (SymBioSe'08)
Coimbra and Aveiro, Portugal
Talk: Molecular biology and the diversity of Protozoa
Abbey-Santander Travel Award

July 2008
International Society of Protistologists (ISOP) Annual Meeting
Dalhousie University, Halifax, Canada
Talk: Diversity of *Planomonas* (Apusozoa)
ISOP Holz-Conner Travel Award + funding from Pembroke College, Oxford

April 2008
British Society for Protist Biology (BSPB) Annual Meeting
University of Swansea, Gregynog, Wales
Poster: Novel strains of marine *Planomonas*

Appendix II: Parliamentary briefing – *Biodiversity offsetting*

Note

I researched and wrote this parliamentary briefing on NERC-funded Secondment to the UK Parliamentary Office of Science and Technology (POST), under the supervision of Dr Jonathan Wentworth (October-December 2010). The paper was peer-reviewed by a range of experts, including from the Department for Environmental, Food and Rural Affairs (DEFRA). Paper copies were sent out to over 2,000 addresses on POST's mailing list and a PDF version of the document is available online. It was launched in conjunction with a seminar held in Parliament, featuring talks by a panel of experts from relevant fields, as well as parliamentarians.

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POSTNOTE

Number 369 January 2011

Biodiversity Offsetting

Given growing recognition of the importance of biodiversity, all sectors are looking for ways to mitigate the environmental costs of development activity. Biodiversity offsetting refers to market-based schemes designed to compensate for losses of biodiversity due to development projects. This POSTnote summarises biodiversity offsetting and examines opportunities and risks of offsets within a UK context.

Overview

- Biodiversity offsetting is a market-based conservation tool that measures negative impacts on biodiversity, replacing the loss through improvements usually nearby.
- Offsets aim to compensate for residual biodiversity loss incurred by development projects by maintaining an equivalent amount of biodiversity elsewhere that would otherwise be lost, or by enhancing biodiversity at an alternate location.
- Several countries currently implement offset law and policy with different levels of regulation and varying success.
- Offsets aim to achieve ‘no net loss’ or a ‘net gain’ of biodiversity.
- Offsetting remains largely undervalued, especially with regard to undervalued or as yet unknown biodiversity.

Biodiversity

Biodiversity is the genetic diversity within species, species diversity within ecosystems, and ecosystem diversity across landscapes. It supports ecosystem functions vital for human well-being, such as agricultural crops, timber, medicinal plants and industrial raw materials. Furthermore, the services provided by healthy ecosystems indirectly benefit humans by, for example, purifying air and water, regulating climate, generating atmospheric oxygen and providing recreational opportunities (POSTnote 281).

Human Activity and Biodiversity Loss

Species are currently being lost up to 10,000 times faster than the natural rate of extinction. The major cause of this relatively recent trend is the alteration, fragmentation and destruction of habitats caused by human activities, including agriculture, forestry, transport, industry and housing.[1] The annual economic cost of global biodiversity and ecosystem degradation is currently estimated to be £1.28-2.88 trillion, or around 7.5% of global GDP, and is likely to remain at that level through 2050 unless appropriate action is taken.[2]

Tackling Biodiversity Loss

Historically, biodiversity loss has been addressed mainly through multilateral conservation agreements as well as domestic protected area legislation (Box 1). Though widely recognised, this strategy has proved unable to stop the persistent and widespread loss and degradation of biodiversity in almost all regions. Participants to the recent intergovernmental meeting of the 193 parties at the Convention on Biological Diversity in Nagoya, Japan, agreed on several measures to reduce the rate of loss of biodiversity by 2020.[3] Key to the success of future conservation efforts is recognition of the social and economic values of biodiversity by decision makers - including its contribution to trade, economic activity, food security and poverty reduction - as well as its role in providing fundamental ecosystem services.

Box 1. Protected Areas in the UK

Multiple legislative layers aim to protect biodiversity from being destroyed by development. The highest level of protection in the UK is the EU’s Natura 2000 framework, limiting damage to biodiversity within Special Protection Areas (SPAs) and Special Areas of Conservation (SACs). Development in the UK is also restricted at thousands of additional sites including Sites of Special Scientific Interest (SSSIs in England, Scotland, Wales) and Areas of Special Scientific Interest (ASSIs in Northern Ireland). Most of these protected sites were designated before awareness of the major environmental perturbations resulting from climate change, making it a priority to factor in such uncertainties in future protected areas policy (POSTnote 341). Enhancing opportunities for species populations to disperse and adapt to changing conditions requires not only efforts to create buffers, increase the size, condition and connectivity of protected areas, but also the creation landscapes which are more ‘permeable’ to the dispersal or movement of species (POSTnote 300).

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Wider Biodiversity

Limitations to scientific knowledge have led to a selection process whereby most protected areas are primarily based on charismatic species and habitats. The current system of protected areas alone is inadequate in conserving these, and, in addition, data are lacking on the status and trends of habitats, ecosystems and less easily observable species, such as some invertebrate and lower plant groups and microbial communities. However, all natural ecosystems depend on these more common, less charismatic, species to maintain their processes and structures.[4]

What is Biodiversity Offsetting?

Given the growing recognition of the importance of biodiversity to vital ecosystem functions and services that support all aspects of human social and economic development, all sectors are looking for ways to compensate for the environmental costs of human activity. Biodiversity offsets have been proposed as a cost-effective means for sharing this burden.

Biodiversity Markets

Biodiversity markets are being increasingly employed as a means of incorporating the cost of nature conservation into development activities. The basic premise is that, through market-based instruments, the positive and negative impacts on biodiversity can be measured and represented as credits and debits. Thus quantified, they are more easily integrated through cost-benefit analysis into economic decision-making (Box 2).[5]

Box 2. Potential Benefits of Biodiversity Offsetting

Commentators agree that offsetting schemes could potentially benefit conservation but only if they are implemented within an appropriate regulatory framework. Potential benefits include:

- improved clarity for developers
- removal of developer's long-term liability for damage to biodiversity, taken on by a third party organisation (habitat banking)
- places value on nature, introducing incentives for conservation
- increased reliability of long-term conservation projects
- flexibility to 'trade up' and create larger conservation networks
- improved conservation awareness amongst developers
- diversified income streams for landowners
- strengthened conservation partnerships
- enhanced public support for conservation.

Wider Context of the "Mitigation Hierarchy"

The "Mitigation Hierarchy" (Figure 1) is a systematic approach to addressing environmental impact and its potential compensation. This is a stepwise approach first seeking to avoid impacts, then to minimise them, then take on-site measures to rehabilitate or restore biodiversity, before finally offsetting residual, unavoidable impacts. Biodiversity offsets should be considered only for the residual impacts that remain. In implementation of offsets, the minimum objective should be no net loss.

Offsetting Principles

Many countries have enacted laws or introduced policies requiring biodiversity offsets or compensation for certain kinds of impacts. Many are market instruments designed to ensure that development projects result in no net loss of biodiversity. Though there are many forms of compensation, best practice biodiversity offsets adhere to internationally recognised principles (Box 3).

Box 3. Principles of Biodiversity Offsetting

The Business and Biodiversity Offsets Programme (BBOP),[6] a partnership between companies, governments, financial institutions and conservation experts, sets out principles underpinning successful offsets. These are important because badly planned offsets could result in a loss of biodiversity by allowing inappropriate development to proceed, or by compensating inadequately.

Offsets should:

- be designed and implemented to result in **no net loss**, or preferably gain, of biodiversity
- achieve **additional** conservation outcomes above and beyond results that would have occurred anyway
- be used only **after** impacts have been avoided, minimised and biodiversity restored on-site
- recognise **limits** to what can be offset (highly irreplaceable or vulnerable biodiversity is hard or impossible to offset)
- be implemented in a **landscape context**, taking into account biological, social, and cultural values
- **involve stakeholders** effectively in design and implementation;
- be designed and implemented in an **equitable** manner
- planned to secure outcomes that last at least as long as the development project's impacts, and preferably in **perpetuity**
- be undertaken in a **transparent** and timely manner, with results communicated to the public
- document the appropriate use of **scientific methodology** and traditional knowledge in offset design.

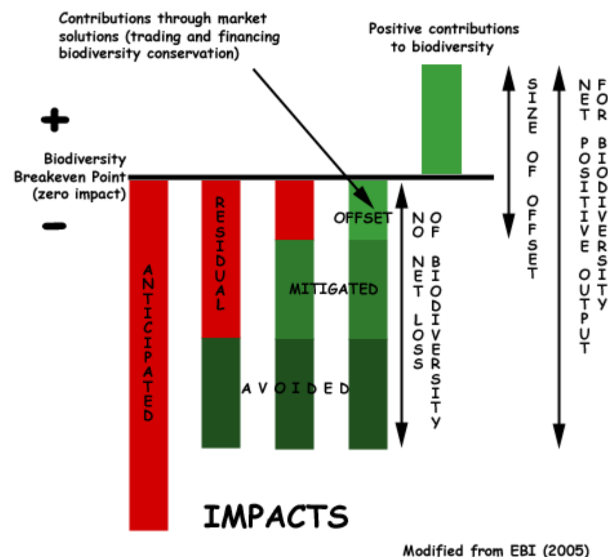

Figure 1. The "Mitigation Hierarchy"

Types of Offsets

The generalised offsetting process involves buyers, sellers, and, in the case of habitat banking, third-party intermediaries.[7] The buyers are developers requiring land for agriculture, industry, housing or other development projects, whereas the sellers are suppliers of the land to be used as an offset for the property to be developed. A range of third-party organisations, including local government, NGOs, insurers, brokers, traders and technical experts, may facilitate interactions between these two parties. Biodiversity offsetting can be executed in three principal ways:

- The **developer** or its partners (such as an NGO) implement the offset
- The developer pays the **government** the amount needed to implement the offset 'in lieu' of implementation itself
- The developer buys **'credits'** from a landowner or conservation bank sufficient to offset its impacts.

The opportunity to aggregate credits into larger, connected offset areas could be beneficial in the establishment of Ecological Restoration Zones, as recommended in the Lawton Review,[8] which, by their nature, need to be extensive in order to deliver ecological functions and services.

Box 4. Biodiversity Offsetting Case Studies

United States: In the US, biodiversity offsetting was initially incorporated into compensatory mitigation laws for wetlands in the United States in the 1970s but market-based schemes, relying on a habitat 'bank' to secure the offset, are now used for a wide range of habitats and individual threatened species. Today, the global annual market size in the US is estimated at minimum £1.15-1.86 billion.[9] The US offsetting strategy is divided into Wetlands and Endangered Species programmes. Wetland or conservation banks may be privately or publicly owned and feature a bank operator allowed to sell habitat or species credits to developers who need to satisfy legal offsetting requirements for compensation.[10] Schemes are controlled by federal policy but implemented regionally in 38 districts of the US Army Corps of Engineers and by offices of the US Fish and Wildlife Services and National Marine Fisheries Service. The system allows buyers and sellers of credits to find each other and agree prices reflecting the cost of land and offset activities. It stimulates third party investment in offset creation as well as standardised units of trading. The market has arisen through strong policy drivers, enforcement, detailed regulation and even an industry association that holds annual conferences (The National Mitigation Banking Association).

Australia: In Australia, offsetting frameworks are encouraged at the federal level under the Environmental Protection and Biodiversity Conservation Act 1999, and reinforced by planning and conservation laws in a number of States and Territories. In 2002, the Victorian government introduced BushBroker®, a system to establish, register and trade native vegetation credits. Under this framework, landowners register their interest in being credit providers. Developers subsequently approach BushBroker® when they need to find an offset. BushBroker® registers all transactions and creates the initial credits by recruiting landowners and conservation bank investors on payment agreement- or land surrender-schemes. In the State of Victoria,[11] there is no explicit legislation for habitat banking but offsets are a legal requirement to protect native vegetation[12] as a prerequisite of planning approval. In New South Wales,[13] a 'biobanking' scheme is regulated by the Department of Environment and Conservation (DEC), allowing developers to voluntarily buy credits to offset the adverse ecological impacts of their development as an alternative to existing threatened species approval schemes.

Operation of Offset Schemes

Existing offset schemes differ according to how they are regulated. However, successful existing strategies, notably in the United States and Australia (Box 4), generally involve an impartial oversight body as well as the various operational actors. Most experts agree that the oversight body is a vital element within the offsetting strategy mechanism.

Offset Size and Quality

In countries where offsetting strategies have been most successful, an important task of the oversight body is to monitor the size and quality of offsets (credits), making sure they are calculated properly so that offset sites are ecologically similar and deliver an amount of biodiversity adequate to offset the impacts. Regulators also facilitate the transparent calculation of offset needs (debits), so developers know how many credits, of which type, they need. Sellers calculate the cost of their credits by estimating the cost of any land purchase or lease needed, habitat creation/restoration, long-term habitat management, compensation procedures, administrative or transaction costs and returns on investment.

Measuring Success

Assessment of the overall success of implemented schemes would involve making sure the execution and consequences of an offset adhere to offsetting principles, deemed as such by an independent scientific body. These outcomes are more difficult to measure at the level of the individual species. With habitats, measures of success may involve vegetation surveys or comparisons of community structure, species diversity or species richness.

Concerns

Biodiversity offsets are controversial. Critics argue that market-based schemes are not effective conservation strategies. Moreover, it is argued, they can even be counterproductive if implemented hastily or in the absence of a proper legislative and regulatory framework. Other concerns include:

- **Perverse incentives** - lowering the threshold of acceptance of conservation outcomes could inadvertently give developers a 'licence to trash'
- **Additionality** - hard to show that 'maintenance' offsets result in outcomes that wouldn't otherwise happen
- **Leakage** - if not chosen properly, offsets could simply displace impacts that would have happened anyway, for example, if you create a protected area to offset the impacts of a mine, those who were previously harming biodiversity in the area (e.g. illegal timber/poaching) move to another location and have the same impact there
- **Restoration difficulties** - some habitats, like grasslands and heathlands, can be difficult to restore in terms of the time and technical skills required, others, such as ancient woodland, are impossible to recreate within human timescales
- **Definition and valuation of biodiversity** - unlike carbon credits, biodiversity measurements cannot easily be based on a single, quantifiable unit. Defining and quantifying biodiversity losses and gains always involves a subjective element, as at present, measuring every component of biodiversity is not achievable and knowledge of biodiversity is incomplete (e.g. at the microbial and genetic level). Other crucial issues may also be overlooked, such as the effects of habitat fragmentation on dispersal, ecosystem function, and the loss of genetic diversity, as well as social views on the definition and value of biodiversity

- **Species recovery lag-time** - long recovery times of some species may not be achieved within the timeframe of the offset and losses may therefore be irreversible
- **Defining 'enhancement'** - the upkeep of the offset site may include actions that could alter the landscape, compromising long-term viability of other species, so enhancement goals should take into account the health of entire ecosystems
- **Difficulties with management and compliance** - proper enforcement is vital. Experience shows that early offsetting schemes abroad suffered from low rates of compliance, despite agreed conditions by key parties. However, in the US the introduction of new performance standards, allowing for fewer alternative ways to implement offsets, have improved enforcement
- **Equity** - offsetting too far from development sites would mean local communities could lose cultural values associated with the biodiversity, access to green spaces and other ecosystem services.

Policy on Offsetting

Several countries in the EU practise a form of offsetting (Box 5). In England, the Lawton Review highlighted the potential importance of offsets in enhancing and preserving coherent, resilient, ecological networks (POSTnote 300). Offsets also featured in the Conservative Party's 2010 Election Manifesto. Proponents argue that measuring the impact of development on nature, and encouraging developers to take responsibility for their footprint, may be the only way to address biodiversity degradation.[14]

Box 5. Offsetting and the European Union
The EU has specific requirements for ecological compensation under the Birds[15] and Habitats[16] Directives, in cases where the integrity of the Natura 2000 network might be compromised. Member States vary in the extent to which they have developed specific laws requiring offsets. Germany has had a system of ecological compensation called the *Eingriffsregelung*, in place since 1976, a framework that is broadly applied to entire ecosystems, their capacity and the resulting natural scenery. The broad coverage of this legislation means it is particularly difficult to evaluate whether offsets are of appropriate quality vis-à-vis conservation goals. In Sweden and France, some offsets are already in place and others are being considered, though these are implemented at the local level.

Regulation

It is vital that offsets are appropriately determined based on predicted losses of biodiversity, requiring rigorous methodology to determine what trade-offs are appropriate or allowable. Furthermore, there must be a strong assurance about delivery, likely requiring an accountability mechanism and funds set aside in every offset transaction to support monitoring and auditing. Most relevant parties in the UK agree that implementing offsets solely on a voluntary basis would not generate enough interest to establish a viable biodiversity market and would lack the rigour necessary to produce the desired 'no net loss' outcome.

Role of Government

Given the necessity of an impartial referee within the offsetting process, one of the main concerns of stakeholders is the role of government within prospective offsetting frameworks. Local Authority representatives, NGOs or the appropriate government conservation agency (Natural England, Scottish National Heritage, Countryside Council for Wales, Northern Ireland Environment Agency) could potentially carry out regulatory duties. However, the degree of government involvement could vary, but may include the establishment of a general offsetting policy framework, collection and publication of information about biodiversity, setting technical standards, establishment of a framework for long-term ecological monitoring or provision of incentives for developers to adhere to policies.

Introduction of Pilot Schemes

In the UK, offsetting schemes are currently only applied on a site-by-site basis, including as part of a pilot study currently underway at the Thames headwaters. Though details of loss/gain calculations and potential delivery mechanisms have yet to be decided, at least one habitat banking organisation is in place[17] and possible strategies of offset quantification have been published.[18] This is in line with the Lawton Review, which concluded that if a formal offsetting system is to be introduced in the UK, "pilot schemes should be first established to test and refine its operation, to ensure it meets the conditions set out for a safe and effective system."[19]

Wider Concerns

Understanding offsetting within the wider context of the mitigation hierarchy, it is clear that avoidance and minimisation need to be given attention equal to that given to compensation or offsetting. Maintaining healthy, viable ecosystems over the long term contributes to human intergenerational well-being. To this end, biodiversity conservation needs to have a large-scale strategic vision, within which biodiversity markets, including carefully constructed and well-managed biodiversity offsetting schemes, may have a role to play.

Endnotes

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11 http://www.dse.vic.gov.au
12 Native Vegetation Regulations under the Planning and Environment Act
13 The Threatened Species Conservation Amendment (Biodiversity Banking) Bill 2006
14 Information note UNEP/CBD/COP/10/INF/27 by the Executive Secretary, Convention on Biological Diversity, Nagoya, Japan, October, 2010
15 Council Directive 79/409/EEC on the Conservation of Wild Birds
16 Council Directive 92/43/EEC of 21 May 1992 on the Conservation of Natural Habitats and of Wild Fauna and Flora
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19 http://www.defra.gov.uk/environment/biodiversity/documents/201009space-for-nature.pdf

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26 January 2011

From Richard Benyon MP
Minister for Natural Environment and Fisheries

Dear Mr Glucksman

Thank you for your email of 14 January about your short seminar on biodiversity offsetting. I am sorry that you did not receive a reply to your invitation before the event, this was due to an unfortunate administrative error.

I know that you have been working with Defra policy officials on this, and they would be attending the seminar. I very much hope that the event was a success.

Yours sincerely

Richard Benyon

RICHARD BENYON MP