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Ultrastructure and molecular phylogeny of *Stephanopogon minuta*: An enigmatic microeukaryote from marine interstitial environments

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Abstract

Although *Stephanopogon* was described as a putative ciliate more than a century ago, its phylogenetic position within eukaryotes has remained unclear because of an unusual combination of morphological characteristics (e.g. a highly multiflagellated cell with discoidal mitochondrial cristae). Attempts to classify *Stephanopogon* have included placement with the Ciliophora, the Euglenozoa, the Heterolobosea and the Rhizaria. Most systematists have chosen, instead, to conservatively classify *Stephanopogon* as incertae sedis within eukaryotes. Despite the obvious utility of molecular phylogenetic data in resolving this issue, DNA sequences from *Stephanopogon* have yet to be published. Accordingly, we characterized the molecular phylogeny and ultrastructure of *Stephanopogon minuta*, a species we isolated from marine sediments in southern British Columbia, Canada. Our results showed that *S. minuta* shares several features with heterolobosean, such as discoidal mitochondrial cristae, a heterolobosean-specific (17_1 helix) insertion in the small subunit ribosomal RNA gene (SSU rDNA) and the lack of canonical Golgi bodies. Molecular phylogenetic analyses of SSU rDNA demonstrated that *S. minuta* branches strongly within the Heterolobosea and specifically between two different tetraflagellated lineages, both named '*Percolomonas cosmopolitus*.' Several ultrastructural features shared by *S. minuta* and *P. cosmopolitus* reinforced the molecular phylogenetic data and confirmed that *Stephanopogon* is a highly divergent multiflagellated heterolobosean that represents an outstanding example of convergent evolution with benthic eukaryovorous ciliates (Alveolata).

Keywords: Discoidal mitochondrial cristae; Heterolobosea; *Percolomonas cosmopolitus*; Vahlkampfiidae; *Stephanopogon*

Introduction

The diversity of eukaryotes consists mainly of microbial lineages that can be confidently classified into one of several well-circumscribed 'supergroups'. Some systematists have advocated six major groups of eukaryotes: Opisthokonta (e.g. animals, fungi), Amoebozoa (e.g. dictyostelids and myxomycetes), Plantae (e.g. green algae, land plants and red algae), Chromalveolata (e.g. stramenopiles and alveolates), Rhizaria (e.g. cercozoans, foraminiferans and radiozoans) and Excavata (e.g. heteroroboseans, euglenozoans, parabasalids, *Carpediemonas*) (Adl et al. 2005; Keeling et al. 2005; Parfrey et al. 2006; Simpson and Roger 2004). Other systematists, however, remain skeptical of these groupings and take a more conservative approach that involves the recognition of many more 'sisterless' supergroups (Patterson 1999). Nonetheless, there are many lineages of eukaryotes that are difficult to classify

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because they are either poorly understood or possess enigmatic combinations of ultrastructural features. The classification presented by Adl et al. (2005), for instance, listed 199 genera that were lumped together as incertae sedis within the Eukaryota. *Stephanopogon* is among these genera and is especially interesting because these organisms are morphologically complex predators that have converged in many characters normally associated with eukaryovorous ciliates (Alveolata).

Stephanopogon possesses several longitudinal rows of flagella (= cilia) and was originally described as a member of the Ciliophora by Entz (1884). Several authors subsequently interpreted this genus to be a plesiomorphic ciliate because all members of the group lacked nuclear dimorphism (i.e. macro- and micronuclei) (Corliss 1979; Lwoff 1923, 1936; Raikov 1969). The presence of isomorphic nuclei in *Stephanopogon* was considered by some authors to be indicative of the ancestral condition of ciliates (Corliss 1979; Raikov 1969). Lipscomb and Corliss (1982) and Patterson and Brugerolle (1988) studied the ultrastructural features of *Stephanopogon apogon* in anticipation that they might be able to reconstruct early stages in the evolution of ciliates. These authors discovered instead that *Stephanopogon* lacks basic ciliate features such as cortical alveoli and recognizable infraciliature. Moreover, *S. apogon* possessed mitochondria with discoidal cristae rather than tubular cristae and lacked conspicuous (canonical) Golgi bodies. Patterson and Brugerolle (1988) suggested that *S. apogon* was most likely related to other eukaryotes with discoidal cristae, such as the Euglenozoa.

These ultrastructural studies led to a great deal of uncertainty about the evolutionary history and taxonomy of *Stephanopogon*; and, over the past 20 years, neither molecular data nor additional ultrastructural data have been published on these enigmatic predators. This is due, in large part, to the difficulty of isolating these organisms from natural samples. Nonetheless, some systematists have used cladistic analyses of morphological character states to infer that *Stephanopogon* should be classified with the Euglenozoa, mainly because both groups possess discoidal mitochondrial cristae (Corliss 1984; Lipscomb 1991). Cavalier-Smith (1991, 1993a, b, 2003), by contrast, used the same evidence and the lack of conspicuous Golgi bodies to intuitively classify *Stephanopogon* as a member of the Heterolobosea (or Percolozoa). Cavalier-Smith (2002) also temporarily moved *Stephanopogon* into the Cercozoa (Rhizaria) based on the presence of *Spongomonas*-like 'sheet structures' attached to the proximal end of each basal body (Hibberd 1983). Hayward and Ryland (1990) and Lei et al. (1999) continued to treat *Stephanopogon* as an unusual member of the Ciliophora. Most modern systematists, however, have taken a more conservative approach and tentatively classify *Stephanopogon* as lacking any clear sister-lineage of eukaryotes (Adl et al. 2005; Alongi 1991; Al-Qassab et al. 2002; Hausmann et al. 2003; Larsen and Patterson 1990; Patterson 1999; Patterson and Zölffel 1991; Patterson et al. 2002b; Simpson 1997).

The confusing taxonomic history of *Stephanopogon* indicates that molecular phylogenetic evidence will be extremely helpful in resolving how this lineage relates to other eukaryotes. After successfully isolating *Stephanopogon minuta* from marine sediments in British Columbia, we were able to investigate the molecular phylogenetic position and novel ultrastructural features of these enigmatic predators.

Material and methods

Isolation and cultivation

S. minuta was collected and manually isolated from marine sediments at low tide in Boundary Bay, British Columbia, Canada (123°03′W, 49°01′N) on May 17, 2007. We were able to establish and temporarily maintain a low-abundance culture of *S. minuta* with a standard f/2 medium prepared with seawater. A small amount of sediment was inoculated into the medium and cultured at 22 °C. A single *S. minuta* cell was isolated by micropipetting from the enrichment culture. The low-abundance culture of *S. minuta* was established with a small pennate diatom (*Nitzschia* sp.) as a food source and maintained at the same conditions as the enrichment culture. All of the molecular and ultrastructural data in this study was obtained from a culture containing only two eukaryotes, namely *S. minuta* and the diatom, *Nitzschia* sp. This approach eliminated any chance of contamination from any other source of aquatic flagellates.

The image of *Percolomonas cosmopolitus* shown in this study was taken from organisms collected from the sediment in Tokyo Bay, Japan on May 28, 2004.

Light and electron microscopy

Light microscopy was performed using a Zeiss Axioplan 2 imaging microscope and a Leica DC500 digital chilled CCD camera. Light micrographs were taken of cells fixed in seawater mixed with an equal volume of 2% glutaraldehyde (GA).

For scanning electron microscopy (SEM), cells of *S. minuta* were mixed with an equal volume of fixative containing 2% GA in 0.1 M sodium cacodylate buffer (SCB) (pH 7.2) and mounted on cover glasses coated with poly-L-lysine at room temperature for 1 h. The cover glasses were rinsed with 0.1 M SCB and fixed in 1% OsO_4 for 30 min. The fixed cells were then rinsed with 0.1 M SCB and dehydrated with a graded ethanol

series from 30% to absolute ethanol. Samples were critical point dried with CO_2 using a Tousimis Critical Point Dryer. Samples were then coated with gold using a Cressington 208HR high Resolution Sputter Coater, and observed with a Hitachi S-4700 field emission scanning electron microscope.

For SEM of *P. cosmopolitus*, cells were mixed with an equal volume of fixative, containing 2.5% GA in 0.1 M SCB (pH 7.2), and were mounted on cover glasses coated by poly-L-lysine at room temperature for 2 h. The cover glasses were rinsed with 0.2 M SCB and fixed in 1% OsO_4 for 30 min. These were then rinsed with 0.2 M SCB and dehydrated with a graded ethanol series from 30% to absolute ethanol. Ethanol was replaced by dehydrated *t*-butanol before samples were freeze-dried with a freeze drier VFD-21S (Shinku-Device, Japan). Samples were coated with platinum/palladium with an E102 ion-sputter coater (Hitachi, Japan), and observed with a JSM-6330F field emission scanning electron microscope (JEOL, Japan).

For transmission electron microscopy (TEM), cell suspensions were mixed with 4% (v/v) GA in 0.2 M SCB (pH 7.2) at room temperature for 1 h. Cells were aggregated into a pellet by centrifugation at 1000*g* for 5 min and then rinsed with 0.2 M SCB (pH 7.2). The pellet of cells was embedded in 2% agarose (Type IX: ultra-low gelling temperature). The specimens were then fixed in 1% (w/v) osmium tetroxide in 0.2 M SCB (pH 7.2) at room temperature for 1 h followed by dehydration through an ethanol series, and substitution with acetone. The specimens were embedded in resin (Epon 812). Ultrathin sections were stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963), and observed using a Hitachi H7600 electron microscope.

DNA extraction, PCR amplification, alignment and phylogenetic analysis

Thirty individual cells of *S. minuta* were isolated from the two-eukaryote culture and washed twice in sterilized f/2 medium. Genomic DNA was extracted from these isolated cells using MasterPure Complete DNA and RNA purification Kit (Epicentre, WI, USA). The polymerase chain reaction (PCR) was performed using a total volume of 25 μl and the PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). Nearly the entire SSU rRNA gene was amplified from genomic DNA using eukaryotic universal primers (PF1: 5′-GCGCTACCTGGTTGATCCTGCCAGT-3′ and R4: 5′-GATCCTTCTGCAGGTTCACCTAC-3′). The PCR protocol had an initial denaturation stage at 95 °C for 2 min; 35 cycles involving 94 °C for 45 s (denaturation), 55 °C for 45 s (annealing), and 72 °C for 1.5 min (extension); and final extension at 72 °C for 5 min. The amplified DNA fragments were purified from agarose gels using UltraClean 15 DNA Purification Kit (MO Bio, CA, USA), and then cloned into the TOPO TA Cloning Kit (Invitrogen, CA, USA). The *S. minuta* sequence was deposited in DDBJ/EMBL/GenBank under the accession number AB365646.

The SSU rRNA sequence of *S. minuta* was manually aligned with taxa representing all of the major groups of eukaryotes, forming a 39-taxon alignment with 1016 unambiguously aligned positions. PhyML (Guindon and Gascuel 2003) was used to analyze this alignment with maximum-likelihood (ML) using a general-time reversible (GTR) model of base substitutions (Rodriguez et al. 1990) incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + G model). Model parameters were estimated from the original dataset. ML bootstrap analysis (100 replicates) was conducted with the same settings described above.

In order to more comprehensively evaluate the phylogenetic position of *S. minuta* within the Heterolobosea, we analyzed an 18-taxon alignment of mostly heterolobosean sequences and 1039 unambiguously aligned positions. The ML phylogenetic analyses described above were repeated on this data set.

Both the 39- and 18-taxon data sets were also analyzed with Bayesian methods using the MrBayes program (Huelsenbeck and Ronquist 2001). The program was set to operate with a gamma distribution and four Monte–Carlo–Markov chains (MCMC) starting from a random tree. A total of 2,000,000 generations were calculated with trees sampled every 50 generations and with a prior burn-in of 100,000 generations (2000 sampled trees were discarded). A majority rule consensus tree was constructed from 38,000 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees.

Results

General morphology and ultrastructure of *Stephanopogon minuta*

S. minuta was 26.5 μm (22.5–30 μm) long and 12.3 μm (10.3–16.5 μm) wide. The cell was vase-shape and curved toward the right when observed from the dorsal side (Fig. 1). The cell was divided into three main regions, from anterior to posterior: ventral barbs (VB), a neck (Ne) and a dorsal hump (DH) (Figs 2A, D–E, 3A, B and 4A–C). *S. minuta* was a eukaryovore that preyed on diatoms in our culture (Figs 1C, 3A and 4A, B) using a pronounced feeding apparatus formed by three barbs and a dorsal transverse lip (DL) (Figs 1 and 2A). The VBs and the dorsal lip defined a slit-shaped cytostome (Figs 2A, E and 3A, B). The cells were capable of crawling forwards and backwards using an array of flagella on the ventral surface (Figs 2B and 5A) and

tended to turn to the right (clockwise) along the substrate. The cells were also able to swim in the water column in a backwards direction when the culture vessel was disturbed. Cysts attached to the substrate of the culture vessel were common (data not shown), and *S. minuta* reproduced by asexual division within the resting cysts. Other possible stages in the life cycle (e.g. sexual reproduction) were not evident.

Two isomorphic nuclei, each with a central nucleolus, were present within the DH (Figs 3A, 4A and E). Endoplasmic reticulum (ER) was positioned beneath a layer of superficial microtubules (Fig. 5D). The mitochondria of *S. minuta* contained discoidal cristae and often contained several densely stained inclusions within the matrix (Fig. 5C). Mitochondria were distributed throughout the cell (Figs 3A, B, 4A, B) and were particularly abundant between the ventral rows of flagella (Fig. 5A). The mitochondria were not surrounded by ER (Fig. 4B), and canonical Golgi bodies were not observed. The feeding apparatus was supported by a pair of microtubular bundles, or rods, that extended posteriorly from electron dense zones present within the dorsal lip and at the base of the VBs (Figs 3A–D and 4B). Moreover, the inside of the cytostome was lined by a supportive microtubular sheet that extended anteriorly before turning posteriorly along the curve formed by the dorsal lip (Fig. 3D). An accumulation of tubular extrusomes was positioned between the microtubular rods and were arranged in linear rows between the microtubules of the supportive sheet (Figs 3A–D and 4B).

Organization of the flagella in *Stephanopogon minuta*

Five flagellar pockets were arranged in a transverse row on the neck just behind the dorsal lip (Fig. 2A and E). One long and one short flagellum emerged from four of the five pockets; the central pocket lacked a long flagellum and only contained a short flagellum (Figs 2A, D, E, 3B, E and 4C). In addition, both a long and a short flagellum inserted at the right-hand side of the neck (Figs 2E and 4C), and only one long flagellum inserted at the left-hand side of the neck (Fig. 2A, D, E). The left-hand side of the DH contained three pairs (or clusters) of long flagella, however, some cells possessed three flagella in the right-hand cluster (Fig. 2A and D). Therefore, a total of 12–13 long flagella were visible on the dorsal surface of *S. minuta* (Fig. 2A). The surface of the DH also contained 8–9 faint longitudinal striations that were each about 2–3 μm wide (Fig. 2A). The cell membrane of the DH was supported by microtubules (Figs 4D and 5D). The ventral surface of *S. minuta* was concave and contained eight longitudinal grooves, each supporting 4–22 flagella (Fig. 2B). Microtubules supported the ridges that were positioned between the flagellar grooves on the ventral side of the cells (Fig. 5A). The total number of flagella on the ventral side was variable and ranged from 100 to 132. Therefore, the total number of flagella on each cell of *S. minuta* ranged from 112 to 144.

The basal bodies were characteristically short at approximately 200 nm in length (Figs 3E and 5B). The proximal end of each basal body was associated with a fibrous sheet and microtubules that supported the wall of the flagellar depression (Figs 3E and 5A, B). The anterior-most basal body in each longitudinal row of ventral flagella had a different configuration of microtubules that extended toward the cytostome region (Fig. 4E). The central pair of axonemal microtubules terminated at their proximal ends on an electron dense globule positioned above the transitional plate (Fig. 5A, B). Flagellar hairs (mastigonemes) were absent.

General morphology of *Percolomonas*

P. cosmopolitus was semi-spherical in shape and possessed a conspicuous ventral groove (Fig. 2C). Four flagella emerged from the subapical region of the cell, at

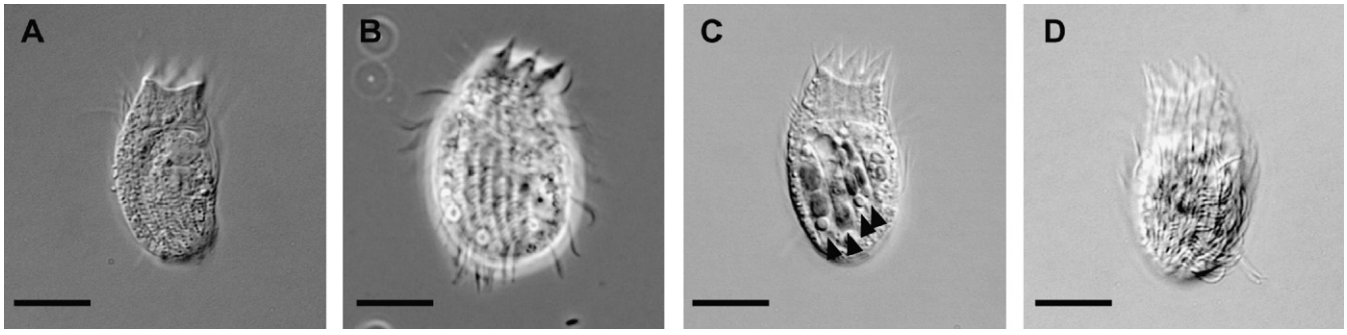

Fig. 1. Light micrographs of *Stephanopogon minuta* showing three ventral barbs at the anterior end of the cell: **A**. Differential interference contrast (DIC) image focused on the dorsal side of the cell. **B**. Phase contrast image showing the three anterior barbs and flagella inserted on both the dorsal and ventral side of the cell. This cell is slightly compressed and enlarged by a cover slip. **C**. DIC image of the cell showing an engulfed diatom (arrowheads) inside of the cell. **D**. DIC micrograph focused on the ventral rows of flagella. Scale bars = 10 μm.

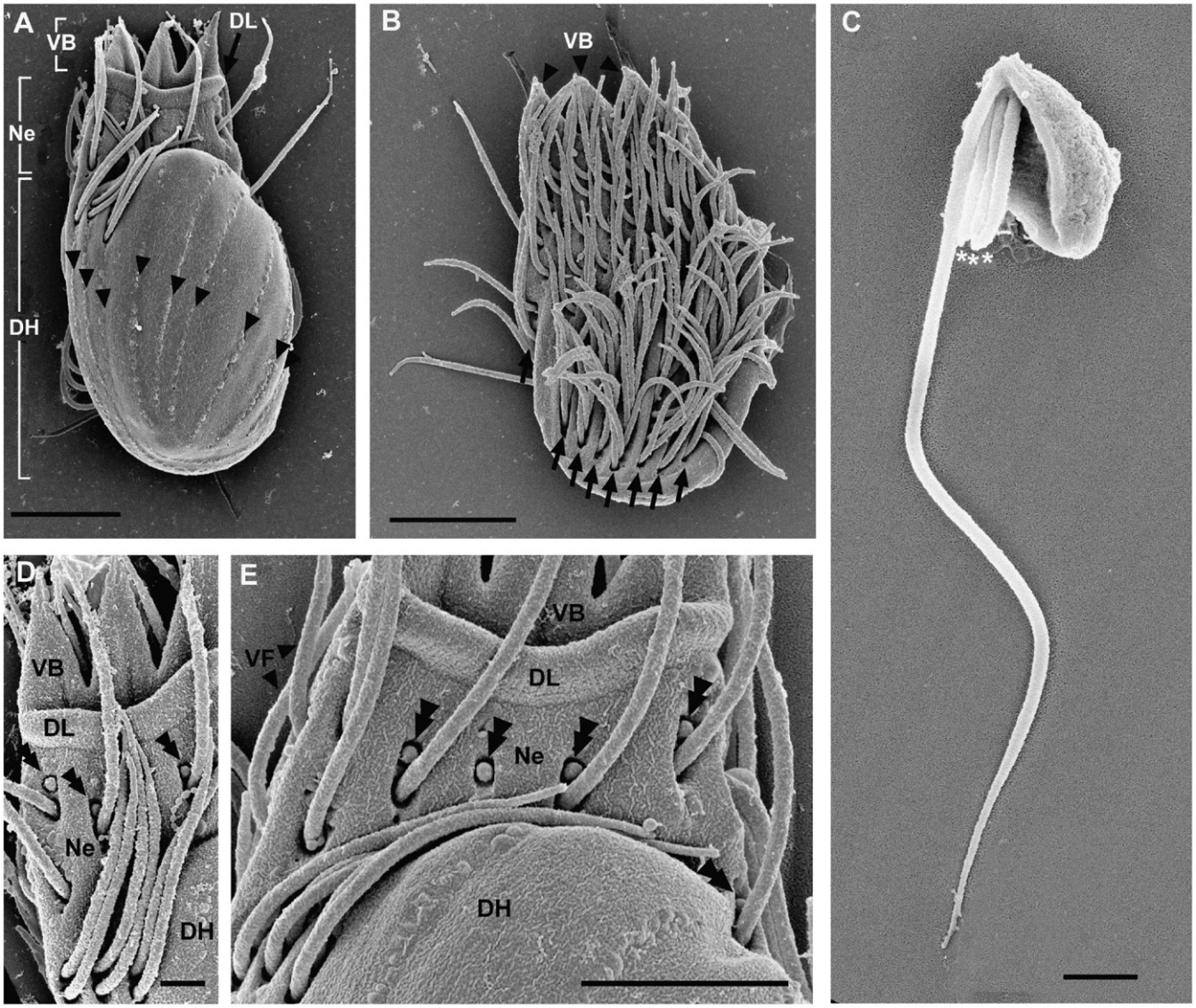

Fig. 2. Scanning electron micrographs (SEM) of *Stephanopogon minuta* (A, B, D, E) and *Percolomonas cosmopolitus* (C): **A**. Dorsal side of the *S. minuta* showing three main regions of the cell: ventral barbs (VB), the neck (Ne) and the dorsal hump (DH). Arrowheads indicate nine rows of longitudinal striations on the dorsal hump (DH). **B**. Ventral side of *S. minuta* showing eight rows of flagella (arrows). **C**. SEM of *Percolomonas cosmopolitus* showing the ventral groove and the three short flagella (asterisks) and one long flagellum inserted in the subapical region of the cell. **D**. Left side view of the ventral barbs, neck and dorsal hump of *S. minuta* showing short flagella (double arrowheads) within distinct flagellar pockets. Three longitudinal rows of two flagella are located on the left anterior side of the dorsal hump (DH). **E**. High magnification view of the neck surface showing five flagellar pockets arranged in a transverse row. Each flagellar pocket contains one short flagellum (double-arrowheads; the left-hand short flagellum is obscured by a long flagellum). The two right-hand flagellar pockets and the two left-hand flagellar pockets each also contain one long flagellum; the central flagellar pocket lacks a long flagellum. Scale bars = 10 μm for Fig. 2A and B; 1 μm for Fig. 2C and D; 2 μm for Fig. 2E. Other abbreviations: DL, dorsal transverse horizontal lip; SF, short flagellum; VF, ventral flagella.

the anterior end of the ventral groove. One flagellum was 3–4 times longer than the other three, which were almost the same length as the cell (2 μm) and were positioned within the ventral groove. These characteristics were concordant with the defining features of *P. cosmopolitus* Fenchel and Patterson (1986). The image in Fig. 2C is presented here for comparative purposes.

Molecular phylogeny of *Stephanopogon minuta* as inferred from SSU rDNA

We determined the nearly complete sequence of the SSU rRNA gene of *S. minuta*. (A sequence from the specific isolate of *P. cosmopolitus* shown in Fig. 2C was not available). As mentioned previously, the genomic

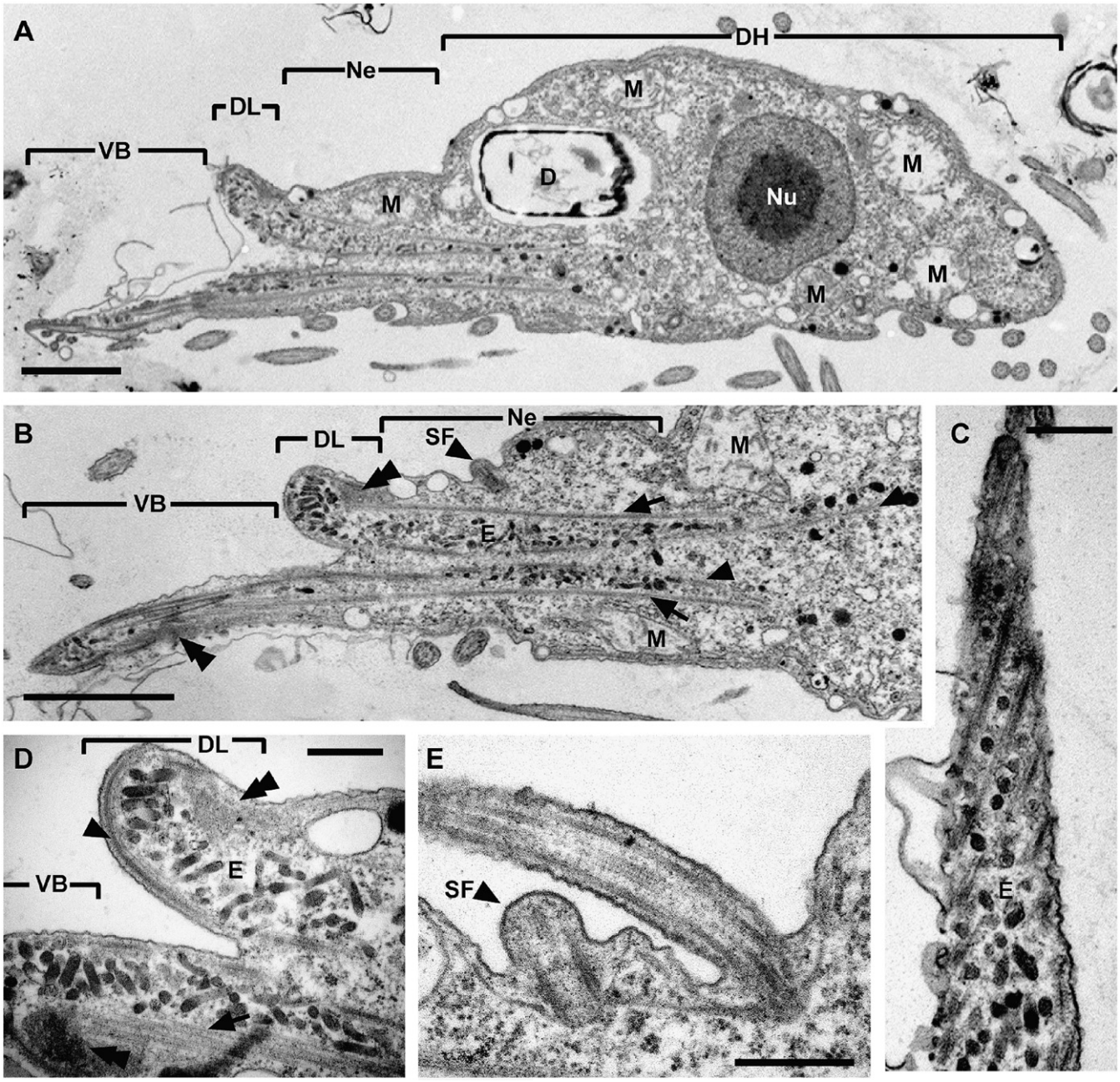

Fig. 3. Transmission electron micrographs (TEM) showing semi-serial sections through *Stephanopogon minuta*: **A**. Sagittal TEM showing a ventral barb (VB), a nucleus (Nu) with a large nucleolus, an engulfed diatom (D) and mitochondria (M). **B**. High magnification TEM through the feeding apparatus showing the short flagellum (SF) that originates from the central pocket on the neck (Ne) and microtubular rods (arrows) that originate from electron-dense zones (double arrowheads). The arrowheads indicate the supportive microtubular sheets that line the inside of the cytostome. Many tubular extrusomes (E) are positioned between the microtubular rods and the microtubular sheet lining the cytostome. **C**. Tangential TEM section showing linear arrays of extrusomes nestled between the microtubular bands of the supportive sheet lining the cytostome. **D**. High magnification TEM of the dorsal lip (DL) showing the organization of the microtubular sheet (arrowhead) and a ventral microtubular rod (arrow) stemming from an electron-dense zone (double arrowheads). **E**. TEM section through the central pair of microtubules and transitional zone of a short flagellum inserted on the dorsal side of the neck (Ne). Scale bars = 2 μm for Fig. 3A and B; 500 nm for Fig. 3C–E. Other abbreviations: DH, dorsal hump; VF, ventral flagella.

DNA from *S. minuta* was extracted from cells that were manually isolated from a culture containing only two eukaryotes (*S. minuta* and the diatom, *Nitzschia* sp.) and washed twice in sterilized f/2 medium. This approach eliminated any chance of contamination from any other source of flagellates, or indeed any other eukaryote. The

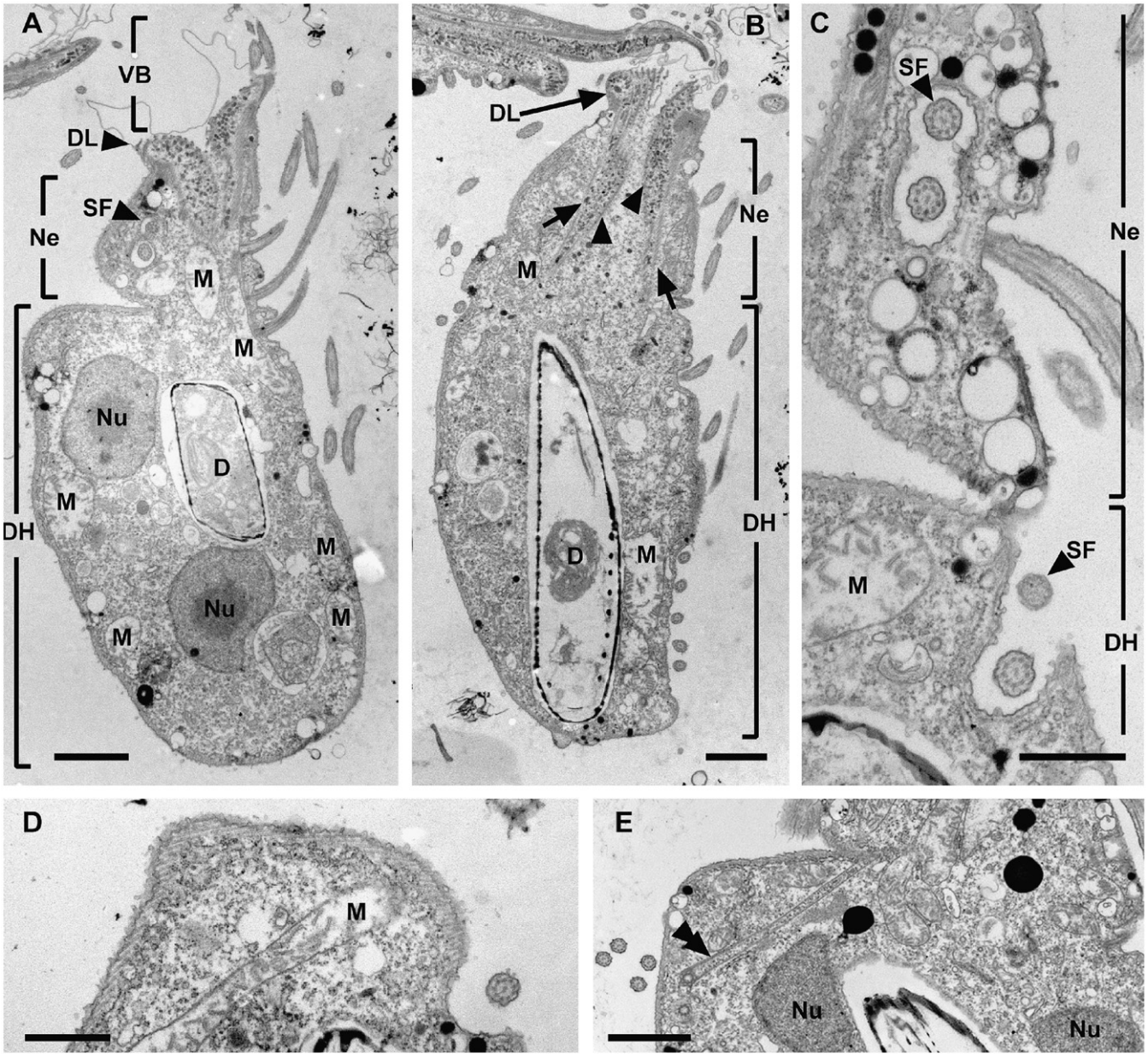

Fig. 4. Transmission electron micrographs (TEM) showing semi-serial sections through *Stephanopogon minuta*: **A**. TEM showing the three main regions of the cell: ventral barbs (VB), the neck (Ne) and the dorsal hump (DH). This micrograph also shows two isomorphic nuclei (Nu), an engulfed diatom (D) and mitochondria (M). **B**. TEM through the cytostome showing the rods (arrows) and the supportive microtubular sheets (arrowheads). **C**. A cross-section through two flagellar pockets containing a short flagellum (SF) and a long flagellum on the right side of the neck. The lower SF is sectioned through the distal tip, and the upper SF is sectioned through the axoneme. **D**. Tangential section through the anterior part of the dorsal hump showing microtubules beneath the cell membrane. **E**. A section through an anterior basal body, that is associated with the left-most ventral row of flagella, showing a long microtubular root (double-arrowhead) extending toward the cytostome. Scale bars = 2 μm for Fig. 4A, B and E; 1 μm for Fig. 4C and D. Other abbreviations: DL, dorsal transverse lip; VF, ventral flagella.

SSU rDNA sequence of *S. minuta* contained a heterolobosean-specific insertion (17_1 helix) of 22 base pairs, starting from position 558 in the V3 region. ML analyses on a 39-taxon alignment including representative sequences from all major groups of eukaryotes, grouped the sequence from *S. minuta* with the heteroloboseans *Percolomonas*, *Naegleria* and *Tetramitus* with very robust statistical support [bootstrap percentage (BP) of 100% and Bayesian posterior probability (PP) of 1.00, Fig. 6]. Although taxonomically related lineages in the 39-taxon alignment were recovered as monophyletic with moderate to high statistical support, the relationships among the major eukaryotic groups (i.e. the backbone) were not well resolved (Fig. 6).

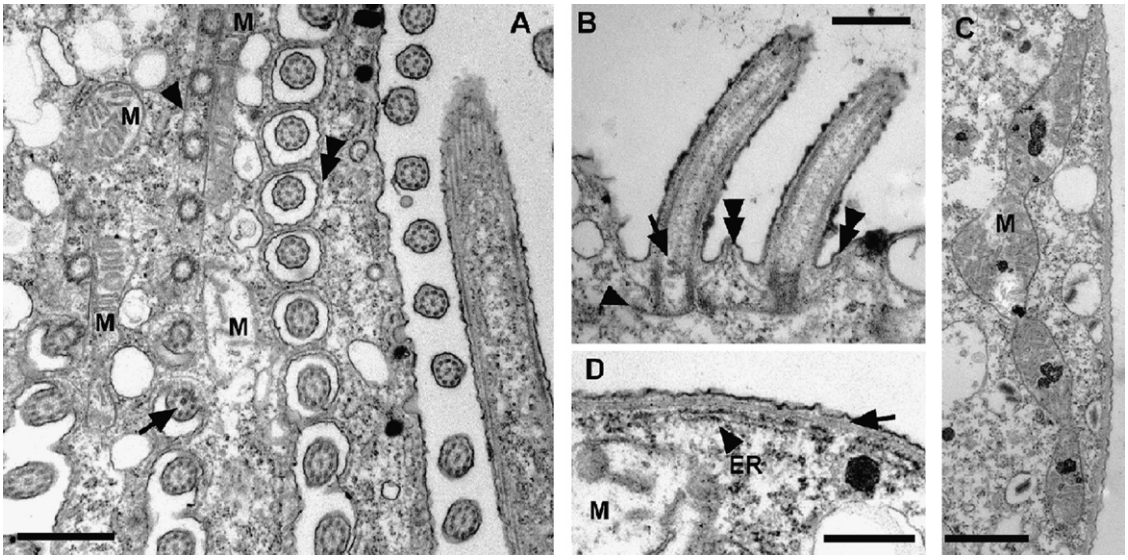

Fig. 5. Transmission electron micrographs (TEM) of *Stephanopogon minuta*: **A**. Transverse section through axonemes forming the ventral rows of flagella showing the presence of mitochondria (M) positioned between the rows. The arrowhead indicates a fibrous link between two basal bodies. The flagellar pockets are supported by microtubules (double arrowhead). The arrow indicates a dense globule near the transitional plate. Note the array of superficial microtubules that support the ridges between rows of flagella (right-hand side of the micrograph). **B**. Longitudinal section through the axonemes of the ventral flagella showing microtubules extending from the flagellar pocket to the proximal part of the basal bodies (double arrowheads). A fibrous plate (arrowhead) is also attached to the proximal end of the basal bodies. The central pair of microtubules terminates on a dense globule (arrow) just above the transitional plate. **C**. The mitochondrion possesses discoidal cristae and often contains several electron-dense inclusions. **D**. A longitudinal section showing microtubules (arrow) and endoplasmic reticulum running underneath the plasma membrane. Scale bars = 1 μm for Fig. 3A and C; 500 nm for Fig. 3B and D. *Abbreviations*: ER, endoplasmic reticulum; M, mitochondrion.

In order to more comprehensively evaluate the phylogenetic position of *S. minuta* within the Heterolobosea, we analyzed an 18-taxon alignment including heterolobosean sequences (ingroup) and euglenozoan sequences (outgroup). In these analyses, *S. minuta* grouped strongly with two different lineages named '*P. cosmopolitus*' (AF011464 and AF519443) and was most closely related to *Percolomonas* sequence AF011464 (BP = 97%; PP = 1.00, Fig. 7). This topology was recovered irrespective of the method used. The data, however, did not provide sufficient phylogenetic signal to confidently assess how the *Stephanopogon*–*Percolomonas* clade relates to the other heterolobosean clades (Fig. 6).

Discussion

Comparative morphology of *Stephanopogon* species

We were able to confidently identify *Stephanopogon* in our culture using three diagnostic features: swimming behavior, three barbs at the anterior end of the cell and a distinctive pattern of longitudinal flagellar rows on the ventral surface (Jones 1974; Jones and Owen 1974). Six species of *Stephanopogon* have been described so far: *S. apogon, S. mesnili*, *S. mobiliensis*, *S. paramesnili*, *S. colpoda* and *S. minuta*. These species are distinguished from one another by differences in the number of barbs, the number of flagellar rows on the ventral surface and cell size (Jones and Owen 1974; Lei et al. 1999). *S. apogon* is easily distinguished from the other five species because it is large (50–90 μm in length), has 12–14 rows of flagella and is the only species without barbs at the anterior end of the cell (Al-Qassab et al. 2002; Borror 1965; Jones and Owen 1974; Patterson and Brugerolle 1988; Larsen and Patterson 1990). *S. mobiliensis* and *S. mesnili* have five and four anterior barbs, respectively (Kahl 1930; Jones and Owen 1974; Lwoff 1936). *S. mobiliensis* is 19–25 μm long and possesses eight rows of flagella (Jones and Owen 1974), while *S. mesnili* is 40–70 μm long and possesses 12 rows of flagella (Dragesco 1963; Kahl 1930).

The remaining three species of *Stephanopogon* have three anterior barbs. Cells of *S. colpoda* are 50–90 μm long and have 12–14 rows of flagella (Entz 1884; Dragesco 1963; Kahl 1930; Hayward and Ryland 1990). The cells of *S. paramesnili* are the largest in this genus, at 60–110 μm long, and have 11–13 rows of flagella (Lei et al. 1999). *S. minuta* are among the smallest in the genus, at 32–35 μm long, and have 7–8 rows of flagella (Lei et al. 1999). Although our isolate is

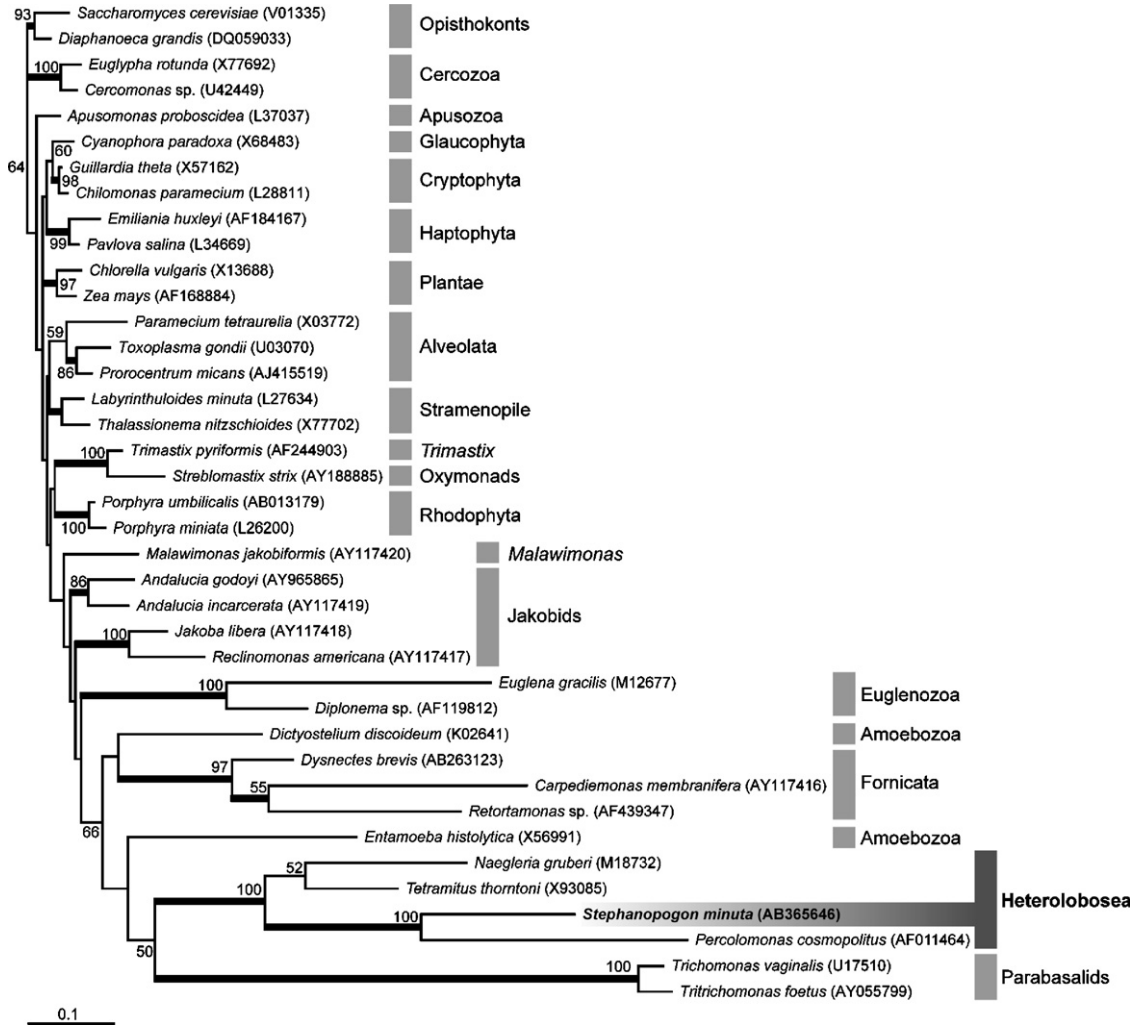

Fig. 6. Phylogenetic position of *Stephanopogon minuta* using SSU rRNA gene sequences. Maximum likelihood (ML) analysis of 39 taxa sampled from phylogenetically diverse eukaryotes. This tree is rooted with opisthokont sequences. ML bootstrap values greater than 50% are shown. Thick branches indicate Bayesian posterior probabilities over 0.90. GenBank accession numbers of the sequences analyzed are shown in parentheses.

slightly smaller (26.5 μm long) than previous reports for *S. minuta*, we attribute this difference to variation within the species. We also suspect that the *Stephanopogon* species identified as *S. colpoda* by Larsen and Patterson (1990) was actually *S. minuta*, because of the size of the cell (18–24 μm) and the presence of eight rows of flagella on the ventral side. It should be noted that Larsen and Patterson (1990) identified this species as '*S. colpoda*' before Lei et al. (1999) formally described *S. minuta* as a new species.

Comparative ultrastructure of *Stephanopogon* within a heterolobosean context

Molecular phylogenetic evidence inferred from SSU rDNA and the presence of a heterolobosean-specific (17_1 helix) insertion demonstrates that *S. minuta* evolved from within the Heterolobosea. These data are consistent with ultrastructural features present in both *Stephanopogon* and other heteroloboseans, such as the lack of Golgi bodies and the presence of discoidal mitochondrial cristae (Cavalier-Smith 1991, 1993a, b, 1998, 1999, 2003a, b). Moreover, these results strongly suggest that *S. minuta* belongs to the Vahlkampfiidae, which is generally recognized as a group of heterotrophic amoeboflagellates (Patterson et al. 2002a). The Vahlkampfiidae contains species characterized by morphologically different life stages. For instance, *Percolomonas*, *Psalteriomonas* and *Pleurostomum* are obligate flagellates; members of *Vahlkampfia* are obligate amoebae; and *Heteramoeba*, *Naegleria* and *Tetramitus* are amoeboflagellates.

Stephanopogon is most closely related to the tetraflagellate *Percolomonas* in molecular phylogenetic analyses of SSU rDNA, and this relationship is consistent

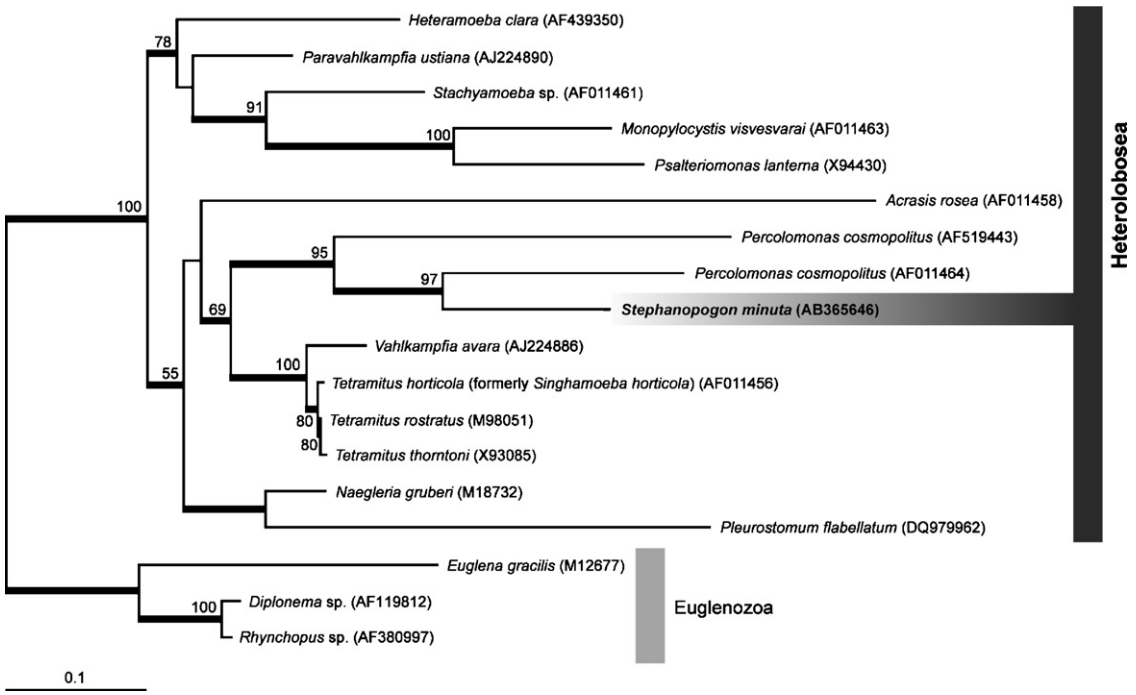

Fig. 7. Maximum likelihood (ML) analysis of 18 taxa focusing on the position of *S. minuta* within the Heterolobosea clade; euglenozoan sequences form the outgroup. ML bootstrap values greater than 50% are shown. Thick branches indicate Bayesian posterior probabilities over 0.90. GenBank accession numbers of the sequences analyzed are shown in parentheses.

with several ultrastructural features shared by members of both genera. For example, the distinctive arrangement of mitochondria and ER found in most heteroloboseans is absent from both *Percolomonas* and *Stephanopogon* (Brugerolle and Simpson 2004; Fenchel and Patterson 1986; Page and Blanton 1985; Patterson et al. 2002a). Moreover, the central pair of axonemal microtubules in both *Stephanopogon* and *Percolomonas* terminates with a ball-like, electron dense globule near the transitional plate (Fig. 3A). The presence of fibrous sheets attached to the proximal ends of the basal bodies is known only in *Stephanopogon* and *Percolomonas* (among heteroloboseans) and is perhaps a synapomorphic feature for the *Percolomonas–Stephanopogon* clade. Overall, these ultrastructural details are concordant with the molecular phylogenetic data and suggest that *Stephanopogon* evolved from *Percolomonas*-like ancestors within the Vahlkamphiidae. However, the large gap between the *Percolomonas* and *Stephanopogon* morphotypes (Figs 2A–C) must be closed with additional research on heterolobosean biodiversity before we can more confidently reconstruct the intervening evolutionary history of these organisms.

Taxonomic considerations

As shown in Fig. 7, the SSU rRNA gene sequence from *S. minuta* branched between two published sequences of '*P. cosmopolitus*': AF519443 and AF011464. This result could be explained in three different ways: (1) the life cycle of *Stephanopogon* includes uncharacterized polymorphic stages that resemble the overall morphology of *Percolomonas* species, (2) the *P. cosmopolitus* morphotype contains cryptic species and forms a paraphyletic stem group from which *Stephanopogon* evolved and (3) our culture of *Stephanopogon* was contaminated with *P. cosmopolitus* which was then sequenced by mistake. Although interpretation '1' cannot be definitively ruled out, it does stand in contrast to previous observations. For instance, except for a resting cyst stage, other life stages were not observed in our culture of *S. minuta*, the ATCC culture of *S. apogon*, or in detailed investigations of *S. mesnili* (Lwoff 1936). Accordingly, we think that interpretation '2' is most consistent with the data, and we can confidently rule out interpretation '3' by reiterating two main points. First, our temporary culture of *S. minuta* contained only diatoms (Stramenopila) as a food source, and all other eukaryotes of any kind were absent. The sequence of *S. minuta* was then derived from 30 cells that were manually isolated from this culture and washed twice prior to DNA extraction. Second, another research group has produced an unpublished SSU rDNA sequence from *S. apogon* (ATCC 50096) that branches with *P. cosmopolitus* in precisely the same phylogenetic position as our sequence of *S. minuta* (Grant and Katz, pers. comm.). These independent and confirmatory results significantly increase the likelihood that both research groups have produced sequences from the correct taxa.

Therefore, because the two sequences from *P. cosmopolitus* do not form a clade exclusive of *Stephanopogon*, they almost certainly represent cryptic species. The long branch-lengths that separate the two sequences of *P. cosmopolitus* also indicate that they represent two very different lineages. Because it would not be reasonable or informative to re-classify *Stephanopogon* within the genus *Percolomonas*, the name of one of the two *P. cosmopolitus* sequences should eventually be changed. Resolution of this taxonomic issue, however, requires further molecular phylogenetic and ultrastructural studies of both *Stephanopogon* species and *Percolomonas* species, especially the organism from which sequence AF011464 was derived.

Convergent evolution of *Stephanopogon* and the multiflagellated state

Stephanopogon was originally described as a member of the Ciliophora, because, superficially, members of both groups look very similar to one another, behave similarly and tend to occupy the same habitats (Entz 1884). Other microeukaryotes with many flagella arranged in distinct rows, like opalinids, were also initially classified within the Ciliophora for essentially the same reasons (Metcalf 1923, 1940). Opalinids, however, are endocommensals that live within the hindguts of amphibians and squamates. Molecular phylogenetic studies and detailed utrastructural data have demonstrated that opalinids have evolved from within the stramenopiles and are closely related to biflagellates known as proteromonads (Cavalier-Smith 1997; Patterson 1985, 1989; Silberman et al. 1996). Although the general appearance of the opalinid flagellar apparatus differs from that of most stramenopiles, both groups share some key flagellar features, such as a transitional helix located just above the flagellar transition zone (Patterson 1985, 1989).

It should also be emphasized that multiflagellated organisms have also been described in several other eukaryotic 'supergroups'. For instance, the multiflagellated amoeba *Multicilia* has been shown to be a member of the Amoebozoa (Mikrjukov and Mylnikov 1998; Nikolaev et al. 2006); hypermastigotes and devescovinids are members of the Parabasalia (Brugerolle and Müller 2000; Carpenter and Keeling 2007); and the multiflagellated (and multicellular) life history stages of poriferans and metazoans (e.g. planulae, trochophores, *Trichoplax* and dicyemids) are members of the Opisthokonta. The distance between the phylogenetic positions of all of these lineages demonstrates that a propulsive apparatus consisting of distinct rows of flagella across the cell surface evolved several times independently across the tree of eukaryotes. In this paper, we have shown that *Stephanopogon* is the only known lineage within the Heterolobosea to have converged in overall morphology with ciliates, opalinids and the other lineages mentioned above. Moreover, the dinuclear (isomorphic) condition in *S. minuta* has also converged with the dinuclear (heteromorphic) condition found in most ciliates. Nonetheless, adaptive explanations for the distinctive organization of the flagella on *S. minuta* (e.g. the short flagella on the neck and the three rows of flagella on the DH) remain enigmatic and await experimental studies focused on behavioral and functional analyses.

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