Protist, Vol. 158, 397—413, July 2007
http://www.elsevier.de/protis
Published online date 14 June 2007

Protist

ORIGINAL PAPER

Ultrastructure and Phylogenetic Placement within Heterolobosea of the Previously Unclassified, Extremely Halophilic Heterotrophic Flagellate *Pleurostomum flabellatum* (Ruinen 1938)

Jong S. Park[a], Alastair G.B. Simpson[b], Won J. Lee[c], and Byung C. Cho[a,1]

[a]Molecular and Microbial Ecology Laboratory, School of Earth and Environmental Sciences, Seoul National University, Seoul 151-742, Korea
[b]Canadian Institute for Advanced Research, Program in Evolutionary Biology, and Department of Biology, Dalhousie University, Halifax B3 H 4J1, Canada
[c]Department of Environmental Engineering, Kyungnam University, 449 Wolyong-dong, Masan 631-701, Korea

Submitted January 15, 2007; Accepted March 23, 2007
Monitoring Editor: Michael Melkonian

Although *Pleurostomum* was described almost a century ago, flagellates assigned to this taxon have been recorded only in very occasional faunistic studies of highly saline habitats, and their phylogenetic position has remained uncertain. We report the cultivation, ultrastructure, and phylogenetic relationships of *Pleurostomum flabellatum* isolated from a Korean saltern pond of 313‰ salinity. This isolate is biflagellated with a cytostomal groove, and is not distinguishable from previous accounts of *P. flabellatum* from saturated brines in India and Australia. *Pleurostomum flabellatum* shows ultrastructural features characteristic of many Heterolobosea: (1) a striated rhizoplast, (2) an absence of stacked Golgi bodies, (3) parallel basal bodies and flagella, and (4) a large number of peripheral microtubules supporting a rostrum. 18S rRNA gene phylogenies strongly confirm the affinities of *P. flabellatum* within Heterolobosea. Furthermore, the 18S rRNA gene of *P. flabellatum* has the heterolobosean-specific helix 17_1, and a group I intron in the same position as in *Acrasis rosea*. Within Heterolobosea, the 'amoeboflagellate' genera *Naegleria* and *Willaertia* were its closest relatives with high bootstrap support and posterior probability. *P. flabellatum* was observed only as a flagellate, and never as an amoeba. Since light microscopy and electron microscopy observations indicate that *P. flabellatum* flagellates are capable both of feeding and division, there might be no amoeba stage. Being morphologically distinct from its closest relatives and phylogenetically distant from other flagellate-only Heterolobosea, *P. flabellatum* cannot be moved into any previously described heterolobosean genus. Instead, we move *Pleurostomum* into Heterolobosea, and assign as the type species *Pleurostomum salinum* Namyslowski 1913, a species that closely resembles *P. flabellatum*. The optimal temperature for growth of *P. flabellatum* is 40 °C. Interestingly, *P. flabellatum* grows optimally at 300‰ salinity and fails to grow below 200‰ salinity,

[1]Corresponding author;
fax +82 2 872 0311
e-mail bccho@snu.ac.kr (B.C. Cho).

doi:10.1016/j.protis.2007.03.004

indicating that it is an 'extreme halophile'. The optimal salinity for growth is the highest for any eukaryote examined to date.

Key words: solar saltern; Heterolobosea; extremely halophilic flagellate; ultrastructure; 18S rRNA; protozoa.

Introduction

A substantial diversity of heterotrophic nanoflagellates (HNFs) has been found in hypersaline environments (Cho 2005). A dozen genera have been known for a long time to appear in saturated brines (Namylowski 1913; Park et al. 2006a; Patterson and Simpson 1996; Post et al. 1983; Ruinen 1938) and may contribute to the consumption of prokaryotes in these habitats (Park et al. 2003). Recently, a HNF newly described as *Halocafeteria seosinensis* was isolated from 300‰ salinity water and was characterized in terms of behavior, autecology, ultrastructure, and 18S rRNA gene sequence (Park et al. 2006a). *Halocafeteria* proved to be a non-mastigoneme-bearing bicosoecid that preferred and tolerated extremely high salinities, qualifying as a borderline 'extreme halophile' according to the categories used routinely for prokaryotes (Kushner 1978). There are several other uncultured HNFs that are apparently restricted to very hypersaline environments, most of which are of uncertain higher phylogenetic affinities, and some of which might be still more halophilic than *Halocafeteria*. One is the genus *Pleurostomum*.

The genus *Pleurostomum* was first created by Namylowski (1913) for cells from the Wieliczka salt mine in Poland. Since then, almost without exception, *Pleurostomum* has been observed only in highly saline brine habitats (Patterson and Simpson 1996; Ruinen 1938). Organisms assigned to *Pleurostomum* have two parallel homodynamic flagella (an unusual arrangement among heterotrophic flagellates) and a conspicuous cytostomal structure that opens laterally. There are currently six described species: *Pleurostomum flabellatum*, *Pleurostomum caudatum*, *Pleurostomum gracile*, *Pleurostomum parvulum*, *Pleurostomum salinum*, and *Pleurostomum turgidum*, which differ primarily in size, cell shape, cell flexibility/plasticity, and flagellar length (Namylowski 1913; Patterson and Simpson 1996; Ruinen 1938). Our previous knowledge of *Pleurostomum* has been restricted to light microscopic observations of cells in natural material or crude cultures. As a result of the lack of monoprotistan cultures the taxonomic position of *Pleurostomum* has not been examined using ultrastructural and molecular phylogenetic data. *Pleurostomum* has not been placed in any higher taxon that is in contemporary usage, and is usually considered as one of several dozen unclassified genera of free-living heterotrophic flagellates (Patterson 1999; Patterson et al. 2000b).

Post et al. (1983) reported that cultivation of HNFs from hypersaline samples had been unsuccessful. However, mixed cultures of bacterivorous HNFs and two pure cultures of HNFs have been obtained from high salinity waters in recent years (Cho 2005; Park et al. 2003). One HNF cultured was microscopically identified as a species of *Pleurostomum*, specifically *P. flabellatum*. Here, the morphological and ultrastructural characteristics, growth physiology, and 18S rRNA gene sequence of this poorly known species from high-salinity water (313‰ salinity) are described in detail. Our study demonstrates that *Pleurostomum* belongs to the taxon Heterolobosea, and is truly an extreme halophile.

Results

Light Microscopy and Scanning Electron Microscopy

Live cells are spindle-shaped, 10—14 μm in length (Fig. 1A, B) and somewhat flexible when in contact with the coverslip or slide. They have two equal flagella, ~1.5—2 times the body length, which insert subapically. The flagella usually beat in parallel with a slow undulating beat, although they often stick to the coverslip or slide. A cytostomal structure originates near the point of flagellar insertion and extends in a spiral for over half the length of the cell (Fig. 1A, B). Food vacuoles are apparently formed at the posterior end of the cytostome and may occupy most of the posterior part of the cell (Fig. 1B). The apical region of the cell (i.e. anterior to the flagellar insertion) is drawn out into a rostrum (Fig. 1B). Some cells occasionally display a fine cytoplasmic projection from the posterior end of the cell body (data not shown). Amoebae, or other distinct alternative life-cycle phases, were not observed. Some protargol-stained cells had one nucleus (Fig. 1C), whereas

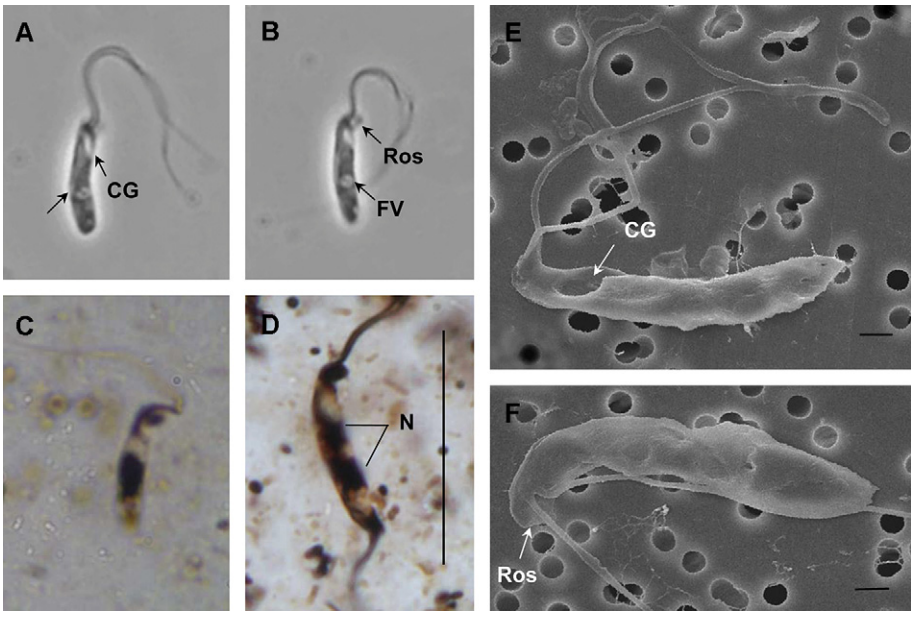


Figure 1. **A,B.** Phase-contrast micrographs of live *Pleurostomum flabellatum* (Ruinen 1938) cultured from a solar saltern, showing cell size and shape, and position of the flagella. Note that a cytostomal structure extends in a spiral for over half the length of the cell (arrow with no abbreviation). CG: cytostomal groove. Ros: rostrum, FV: food vacuole. **C,D.** Protargol-stained *P. flabellatum*. N: Nucleus. **E,F.** Scanning electron micrographs of *P. flabellatum* showing the positions of cytostomal groove and rostrum. A—D: Scale bars: 20 μm; E,F: Scale bars: 1 μm.

some cells with two nuclei and two additional long flagella were observed, presumably representing pre-division stages (Fig. 1D). Scanning electron micrographs (SEMs) confirm that *Pleurostomum flabellatum* has a conspicuous cytostomal structure with a broad opening in the anterior half of the cell (Fig. 1E). Two flagella insert at the anterior end of the cell (Fig. 1E). The flagella are closely arranged as a parallel pair below the rostrum (Fig. 1F). No mastigonemes or other flagellar elaborations were observed on either flagellum.

Transmission Electron Microscopy

The nucleus, with central nucleolus, is located toward the anterior end of the cell (Fig. 2A, F), but posterior to the opening of the cytostome. Mitochondria with rounded profiles and electron-dense matrix material are common (Fig. 2A—C). The mitochondria appear to lack cristae, although we cannot exclude the possibility that these are present, but not well preserved in our fixation. The mitochondria are not typically associated with endoplasmic reticulum. Food vacuoles, some with recognizably prokaryotic contents, are present toward the posterior end of the cell (Fig. 2A, B). Apparently empty vacuoles are also common (Fig. 2C). Small (approximately 100 nm diameter) electron-dense microbody-like organelles were occasionally observed (Fig. 2D). These appear to have a single bounding membrane. Discrete stacked Golgi bodies were not observed. The major cytoskeletal elements are concentrated at the anterior end of the cell (Fig. 2E). The apical rostrum is closely associated with the flagellar apparatus (Fig. 2C). Below the flagellar apparatus is the opening of the feeding apparatus, which extends into the cell as an elongate curved tube-like cytostome (Fig. 2F). Posterior to the opening of the feeding apparatus, the cell surface is subtended by a complete corset of spaced microtubules (data not shown).

The two flagella insert subapically in a near-parallel configuration, to the left of the apical rostrum (Figs 3A—C, 5A, B). In many (but not all) cells, two shorter, non-flagellated basal bodies are observed to the right of the flagellated basal bodies and lying at an angle to them (Fig. 3C). The flagella have a normal 9+2 axoneme organization, with no conspicuous paraxonemal structures, flagellar hairs, or other elaborations (Fig. 3D). The transition zone appears unremarkable (data not shown) and the flagellated basal bodies are about 420 nm long and have conventional

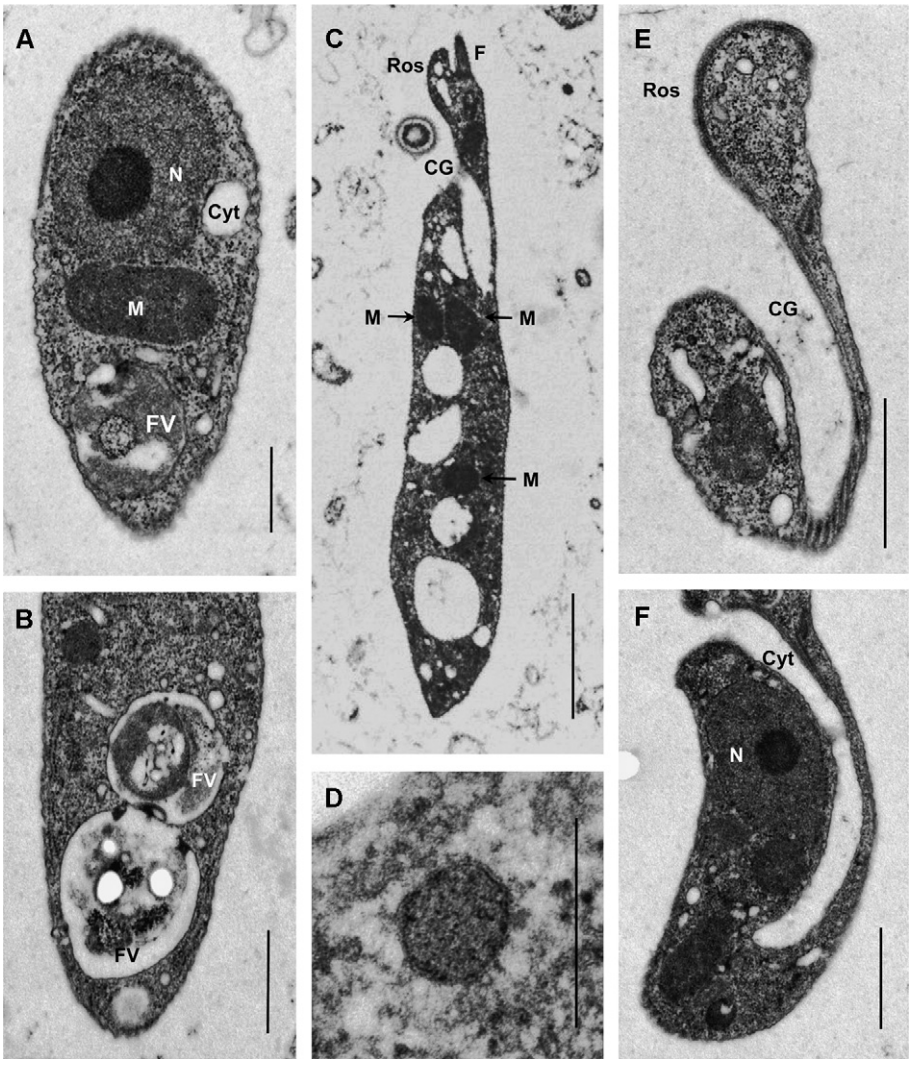


Figure 2. *Pleurostomum flabellatum*, transmission electron micrographs, ultra-thin sections. **A,B.** Oblique sections through the cell. N: nucleus; M: mitochondrion; FV: food vacuoles containing prokaryotic cells, Cyt: cytostome. (A) Scale bar: 500 nm; (B) Scale bar: 1000 nm. **C.** Longitudinal section through the cell. CG: cytostomal groove; F: flagellum. Scale bar: 2000 nm. **D.** Small microbody-like organelle. Scale bar: 200 nm. **E.** Longitudinal section of the anterior part of the cell, showing the opening of the cytostomal groove and rostrum (Ros). Scale bar: 1000 nm. **F.** Longitudinal section of the elongate, curved tube-like cytostome (Cyt). Scale bar: 1000 nm.

cartwheels (Fig. 3A, E—G). The basal bodies are connected by delicate striated fibers and are separated by approximately 70 nm at their bases (Fig. 3E—G).

There is one major microtubular root, labeled R1 (following Brugerolle and Simpson 2004). R1 originates in association with the right side of basal body 1, at an oblique angle to the basal body (Fig. 3B, C, E—G). R1 is associated with a striated rhizoplast, which originates in association with the left side of the basal bodies, especially basal body 1 (Fig. 3B, F). The rhizoplast has conspicuous lateral striations every 36 nm, which alternate with fine striations (Figs 3F, 4C). A second microtubular root is located in a similar position and orientation relative to basal body 2 as R1 is to basal body 1. This second root is very short — approximately 100 nm (Fig. 3E, F). This root is identified as R1′ (see Brugerolle and Simpson 2004). R1 consists of 12 immediately adjacent microtubules, while R1′ contains approximately seven microtubules (Fig. 4A, B).

Microtubular root R1 and the rhizoplast travel away from the basal bodies to form part of the

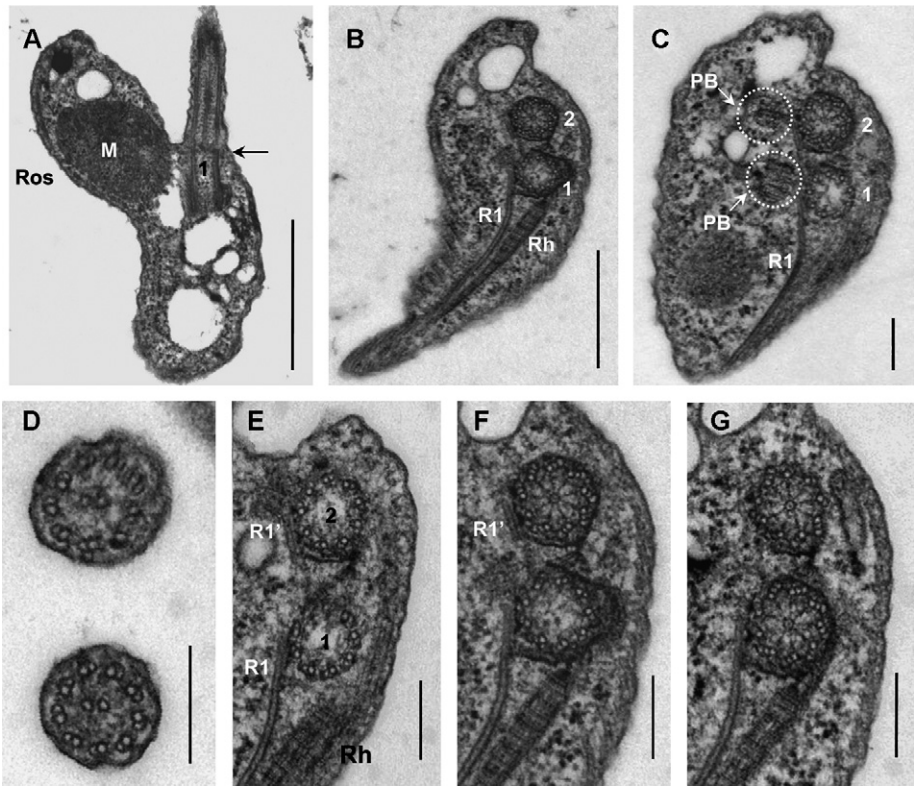


Figure 3. *Pleurostomum flabellatum*, transmission electron micrographs, ultra-thin sections. **A.** Anterior end of the cell. 1: basal body 1; Ros: rostrum; arrow: transition zone of flagellum 1; M: mitochondrion. Scale bar: 1000 nm. **B,C.** Transverse sections of the basal bodies. 2: basal body 2; R1: root 1 originating in association with basal body 1; Rh: rhizoplast originating on the left side of basal body 1; PB: two proto-basal bodies forming prior to cell division. (B) Scale bar: 500 nm; (C) Scale bar: 200 nm. **D.** Normal 9+2 axoneme structure. Scale bar: 200 nm **E—G.** Transverse serial sections of the basal bodies. Note the cartwheels and fine connecting fibers. R1′: root 1′ originating in association with basal body 2. Scale bars: 200 nm.

support for the feeding apparatus (Fig. 4C). The feeding apparatus opens as a groove below the flagellar apparatus, with its opening directed to the right. The outer/left margin of the groove is initially supported by R1 and the rhizoplast. As the groove opens the rhizoplast becomes reduced in width and a group of four microtubules diverges from the right-most/dorsal-most side of R1 (Fig. 4D). Hereafter, this group is called R1b and the remainder of the R1 is called R1a. R1a and the remnant of the rhizoplast support the 'outer' margin of the groove, while R1b forms part of the support of the floor of the groove (Fig. 4D). The remainder of the groove is supported by an array of microtubules originating from the rostrum (Fig. 4D, E; see below). Posteriorly, the groove becomes enclosed to form a cytostome, which continues to be supported by longitudinal microtubules (Fig. 4F). The cytostome lies very close to the margin of the cell and is often denoted by a slight bulge in the profile of the cell, at least in cells fixed for TEM (Fig. 4F).

A pair of slightly separated microtubules associated with a small structure of dense material originates in association with the left side of basal body 2, and extends past the left side of basal body 1 and the origin of the rhizoplast (Figs 3E, 5A—C). Several other apparently single microtubules originate in this general area. The pair of microtubules, and at least two other microtubules, continue away from the basal bodies to support the cell membrane on the outside of the outer margin of the groove (i.e. to the outside of R1a, Fig. 4D, E).

A large sheet of spaced peripheral microtubules (PMts) is located to the left of the basal bodies, closely associated with, but orthogonal to the pair of microtubules described in the previous paragraph (Figs 4E, 5A, B, D). Adjacent microtubules in the sheet are linked to each other, via connections

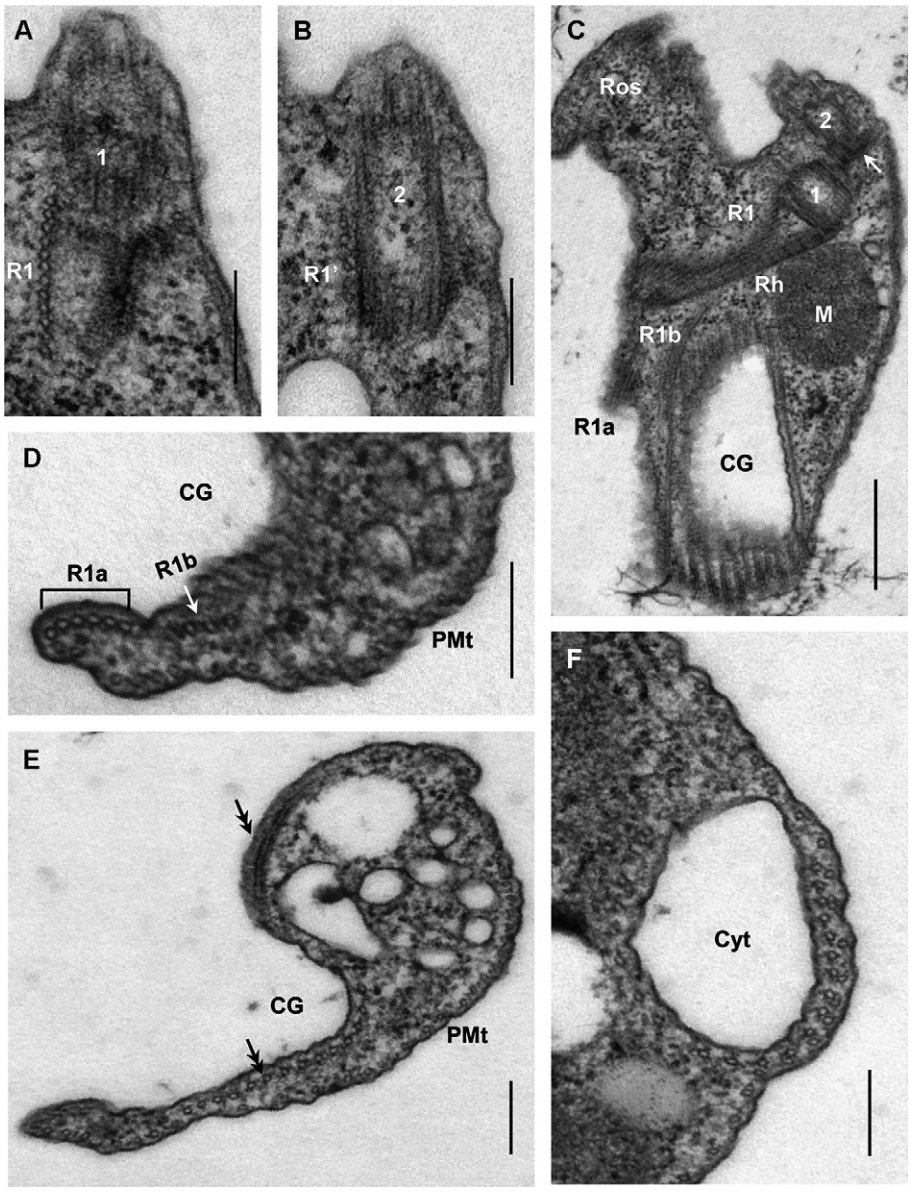


Figure 4. *Pleurostomum flabellatum*, transmission electron micrographs, ultra-thin sections. **A,B.** Transverse sections of R1 and R1′. R1 and R1′ consist of 12 and ~7 microtubules, respectively. 1: basal body 1, 2: basal body 2. Scale bars: 200 nm. **C.** Anterior end of the cell showing R1 and the rhizoplast (Rh), with R1 diverging into two parts, R1a and R1b. Note the association of R1b with the cytostomal groove (CG), M: mitochondrion. Scale bar: 500 nm. **D.** A group of four microtubules (R1b) diverging from the remainder of R1 (R1a). R1a (eight microtubules) supporting the margin of the cytostomal groove. PMt: peripheral microtubules supporting the cell membrane. Scale bar: 200 nm. **E.** The cytostomal groove is partially supported by microtubules originating from the rostrum (double-headed arrows). Scale bar: 200 nm. **F.** The groove after enclosure to form the cytostome, which continues to be supported by longitudinal microtubules. Cyt: cytostome. Scale bar: 200 nm.

to a fine filament running between them (Fig. 5D). As it runs posteriorly from the region of the basal bodies, this sheet of microtubules forms the main corset of microtubules supporting the cell. However, slightly below the basal bodies, the dorsal-most three microtubules from this sheet are slightly separated from the remainder, and become associated with additional dense material (Fig. 5C, D). If followed anteriorly, these three microtubules travel dorsally relative to the basal

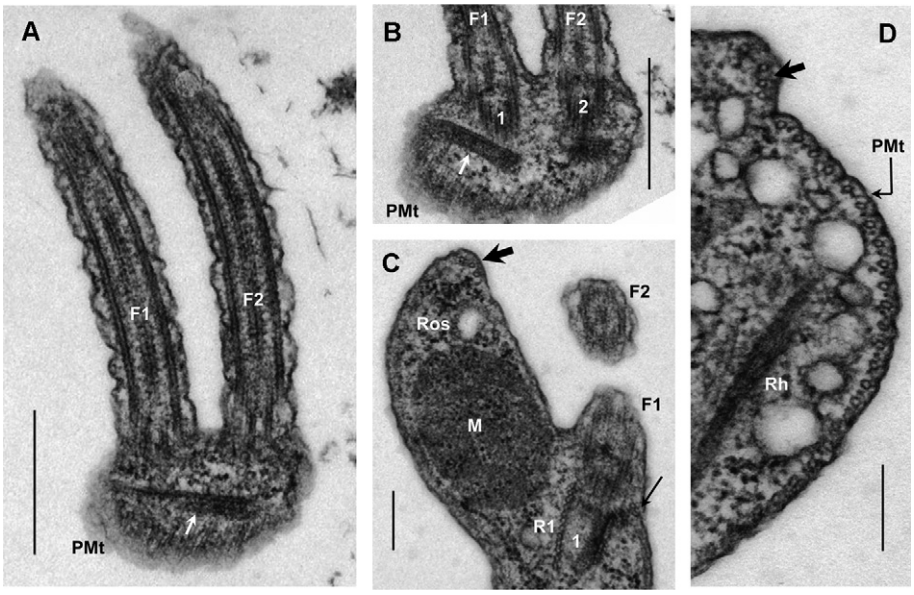


Figure 5. *Pleurostomum flabellatum*, transmission electron micrographs, ultra-thin sections. **A,B.** Consecutive sections showing parallel flagella, and two microtubules associated with dense material (arrow) between the flagella/basal bodies and the main band of peripheral microtubules (PMt). F1 and F2: flagellum 1 and 2, respectively. Scale bars: 500 nm. **C**. Transverse section of the pair of microtubules near the flagellar insertion (thinner arrow). Note also the three microtubules that support the margin of the rostrum (thicker arrow). 1: basal body 1; M: mitochondrion; R1: root 1; Ros: rostrum. Scale bar: 200 nm. **D.** The main sheet of spaced peripheral microtubules (PMt) immediately posterior to the base of the basal bodies. Note that the rostrum-supporting microtubules (thicker arrow) are originally the dorsal-most microtubules of the PMt. Rh: rhizoplast. Scale bar: 200 nm.

bodies. They then extend anteriorly relative to the flagellar insertion and follow a gradual, arcing curve to form the anterior margin of the rostrum (Fig. 5C). They terminate at the ventral 'end' of the rostrum, close to the R1 and rhizoplast.

A second major series of submembranous microtubules originates along the right side of the rostrum, probably in association with the edges of the rostrum margin microtubules described in the previous paragraph (Fig. 2E). They travel ventrally to constitute the bulk of the microtubules associated with the feeding apparatus (i.e. alongside R1b; Fig. 4D, E).

An interpretation of the proximal flagellar apparatus, and associated microtubular structures, is shown in Figure 6.

18S rRNA Gene Sequence Analysis

The 18S rRNA gene sequence amplified from *P. flabellatum* was 2598 bp long. The most similar sequence returned by a BLASTN search of the GenBank database was the heterolobosean *Acrasis rosea*. The sequence includes two unusual features. First, a group I intron 388 bp in length is inserted after position 1486 in the amplified sequence, which is within the V8 region. This is the same position as the third of three group I introns in *A. rosea*, and a group 1 intron in the 18S rRNA gene of an undescribed heterolobosean (J. D. Silberman, pers. comm.).

Second, in the V3 region of the 18S rRNA gene sequence, an insertion corresponding to helix 17_1 was identified (see supplementary material, Fig. 1). Helix 17_1 is thought to be a unique feature of Heterolobosea, and is present in all studied Heterolobosea except the basal '*Macropharyngomonas halophila*' nomen nudum (Nikolaev et al. 2004).

Phylogenetic trees estimated from 18S rRNA gene sequence data using maximum likelihood distance (minimum evolution; ME), maximum parsimony (MP), maximum likelihood (ML), and Bayesian analysis (BA) methods all recovered Heterolobosea, including *P. flabellatum,* as a monophyletic group with high bootstrap support or posterior probability (ME 96%, MP 91%, ML 100%, BA 1.00; Fig. 7A). As in previous analyses

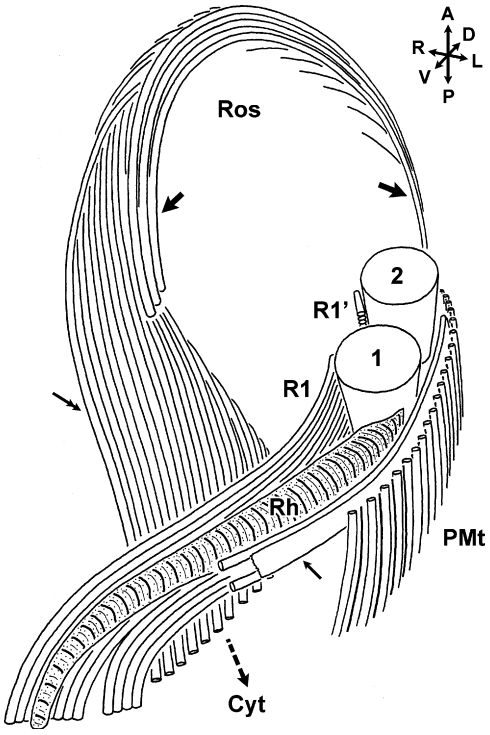


Figure 6. Interpretative diagram showing the flagellar apparatus and other cytoskeletal elements at the anterior end of the cell. Thicker arrows: the band of three microtubules supporting the margin of the rostrum; thin arrow: two microtubules associated with dense material; double-headed thin arrow: the series of microtubules originating from the rostrum that form part of the support for the feeding apparatus; Ros: rostrum; R1: root 1; R1′: root 1′; 1: basal body 1; 2: basal body 2; PMt: peripheral microtubules; A: anterior; P: posterior; R: right; L: left; D: dorsal; V: ventral.

(Nikolaev et al. 2004), '*M. halophila*' was the basal divergence within Heterolobosea (ME 100%, MP 93%, ML 99%, BA 1), while other heterolobosean taxa were divided into various relatively robust clades (Fig. 7A). These clades included (1) *Tetramitus* and *Vahlkampfia*, (2) *Naegleria* and *Willaertia*, (3) *Sawyeria*, *Psalteriomonas*, *Monopylocystis*, and *Stachyamoeba*, and (4) *Heteramoeba* and '*Plaesiobystra hypersalinica*' nomen nudum. These first two clades grouped weakly with each other and with *Acrasis* and *Percolomonas*, while the second two grouped weakly with each other and with *Paravahlkampfia* and the environmental sequence 'RT5in38'. *Neovahlkampfia* was not strongly allied to any other group in particular. Within this framework, *P. flabellatum* specifically formed the sister group of the clade composed of *Naegleria* and *Willaertia* with high bootstrap support (ME 100%, MP 91%, ML 99%) and posterior probability 1.00. When analyses were conducted with heterolobosean sequences only, *P. flabellatum* still formed a clade with *Naegleria* and *Willaertia* with high bootstrap support values (ME 100%, MP 100%, ML 98%) and posterior probability 1.00 (Fig. 7B).

Optimal Temperature and Salinity

The optimum temperature for *P. flabellatum* at 313‰ salinity was 40 °C, close to the highest in situ temperature in summer in the saltern from which this culture was isolated (Fig. 8A, Park et al. 2006b). At this temperature, *P. flabellatum* grew with a doubling time of 55 h in 313‰ salinity media (Fig. 8A). *P. flabellatum* could not grow at 15 °C or above 50 °C and grew at much lower rates at 20—30 °C or 45 °C. Within the range of the experiment, the optimum salinity of *P. flabellatum* for growth at 40 °C was 300‰—the highest salinity examined (Fig. 8B). Cultures could not grow (i.e. cell abundance decreased) at or below 150‰ salinity, and growth rates of *P. flabellatum* were substantially higher at salinities ranging from 200‰ to 300‰ (Fig. 8B). At optimal temperature and optimal salinity (i.e. at 40 °C and 300‰ salinity), a doubling time of 40 h was recorded.

Discussion

Taxonomy and Species Identification

Pleurostomum species have been observed occasionally in natural saturated brines (Namyslowski 1913; Patterson and Simpson 1996; Ruinen 1938), but never, it seems, were established in long-term culture. Therefore, the phylogenetic/systematic position and growth physiology of *Pleurostomum* have been unknown for a long time. Four of the six nominal species of *Pleurostomum* (*Pleurostomum caudatum*, *Pleurostomum salinum*, *Pleurostomum gracile* and *Pleurostomum parvulum*) were described along with the genus by Namyslowski (1913); however, no type species for the genus was specifically nominated, and the illustrations accompanying the text are of poor quality. Ruinen (1938) recorded further observations of most of Namylowski's species, but also described a new species, *Pleurostomum flabellatum*, with more detailed accompanying illustrations. The final species, *Pleurostomum turgidum*, was described by Patterson and Simpson (1996), who also observed organisms they identified with Ruinen's *P. flabellatum*,

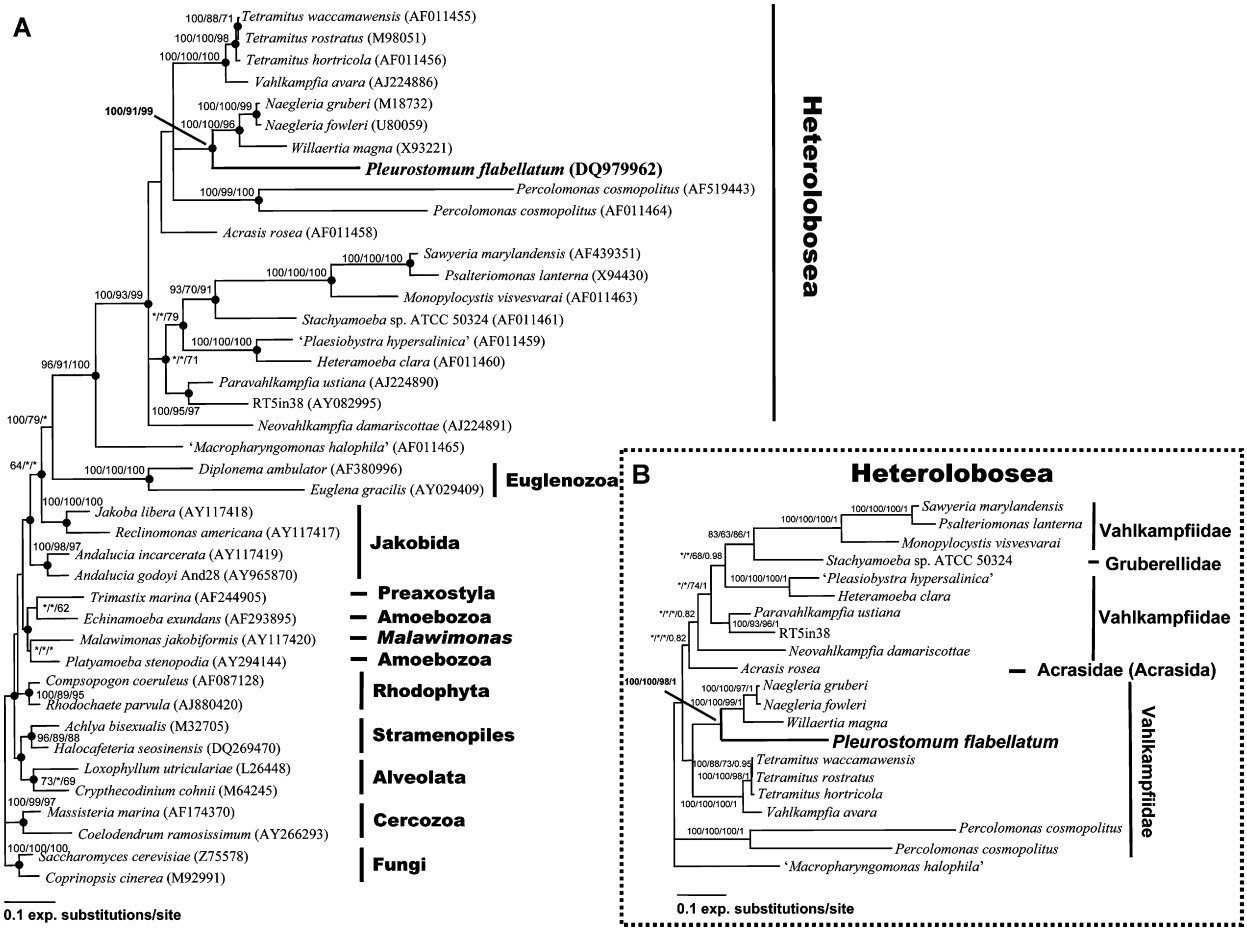


Figure 7. **A.** 18S rRNA gene tree (the tree of the Bayesian analysis) showing the phylogenetic position of *Pleurostomum flabellatum* relative to 40 other eukaryotes. Bootstrap values (>60%) from minimum evolution (ME; 10,000 replicates), maximum parsimony (MP; 10,000 replicates), and maximum likelihood (ML; 500 replicates) analyses are shown at the nodes. Bootstrap values are presented in the order ME/MP/ML. Solid circles indicate a Bayesian posterior probability of 0.95 or greater. Accession numbers of each taxon are presented in parentheses. *: Bootstrap value of <60%. **B.** 18S rRNA gene tree (the tree of the Bayesian analysis) showing the phylogenetic position of *P. flabellatum* relative to Heterolobosea only. Bootstrap values (>60%) from minimum evolution (ME; 10,000 replicates), maximum parsimony (MP; 10,000 replicates), maximum likelihood (ML; 500 replicates), and Bayesian posterior probability (MB) analyses are shown at the nodes. The bootstrap values are presented in the order ME/MP/ML and followed by Bayesian posterior probabilities. *: Bootstrap value of <60%.

and published light micrographs of live cells of both species.

On the basis of light-microscopic observations, our isolate from 313‰ salinity water is clearly consistent with *Pleurostomum*. The shape of the cell body, the number and length of the flagella, beating motion of the flagella, and length of the cytostomal structure in our species are not distinguishable from Patterson and Simpson's (1996) observations under the name *P. flabellatum*, and are similar in most respects to Ruinen's (1938) original description. Some cells studied here showed a cytoplasmic projection at the posterior end of the cell as previously reported (Ruinen 1938), although this might be a stress artifact. Furthermore, the presence of a rostrum at the anterior end of the cell is consistent with the original description of *P. flabellatum*, though not specifically noted by Ruinen (1938). Our cells are slightly smaller than those reported in the original description (16—30 μm in length, Ruinen 1938), but overlap with those reported by Patterson and Simpson (1996). Ruinen reports that *P. flabellatum* is flattened, with the part of the cell that overlies

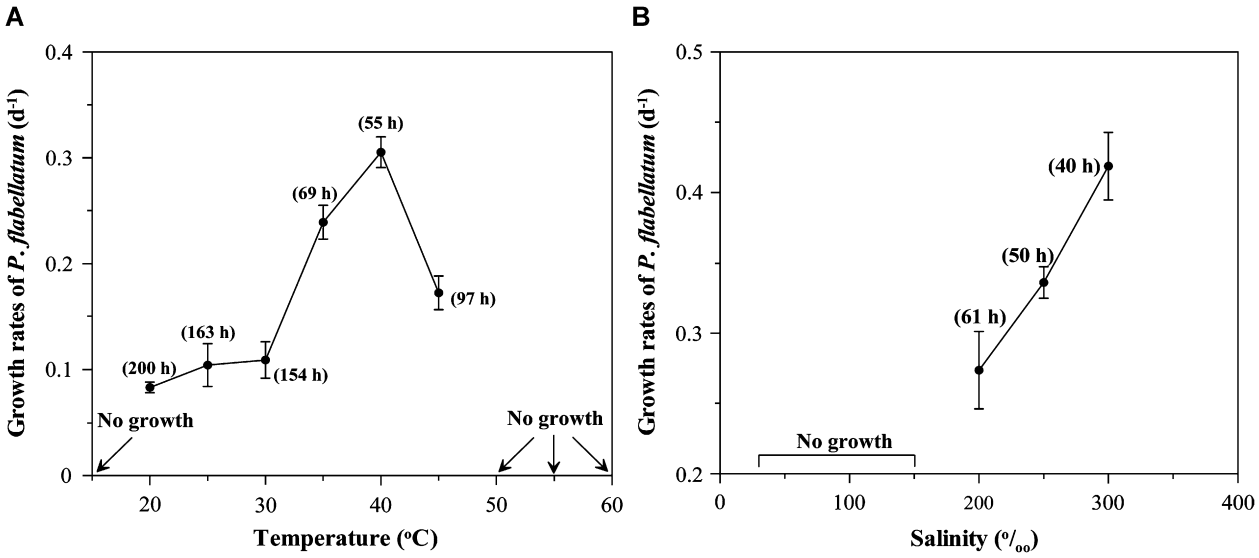


Figure 8. Specific growth rates of *Pleurostomum flabellatum* determined for a range of temperatures from 15 °C to 60 °C at 313‰ salinity (**A**) and a range of salinities from 30‰ to 300‰ salinity at 40 °C (**B**). Doubling times of *P. flabellatum* are represented in parentheses. Error bars show 1 SD.

the cytostomal structure being raised slightly to form a 'fin'. Following the cytostomal structure, this fin usually spirals around the cell. Neither the cells we observed, nor those reported by Patterson and Simpson (1996) appear markedly flattened, but we did observe a raising of the cell surface coincident with the cytostome in our transmission electron micrographs. It is possible that the flattest and most twisted cells reported by Ruinen (1938) were starving cells.

Three of the other nominal *Pleurostomum* species are readily distinguishable from our isolate: Unlike our isolate, the two flagella in *P. gracile* are usually unequal in length (Ruinen 1938). In addition, the posterior end of *P. gracile* is narrowed and the anterior end is rounded. The more ovoid cell of *P. turgidum* and greater length of its flagella (2.5—3.5 times cell length) distinguish it from our isolate. At 6 μm in length, *P. parvulum* is much smaller than our isolate.

Other than *P. flabellatum*, the two nominal species most similar to our isolate are *P. caudatum* and *P. salinum.* Like *P. flabellatum*, these species are basically elongate, and have equal flagella of body length or somewhat longer (Namyslowski 1913; Ruinen 1938). *Pleurostomum caudatum* (20 μm) and *Pleurostomum salinum* (20—22 μm) are both somewhat larger than our isolate, but within the size range for *P. flabellatum* reported by Ruinen (1938). The cells differ slightly in shape — the posterior end of *P. caudatum* forms an elongate point that often forms a fine extension (similar to *P. flabellatum*) while *P. salinum* is rod-shaped — long and thin without strongly tapering ends (Namylslowski 1913; Ruinen 1938). Furthermore, Namyslowski (1913) reports that *P. caudatum* flexes quite markedly, such that the ends of the cell can touch, while *P. salinum* is inflexible — our isolate appears to be intermediate between these two conditions. Despite the close morphological similarity between *P. flabellatum*, *P. caudatum*, and *P. salinum*, we elect to maintain recognition of all three species, and consider *P. flabellatum* the most appropriate assignation for our isolate. Future work might demonstrate that one of more of these species should be synonymized; however, we believe that this decision should be preceded by examination of additional isolates, using molecular sequence data in addition to detailed morphological examination.

There does not appear to be a nominated type species for *Pleurostomum*. We here use 'subsequent designation' to propose *P. salinum* Namylslowski, 1913 as the type species for *Pleurostomum* Namylslowski, 1913. As noted in the previous paragraph, *P. salinum* is clearly a very similar organism to *P. flabellatum* as studied here. We are not examining the type species of *Pleurostomum* in the current paper, but at this time, we contend that inferences about the broad-scale phylogenetic placement and identity of

P. flabellatum could be applied safely to the very similar *P. salinum*, and therefore to the genus *Pleurostomum* as an entity.

Phylogenetic and Systematic Position of *Pleurostomum*

Prior to this study *Pleurostomum* has been examined only by light microscopy and is currently not assigned to a contemporary higher taxon within eukaryotes (Patterson et al. 2000b). Our phylogenetic analysis of 18S rRNA gene sequences places *P. flabellatum* within Heterolobosea, with high statistical support, irrespective of methods. In addition, the Heterolobosea-specific helix 17_1 was identified in the 18S rRNA sequence of *P. flabellatum* (Nikolaev et al. 2004). Further, a group I intron was found. Group I introns are particularly common in heterolobosean 18S rRNA genes (though far from unique to this group), and the intron in *P. flabellatum* inserts in the same position within the 18S rRNA sequence as some other heterolobosean introns. Ultrastructural studies of *P. flabellatum* reveal several features that are characteristic of some or all Heterolobosea, especially flagellated forms (Brugerolle and Simpson 2004; Fenchel and Patterson 1986). These include (1) a striated rhizoplast, (2) absence of stacked Golgi bodies, (3) parallel basal bodies and flagella, and (4) a rostrum supported by a large number of PMts. A conspicuous rostrum is absent in most studied heterolobosean flagellates that have complex microtubular cytoskeletons (e.g. *Percolomonas*, *Lyromonas*, *Psalteriomonas*), but is present in *Tetramitus rostratus* (Balamuth et al. 1983). Thus, both our molecular and morphological data demonstrate clearly that *P. flabellatum* is a heterolobosean.

What is the phylogenetic and systematic position of *P. flabellatum* within Heterolobosea? Traditionally, the class Heterolobosea is divided into two orders — Acrasida (e.g. *Acrasis*) and Schizopyrenida — based on the presence of fruiting bodies in Acrasida (Page and Blanton 1985; Patterson et al. 2000a). Schizopyrenida (amoeboflagellates, Fig. 7B) is usually subdivided into the families Vahlkampfiidae and Gruberellidae (e.g. *Stachyamoeba* and *Gruberella*) primarily on the basis of whether the nucleolus persists or fragments during mitosis, but also on the morphology of the amoebae, and the presence of flagellated stages in the life history of many Vahlkampfiidae (Patterson et al. 2000a). In recent years, molecular phylogenies have indicated that both *Percolomonas* and *Psalteriomonas* fall within Heterolobosea (Nikolaev et al. 2004; O'Kelly et al. 2003; Silberman et al. 2002) and improved morphological studies have eroded previously proposed phenotypic distinctions between *Percolomonas*, *Psalteriomonas* and *Lyromonas*, and other Heterolobosea (Brugerolle and Simpson 2004). Most recent treatments of Heterolobosea implicitly or explicitly have a broad concept of Vahlkampfiidae that includes *Psalteriomonas*, and where considered, *Lyromonas* and *Percolomonas* (De Jonckheere et al. 2005; Patterson et al. 2000a). By contrast, Nikolaev et al. (2004) use Vahlkampfiidae in a restricted sense to refer to *Vahlkampfia* sensu Brown and De Jonckheere (1999), *Tetramitus* sensu De Jonckheere et al. (2005), *Naegleria*, *Willaertia*, and *Percolomonas*. They use the name Lyromonadidae for a group within Heterolobosea consisting of *Psalteriomonas*, *Sawyeria, Monopylocystis*, and, implicitly, *Lyromonas* [this implies that Psalteriomonadidae and Lyromonadidae are synonyms, although Psalteriomonadidae appears to have been created first — see Cavalier-Smith (1992, 1993)] and leave other taxa, including *Paravahlkampfia* and *Neovahlkampfia*, apparently without family assignation.

The closest relatives of *P. flabellatum* are *Naegleria* and *Willaertia*, which are consistently assigned to Vahlkampfiidae. It has been reported that some species of *Naegleria* and *Willaertia* are able to divide in the flagellated stages (De Jonckheere and Brown 1995; Dobson et al. 1993). Thus, it is possible that the strong clade of *Pleurostomum*, *Naegleria*, and *Willaertia* shares a common phenotypic characteristic — proliferating in the flagellated stages. These results collectively suggest that it is appropriate to assign *Pleurostomum* to Vahlkampfiidae.

We caution, however, that current concepts of the taxon Vahlkampfiidae are not satisfactory. Most trivially, *Pleurostomum*, *Percolomonas*, and perhaps *Lyromonas* seem to lack an amoeba stage, and thus do not conform to the traditional definition of Vahlkampfiidae. More importantly, under the broad concept of the family, Vahlkampfiidae is always recovered as a non-monophyletic group, with studied members of Acrasida and Gruberellidae falling cladistically within Vahlkampfiidae (Fig. 7B; Nikolaev et al. 2004). Restriction of Vahlkampfiidae to represent a monophyletic group (e.g. Nikolaev et al. 2004) would seem a possible solution. It must be noted, however, that there is ambiguity over the identity of *Vahlkampfia*, the type genus of family

Vahlkampfiidae. *Vahlkampfia* has traditionally been distinguished by the absence of a flagellate stage, a feature that could be lost repeatedly in evolution. Species originally assigned to *Vahlkampfia* form several different clades within Heterolobosea (Brown and De Jonckheere 1999; Nikolaev et al. 2004). Recognizing this, Brown and De Jonckheere (1999) divided *Vahlkampfia* into several taxa, creating the genera *Paravahlkampfia* and *Neovahlkampfia*. The name *Vahlkampfia* was retained for *Vahlkampfia avara* and its specific relatives, and *Vahlkampfia avara* Page, 1967 was nominated as the 'new type' for the genus (Brown and De Jonckheere 1999). This conflicts with Page's (1967) earlier analysis, which identified *Vahlkampfia vahlkampfi* Chatton, 1910 as the type species [in the original creation of *Vahlkampfia* by Chatton and Lalung-Bonnnaire (1912), several species were assigned to the genus but no type species was specified]. The former action might be more stabilizing since *V. avara* is available in culture, but does not conform to the Code of Zoological Nomenclature, while Page's treatment could constitute a legitimate, and therefore protected, act of subsequent designation under the International Code of Zoological Nomenclature (Art. 69; Ride et al. 1999). Unfortunately, there appears to be no reason to believe that *V. vahlkampfi* is any more likely to be related to *V. avara* than it is to be related to organisms currently assigned to *Paravahlkampfia* or *Neovahlkampfia* (for example). At present, defining Vahlkampfiidae in a restrictive way may be unstable, by virtue of the nomenclatural uncertainty surrounding its type genus *Vahlkampfia.*

Morphological Comparisons

Pleurostomum flabellatum is one of relatively few members of Heterolobosea in which the cytoskeleton of the flagellate stages has been examined in detail. The presence of a single major microtubular root (R1) supported by the rhizoplast is characteristic of heterolobosean flagellates with feeding apparatuses, as is the placement of a short R1′ root next to basal body 2 (Brugerolle and Simpson 2004). Unlike previously studied heterolobosean flagellates, an 'I' fiber was not observed associated with R1 (Simpson 2003). Most heterolobosean flagellates have four basal bodies; however some, including the closely related *Naegleria,* usually have two basal bodies. As in *Naegleria* flagellates, the new basal bodies in *P. flabellatum* appear to the right of the existing basal bodies (Brugerolle and Simpson 2004). *Willaertia*, on the other hand, has four basal bodies.

Most studied heterolobosean flagellates have a longitudinal groove-like feeding apparatus supported in part by the R1, which splits into two parts (Broers et al. 1990, 1993; Brugerolle and Simpson 2004; Simpson 2003). The one well-studied exception is *Tetramitus rostratus*, in which the open portion of the groove is shorter, and the feeding apparatus curves into the cell as a microtubule-supported tubular cytostome (Balamuth et al. 1983). The tubular cytostome with the broad groove-like opening seen in *P. flabellatum* is similar to that of *T. rostratus*. As in most heterolobsean flagellates the R1 of *P. flabellatum* supports the groove, and splits into two parts; however, most of the microtubules that support the cytostome seem to originate from the rostrum, rather than from between the two halves of the R1. It would be interesting to have more detailed data from *T. rostratus* to compare the microtubular structures that support the feeding apparatus, and also those that support or originate from the rostrum in *P. flabellatum* (see above).

Physiology and Ecology

Pleurostomum flabellatum was isolated from high salinity (313‰ salinity) waters and has an optimal salinity of 300‰ (possibly higher). It is striking that the doubling time of *P. flabellatum* is about 55 h in 313‰ salinity at 40 °C. In addition, the doubling time of *P. flabellatum* under optimal conditions (i.e. 300‰ salinity and 40 °C) is about 40 h (Fig. 8B). These results suggest that the energetic costs of osmoregulation are high, as proposed previously for the borderline extreme halophile — '*Halocafeteria seosinensis*' — (Park et al. 2006a). The optimal salinity of *P. flabellatum* for growth is the highest among the eukaryotes examined so far. In fact, *P. flabellatum* rates as an 'extremely halophilic eukaryote' if the same criterion used for halophilic prokaryotes is applied (Kushner 1978). Recently, the halophilicity of *H. seosinensis* was examined based on material from the same sampling site. A borderline extreme halophile, *H. seosinensis* that was isolated from 300‰ salinity water survived in saturated salt conditions (a measured 363‰ salinity) but grew optimally at 'only' 150‰ salinity (Park et al. 2006a). Likewise, the chlorophyte alga *Dunaliella salina*, commonly found in hypersaline environments, grows optimally at 120‰ salinity (Javor 1989). Interestingly, the optimal salinity of *P. flabellatum* for growth is higher than that of most extremely halophilic

prokaryotes. The halophilicity of *P. flabellatum* is similar to that of two extremely halophilic prokaryotes, *Halorhodospira halophila* and *Salinibacter ruber*, which grow optimally in 110—320‰ and 150—300‰ salinities respectively (Antón et al. 2002; Imhoff and Süling 1996). However, those prokaryotes grew below 200‰ salinity, whereas our *P. flabellatum* isolate failed to grow under similar conditions. Similarly, Ruinen (1938) observed *P. flabellatum* only at salinities of 160‰ and above, while Patterson and Simpson's (1996) observations were from saturated brine.

We did not observe the discoidal mitochondrial cristae typical of Heterolobosea in *Pleurostomum*. In fact, while the mitochondrial organelles were bounded by two membranes, no crista-like structures were observed. This may be related to the low oxygen tension of warm hypersaline water. *P. flabellatum* grew optimally at 40 °C in 300‰ salinity water where dissolved oxygen (DO) concentration measured by an oxygen probe (DO meter, YSI 58) was $1.8 \pm 0.2\,\mathrm{mg\,O_2\,l^{-1}}$ (data not shown), which is about 3 times lower than that of ordinary natural seawater (Garrison 1993). Thus, it is likely that *P. flabellatum* is routinely exposed to, and can tolerate, somewhat hypoxic conditions. One major clade of Heterolobosea, including *Sawyeria*, *Psalteriomonas*, *Monopylocystis*, and probably *Lyromonas*, lack classical mitochondria, and are either anaerobes or microaerophiles (Broers et al. 1990, 1993; O'Kelly et al. 2003), while the amoeba *Vahlkampfia anaerobica* is also anaerobic/microaerophillic (Smirnov and Fenchel 1996), but of unknown affinities within Heterolobosea. Where studied biochemically, the mitochondrial-like organelles of these Heterolobosea have proven to be hydrogenosomes (Broers et al. 1993). The biochemistry of the mitochondrial organelles of *P. flabellatum* is currently unknown, but merits further examination, especially since *P. flabellatum* is clearly not closely related to the main anaerobe/microaerophile clade within Heterolobosea.

Finally, it is notable that numerous heterolobosean taxa seem to survive in extreme environments, such as high salinity, low pH, and anoxic areas. Several hypersaline or anaerobic/microaerophillic taxa have already been listed above (i.e. *Pleurostomum*, *Sawyeria*, *Psalteriomonas*, *Monopylocystis*, and *Lyromonas*). In addition, a heterolobosean amoeba was isolated recently from our studied saltern (JSP and BCC unpubl.), and has a ribosomal RNA gene sequence similar to that deposited in Genbank under the nomen nudum '*Plaesiobystra hypersalinica*'. Larsen and Patterson (1990) hold that the halophillic species *Percolomonas salinus* (previously *Trichomastix salinus*, thence *Tetramitus salinus* — see also Ruinen 1938) is a heterolobosean on the basis of unpublished ultrastructural studies by another researcher (J. Gunderson). We speculate that *Percolomonas salinus* is the same as, or closely related to, the basal heterolobosean studied under the nomen nudum '*Macropharyngomonas halophila*', which is presumably also halophillic. Furthermore, an environmental sequence ('RT5in38') belonging to a heterolobosean has been found from a river of pH 2 (Amaral Zettler et al. 2002).

In conclusion, ultrastructural characteristics and molecular data show that the previously unassigned HNF *P. flabellatum* is a heterolobosean, and it is confirmed that this species is bacterivorous and a genuine extreme halophile.

Taxonomic Summary

Redescription of *Pleurostomum* (Namyslowski 1913)

Cells with two parallel flagella arising near the anterior end, normally beating homodynamically. With a cytostomal structure opening laterally, posterior to the point of flagellar insertion. Restricted to hypersaline habitats.

Type species: Namyslowski (1913) did not identify a type species for *Pleurostomum* when creating this genus, and we are not aware of any author since then fixing the type species of this genus by an act of subsequent designation. Therefore, under Article 69 of the International Code of Zoological Nomenclature (Ride et al. 1999), we fix *Pleurostomum salinum* (Namyslowski 1913) as the type species of *Pleurostomum,* by subsequent designation.

Redescription of *Pleurostomum flabellatum* (Ruinen 1938**):** Description: spindle-shaped cells 10—30 μm in length, with two parallel flagella of equal length (~1.5—2 times the cell length in living cells). Only flagellate forms, which can divide. With a rostrum located alongside and anterior to the flagellar insertion. Cell slightly flexible when in contact with surfaces. Restricted to very hypersaline habitats (>150‰ salinity).

Preserved material: A slide of preserved cells from a monoprotistan culture of *Pleurostomum flabellatum* is deposited in the Protist Type Specimen Slide Collection, US Natural History Museum, Smithsonian Institution, Washington, DC (USNM

slide USNM 1099761). This culture has also been deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession number CCAP 1959/1.

Assignation: Eukaryota; Heterolobosea; Vahlkampfiidae.

Methods

Isolation, cultivation, and light microscopy: *Pleurostomum flabellatum* was isolated from high salinity water (313‰ salinity) collected in March 2003 from multi-pond systems located at Seosin on the west coast of Korea (37° 09′ 36″N, 126° 40′ 44″E). Salinity in the solar saltern was measured by diluting saltern waters with distilled water to fall within the scale of a Temperature/Conductivity/Salinity Instrument (YSI 30, YSI, OH). The cultivation of *P. flabellatum* was carried out in the same manner as described by Park et al. (2006a). Live *P. flabellatum* cells were observed with phase contrast microscopy using a Zeiss Axiovert 200 M microscope equipped with an Axiocam HR digital camera. Protargol-stained cells were prepared as described by Foissner (1991), and photographed using digital camera equipment (3 megapixel, coolpix995, Nikon). A permanent slide of protargol-stained cells has been deposited in the Protist Type Specimen Slide Collection, National Museum of Natural History, Smithsonian Institution, Washington D.C (USNM 1099761). Also, a culture of this isolate of '*Pleurostomum flabellatum*' has been deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession number CCAP 1959/1.

Scanning electron microscopy: For scanning electron microscopy (SEM), cultures were fixed at 4 °C in 1% v/v electron microscopic grade glutaraldehyde (Sigma) in 0.05 M cacodylate buffer (pH 7.8). Fixed cells (1—2 ml) were collected on 0.8 μm polycarbonate filters (25 mm in diameter) under a vacuum not exceeding 100 mm Hg, and then immediately stored at −70 °C before freeze drying. Collected cells on polycarbonate filters were dried in a freeze drier at −50 °C and coated with gold/platinum with an ion sputter system. Cells were examined with a field emission scanning electron microscope (JSM-6700F, Japan).

Transmission electron microscopy: For ultrathin sectioning, cells were grown in 250‰ salinity media. The cells were centrifuged at 5000 *g* for 3 min and fixed for 30 min at room temperature in a cocktail containing 4% v/v glutaraldehyde, 22.4% w/v NaCl, and 0.1 M cacodylate buffer (pH 7.4). After rinsing the cells three times with 22.4% w/v NaCl in 0.1 M cacodylate buffer, cells were post-fixed for 1 h in 0.8% w/v OsO_4 and 21% w/v NaCl in 0.1 M cacodylate. After being rinsed free of post-fixative, cells concentrated by centrifugation were trapped in 1.5% (w/v) agarose. Agarose blocks were dehydrated by applying a graded series of ethanol-water mixtures, and then embedded in Spurr's resin. Serial sections were cut with a diamond knife on a Leica UC6 ultramicrotome (Leica, UK) and were subsequently stained with saturated uranyl acetate in 50% ethanol and with lead citrate. Sections were observed using a Tecnai 12 electron microscope (Philips). A goniometer stage was used for about half of the observations.

Molecular sequencing: Nucleic acid from *P. flabellatum* cultures was prepared in the same manner as reported by Park et al. (2006a). Amplification of 18S rRNA genes was performed using standard polymerase chain reaction (PCR) protocols with eukaryote-specific primers EukA and EukB (Medlin et al. 1988). The reaction mixture contained 50—100 ng of DNA, 0.2 mM deoxynucleoside triphosphate, each primer at a concentration of 0.5 μM, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (Invitrogen, Canada). The reaction was cycled 40 times. The size of the PCR products (~2.6 kb) was determined by agarose gel electrophoresis. Amplicons were cloned into a TA vector (TOPO 2.1, Invitrogen), four positive clones were partially sequenced, and then one positive clone was completely sequenced using various eukaryotic sequencing primers. The 18S rRNA gene sequence from *P. flabellatum* has been deposited in GenBank under the accession number DQ979962.

Phylogenetic analysis: The 18S rRNA gene sequence from *P. flabellatum* was compared to the sequences in the GenBank database using a BLASTN search. Sequences were manually aligned with those from Heterolobosea and selected other eukaryotes based on a seed alignment kindly provided by C. Berney (Oxford University) and augmented by sequences obtained from GenBank. A total of 1164 of unambiguously aligned sites was retained for phylogenetic analysis. We also constructed a data set including only Heterolobosea with 1179 unambiguously aligned sites. These alignments are available on request.

Phylogenetic trees were inferred by maximum likelihood (Felsenstein 1981), maximum likelihood distance, and maximum parsimony methods using PAUP* 4b10 (Swofford 1998), and by

Bayesian analysis using MrBAYES 3.0 (Huelsenbeck and Ronquist 2001). For the analyses except parsimony, the Tamura-Nei+gamma+I model (Tamura and Nei 1993) was used (this model was chosen over similar models by likelihood ratio tests). For the distance and likelihood analyses, the parameter values were estimated from a test tree using PAUP*. For each distance analysis, the minimum evolution tree was found using 20 random additions and tree bisection-reconnection (TBR) branch-swapping and a bootstrap analysis (Felsenstein 1985) was performed with 10,000 replicates (five random additions and TBR). For each maximum likelihood analysis, the best tree was found using 20 random additions and TBR, and a 500 replicate bootstrap analysis was performed (neighbor-joining starting trees, then TBR). To estimate Bayesian posterior probabilities, four simultaneous Markov Chain Monte Carlo (MCMC) chains were run for 1,000,000 generations and sampled every 500 generations (burn-in 200,000 generations).

Cell abundance, optimal temperature, and optimal salinity: The procedure for measuring abundance, optimal temperature, and optimal salinity was the same as reported by Park et al. (2006a). Briefly, to determine the optimal growth temperature, culture flasks containing 30 ml of FAHS medium (0.2 μm filtered and autoclaved high salinity water) supplemented with heat-killed bacteria (*Idiomarina seosinensis*, Choi and Cho 2005) were inoculated with actively growing *P. flabellatum*, and maintained in the dark at temperatures ranging from 15 to 60 °C at 5 °C intervals (two cultures per temperature). Heat-killed bacteria were centrifuged at 3,000 rpm for 5 min, and washed once with 1 ml of FAHS medium. Cell abundance was monitored daily for 9—13 d with additional heat-killed bacteria added at 2—3 d intervals (final conc. of $1.4—2.4 \times 10^8$ cells ml^{-1}). Growth rates at each temperature were calculated during exponential growth.

To determine optimal growth salinity, an artificial seawater stock (AS medium, 306‰ salinity; 243.2 g NaCl, 7.6 g KCl, 54.4 g $MgCl_2 \cdot 6H_2O$, 59.4 g $MgSO_4 \cdot 7H_2O$, 1.4 g $CaCl_2 \cdot 2H_2O$ l^{-1} double-distilled water) was used as described by Park et al. (2006a). Heat-killed bacteria were centrifuged at 3000 rpm for 5 min, and washed once with 1 ml of PBS solution (0.05 M Na_2HPO_4-0.85% NaCl, pH 9). After centrifugation, the heat-killed bacteria were resuspended in AS media of the appropriate salinity (30—300‰) to avoid salinity changes in the experimental culture. Various salinity AS media (40 ml) were inoculated with actively growing *P. flabellatum* (final conc. of 1.7×10^3 cells ml^{-1}) and heat-killed bacteria (final conc. of $1.3—1.5 \times 10^8$ cells ml^{-1}), and incubated in the dark at 40 °C for 9 d, with additional heat-killed bacteria added at 2—3 d intervals. The growth rates of *P. flabellatum* were determined as above.

Acknowledgements

The authors thank C. Berney (Oxford University) and J. Silberman (University of Arkansas) for providing 18S rRNA gene sequence alignments, G. Tarrant for German translation, and A. Heiss (Dalhousie University) for proof-reading. The present study was supported by the BK 21 project of the Korean Government, the Korea Sea Grant Program (BCC), and NSERC Grant 298366-04 to AGBS. Some computational resources were provided by Genome Atlantic, Project in Microbial Genomics. AGBS thanks the Canadian Institute for Advanced Research (CIAR) for support as a 'scholar' in the Evolutionary Biology Program.

Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.protis.2007.03.2004.

References

Amaral Zettler LA, Gómez F, Zettler E, Keenan BG, Amils R, Sogin ML (2002) Eukaryotic diversity in Spain's river of fire. Nature **417**: 137

Antón J, Oren A, Benlloch S, Rodríguez-Valera F, Amann R, Rosselló-Mora R (2002) *Salinibacter ruber* gen. nov., sp. nov., a novel extreme halophilic bacterium from saltern crystallizer ponds. Int J Syst Evol Microbiol **52**: 485—491

Balamuth W, Bradbury PC, Schuster FL (1983) Ultrastructure of the amoeboflagellate *Tetramitus rostratus*. J Protozool **30**: 445—455

Broers CAM, Stumm CK, Vogels GD, Brugerolle G (1990) *Psalteriomonas lanterna* gen. nov., sp. nov., a free-living amoeboflagellate isolated from freshwater anaerobic sediments. Europ J Protistol **25**: 369—380

Broers CAM, Meyers HHM, Seymens JC, Stumm CK, Vogels GD, Brugerolle G (1993) Symbiotic association of *Psalteriomonas vulgaris* n. spec. with *Methanobacterium formicicum*. Europ J Protistol **29**: 98—105

Brown S, De Jonckheere JF (1999) A reevaluation of the amoeba genus *Vahlkampfia* based on SSU rDNA sequences. Europ J Protistol **35**: 49—54

Brugerolle G, Simpson AGB (2004) The flagellar apparatus of heteroloboseans. J Eukaryot Microbiol **51**: 96—107

Cavalier-Smith T (1992) Percolozoa and the Symbiotic Origin of the Metakaryotic Cell. In Sato S, Ishida M, Ishikawa H (eds) Endocytobiology V. University Press, Tübingen, pp 399—406

Cavalier-Smith T (1993) The kingdom Protozoa and its 18 phyla. Microbiol Rev **57**: 953—994

Chatton E, Lalung-Bonnarie (1912) Amibe limax (*Vahlkampfia* n. gen.) dans l'intestin humain. Son importance pour l'interpretation des amibes de culture. Bull Soc Pathol Exot **5**: 135—143

Cho BC (2005) Heterotrophic Flagellates in Hypersaline Waters. In Gunde-Cimerman N, Oren A, Plemenitaš A (eds) Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya. Springer-Verlag, Berlin, Heidelberg-, New York, pp 543—549

Choi DH, Cho BC (2005) *Idiomarina seosinensis* sp. nov., isolated from hypersaline water of a solar saltern in Korea. Int J Syst Evol Microbiol **55**: 379—383

De Jonckheere JF, Brown S (1995) *Willaertia minor* is a species of *Naegleria*. Evidence that *Naegleria* flagellates can divide. Europ J Protistol **31**: 58—62

De Jonckheere JF, Brown S, Walochnik J, Aspöck H, Michel R (2005) Morphological investigation of three *Tetramitus* spp which are phylogenetically very closely related: *Tetramitus horticolus*, *Tetramitus russelli* n. comb. and *Tetramitus pararusselli* n. sp. Europ J Protistol **41**: 139—150

Dobson PJ, Robinson BS, Christy P, Hayes SJ (1993) Low genetic diversity in *Willaertia magna* from wide geographical sources, and characterization of *Willaertia minor* n. sp. (Heterolobosea, Vahlkampfiidae). J Eukaryot Microbiol **40**: 298—304

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol **17**: 368—376

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**: 783—791

Fenchel T, Patterson DJ (1986) *Percolomonas cosmopolitus* (Ruinen) n. gen., a new type of filter-feeding flagellate from marine plankton. J Mar Biol Assoc UK **66**: 465—482

Foissner W (1991) Basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa. Europ J Protistol **27**: 313—330

Garrison T (1993) Oceanography: An Invitation to Marine Science. Wadsworth, Belmont, CA, p 132

Huelsenbeck JP, Ronquist F (2001) MrBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17**: 754—755

Imhoff JF, Süling J (1996) The phylogenetic relationship among Ectothiorhodospiraceae: a reevaluation of their taxonomy on the basis of 16S rDNA analyses. Arch Microbiol **165**: 106—113

Javor B (1989) Hypersaline Environments: Microbiology and Biogeochemistry. Springer-Verlag, New York, pp 147—158

Kushner DJ (1978) Microbial Life in Extreme Environments. Academic Press, London and new York, p 323

Larsen J, Patterson DJ (1990) Some flagellates (Protista) from tropical marine sediments. J Nat Hist **24**: 801—937

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. Gene **71**: 491—499

Namyslowski B (1913) Über unbekannte halophile Mikroorganismen aus dem Innern des Salzbergwerkes Wieliczka. Bull Int Acad Sci Krakow B **3/4:** 88—104

Nikolaev SI, Mylnikov AP, Berney C, Fahrni J, Pawlowski J, Aleshin VV, Petrov NB (2004) Molecular phylogenetic analysis places *Percolomonas cosmopolitus* within Heterolobosea: evolutionary implications. J Eukaryot Microbiol **51**: 575—581

O'Kelly CJ, Silberman JD, Amaral Zettler LA, Nerad TA, Sogin ML (2003) *Monopylocystis visvesvarai* n. gen., n. sp. and *Sawyeria marylandensis* n. gen., n. sp.: two new amitochondrial heterolobosean amoebae from anoxic environments. Protist **154**: 281—290

Page FC (1967) Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmanella* and 3 of *Vahlkampfia*. J Protozool **14**: 499—521

Page FC, Blanton RL (1985) The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). Protistologica **12**: 37—53

Park JS, Cho BC, Simpson AGB (2006a) *Halocafeteria seosinensis* gen. et sp. nov. (Bicosoecida) A halophilic bacterivorous nanoflagellate isolated from a solar saltern. Extremophiles **10**: 493—504

Park JS, Kim HJ, Choi DH, Cho BC (2003) Active flagellates grazing on prokaryotes in high salinity waters of a solar saltern. Aquat Microb Ecol **33**: 173—179

Park JS, Choi DH, Hwang CY, Park GJ, Cho BC (2006b) Seasonal study on ectoenzyme activities, carbohydrate concentrations, prokaryotic abundance and production in a solar saltern in Korea. Aquat Microb Ecol **43**: 153—163

Patterson DJ (1999) The diversity of eukaryotes. Am Nat **154**: S96—S124

Patterson DJ, Simpson AGB (1996) Heterotrophic flagellates from coastal marine and hypersaline sediments in Western Australia. Europ J Protistol **32**: 423—448

Patterson DJ, Rogerson A, Vørs N (2000a) Class Heterolobosea. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa, 2nd edn. Society of Protozoologists, Lawrence, Kansas, pp 1104—1111

Patterson DJ, Vørs N, Simpson AGB, O'Kelly CJ (2000b) Residual Free-living and Predatory Heterotrophic Flagellates. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa, 2nd edn. Society of Protozoologists, Lawrence, Kansas, pp 1302—1328

Post FJ, Borowitzka LJ, Borowitzka MA, Mackay B, Moulton T (1983) The protozoa of a Western Australian hypersaline lagoon. Hydrobiologia **105**: 95—113

Ride WEL, Cogger HG, Dupuis C, Kraus O, Minelli A, Thompson FC, Tubbs PK (1999) International Code of Zoological Nomenclature, 4th edn. The International Trust for Zoological Nomenclature, The Natural History Museum, London, UK

Ruinen J (1938) Notizen über Salzflagellaten. II. Über die Verbreitung der Salzflagellaten. Arch Protistenkd **90**: 210—258

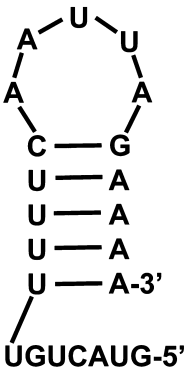
Silberman JD, Simpson AGB, Kulda J, Cepicka I, Hampl V, Johnson PJ, Roger AJ (2002) Retortamonad flagellates are closely related to diplomonads — implications for the history of mitochondrial function in eukaryote evolution. Mol Biol Evol **19**: 777—786

Simpson AGB (2003) Cytoskeletal organization, phylogenetic affinities and systematics in the contentious taxon Excavata (Eukaryota). Int J Syst Evol Microbiol **53**: 1759—1777

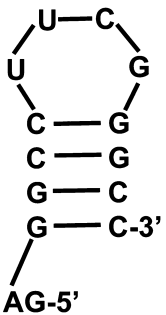
Smirnov AV, Fenchel T (1996) *Vahlkampfia anaerobica* n. sp. and *Vannella peregrina* n. sp. (Rhizopoda) — anaerobic amoebae from a marine sediment. Arch Protistenkd **147**: 189—198

Swofford DL (1998) *PAUP**4.0. Sinauer Associates, Sunderland, MA

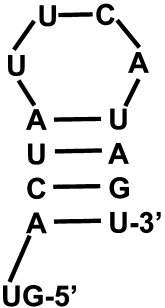
Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol **10**: 512—526



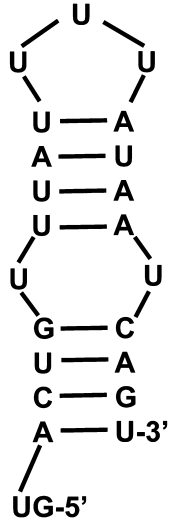
Pleurostomum flabellatum



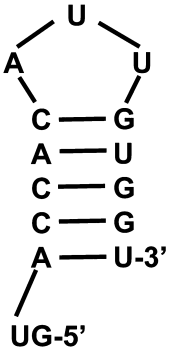
Percolomonas cosmopolitus



Acrasis rosea



Psalteriomonas lanterna



Tetramitus rostratus

Supplementary Figure 1