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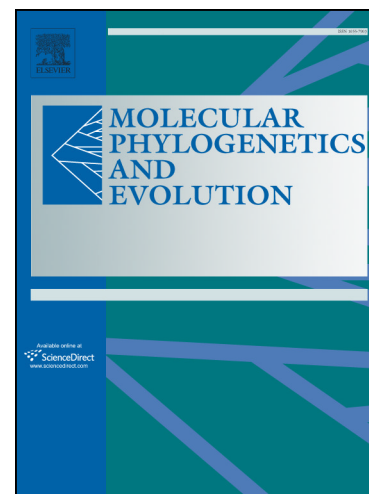
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# Multiple origins and strong phenotypic convergence in fish-cleaning palaemonid shrimp lineages

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## Abstract

Several species of palaemonid shrimps are known to act as fish-cleaning symbionts, with cleaning interactions ranging from dedicated (obligate) to facultative. We confirmed five evolutionarily independent origins of fish cleaning symbioses within the family Palaemonidae based on a phylogenetic analysis and the ancestral state reconstruction of 68 species, including 13 fish-cleaners from the genera *Ancylomenes*, *Brachycarpus*, *Palaemon*, *Periclimenes*, and *Urocaridella*. We focus in particular on two distantly related lineages of fish cleaning shrimps with allopatric distributions, the Indo-West Pacific *Ancylomenes* and the western Atlantic monophyletic *Ancylomenes*/*Periclimenes* group, which exhibit striking similarities in morphology, colouration and complex behaviour. Specifically, representatives of both lineages are similar in: (1) the general body shape and colour pattern; (2) the utilization of sea anemones as conspicuous cleaning stations; and (3) the use of sideways body swaying to visually promote their bright colour spots in order to attract fish clients. Such morphological, ecological and ethological convergences are apparently due to adaptations to fish cleaning linked to the establishment of similar modes of communication with fish clients in these species.

## Keywords

Crustacea; Palaemonidae; cleaner shrimps; behaviour; evolution.

## 1. Introduction

Cleaning is an ecological and behavioural phenomenon widely occurring among animals in terrestrial as well as aquatic ecosystems (Dickman, 1992). The understanding of

the nature of cleaning symbioses has considerably changed over the years, from explaining them as ectoparasitism, through one-sided exploitation and selfless cooperation, to the currently prevailing view as a mutually beneficial interaction (Poulin and Grutter, 1996; Cheney and Côté, 2005; Vaughan et al., 2017). Cleaning symbiosis has been recently defined by Vaughan et al. (2017) as a cooperative interspecific behaviour whereby a cleaner removes and consumes materials that negatively impact a client, and is preceded by communication between the cleaner and client.

Cleaning interactions at marine locations with a high ectoparasitic load result in a higher removal rate of ectoparasites and a concurrent lower rate of removal of other material such as mucus and tissue from the body surface of client fishes, while the opposite is observed when ectoparasite abundance is low (Arnal et al., 2001; Becker and Grutter, 2004, 2005; Feder, 1966). These interactions vary according to multiple factors, from mutualistic (Bshary et al., 2007), to neutral, or even parasitic in nature (Becker and Grutter, 2004, 2005; Grutter, 2000; Cheney and Côté, 2005), and represent a complex array of relationships (e.g., Huebner and Chadwick, 2012a, 2012b; Cheney and Côté, 2005; Titus et al., 2015). Driven by fitness benefits for both partners (Cheney and Côté, 2005; Poulin and Grutter, 1996; Poulin and Vickery, 1995), cleaners may have evolved a reward system to entice hosts to visit them for stimulation and stress reduction, while at the same time providing food for the cleaners (Losey, 1972). As stated by Vaughan et al. (2017), communication between client and cleaner is the catalyst for cleaning cooperation, and we generally distinguish ‘dedicated’ (termed ‘obligate’ in older literature) and facultative cleaning symbioses, and ‘incidental’ cleaning – as a separate form of mutualistic interactions.

In marine habitats, global cleaner diversity is dominated by fish species, recently estimated to comprise of at least 208 species from 36 families (Vaughan et al., 2017), while in decapod crustaceans cleaning symbiosis has only been observed in some 50 species of caridean shrimps of the families Palaemonidae (Fig. 1), Lysmatidae, and Alpheidae, as well as in some stenopodidean shrimps (Vaughan et al., 2017). The majority of palaemonid cleaners are associated with sea anemones that are used as conspicuous cleaning stations, with cleaners mostly occurring in groups of several individuals (Chadwick et al., 2008; Chen and Huang, 2012; Sargent and Wagenbach, 1975). The family Palaemonidae at present comprises over 1,000 described species in 150 genera (De Grave and Fransen, 2011), but dedicated cleaner interactions have only been documented for species belonging to three genera (*Ancylomenes* – 2 spp., *Periclimenes* – 1 sp., *Urocaridella* – 1 sp.). On the other hand, facultative cleaning has been documented in further two genera (*Brachycarpus* – 1 sp., *Palaemon* – 3 spp.) (see e.g., Becker and Grutter, 2005; Chadwick et al., 2008; Östlund-Nilsson et al., 2005; Titus et al., 2015, 2017b; Vaughan et al., 2017).

The majority of palaemonid cleaner species are remarkable for their similarities in bright colouration, body shape, and complex behavioural acts preceding the cleaning. For example, the western Atlantic (WA) *Ancylomenes pedersoni* and *Periclimenes yucatanicus*

wave their long white antennae (Limbaugh et al., 1961; Titus et al., 2017b), while the Indo-West Pacific (IWP) members of the genus *Ancylomenes* ‘dance’ (Becker et al., 2005) or signal with their white-banded clawed legs (chelipeds) to advertise their services (Chadwick et al., 2008; Okuno and Nomura, 2002), or to reduce client aggression (Chapuis and Bshary, 2010).

In our study, we used phylogenetic and ancestral state reconstruction analyses to test the hypothesis that cleaning symbioses evolved independently in multiple lineages of palaemonid shrimps in two geographically isolated (IWP and WA). Furthermore, we demonstrate a striking phenotypic convergence of IWP and WA actiniarian-associated cleaners presently assigned to the genus *Ancylomenes*.

## 2. Material and methods

### 2.1 Sampling

We analysed 39 genera (68 spp.) of palaemonid shrimps, including 13 species of the genera *Ancylomenes*, *Periclimenes*, *Brachycarpus*, *Palaemon*, and *Urocaridella*, for which fish-cleaning behaviour has been reported in the literature or observed in the field by the authors. Further taxa included in the phylogenetic analysis were selected to represent palaemonid shrimps related to the above-mentioned genera, particularly species associated with anemones and other cnidarians. As the key group of interest consists of species which are related to the polyphyletic genus *Periclimenes* (see Horká et al., 2016a), our dataset largely focuses on these taxa to encompass their diversity and host associations with other taxa. Apart from 13 species still formally assigned to *Periclimenes*, 26 additional species belong to genera already separated from it, e.g., *Actinimenes*, *Ancylocaris*, *Ancylomenes*, *Brucecaris*, *Cristimenes*, *Cuapetes*, *Exoclimenella*, *Laomenes*, *Manipontonia*, *Periclimenella*, *Phycomenes*, *Rapimenes*, *Zenopontonia*. These are predominantly ectosymbionts on Cnidaria and Echinodermata, or free-living forms. Endosymbiotic species in molluscs or sponges have been included in our study to a lesser extent, while ascidian endosymbionts were omitted (as they are closely related to mollusc endosymbionts, according to the phylogeny in Horká et al., 2016a). Present phylogenetic dataset thus covers all main Indo-West Pacific and Atlantic palaemonid taxa relevant for this aspect of shrimp biology, containing a wide spectrum of species across the family Palaemonidae, as currently recognized (De Grave et al., 2015).

Specimens of shrimp species were mostly collected by ZĐ during research expeditions to the Great Barrier Reef (*CReefs Lizard Island 2010*) and Papua New Guinea (*Papua-Niugini 2012*), with further samples obtained from research trips to the Caribbean Sea, the Mediterranean Sea, the Red Sea, and the South China Sea. Shrimps were collected directly by scuba diving or by other standard sampling methods and devices (e.g., trawl, grab, suction pump, rock brushing), photographed, and preserved in 70-80% ethanol for subsequent

identification and analyses. The majority of specimens were identified to species level by ZĐ, with complementary material supplied and/or identified by CF and SDG from various regions. Pieces of tissue (pleonal muscle) or eggs were directly preserved in the field in 96-99% ethanol for subsequent molecular analyses. Details of all taxa including sampling locations, voucher numbers, and GenBank accession numbers, are listed in Supplementary Table S1. Information of host associations and cleaning interactions are provided in Table 1.

In-situ movies documenting various elements of cleaning behaviour, such as claw signalling, body swaying or dancing in front of potential clients, were recorded with either a Sony HDV 1080i digital camera housed in an underwater case, or an Olympus Tough TG-4 camera (see Supplementary videos). These movies were recorded on various occasions during shrimp-collecting scuba dives, and were not primarily devoted to research fish-cleaning interactions.

## 2.2 DNA analysis

A total of 261 partial sequences of two nuclear (18S rRNA, histone H3) and two mitochondrial (16S rRNA and cytochrome *c* oxidase subunit I, COI) markers were analysed. Most sequences originated from our previous studies (Fransen, 2014; Horká et al., 2014, 2016a, 2016b), with additional 15 sequences obtained from GenBank, and six taxa newly sequenced. For most taxa we had all four markers available, but we failed to obtain 11 sequences, mostly of 18S (Suppl. Tab. S1).

Total genomic DNA was isolated from posterior pleopods, pleonal muscle tissue or rarely from eggs (with developed embryos) in very small females. The isolation was performed with a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The markers were amplified by the polymerase chain reaction (PCR) technique using the primers: 18Sa2.0 (5'-ATGGTTGCAAAGCTGAAAC-3') and 18S9R (5'-GATCCTTCCGCAGGTTCA CCTAC-3') (Whiting et al., 1997) for 18S rRNA; H3af (5'-ATGGCTCGTACCAAGCAGA CVGC-3') and H3ar (5'-ATATCCTTRGGCATRATRG TGAC-3') (Colgan et al., 2008) for histone H3; LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994) for COI; and either 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al., 2002) or 1471B (5'-CCTGTTTANCAAAAACATGTCTG-3') and 1472B (5'-AGATAGAAACCAACCTGGCTCAC-3') (Liu et al., 2007) for 16S rRNA.

Polymerase chain reactions of newly sequenced taxa were performed in 20 µl volumes containing 1 µl of DNA template, 0.4 µM of each primer, MyTaq Red PCR buffer and 1 U of MyTaq<sup>TM</sup> Red DNA polymerase (Bioline Reagents, London, UK).

Thermal cycling profiles followed Horká et al. (2016a) and Li et al. (2011), and the amplified DNA was purified using the GenElute PCR Clean-up Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol.

Sanger sequencing reactions were performed using an ABI3730XL DNA Sequencer by Macrogen (Amsterdam, The Netherlands). For the mitochondrial marker (COI), the majority of samples were sequenced in both directions. Sequencing of the nuclear markers (H3, 18S) was performed mainly in 5' to 3' directions, but, as with the mitochondrial markers, usually with 2-3 samples sequenced to confirm consistency of the data within a species. The chromatograms were visually checked and manually edited where appropriate using the software Chromas v2.6.4 (Technelysium, Austria).

### 2.3 Phylogenetic analyses

Multiple sequence alignments were constructed in MEGA6 (Tamura et al., 2013) using the MUSCLE algorithm (Edgar, 2004). In the same software, the protein coding sequences (COI, H3) were translated into amino acids to check for potential stop codons within the open reading frame. The substitution saturation for COI and H3 genes was tested in DAMBE v5.3 (Xia and Lemey, 2009) using the index proposed by Xia et al. (2003). The 3<sup>rd</sup> codon position of the COI marker was saturated ( $P < 0.05$ ), and thus excluded from subsequent analyses. Gblocks v0.91b (gap selection criterion = allowed gap positions with half) was used for the detection and elimination of the poorly aligned and highly divergent regions in 16S and 18S rRNA alignments that may not be homologous or may have been saturated by multiple substitutions (Talavera and Castresana, 2007).

Bayesian inference (BI) and Maximum Likelihood (ML) analyses were used to estimate phylogenetic relationships, both via the on-line CIPRES Science Gateway v3.3 (Miller et al., 2010). In both analyses, the four molecular markers used were analysed according to two partition schemes: all markers individually (four partitions), and with mitochondrial coding loci divided by codon position (excluding the third saturated position in COI; seven partitions). The best fit model of nucleotide substitution for each data partition was selected under the Akaike information criterion in PartitionFinder v2.1.1 (Lanfear et al., 2016).

Bayesian analysis was conducted with MrBayes v3.2.6 on XSEDE (Ronquist et al., 2012). The posterior probabilities of topologies, branch lengths and model parameters were inferred independently for each partition (Suppl. Tab. S2) using the Markov chain Monte Carlo (MCMC) algorithm. Two independent MCMC runs of four chains were run for  $20 \times 10^6$  generations with trees sampled and saved every 500 generations, 25% of them were discarded as burn-in. The remaining trees were used to construct the 50% majority rule consensus tree and to estimate the Bayesian posterior probabilities. The convergence of chains was confirmed by analysis of output parameters in Tracer v1.6 (Rambaut et al., 2014). The resulting tree partitioned by markers is presented in Figure 2 (a corresponding tree partitioned



by codon positions can be found in supplementary information). The ML analysis using GTR+I+ $\Gamma$  nucleotide model was conducted in RAxML-HPC BlackBox v8.2.6 (Stamatakis, 2014), ML trees according to both partition schemes are presented in supplementary information. All obtained trees were rooted by outgroup taxa from the family Pandalidae and displayed using ITOL (Interactive Tree Of Life) v3.5.3 (Letunic and Bork, 2016).

Ancestral state character reconstruction (ASR) was performed to evaluate evolutionary pathways of selected phenotypic traits potentially related to the cleaning life style of shrimps, using Mesquite v3.31 (Maddison and Maddison, 2017). A maximum likelihood analysis of ancestral states was based on the BI topology of the final four-marker tree, taking into account branch lengths and using the Markov k-state one-parameter model, which is a generalization of the Jukes-Cantor model (Lewis, 2001), and assumes a single rate for all transitions between character states. The same ASR analysis was also carried out with the ML tree topology, with the results being identical to those from the BI topology, thus only the BI-derived topology is presented.

Host affiliations were coded into five states, as follows: free living shrimps (0), and symbiotic species associated with Cnidaria (1), Echinodermata (2), Mollusca (3), and Porifera (4). Further, we defined categories for the following aspects potentially related to the cleaning life style: shrimp-anemone association (0 – absent, 1 – present), ‘body swaying’ (0 – absent, 1 – present), and ‘advanced signalling’ for cleaning, i.e. claw signalling, antennae whipping, and/or display swimming in front of a potential client (0 – absent, 1 – present), and fish-cleaning (0 – unknown, 1 – reported), with the question mark (?) used for the latter two where such behaviour is suspected, but not yet demonstrated in the literature. The representative colour characters potentially associated with the cleaning life style in palaemonids were delineated as follows: white antennae, white banded chelipeds, pleonal signal spot, and tail eye-spots; with all coded as 0 (absent) or 1 (present). The matrix of all character states is presented in Supplementary Table S3. For estimating the evolutionary correlation between characters, we used the phylogenetically independent contrasts method (Felsenstein, 1985) based on the BI tree (Fig. 2). Analyses were conducted in R using the package “ape”.

### 3. Results and Discussion

Based on our four-marker phylogenetic analysis using two nuclear (18S rRNA, histone 3) and two mitochondrial (16S rRNA and COI) markers, the fish-cleaning palaemonid shrimps are nested in five evolutionary lineages (Fig. 2, Suppl. Figs S1, S2). Further, the ecological and ethological aspects analysed here have clearly independently evolved, as demonstrated by the ASR analyses (Fig. 3, Suppl. Fig. S3).

The most basal-positioned clade (Clade 1, Fig. 2) includes the Indo-West Pacific (IWP) genus *Urocaridella*, while the lineages nested in two further clades (Clades 2 and 3, Fig. 2)

include the eastern Atlantic *Palaemon elegans* and the pantropic/temperate species *Brachycarpus biunguiculatus*. These taxa (Tab. 1) are either dedicated cleaners (*U. antonbruunii*), or facultative cleaners (*Palaemon*, *Brachycarpus*, *U. pulchella*).

Of particular interest are two further evolutionary branches (clades 4 and 5, Fig. 2) that show remarkable similarities in behaviour, morphology and colour patterns of anemone-associated palaemonids. Clade 4 contains the IWP species of the genus *Ancylomenes* placed within a wider assemblage of cnidarian-associated (e.g., *Ancylocaris*, *Rapimenes*, *Pliopontonia*, and their relatives), echinoderm-associated (*Laomenes*, *Cristimenes*), or free-living (*Phycomenes sulcatus*) shrimps. From the ten analysed IWP *Ancylomenes* species, six can be regarded as associated with anemones and exhibiting cleaning interactions (Tab. 1). One of them, *A. longicarpus*, is presently regarded as a dedicated fish cleaner (Vaughan et al., 2017). Cleaning behaviour has not been directly documented in the literature for *A. aqabai*, *A. kuboi*, *A. luteomaculatus* and *A. venustus*, but it seems likely that dedicated or perhaps facultative cleaning also occurs in these species. However, as in Vaughan et al. (2017), we caution against making this assumption merely based on their phylogenetic placement.

A separate clade of fish-cleaners (clade 5) is represented by two western Atlantic (WA) species, *Ancylomenes pedersoni* and *Periclimenes yucatanicus* (see Jonasson, 1987; Limbaugh et al., 1961; Mahnken, 1972; Titus et al., 2015, 2017b). Both are considered as dedicated fish cleaners (Vaughan et al., 2017). *Ancylomenes pedersoni* has recently been indicated by Titus et al. (2017a) to be a complex of at least three WA species of cleaner shrimps covering also a previously recognized *A. anthophilus* (see Holthuis and Eibl-Eibesfeldt, 1964). However, as a proper systematic re-arrangement has not yet been performed, the above-mentioned species, *A. pedersoni* and *P. yucatanicus*, together with the phylogenetically closely related *P. rathbunae*, are still formally the only known WA anemone-associated palaemonid shrimps.

### 3.1 Independent evolution of fish-cleaning lineages

The cleaner shrimps of the genera *Urocaridella*, *Palaemon* and *Brachycarpus* have already been, to various extents (Gan and Li, 2014; Kou et al., 2013), resolved as three independent evolutionary lineages, basally separated from the majority of the major lineage of predominantly symbiotic palaemonids. This is consistent with our phylogenetic analysis (Fig. 2, clades 1–3).

Species of the IWP genus *Urocaridella* (Clade 1) have been frequently referred to as fish cleaners that are both diurnally or nocturnally active (e.g., Becker et al., 2005; Becker and Grutter, 2004; Bonaldo et al., 2015; Bos and Fransen, 2018; Chen and Huang, 2012), and *U. antonbruunii* has been regarded as a dedicated cleaner (Vaughan et al. 2017). They are somewhat similar in general appearance to shrimps of the genus *Ancylomenes* (Clade 4), both in their slender-build transparent body and their colour pattern, but are not associated with sea anemones (Fig. 3, Suppl. Fig. S3). In their morphology, colour pattern and behaviour, the



facultative cleaners *Brachycarpus* and *Palaemon* are different from the dedicated *Ancylomenes*/*Periclimenes* cleaners. *Brachycarpus biunguiculatus* has been noted to act as a nocturnal fish-cleaner (Corredor, 1978), while only three species of the species-rich genus *Palaemon* have been occasionally observed cleaning fish (Östlund-Nilsson et al., 2005; Vaughan et al., 2017), although their diet is largely derived through predation of small-sized prey (Ashelby et al., 2016).

The genus *Ancylomenes*, as presently recognized, contains 22 species (Bruce, 2013), of which only *A. aesopius*, *A. lucasi* and *A. pedersoni* are found outside IWP, in the southern Australia, eastern Pacific and WA, respectively (Okuno and Bruce, 2010). Eight species, namely *A. adularans*, *A. holthuisi* (Fig. 1B), *A. kobayashii*, *A. longicarpus*, *A. lucasi*, *A. magnificus* (Fig. 1C), *A. pedersoni* (Fig. 1D), and *A. speciosus*, have been observed to act as fish cleaners (e.g., Becker et al., 2005; Chapuis and Bshary, 2009; Okuno, 2004, 2005; Okuno and Bruce, 2010; Okuno and Nomura, 2002; Titus et al., 2015). From the IWP species of the genus, only the Red Sea species *A. longicarpus* has been qualified as a dedicated cleaner (Vaughan et al. 2017), although it seems likely that the majority of members of this genus are either dedicated or facultative cleaners.

The remaining two dedicated cleaners in Vaughan et al. (2017) are the WA anemone-associated *P. yucatanicus* and *A. pedersoni*. The suggested evolutionary link between the tropical WA *A. pedersoni* and its IWP counterparts, expressed by the incorporation of those taxa into a single genus by Okuno and Bruce (2010), has already been refuted in the phylogenetic analysis by Horká et al. (2016a), further supported by the present study (Fig. 2). These analyses clearly indicate that *A. pedersoni* and *P. yucatanicus* are closely related to the sympatric, non-cleaning but anemone-associated, *P. rathbunae*.

Based on our ASR analyses (Fig. 3), the cleaning life style evolved separately at the terminal branches within each of the clades. The absence of deeper evolutionary links within the studied attributes suggests the relatively recent emergence of cleaning symbioses in palaemonid shrimps.

### 3.2 Similarities and differences in shape and colour

Representatives of both the Indo-Pacific and Atlantic lineages of fish-cleaning palaemonid shrimps currently assigned to the genus *Ancylomenes* (Clades 4, 5), as well as WA *P. yucatanicus*, share a slender body with a large pleon typical for good swimmers. Their chelipeds are slender and similar in shape, generally equal in size, with elongate chelae. However, the most prominent character shared by both groups is the strongly humped pleon with a species-specific distinct dorsal coloration (Figs 1B-D, 2E-G; Bruce, 1990; Chace, 1958). In contrast to the striking species-specific colour pattern, representatives of the genus *Ancylomenes* differ only in relatively minor morphological details, particularly in the shape of the rostrum, lower orbital angle, interocular process (béc ocellaire), second chelipeds, and the

female pleon (e.g., Holthuis and Eibl-Eibesfeldt, 1964; Chace, 1958; Okuno and Bruce, 2010; Okuno and Nomura, 2002).

All IWP members of *Ancylomenes*, and especially the fish-cleaning species, have a purple-red and white spotted carapace, a pleonal hump with a distinct large colour spot, and a tail fan with large eyespots (Suppl. Fig. S3). Both WA fish-cleaning shrimp species (*A. pedersoni*, *P. yucatanicus*) are brightly coloured as well but differ from the IWP *Ancylomenes* species with regards to their colour pattern. Both WA species (Fig. 2G,H) have the second pair of chelipeds purple-white banded or spotted, like their IWP counterparts (Fig. 2E,F). As in the IWP *Ancylomenes* spp., the WA *P. yucatanicus* (Fig. 2H) also has a large conspicuous colour spot on the dorsally produced pleonal hump, but the dorsum and tail fan of *A. pedersoni* (Figs 1D, 2G) are densely purple-dotted. Similarly, the ‘eye-spots’ on the tail-fan are well-developed in *P. yucatanicus* (e.g., Holthuis and Eibl-Eibesfeldt, 1964; Ritson-Williams and Paul, 2007; Spotte and Bubucis, 1997) but not in *A. pedersoni*, while they are consistently present in all IWP *Ancylomenes* spp. (pers. obs.). The antennal flagella are white, or white-banded, in the WA cleaners (Figs 1D, 2H; see also Spotte, 1997), but mostly transparent in the IWP *Ancylomenes* spp.

### 3.3 Behaviour

In general, the palaemonid cleaning shrimps from both the Indo-Pacific and Atlantic behave in a rather uniform manner. The shrimp attract the attention of a variety of fishes by swaying their body while standing on a conspicuous part of the anemone or substrate. The fish clients will then assume a stationary position close to the shrimp, which boards and cleans the fish by removing parasites, mucus, and infected tissues (Limbaugh et al., 1961; Sargent and Wagenbach, 1975). If direct cleaning of parasites is not done on each occasion, then tactile stimulation from the ‘cleaner’ might be an alternative reward for the fish, as it keeps the clients satisfied and thereby also provides future feeding opportunities for cleaners by returning clients (Losey, 1987; Poulin and Grutter, 1996).

In many cases, shrimps have been reported, to prefer feeding on fish mucus instead of the removal of parasites; this phenomenon known as ‘cheating’ is particularly well studied in the IWP cleaner wrasse *Labroides dimidiatus* (see Bshary and Grutter, 2002). Chapuis and Bshary (2009) suggest that cheating by the IWP cleaner shrimp *Ancylomenes longicarpus* might be less frequent than in cleaner wrasse, as the shrimp is more vulnerable to aggressive responses by clients. On the other hand, both WA cleaner shrimps, *A. pedersoni* and *P. yucatanicus*, were frequently observed cheating on their clients (Sargent and Wagenbach, 1975; Titus et al., 2017b). Therefore, cheating has also convergently evolved in both ‘cleaning’ shrimp clades focussed on herein.

**Cleaning stations.** Cleaners belonging to Clades 4 and 5 are consistently associated with sea anemones as their ‘cleaning stations’ (reviewed in Spotte et al., 1991; see Fig. 3B).

Sea anemones are highly visible and recognizable objects on any reef (Huebner and Chadwick, 2012a), and are exposed during the daytime when most client fish are active. Ancestral taxa of both phylogenetic lineages of shrimp fish cleaners are generally associated with cnidarians (Fig. 3A; see also Gan et al., 2015; Horká et al., 2016a; Kou et al., 2015). ASR analysis reveals an independent switch in both lineages to sea-anemones from other cnidarian hosts (Fig. 3A,B), and subsequently developing cleaning relations with fish (Fig. 3E). In contrast, the dedicated cleaner *U. antonbruunii* and the facultative cleaners of *Palaemon*, *Brachycarpus*, and *Urocaridella* evolved directly from free-living ancestors (Fig. 3A).

**Dancing & colour pattern.** Sideways body swaying (Suppl. Videos S1-S4) is characteristic for most of the *Ancylomenes* fish cleaners (Fig. 3C), e.g., *A. longicarpus*, *A. holthuisi*, *A. magnificus*, *A. venustus*, *A. speciosus* (Chadwick et al., 2008; Chapuis and Bshary, 2010; Okuno and Nomura, 2002), but also for the secondarily free-living congeneric species *A. kubo* (Suppl. Video S1), for which fish cleaning has not yet been observed. Bruce (1977) highlighted a major enlargement of the postero-dorsal lobe of the third pleonal segment with conspicuous colour markings in *A. aesopius* and closely related species involved in fish-cleaning symbioses, and postulated that the pleonal hump was instrumental in the effectiveness of the sideways swaying movement of the body as a signal to advertise fish-cleaning services.

Body swaying and luring fish has also been reported for the WA *A. pedersoni*. Sargent and Wagenbach (1975) and Limbaugh et al. (1961) already described this movement as lateral ‘rocking’ on the last three pairs of pereopods or back and forth swaying, respectively, always together with antennal whipping (Titus et al., 2017; Suppl. Video S4). Such behaviour, however, is not exclusive to *Ancylomenes*, as similar body swaying has been observed for *Urocaridella* species (Becker et al., 2005, pers. obs. IH, ZĐ; Suppl. Video S1).

Our ASR analysis indicates that body swaying (Fig. 3C), as described above, is related to sea anemone host usage, although currently undocumented for the anemone-associated genus *Actinimenes* as well as the majority of the Atlantic *Periclimenes* species living on anemones. As fish cleaning still has not been documented for the entire cleaning lineages, it is clear that the knowledge on the extent of dedicated cleaning and behavioural acts remains incomplete. Independent contrast analysis performed for anemone association and body swaying, based on the ASR topology, thus did not confirm a statistical correlation ( $r^2 = 0.134$ ;  $p = 0.288$ ).

However, body swaying behaviour might not be exclusively linked to fish-cleaning evolution, as it has also been documented for non-cleaning anemone-associated species, such as the IWP *Ancyllocaris brevicarpalis* (see Bruce and Svoboda, 1983; Suppl. Video S1) and the Mediterranean *Periclimenes aegylios* (Suppl. Video S1). Only the former is relatively closely related to *Ancylomenes* (see Fig. 2; Horká et al., 2016a). This may thus be best interpreted as a more ancestral behaviour associated with sea anemones, and subsequently

adopted in a more pronounced form for cleaning advertisement. It is, however, retained in species like *A. kuboi* (Suppl. Video S1) which have secondarily abandoned an association with anemones (Fig. 3A).

**Advanced signalling.** The claw signalling that precedes the cleaning by some IWP palaemonid shrimps consists of vigorous sideways flicking by the second, purple-white banded, chelipeds. Up to now such signalling has been reported for *A. kobayashii* by Okuno and Nomura (2002), *A. longicarpus* (Suppl. Video S2; Chadwick et al., 2008; Chapuis and Bshary, 2010), and was recently observed in *A. venustus* (Suppl. Video S2). According to Chapuis and Bshary (2010), however, this signalling does not primarily serve to attract clients but to convey information about identity as a preconflict management to avoid predation by the client fish. *Ancylomenes magnificus* does not signal by its chelae, but rather performs a striking wave-like swimming display in front of potential clients (Suppl. Video S3), which in this respect might be regarded as a substitute mode for advertising cleaning. Claw signalling, antennal whipping and display swimming are here all regarded as ‘advanced forms of signalling’ by shrimp performed at a safe distance from the potential client. The ASR analysis (Fig. 3D) shows that this behaviour is restricted to our representatives of three cleaner shrimps lineages (i.e. except *B. biunguiculatus* and *P. elegans*), but remains unknown for several species of *Ancylomenes*, one species of *Urocaridella*, and *P. yucatanicus*. It is most likely present in more species but has not yet been observed, or perhaps alternative signalling displays are performed, like extending antennae and tactile contact with clients in *B. biunguiculatus* (see Corredor, 1978).

#### 4. Conclusions

Our phylogenetic and ancestral state reconstruction analyses demonstrate that two geographically and evolutionary distinct clades of fish-cleaning palaemonid shrimps, the Indo-West Pacific *Ancylomenes*, and the western Atlantic *Ancylomenes*/*Periclimenes* group, independently evolved from anemone-associated symbionts to species using their anemone hosts as protective, well-advertised ‘cleaning stations’ on coral reefs. Representatives of both lineages developed similar fish-attracting behavioural and morphological traits to advertise cleaning services and reduce client aggression. Despite this independent evolution, in many aspects they are strikingly similar (including the use of anemone hosts as cleaning stations, sideways swaying body with bent and brightly coloured pleon, and communications with client fish). Both lineages contain efficient cleaners capable of removing high loads of ectoparasites, i.e., IWP *Ancylomenes holthuisi* and WA *A. pedersoni*.

Based on ancestral state reconstruction analyses of partial behavioural acts of the cleaners and their relatives, dedicated cleaning is likely more prevalent among IWP *Ancylomenes* species than currently documented. The easily recognizable ‘advanced forms of signalling’ examined in this study, may be a precursor to dedicated cleaning in shrimps,

allowing researchers to explore this phenomenon further by *in-situ* observations or experiments.

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## Figure and table captions

**Table 1.** Summary of host associations and cleaning interactions in analysed palaemonid shrimps. Terms and symbols for Cleaning interactions: dedicated, facultative – as defined by Vaughan et al. (2017), none – without fish-cleaning interactions, pers. obs. – personally observed by authors; reported – type of cleaning not specified; unreported – related to cleaners but cleaning not reported.

**Figure 1.** Palaemonid cleaner shrimps in interactions with fish clients, A-C Indo-West Pacific, D Western Atlantic. A, *Urocaridella pulchella* on the fimbriated moray *Gymnothorax fimbriatus*; B, *Ancylomenes holthuisi* on the lizardfish *Synodus* sp.; C, *A. magnificus* on a snake eel (Ophichthidae); D, *A. pedersoni* on the glasseye snapper *Heteropriacanthus cruentatus*. Authors of photographs: A.H. Hadi (A); T. Gurney (B); J. Belanger (C) (all with the permission of Shutterstock); B. Kráčmar (D).

**Figure 2.** Phylogenetic tree (based on Bayesian inference) of palaemonid shrimps showing five lineages of fish cleaners highlighted (clades 1–5); fish-cleaner shrimps (see Table 1), are marked with a fish symbol. Bayesian posterior probabilities and RAxML bootstrap supports expressed as percentages, respectively, are indicated on nodes. A dash (–) indicates RAxML values <50; an asterisk (\*) indicates different topology of the RAxML tree. The presented data are based on analysis of the dataset partitioned by markers; a

Bayesian tree based on the dataset partitioned by codon positions, and those based on RAxML analyses, are provided in Suppl. Figs 1 and 2, respectively. Letters (A-H) after selected species names refer to inset photographs (courtesy of D. Van Beeck (A), F. Sumolang (B) (both with permission of Shutterstock), P. Wirtz (C, D), A. Ryanskiy (E, F), C.H.J.M. Fransen (G), Z. Ďuriš (H).

**Figure 3.** Phylogenetic hypothesis with ancestral state reconstruction assessed by maximum likelihood criterion for selected ecological and behavioural aspects related to the cleaning life style of palaemonid shrimps, based on the topology of the Bayesian tree (Fig. 2). Pie charts predict the character state in nodes.

### Supplementary information

**Supplementary Table S1.** Details of material used in the phylogenetic analyses, including sequences downloaded from GenBank. Newly obtained sequences are marked in bold. MNHN: National Museum of Natural History, France. MTQ: Museum of Tropical Queensland, Australia. NTOU: National Taiwan Ocean University. PNG: Papua New Guinea. RMNH: Naturalis Biodiversity Center, Leiden, the Netherlands. UO: University of Ostrava, Czech Republic. USVI: U.S. Virgin Island; N/A - sequence not available.

**Supplementary Table S2.** Substitution models selected in Partition Finder for H3, COI, 16S rRNA, and 18S rRNA genes based on the Akaike information criterion, and alignment lengths. Models for protein-coding genes (H3, COI) are shown for the 1<sup>st</sup>, 2<sup>nd</sup> or 3<sup>rd</sup> codon positions; saturation test was applied according to Xia et al. (2003).

**Supplementary Table S3.** Matrix of characters associated with the cleaning life style of the palaemonid shrimps used in ancestral character reconstruction.

**Supplementary Figure S1.** Phylogenetic tree of palaemonid shrimps showing five independent lineages of fish cleaners resolved by Bayesian analysis based on the combined dataset for four markers (COI, 16S, H3, 18S) partitioned by codon positions.

**Supplementary Figure S2.** Phylogenetic trees of palaemonid shrimps showing five independent lineages of fish cleaners resolved by RAxML analysis based on the combined dataset for four markers (COI, 16S, H3, 18S) partitioned by genes (A) and codon positions (B). Bootstrap supports  $\geq 50$  are displayed.

**Supplementary Figure S3.** Phylogenetic hypothesis with ancestral state reconstruction assessed by the maximum likelihood criterion for selected colour characters associated with the cleaning life style of the palaemonid shrimps, based on the topology of the Bayesian tree (Fig. 2). Only terminal branches are presented for the trees B – E. Pie charts predict the character state in nodes.

**Supplementary Video S1.** Sideways body swaying of palaemonid shrimps: *Ancylomenes holthuisi*, *A. venustus*, *A. kuboi*, *Ancylocaris brevicarpalis*, and *Urocaridella antonbruunii*, Vietnam, 2008, and *Periclimenes aegylios*, Turkey, 2011 [video by Z. Ďuriš].



**Supplementary Video S2.** Sideways body swaying and claw signalling by shrimps

*Ancylomenes longicarpus*, Aqaba, Jordan, 2008, and *A. venustus*, Great Barrier Reef, Australia, 2010 [video by Z. Ďuriš].

**Supplementary Video S3.** Sideways body swaying and display swimming by the shrimp

*Ancylomenes magnificus*, Vietnam, 2008, and *A. aqabai*, Jordan, Red Sea, 2009 [video by Z. Ďuriš].

**Supplementary Video S4.** Whipping by antennae, swimming, and sideways body swaying of

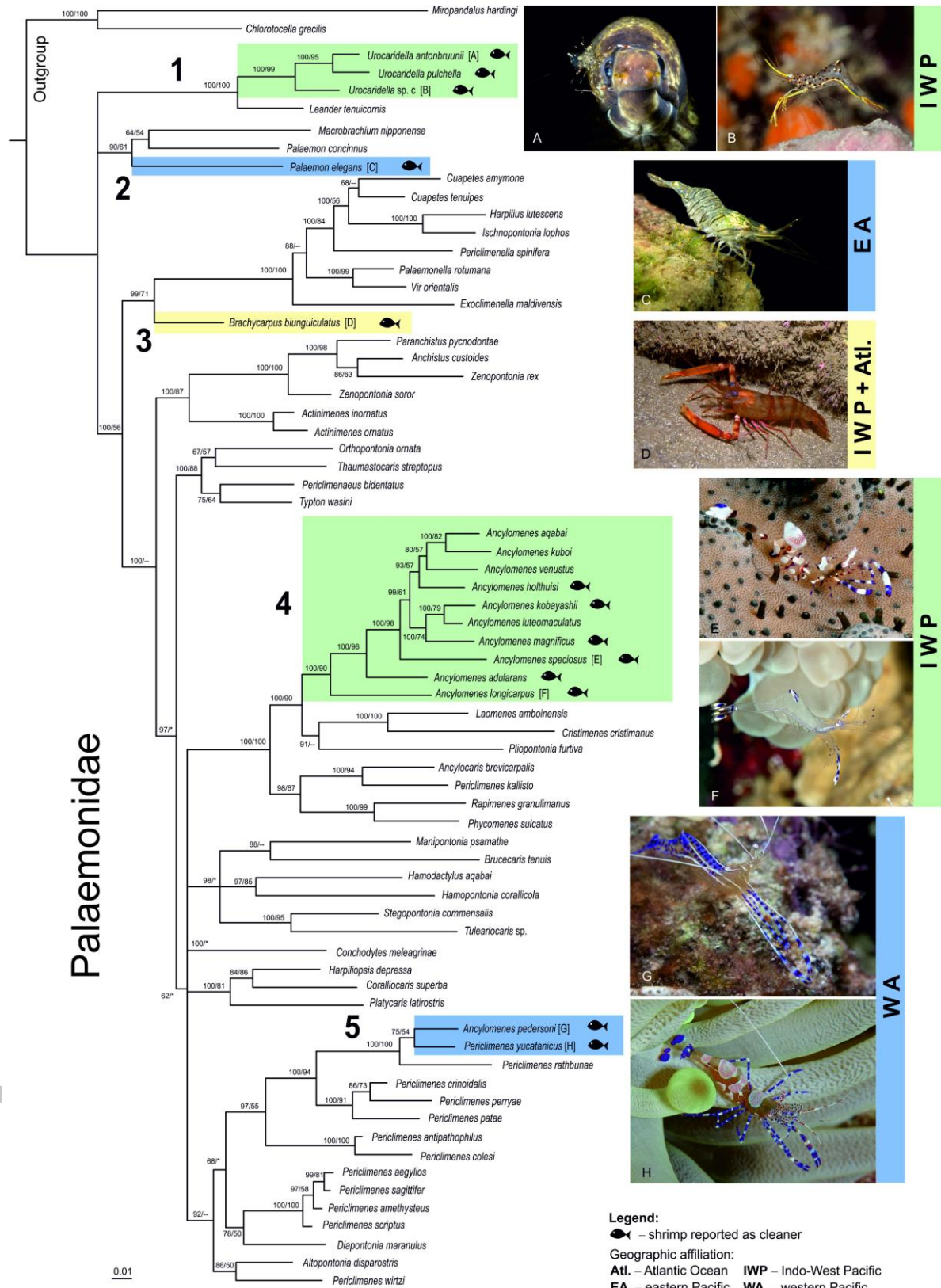
shrimp *Ancylomenes pedersoni*, Martinique, Caribbean Sea, 2016 [video by Z. Ďuriš].

**Supplementary Video S5.** Fish cleaning and attempt to clean diver's hand by shrimp

*Ancylomenes longicarpus*, and cleaning of hand and fish by *Urocaridella pulchella*, Jordan, Red Sea, 2008 and 2009 [video by Z. Ďuriš].









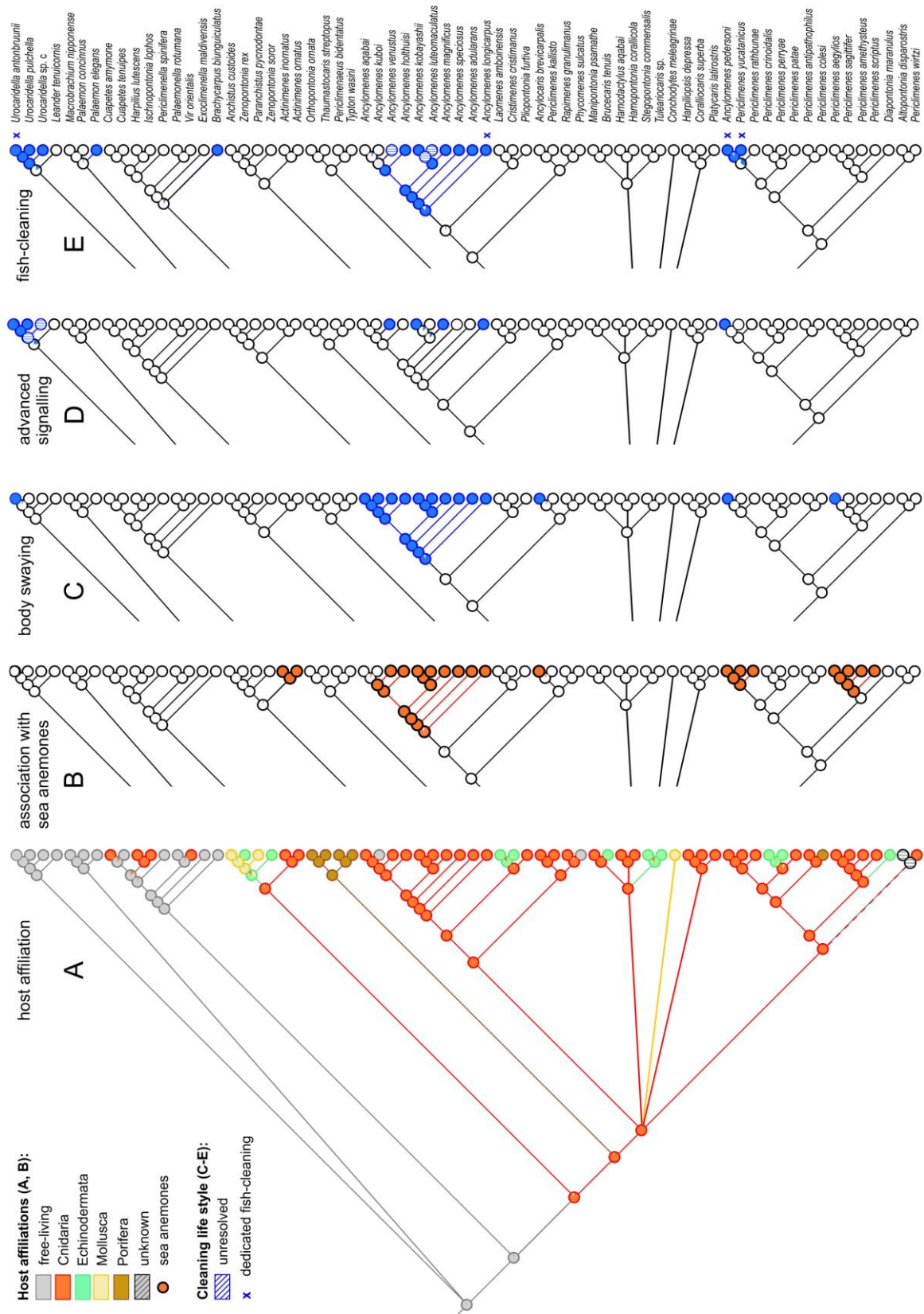


Table 1. Summary of host associations and cleaning interactions in analysed palaemonid shrimps. Terms and symbols for Cleaning interactions: dedicated, facultative - as defined by Vaughan et al. (2017), none - without fish-cleaning interactions, reported - cleaning reported, type not specified; unreported - related to cleaners but cleaning not reported.

Species (Palaemonidae)	Most frequent host	Occasional hosts	Relation to anemones	References	Cleaning interactions	References
<i>Actinimenes inornatus</i> (Kemp, 1922)	Cnidaria: Actiniaria	unreported	obligatory	Ch08	none	
<i>Actinimenes omatus</i> (Bruce, 1969)	Cnidaria: Actiniaria	unreported	obligatory	Ch08	none	
<i>Altopontonia disparostris</i> Bruce, 1990	unreported	unreported	none		none	
<i>Ancyllocaris brevicarpalis</i> Schenkel, 1902	Cnidaria: Actiniaria	unreported	obligatory	Ch08	none	
<i>Ancylomenes adularans</i> (Bruce, 2003)	Cnidaria: Actiniaria	Ceriantharia	facultative	H16a, OB10	reported	Ok05
<i>Ancylomenes aqabai</i> (Bruce, 2008)	Cnidaria: Scyphozoa (benthic)	unreported	none	H16a	unreported	
<i>Ancylomenes holthuisi</i> (Bruce, 1969)	Cnidaria: Actiniaria	Scleractinia, Scyphozoa	facultative	OB10	reported	BG04, ts (Fig. 1B)
<i>Ancylomenes kobayashii</i> (Okuno & Nomura, 2002)	Cnidaria: Actiniaria	unreported	obligatory	OB10	reported	ON02
<i>Ancylomenes kuboi</i> Bruce, 2010	free-living	Cnidaria: Actiniaria (bottom near host)	facultative	H16a, B10, pers. obs.	unreported	
<i>Ancylomenes longicarpus</i> (Bruce & Svoboda, 1983)	Cnidaria: Actiniaria	unreported	obligatory	Ch08	dedicated	ChB09, V17, pers. obs.
<i>Ancylomenes luteomaculatus</i> Okuno & Bruce, 2010	Cnidaria: Actiniaria	Ceriantharia, Corallimorpharia	facultative	OB10, pers. obs.	unreported	
<i>Ancylomenes magnificus</i> (Bruce, 1979)	Cnidaria: Actiniaria	Alcyonacea, Ceriantharia, Hydrozoa, Scleractinia	facultative	OB10	reported	B05, ts (Fig. 1C)
<i>Ancylomenes pedersoni</i> (Chace, 1958)	Cnidaria: Actiniaria	Ceriantharia, Scyphozoa (benthic)	facultative	Cr84	dedicated	OB10, T15, V17, ts (Fig. 1D)
<i>Ancylomenes speciosus</i> (Okuno, 2004)	Cnidaria: Actiniaria	Scleractinia	facultative	OB10	reported	Ok04
<i>Ancylomenes venustus</i> (Bruce, 1989)	Cnidaria: Actiniaria	Scleractinia	facultative	OB10	unreported	
<i>Anchistus custoides</i> Bruce, 1977	Mollusca: Bivalvia	unreported	none		none	
<i>Brachycarpus biunguiculatus</i> (Lucas, 1846)	free-living	Porifera; Scleractinia	none	pers. obs.	facultative	C78
<i>Bucecaris tenuis</i> (Bruce, 1969)	Echinodermata: Crinoidea	unreported	none		none	
<i>Conchodytes meleagrinae</i> Peters, 1852	Mollusca: Bivalvia	unreported	none		none	
<i>Coralliocaris superba</i> (Dana, 1852)	Cnidaria: Scleractinia	unreported	none		none	
<i>Cristimenes cristimanus</i> (Bruce, 1965)	Echinodermata: Echinoidea	unreported	none		none	
<i>Cuapetes amymone</i> (De Man, 1902)	Cnidaria: Scleractinia	unreported	none		none	
<i>Cuapetes tenuipes</i> (Borradaile, 1898)	free-living	Cnidaria: Actiniaria, Echinodermata: Echinoidea (bottom under host)	facultative	H16a, pers. obs.	none	
<i>Diapontonia maranulus</i> Bruce, 1986	Echinodermata: Echinoidea	unreported	none		none	
<i>Exoclimenella maldivensis</i> Ćuriš & Bruce, 1995	free-living	Scleractinia	none	DB94	none	
<i>Hamodactylus aqabai</i> Bruce & Svoboda, 1983	Cnidaria: Alcyonacea	unreported	none		none	
<i>Hamopontonia corallicola</i> Bruce, 1970	Cnidaria: Scleractinia	unreported	none		none	
<i>Harpiliopsis depressa</i> Stimpson, 1860	Cnidaria: Scleractinia	unreported	none		none	
<i>Harpilius lutescens</i> Dana, 1852	Cnidaria: Scleractinia	unreported	none		none	
<i>Ischnopontonia lophos</i> (Barnard, 1962)	Cnidaria: Scleractinia	unreported	none		none	
<i>Laomenes amboinensis</i> (De Man, 1888)	Echinodermata: Crinoidea	unreported	none		none	
<i>Leander tenuicornis</i> (Say, 1818)	free-living	unreported	none		none	
<i>Macrobrachium nipponense</i> (De Haan, 1849)	free-living	unreported	none		none	
<i>Manipontonia psamathe</i> (De Man, 1902)	Cnidaria: Antipatharia	unreported	none		none	
<i>Orthopontonia ornata</i> (Bruce, 1970)	Porifera: Demospongiae	unreported	none		none	
<i>Palaemon concinnus</i> Dana, 1852	free-living	unreported	none		unreported	
<i>Palaemon elegans</i> Rathke, 1837	free-living	unreported	none		facultative	Os05, V17
<i>Palaemonella rotumana</i> (Borradaile, 1898)	free-living	unreported	none		none	
<i>Paranchistus pycnodontae</i> Bruce, 1978	Mollusca: Bivalvia	unreported	none		none	

Table 1 (continued)

Species (Palaemonidae)	Most frequent host	Occasional hosts	Relation to anemones	References	Cleaning interactions	References
<i>Periclimenaeus bidentatus</i> Bruce, 1970	Porifera: Demospongiae	unreported	none		none	
<i>Periclimenella spinifera</i> (De Man, 1902)	free-living	unreported	none		none	
<i>Periclimenes aegylios</i> Grippa & d'Udekem d'Acoz, 1996	Cnidaria: Actiniaria	unreported	obligatory	GU96	none	
<i>Periclimenes amethysteus</i> (Risso, 1827)	Cnidaria: Actiniaria	unreported	obligatory	GU96	none	
<i>Periclimenes antipathophilus</i> Spotte, Heard & Bubucis, 1994	Cnidaria: Antipatharia	unreported	none		none	
<i>Periclimenes colesi</i> De Grave & Anker, 2009	Porifera: Demospongiae	unreported	none		none	
<i>Periclimenes crinoidalis</i> Chace, 1969	Echinodermata: Crinoidea	unreported	none		none	
<i>Periclimenes kallisto</i> Bruce, 2008	Cnidaria: Antipatharia	unreported	none		none	
<i>Periclimenes patae</i> Heard & Spotte, 1991	Cnidaria: Antipatharia	unreported	none		none	
<i>Periclimenes perryae</i> Chace, 1942	Echinodermata: Ophiuroidea	unreported	none		none	
<i>Periclimenes rathbunae</i> Schmitt, 1924	Cnidaria: Actiniaria	Anthozoa (varia), Scyphozoa (benthic)	facultative	BF16, Cr84, RP07, S91	unreported	
<i>Periclimenes sagittifer</i> (Norman, 1861)	Cnidaria: Actiniaria	unreported	obligatory	GU96	none	
<i>Periclimenes scriptus</i> (Risso, 1822)	Cnidaria: Actiniaria	Alcyonacea	facultative	U05	none	
<i>Periclimenes wirtzi</i> d'Udekem d'Acoz, 1996	Cnidaria: Antipatharia	unreported	none		none	
<i>Periclimenes yucatanicus</i> (Ives, 1891)	Cnidaria: Actiniaria	Scyphozoa (benthic), Corallimorpharia	facultative	Cr84, RP07	dedicated	L61, T17, V17
<i>Phycomenes sulcatus</i> (Đuriš, Horká & Marin, 2008)	free-living	unreported	none		none	
<i>Platycaris latirostris</i> Holthuis, 1952	Cnidaria: Scleractinia	unreported	none		none	
<i>Pliopontonia furtiva</i> Bruce, 1973	Cnidaria: Corallimorpharia	unreported	none		none	
<i>Rapimenes granulimanus</i> (Bruce, 1978)	Cnidaria: Hydrozoa	unreported	none		none	
<i>Stegopontonia commensalis</i> Nobili, 1906	Echinodermata: Echinoidea	unreported	none		none	
<i>Thaumastocaris streptopus</i> Kemp, 1922	Porifera: Demospongiae	unreported	none		none	
<i>Tuleariocaris</i> sp.	Echinodermata: Echinoidea	unreported	none		none	
<i>Typton wasini</i> Bruce, 1977	Porifera: Demospongiae	unreported	none		none	
<i>Urocaridella</i> sp. c	free-living	unreported	none		reported	B05, BG04
<i>Urocaridella antonbruunii</i> (Bruce, 1967)	free-living	unreported	none		dedicated	C78, V17
<i>Urocaridella pulchella</i> Yokes & Galil, 2006	free-living	unreported	none		facultative	YG06, ts (Fig. 1A, Suppl. Video S5)
<i>Vir orientalis</i> (Dana, 1852)	Cnidaria: Scleractinia	unreported	none		none	
<i>Zenopontonia rex</i> (Bruce, 1967)	Echinodermata: Holothuroidea	Echinodermata: Asteroidea, Gastropoda	none		none	
<i>Zenopontonia soror</i> (Nobili, 1904)	Echinodermata: Asteroidea	unreported	none		none	

Abbreviated references: B05 - Becker et al. (2005), B10 - Bruce (2010), BF16 - Brinkmann and Fransen (2016), BG04 - Becker and Grutter (2004), C78 - Corredor (1978), Ch08 - Chadwick et al. (2008), ChB09 - Chapuis and Bshary (2009), Cr84 - Criaes (1984), DB94 - Đuriš and Bruce (1995), GU96 - Grippa and d'Udekem d'Acoz (1996), H16a - Horká et al. (2016a), L61 - Limbaugh et al. (1961), OB10 - Okuno and Bruce (2010), Ok04 - Okuno (2004), Ok05 - Okuno (2005), ON02 - Okuno and Nomura (2002), Os05 - Östlund-Nilsson et al. (2005), pers. obs. - personally observed by authors; RP07 - Ritson-Williams and Paul (2007), S91 - Spotte et al. (1991), T15 - Titus et al. (2015), T17 - Titus et al. (2017), ts - this study, U05 - d'Udekem d'Acoz (2005), V17 - Vaughan et al. (2017), YG06 - Yokes and Galil (2006).

**Highlights**

- Five evolutionary origins of fish-cleaning symbioses within palaemonid shrimps.
- Two lineages of cleaning shrimps strikingly similar in morphology, colouration and behaviour.
- Advanced signalling by cleaner shrimps with antennae, claws, and display swimming.



