Protist, Vol. 160, 265—283, May 2009
http://www.elsevier.de/protis
Published online date 29 December 2008

Protist

ORIGINAL PAPER

Ultrastructure and Molecular Phylogeny of two Heterolobosean Amoebae, *Euplaesiobystra hypersalinica* gen. et sp. nov. and *Tulamoeba peronaphora* gen. et sp. nov., Isolated from an Extremely Hypersaline Habitat

Jong Soo Park[a], Alastair G.B. Simpson[a,1], Susan Brown[b], and Byung Cheol Cho[c]

[a]Canadian Institute for Advanced Research, Program in Integrated Microbial Diversity, and Department of Biology, Dalhousie University, Halifax, Canada B3H 4J1
[b]Dorchester, Dorset, UK
[c]Molecular and Microbial Ecology Laboratory, School of Earth and Environmental Sciences, Seoul National University, Seoul 151-742, Republic of Korea

Submitted July 7, 2008; Accepted October 11, 2008
Monitoring Editor: Michael Melkonian

We isolated two amoebae, *Tulamoeba peronaphora* gen. et sp. nov. and *Euplaesiobystra hypersalinica* gen. et sp. nov. from the high salinity waters (293—300‰ salinity) of a Korean solar saltern. These new species show features typical of Heterolobosea — a limax form with eruptive pseudopodial formation, flattened/discoidal mitochondrial cristae, cysts with plugged pores, and no discrete, stacked dictyosomes. 18S rRNA gene phylogenies place both species within the Heterolobosea. *Tulamoeba peronaphora* appears to lack a flagellate phase, and has one cyst pore that penetrates the cyst wall. In 18S rRNA gene trees, *Tulamoeba peronaphora* is specifically related to *Pleurostomum flabellatum*, an extreme halophile that is observed only as a flagellate. Its next closest relatives are *Naegleria* and *Willaertia. Euplaesiobystra hypersalinica* has 2—4 cyst pores in the ectocyst wall (only), and has a bi-flagellated flagellate phase with no obvious cytostome. Its closest described relative is *Heteramoeba clara*, which is marine, has a cytostome, lacks cyst pores, and has a different nucleolus organization. The *Euplaesiobystra hypersalinica* 18S rRNA gene is 99.5% identical to a sequence accessed under the nomen nudum '*Plaesiobystra hypersalinica*' — we consider them the same species. *Tulamoeba peronaphora* grows at 75—250‰ salinity, while *E. hypersalinica* grows at 100—300‰ (at least) salinity. Both amoebae seem to be 'extreme halophiles', and their ancestors invaded high salinity environments independently of each other. These results provide more evidence that there is a substantial ecological and phylogenetic diversity of heterotrophic eukaryotes capable of growing in very high salinity environments, and these ecosystems may be more complex than usually assumed.

Key words: amoeba; cyst; Heterolobosea; halophile; molecular phylogeny; protozoa.

[1]Corresponding author; fax +1 902 494 3736.
e-mail Alastair.Simpson@dal.ca (A.G.B. Simpson).

doi:10.1016/j.protis.2008.10.002

Introduction

Extremely hypersaline habitats (>300‰ salinity) are widely, but sparsely distributed across Earth (Javor 1989). Microorganisms known to inhabit these environments are mainly prokaryotes (e.g. Haloarchaea and *Salinibacter*, Antón et al. 2000; Guixa-Boixareu et al. 1996) and the autotrophic eukaryote *Dunaliella* (Javor 1989). Heterotrophic eukaryotes are often regarded as absent from high salinity waters (Guixa-Boixareu et al. 1996; Pedrós-Alió et al. 2000), implying that these ecosystems may lack typical predators. This view, however, sits uncomfortably with several anecdotal/faunistic accounts of various heterotrophic protists living in saturated or near-saturated brines (Hauer and Rogerson 2005a; Kirby 1932; Namyslowski 1913; Patterson and Simpson 1996; Post et al. 1983; Ruinen 1938a, b; Volcani 1945). Recently Park et al. (2003) demonstrated that heterotrophic eukaryotes, specifically small flagellates, were causing significant prokaryote mortality in a Korean saltern with a salinity of >300‰. Two extremely halophilic, bacterivorous heterotrophic flagellates — *Halocafeteria seosinensis* and *Pleurostomum flabellatum* — were subsequently isolated from this environment (Park et al. 2006a, 2007). On the basis of ultrastructural characteristics and molecular phylogeny, *Halocafeteria seosinensis* and *Pleurostomum flabellatum* were identified as belonging to Bicosoecida and Heterolobosea, respectively (Park et al. 2006a, 2007). Both species actively grow in media of >300‰ salinity, and *Pleurostomum flabellatum* actually seems to grow optimally at this salinity level (Park et al. 2007).

It seems unlikely, however, that flagellates are the only ecologically relevant heterotrophic eukaryotes in extremely hypersaline habitats. Prokaryotic mortality caused by heterotrophic flagellates was estimated to be 25—85% in >300‰ salinity waters (Park et al. 2003). Considering that viral lysis was estimated to be responsible for <5% of total prokaryotic losses in high salinity water (Guixa-Boixareu et al. 1996; Pedrós-Alió et al. 2000), other factors, perhaps other predatory or parasitic organisms, could be contributing significantly to prokaryotic mortality. In other aquatic environments, ciliates and amoebae consume prokaryotes. Recently, Cho et al. (2008) reported the isolation and culture of an extremely halophilic ciliate that ingests both prokaryotes and *Dunaliella* sp. and through morphological and molecular phylogenetic studies identified this organism as a new species, *Trimyema koreanum*, in the class Plagiopylea (Cho et al. 2008). However, we are unaware of any comparable polyphasic studies of amoebae that have been cultured from extremely hypersaline habitats.

There are several reports of bacterivorous amoebae of various kinds in moderately hypersaline or very hypersaline environments (e.g. Hamburger 1905; Hauer and Rogerson 2005a; Hauer et al. 2001; Post et al. 1983; Rogerson and Hauer 2002; Ruinen and Baas Becking 1938; Volcani 1943), collectively representing dozens of distinguishable species (Hauer and Rogerson 2005b; Rogerson and Hauer 2002). Ruinen and Baas Becking (1938) summarized previous studies and recorded new observations of amoebae from saline habitats. Included along with polypodial lobose amoebae and forms with filose pseudopodia are accounts of two nominal species of limax amoebae with eruptive pseudopodia that likely represent heteroloboseans. Volcani (1943) reported that an amoeba isolated from the Dead Sea at >300‰ salinity grew optimally at 150—180‰ salinity and tolerated >330‰ salinity. Volcani's amoeba could transform into a flagellate with two flagella, and may therefore also have been a heterolobosean. Post et al. (1983) observed amoebae that they identified as members of the heterolobosean genera *Heteramoeba* and *Naegleria*, plus three other unclassified species, in samples with salinities of 170—200‰, with the '*Naegleria* sp.' observed in cultures up to saturation. More recently, Rogerson and Hauer (2002) isolated several amoebae identified by light microscopy as Heterolobosea (e.g. *Vahlkampfia*) from samples of ~160‰ salinity from a pond at the margin of the hypersaline Salton Sea, California. Furthermore, an 18S rRNA gene sequence that clearly branches within the Heterolobosea has been accessed to Genbank under the name '*Plaesiobystra hypersalinica*' and appears under this name in several published phylogenies (Nikolaev et al. 2004; Park et al. 2007). There are no published morphological data, nor a formal description of this genus or species, but the organism is an amoeba that was isolated from ~140‰ salinity water (T.A. Nerad, pers. comm.). Thus, there is good evidence that a variety of amoebae exist in hypersaline environments (sensu lato), and that many of them might be members of Heterolobosea.

Here, we investigate the light microscopical and ultrastructural characteristics, and 18S rRNA gene sequences, of two amoebae that have been isolated from extremely high salinity waters

(293—300‰ salinity), and maintained as monoprotistan cultures. Both are bacterivorous, produce cysts with plugged pores and can grow in very hypersaline media. Our study demonstrates that both amoebae belong to the Heterolobosea, but are not closely related to each other. One might never been observed before in detail, and is described as *Tulamoeba peronaphora* gen. et sp. nov. Its closest known relative is the extremely halophilic flagellate *Pleurostomum flabellatum*. The other amoeba (which also has a flagellate phase) is 99.5% identical to '*Plaesiobystra hypersalinica*' in 18S rRNA gene sequence. Given the nomenclatural unavailability of the name '*Plaesiobystra*' this second species is described as *Euplaesiobystra hypersalinica* gen. et sp. nov.

Results

Tulamoeba peronaphora

Light Microscopy: The length and width of the actively moving trophozoites (amoebae) were 6—17 μm (mean ± SD of 8.8 ± 2.3 μm, $n = 30$) and 2—6 μm (mean ± SD of 4.1 ± 0.9 μm, $n = 30$), respectively. The average ratio of length to width of the trophozoites was 2.2 (range 1.4—3.4). The trophozoites generally had a monopodial limax appearance (Fig. 1A—D). Pseudopodial progression was markedly eruptive. Some trophozoites occasionally showed a long hyaloplasm (up to half of total cell length) when feeding on prokaryotes (Fig. 1E). Fine uroidal filaments were sometimes visible, but no distinct, bulbous uroid (Fig. 1F). No flagellate form was observed.

The isolate formed cysts in both old and young cultures. The cysts were commonly observed associated with prokaryotic aggregates (Fig. 1G). The cysts typically showed a spherical (Fig. 1H, I) or slightly ellipsoidal (Fig. 1J) shape. The diameter of the cysts was between 6 and 10 μm (mean ± SD of 7.9 ± 1.2 μm, $n = 30$). The cytoplasm often contained a single rounded inclusion that constituted a large fraction of the total volume of the cyst (Fig. 1H—J). The main portion of the cyst wall was always < 1 μm thick (Fig. 1H, I). Cysts had a single pore that penetrated the cyst wall (Fig. 1H—J) and the cyst wall around this pore was somewhat thickened (Fig. 1H—J). The pore contained a plug that was difficult to see but which extended markedly into the interior of the cyst (Fig. 1H, I). Sometimes we did not observe cytoplasm within the confines of the cyst wall (Fig. 1G), perhaps due to excystment. No ectocyst was observed.

Ultrastructure: Trophozoites of *Tulamoeba peronaphora* had a single nucleus with a single, central, and electron-dense nucleolus (Fig. 2A, B). In most cells hyaloplasmic regions were observed — these regions were devoid of large organelles and other inclusions and represent the progressing pseudopod (Fig. 2A, B). Fine pseudopodial projections were sometimes observed (Fig. 2B). Numerous mitochondrial profiles were observed, especially near the nucleus (Fig. 2A, B). Mitochondria had flattened/discoidal cristae and were commonly surrounded by a portion of the rough endoplasmic reticulum (Fig. 2C). Within the cytoplasm food vacuoles were usually observed, with some containing partially digested prokaryotic prey (Fig. 2A, D). Discrete dictyosomes were not observed in the trophozoites (nor in the cysts — see below), although concentrations of unstacked endomembranes of unknown identity were seen within the cell, likely inflated by fixation conditions (arrows in Fig. 2A, B).

Cysts had a single very large inclusion with a granular consistency (Fig. 2E, F). The nucleus was located close to the cell membrane (Fig. 2G). In each cyst there were several round vesicles with numerous bound ribosomes (Fig. 2F, H). In the fixed material there was a space between cyst wall and cytoplasm (Fig. 2E, F), most likely a fixation artefact. In isolated places the cell membrane had a highly rugose or dimpled appearance — clusters of small vesicular structures seen near the cell membrane might largely or entirely represent invaginations of the cell membrane rather than separate vesicles (Fig. 2F).

The cyst wall presented as a moderately electron-dense, fibrous matrix (Fig. 2E—G, I). The inner half of the cyst wall could appear less fibrous and more electron-dense than the outer half — this was most noticeable in grazing sections of the cyst wall (Fig. 2J). As with light microscopic observations, discrete ectocyst material was not observed (Fig. 2E—G, I). Cysts had one pore that penetrated the cyst wall (Figs 2K, 3B). The cyst wall was noticeably thickened around the pore (Figs 2E, K, 3B). The pore was filled by a plug of weakly-straining material that extended deeply into the interior of the cyst, to be closely associated with the encysted cell at its base (Fig. 2F). The outer surface of the plug was dome-shaped and less electron-dense than the remainder of the plug.

Scanning electron micrographs showed the surface of the cysts to be somewhat rugose

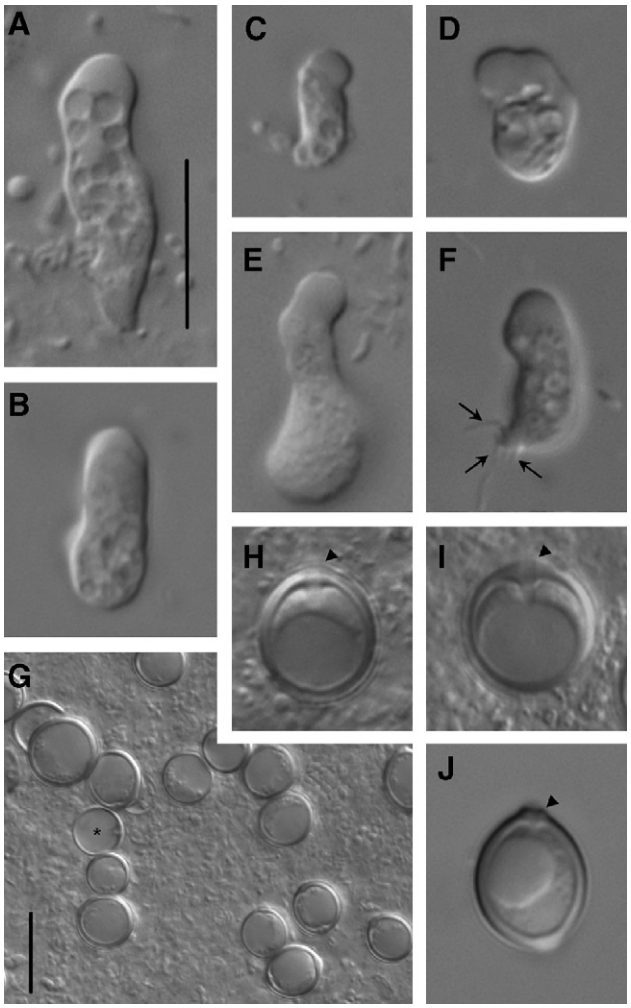

Figure 1. *Tulamoeba peronaphora*, n. gen., n. sp., cultured from high salinity (300‰) water, Differential Interference Contrast (DIC) micrographs. **A—F**. Amoebae (trophozoites) showing size, shape, and pseudopod formation. Note the uroidal filaments in F (arrows). **G—J**. Cysts. Asterisk in G denotes an empty cyst. Arrowheads in H—J denote the single cyst pore with intrusive plug. Note also the single large inclusion of most cysts. Scale bar in A represents 10 μm for all figures except G. Scale bar in G represents 10 μm.

(Fig. 3A, B). The cyst pore could be identified clearly (Fig. 3B), however the plug may not survive specimen preparation.

Euplaesiobystra hypersalinica

Light microscopy: The length of the trophozoite of the isolate of *Euplaesiobystra hypersalinica* ranged from 19 to 41 μm (mean ± SD of 30.5 ± 5.6 μm, $n = 30$), and the width was between 9 and 16 μm (mean ± SD of 12.0 ± 1.9 μm, $n = 30$). The average ratio of length to width was approximately 2.6 (range; 1.6—3.5). Trophozoites were generally limax and monopodial, and showed eruptive pseudopodial formation (Fig. 4A—D). Some trophozoites showed fine uroidal filaments when they moved, but no distinct, bulbous uroid (Fig. 4E). Cells contained presumed food vacuoles, and large rounded inclusions with homogenous contents, possibly oil droplets (Fig. 4A—E). Pink particles were observed in vacuoles in cells from old cultures (Supplementary Fig. S1).

Interestingly, we occasionally observed a flagellate form in cultures of *E. hypersalinica* (Fig. 4F). The flagellates had two equal flagella that moved homodynamically. The flagellar length was about two times body length. The cell body showed a more-or-less spherical shape with a diameter of ~20 μm (Fig. 4F). We did not observe the flagellates ingesting prokaryotes.

This isolate also formed spherical cysts, which were associated with prokaryotic aggregates in the culture (Fig. 4G). The diameter of the cysts ranged from 14 to 20 μm (mean ± SD of 16.8 ± 1.7 μm, $n = 30$), and most cyst walls were 1—2 μm in thickness (Fig. 4I, J). A distinct ectocyst was usually visible. Sometimes the cyst had an irregular outline (Fig. 4G, K). Cysts had 2—4 visible pores (mean ± SD of 2.6 ± 0.6, $n = 20$) that did not penetrate the whole cyst wall (Fig. 4G—J). The pores were rounded-to-oval and surrounded by thickened ectocyst material. Sometimes rounded cells without a cyst wall were observed in the culture — these may represent encysting cells (Fig. 4L).

Ultrastructure: A single nucleus with an electron-dense central nucleolus was observed in trophozoites (Fig. 5A, B). Mitochondria had discoidal cristae and were encircled by rough endoplasmic reticulum (Fig. 5C). In most cases, food vacuoles included partially digested prokaryotic prey (Fig. 5D). Some food vacuoles contained a particle of starch from the culture medium (Fig. 5E). The cytoplasm also contained many rounded lipid droplets (Fig. 5F). Dictyosomes were not observed in the trophozoites, nor in cysts.

Like the trophozoites, cysts had a single nucleus with an electron-dense central nucleolus (Fig. 5G, H). Mitochondria were similar in appearance to those in trophozoites, but better preserved under the fixation conditions employed (Fig. 5L). The cytoplasm could also contain numerous lipid

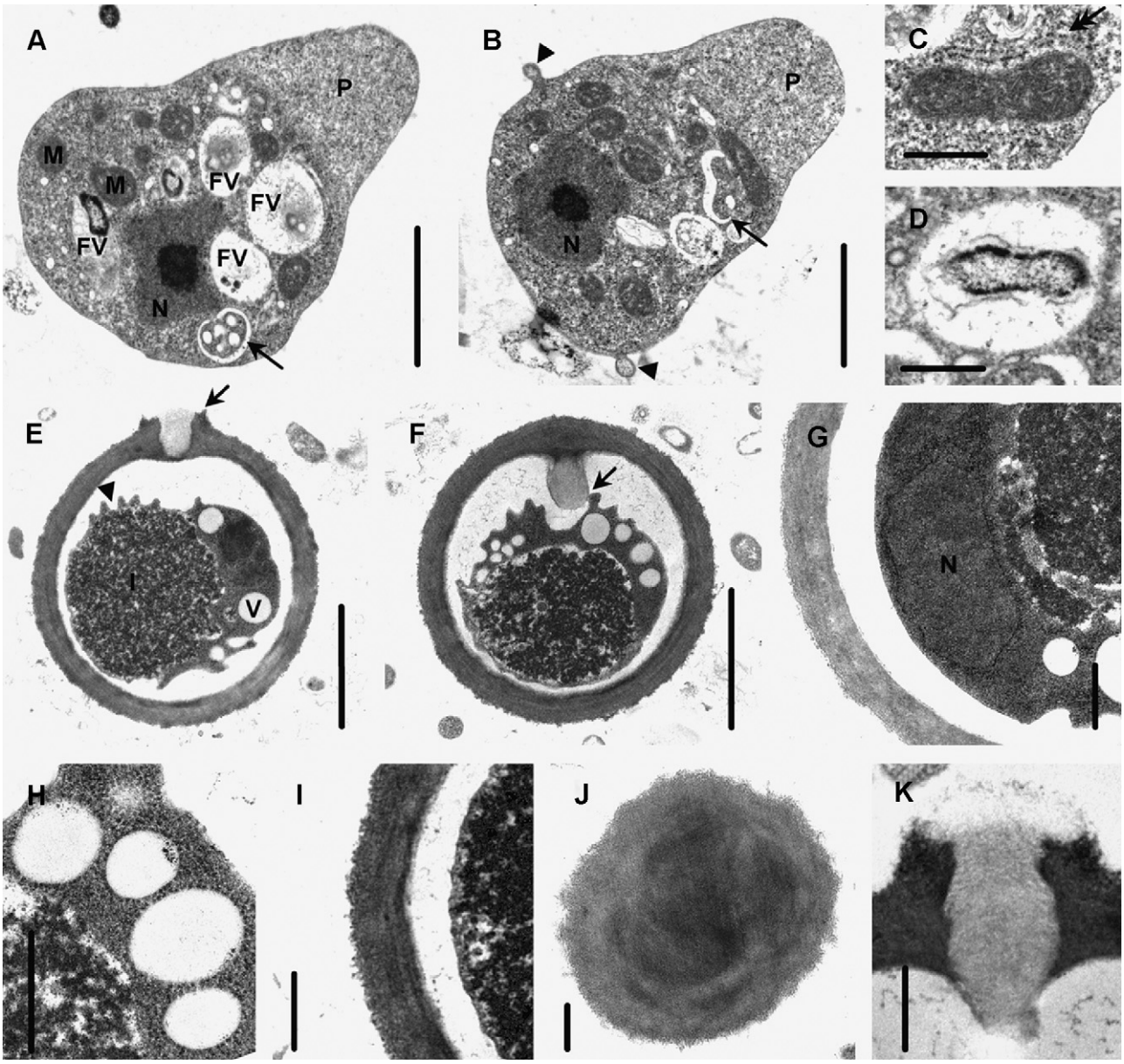

Figure 2. *Tulamoeba peronaphora*, transmission electron micrographs, ultra-thin sections. **A—D**. Trophozoites. **A, B**. Two views of one cell. N: nucleus, M: mitochondria, FV: food vacuoles, P: pseudopod hyaloplasm. Arrows: endomembrane-rich regions. Arrowheads: fine pseudopodial structures. **C**. Mitochondrion associated with rough endoplasmic reticulum (double-headed arrow). **D**. Partially digested prokaryote in a food vacuole. **E—K**. Cysts. **E, F**. Low-power views of cysts. I: inclusion with granular consistency, V: vesicles, Arrowheads: the cell membrane with dimpled appearance. Arrow: cyst pore and plug. **G**. Nucleus (N) in peripheral location within cyst. **H**. Cyst vesicles; note the numerous bound ribosomes. **I**. Cyst wall in cross-section. **J**. Grazing sectioning of cyst wall, showing variation in appearance of the inner and outer portions of the wall. **K**. Cyst pore, showing plug, with delicate cap. Scale bars for A, B and E, F: 2 μm, Scale bars for C, D and G—K: 500 nm.

droplets (Fig. 5H, M). In most cysts, much of the cell volume consisted of granular material, less electron-dense than the cyst cytoplasm, but with some more electron-dense inclusions, all enclosed within biological membranes. Some sections indicated that the material may be divided into several distinct compartments (Fig. 5I).

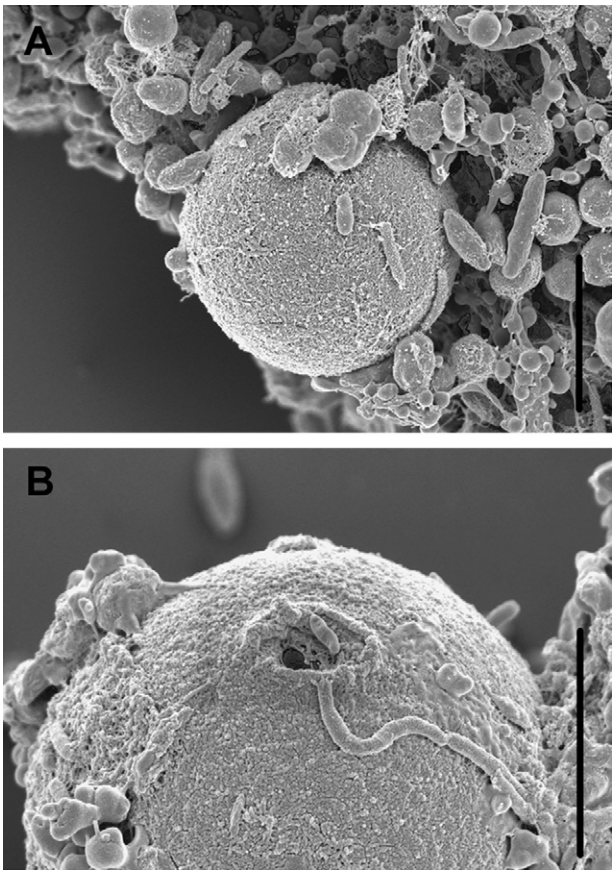

Figure 3. *Tulamoeba peronaphora* cysts, scanning electron micrographs, showing the cyst shape, surface appearance and pore (without plug). Scale bars: 3 μm.

The cyst wall consisted of a thick endocyst, and, usually, a much thinner ectocyst of variable appearance (Fig. 5G, H, J, K). The endocyst was of even thickness, and presented as weakly stained fibrous material (Fig. 5N). In general, the endocyst closely adhered to the encysted cell. The ectocyst was undetectable in some cysts; in others it was a thin envelope adhering to the endocyst (Fig. 5H); whilst in others the envelope was conspicuous and moderately electron-dense (Fig. 5G), was sometimes detached from the endocyst, and sometimes was coated with filamentous fibrous material (Fig. 5J). These forms probably represented a maturity series. Where observed, cyst pores penetrated most of the thickness of the ectocyst wall but did not penetrate at all into the inner fibrous material of the endocyst (Figs 5G, K, O, P, 6A, B). The outer cyst wall around the pore appeared as several leaves that diverged to form a raised margin to the pore (Fig. 5P). The pore was plugged by a rounded mass of granular material that did not stain strongly.

In some cysts the ectocyst had an irregular outline while the endocyst appeared detached from the internal material, and the internal material appeared to be cell debris rather than a complete cell (Fig. 5K). These might represent post-excystment stages or dead cysts.

Under scanning electron microscopy the surface of the cysts (i.e. the ectocyst) was fairly smooth (Fig. 6A, B). The walls of the cyst pores had a laminate appearance (Fig. 6A, B), consistent with the multiple leaves of material seen by TEM. The pore plugs did not survive fixation.

Molecular Phylogenetics

The 18S rRNA gene sequences amplified from *Tulamoeba peronaphora* (isolate A1) and *Euplaesiobystra hypersalinica* (isolate A2) were 1,964 and 1,972 bp long, respectively. The most similar sequences to isolate A1 (as revealed by BLASTN search of the Genbank database) were of the genus *Naegleria*, whereas isolate A2 shared 99.5% identity with Genbank sequence AF011459, which is accessed under the nomen nudum '*Plaesiobystra hypersalinica'.* Both *T. peronaphora* and *E. hypersalinica* had a predicted helix 17_1 structure within the V3 region of the inferred 18S rRNA (see Supplementary Fig. S2). This is an unique feature of heterolobosean 18S rRNA gene sequences (Nikolaev et al. 2004; Park et al. 2007; Yubuki and Leander 2008).

Phylogenetic trees estimated for the 18S rRNA gene sequences showed clearly that the two amoebae are nested within the Heterolobosea (Fig. 7). As in previous studies (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004; Park et al. 2007), '*Macropharyngomonas halophila'* (nomen nudum) was basal within Heterolobosea, with strong statistical support. *Tulamoeba peronaphora* (isolate A1) formed a strong clade with *Pleurostomum flabellatum* (bootstrap support — ML: 100%, MP: 92%, ME: 99%, and posterior probability of 1.00, Fig. 7). The *Pleurostomum+Tulamoeba* clade was, in turn, sister to *Naegleria* and *Willaertia* with strong bootstrap support (ML: 100%, MP: 100%, ME: 100%) and posterior probability of 1.00. The *Euplaesiobystra hypersalinica* (isolate A2) sequence and the '*Plaesiobystra hypersalinica'* sequence (AF011459) differed at only one of the analysed sites and formed a maximally supported clade. These two sequences in turn formed a

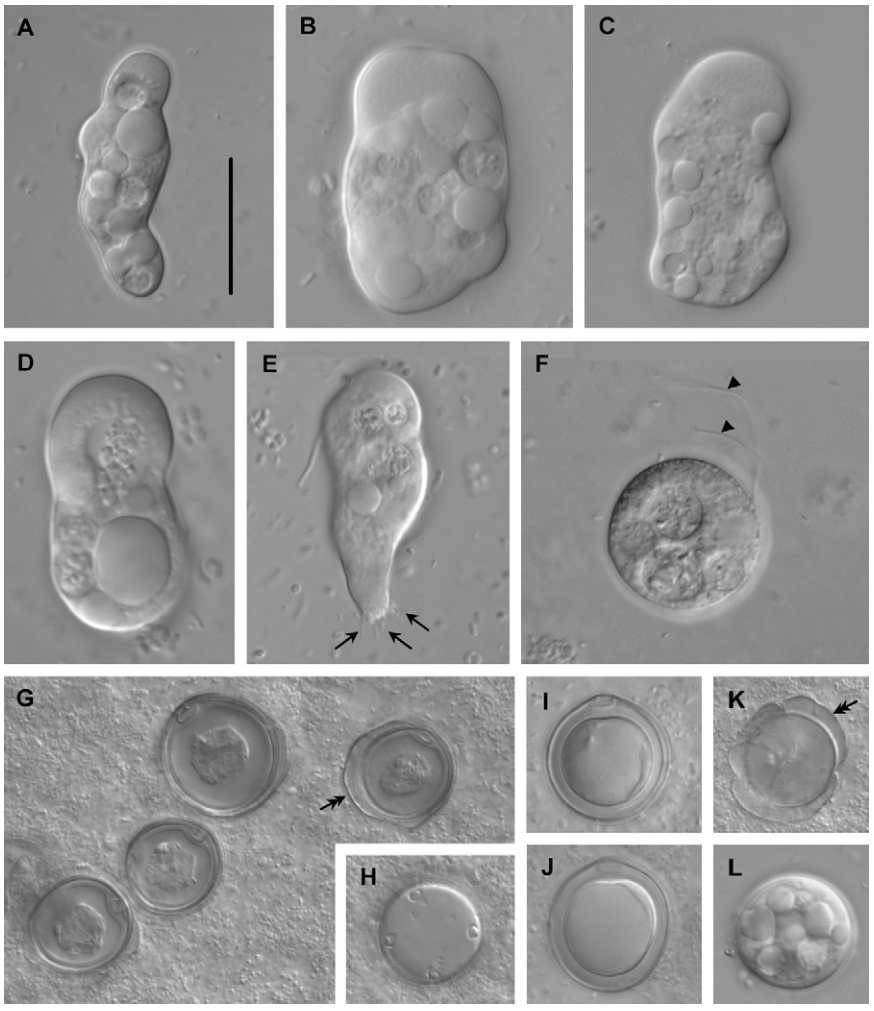

Figure 4. *Euplaesiobystra hypersalinica* n. gen., n. sp., cultured from high salinity (293‰) water, Differential Interference Contrast (DIC) micrographs. **A—E**. Amoebae (trophozoites), showing size and shape, and pseudopod formation. Arrows in E denote uroidal filaments. **F**. Flagellate phase, with arrowheads denoting the two flagella. **G—K**. Cysts. Note the four cyst pores visible in H. Double-headed arrows indicate ectocysts with irregular outlines. **L**. Rounded cell without a cyst wall, possibly an encysting cell. Scale bar: 20 μm for all figures.

strongly supported monophyletic group with *Heteramoeba clara* (Bootstrap support — ML: 100%, MP: 100%, ME: 100%; posterior probability: 1.00, Fig. 7).

Autoecology

We recorded growth of *Tulamoeba peronaphora* culture lines at salinities ranging from 75‰ to 250‰ (Table 1). *Euplaesiobystra hypersalinica* was observed to grow at salinities in the range of 100‰ to 300‰ (Table 1), although growth at 300‰ was extremely slow. In both isolates growth appeared to be most rapid, and resulted in the most dense cultures, at salinities of 150‰ and 200‰ (based on our qualitative observations).

We also examined the ability of the organisms to grow at high temperatures (in 200‰ salinity media). *Tulamoeba peronaphora* grew at 43 °C but failed to grow at 46 °C, while *Euplaesiobystra hypersalinica* grew at 50 °C but failed to grow at 55 °C.

To test whether the cysts could tolerate marine conditions, two cyst-containing cultures of each species were stored for 2 months at 30‰ salinity at room temperature. Only cysts were observed

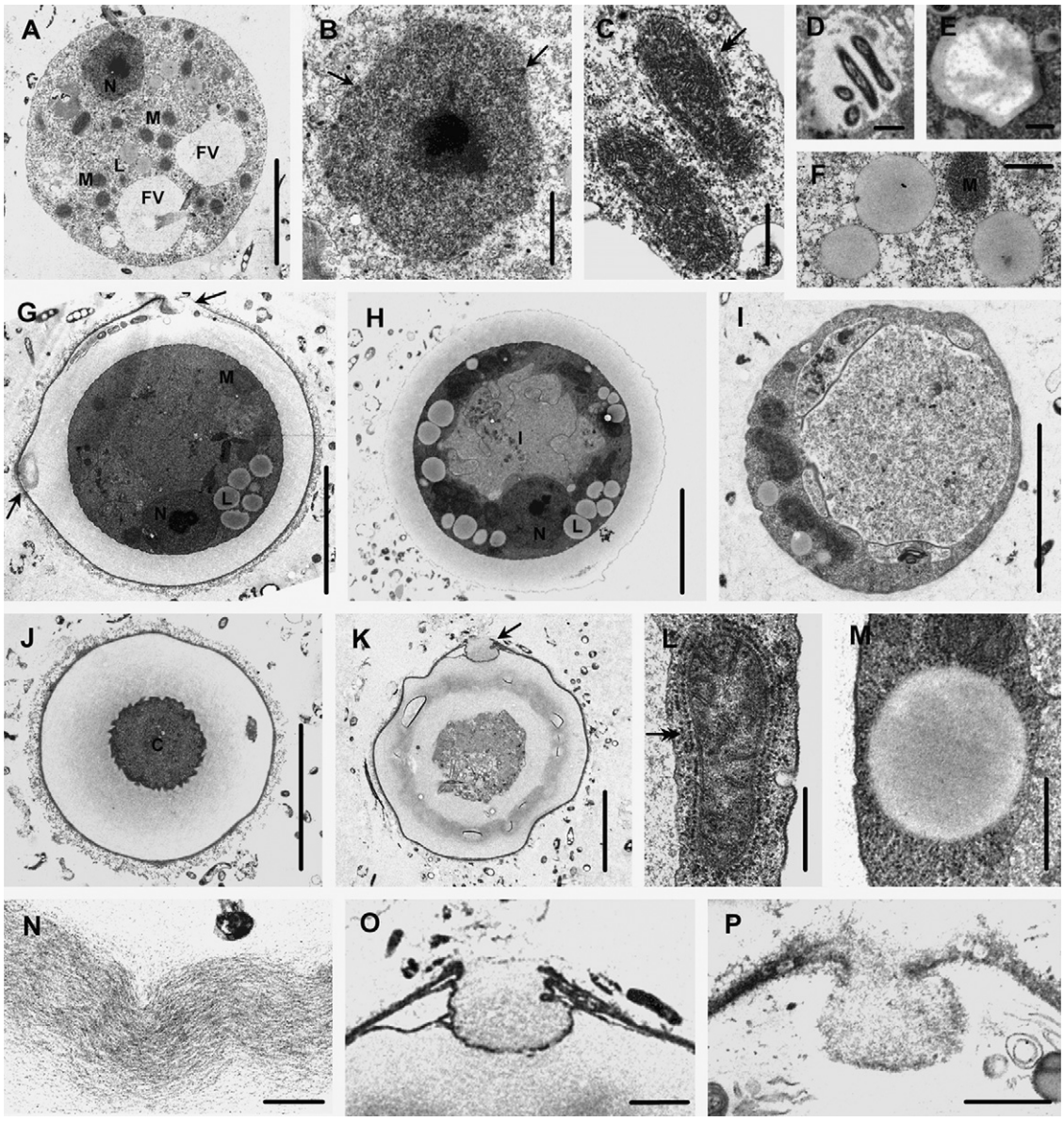

Figure 5. *Euplaesiobystra hypersalinica*, transmission electron micrographs, ultra-thin sections. **A—F**. Trophozoites. **A**. Portrait view. N: nucleus, M: mitochondria, FV: food vacuoles, L: lipid droplets. **B**. Nucleus, with conspicuous nuclear pores (arrows). **C**. Mitochondria associated with rough endoplasmic reticulum (double arrow). **D**. Food vacuoles containing prokaryotes. **E**. Food vacuole containing starch from the culture medium. **F**. Rounded lipid droplets in the cytoplasm. M: mitochondrion. **G—P**. Cysts and encysting cells. **G, H**. Well developed cyst with ectocyst and endocyst. N: nucleus, M: mitochondria, L: lipid droplets, I: inclusion in the cytoplasm. Arrows: cyst pores. **I**. Presumed encysting cell with a large inclusion but no cyst wall. **J**. Tangential section of cyst with conspicuous fibrous material attaching to ectocyst. C: cytoplasm. **K**. Ectocyst with an irregular outline. Note: the internal material appears to be cell debris and may represent a dead cyst or post-excystment remnant. Arrow: cyst pore. **L**. Mitochondrion in the cyst, with the well-resolved cristae. Double arrow: rough endoplasmic reticulum. **M**. Lipid droplet in the cyst. **N**. High magnification view of endocyst material. **O, P**. Cyst pore. Note: The cyst pore does not intrude through to the endocyst. Scale bars for A, and G-K: 5 μm, scale bars for B and O: 1 μm, and scale bars for C—F and L—N, and P: 500 nm.

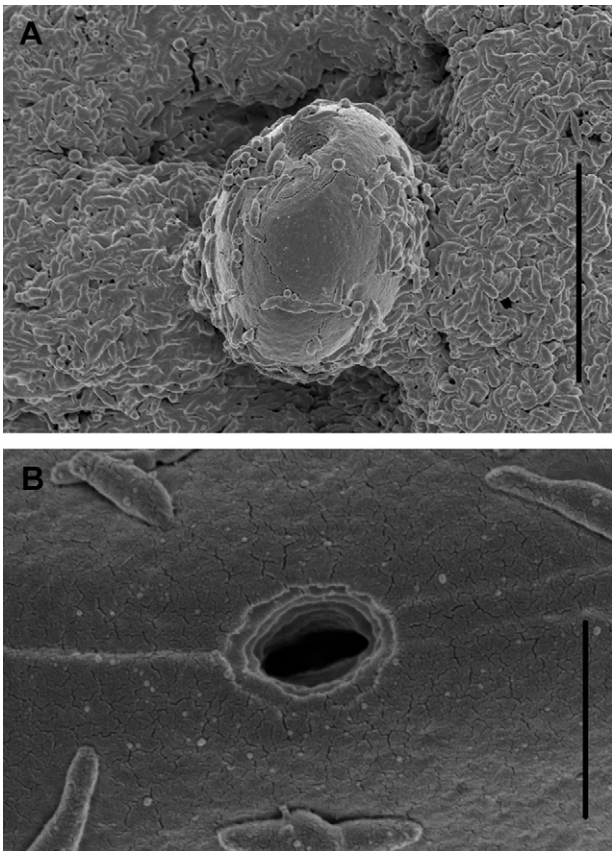

Figure 6. *Euplaesiobystra hypersalinica* cysts, scanning electron micrographs, showing the cyst shape, surface morphology and pores (plugs apparently absent). Scale bar for **A**: 10 μm, and scale bar for **B**: 2 μm.

during storage. Numerous motile trophozoites were found in all culture lines within 10 days after the cysts were returned to 200‰ salinity media.

Discussion

Identity and Taxonomy

Tulamoeba peronaphora and *Euplaesibystra hypersalinica* clearly share numerous characteristics with previously examined heterolobosean amoebae (Page 1988; Page and Blanton 1985; Patterson et al. 2000). Both *T. peronaphora* and *E. hypersalinica* are limax amoebae with eruptive pseudopodial formation. The average ratio of length to width is <3. Their mitochondria have flattened/discoidal cristae encircled, at least partly, by rough endoplasmic reticulum. Discrete stacked dictyosomes were observed neither in trophozoites nor in cysts. Both species form cysts with plugged pores, which is a common feature of the Heterolobosea, though not universal (see below). *Euplaesiobystra hypersalinica*, but not *T. peronaphora*, has a flagellate phase with homodynamic flagella. Phylogenies of 18S rRNA genes place both isolates deeply within the Heterolobosea with strong statistical support. Helix 17_1, an 18S rRNA secondary structure feature diagnostic for Heterolobosea (Nikolaev et al. 2004; Park et al. 2007; Yubuki and Leander 2008) was also found in both *T. peronaphora* and *E. hypersalinica*. Thus, our data demonstrate convincingly that these two amoebae isolated from hypersaline environments are members of the Heterolobosea.

We have not observed a flagellate phase in *Tulamoeba peronaphora*. Previously described Heterolobosea with no known flagellate phase (and no demonstated close affinity to flagellate-producing forms — see Brown and De Jonckheere 1999; De Jonckheere and Brown 2005) have been placed in the genera *Vahlkampfia*, *Paravahlkampfia*, *Neovahlkampfia*, *Pseudovahlkampfia*, *Monopylocystis*, *Sawyeria*, *Gruberella, Stachyamoeba, Pernina* and *Rosculus* (Brown and De Jonckheere 1999, 2004; Garstecki et al. 2005; O'Kelly et al. 2003; Patterson et al. 2000). *Tulamoeba* differs from *Vahlkampfia*, *Paravahlkampfia*, and *Neovahlkampfia* (i.e. organisms traditionally assigned to *Vahlkampfia*) because of the presence of a cyst pore (Brown and De Jonckheere 1999; Page 1967; Patterson et al. 2000). *Stachyamoeba* also lacks cyst pores, and also has parietal, rather than central nucleolar material (Patterson et al. 2000). Although *Monopylocystis* has a single protruding pore in the cyst wall, both *Monopylocystis* and *Sawyeria* (which reportedly does not form cysts) belong to the 'anaerobic clade' of Heterolobosea (Lyromonadea) and lack cristae in their mitochondrion-like organelles (O'Kelly et al. 2003). Both also have peripheral nucleolar material in different arrangements (O'Kelly et al. 2003). Trophozoites of *Tulamoeba* are only $\sim$9 μm long on average, smaller than those of most other heterolobosean amoebae ($>$15 μm, Brown and De Jonckheere 2004; Garstecki et al. 2005; O'Kelly et al. 2003; Page 1988; Patterson et al. 2000), and substantially smaller than the type species of the monotypic genera *Pseudovahlkampfia*, which is also commensal, *Gruberella*, which is also multinucleate (Patterson et al. 2000), and *Pernina*, which has cysts with distinct ecto- and endocyst components and 3—7 cyst pores (Kadiri et al. 1992).

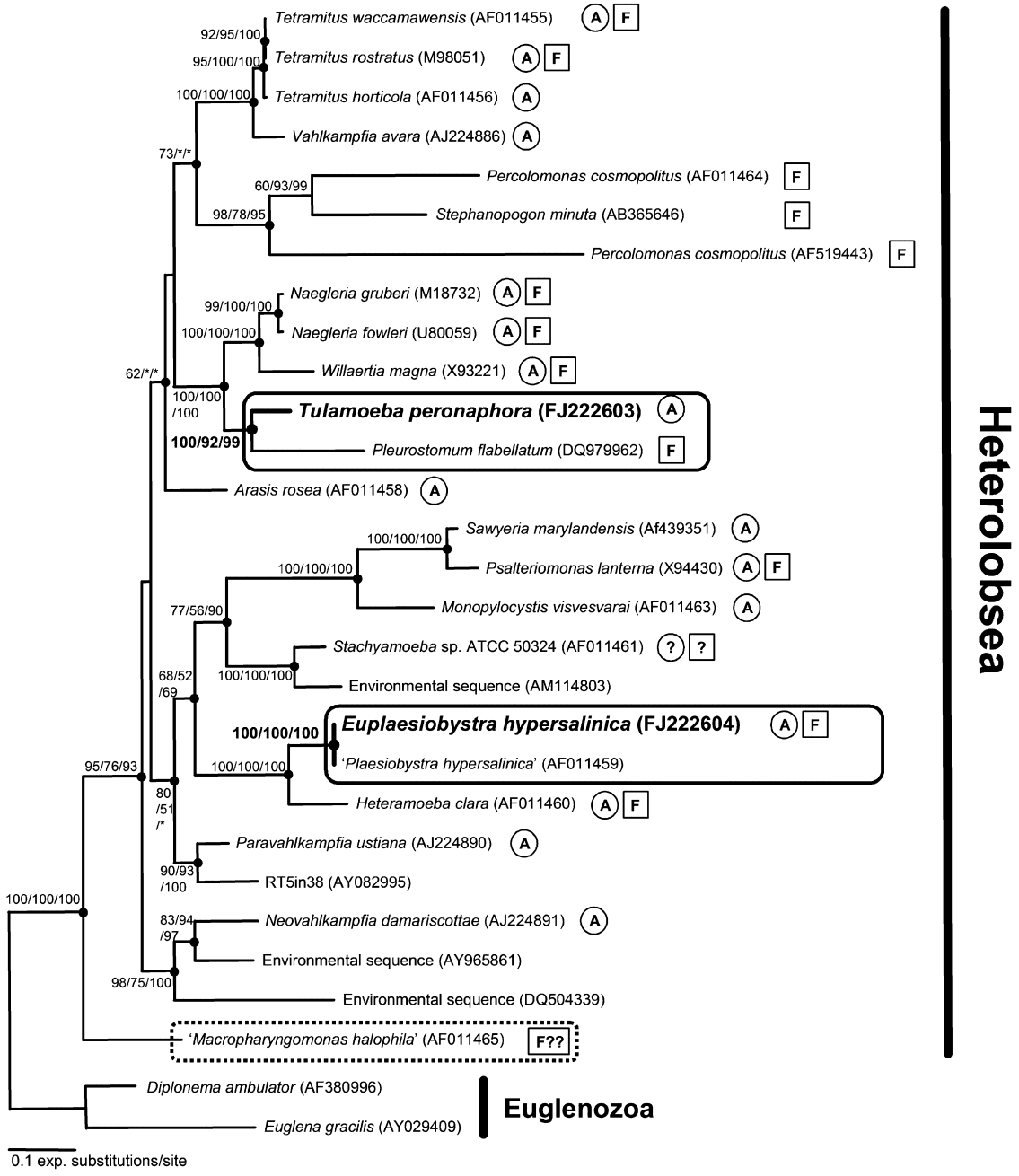

Figure 7. 18S rRNA gene tree showing the phylogenetic position of *Tulamoeba peronaphora* and *Euplaesiobystra hypersalinica* relative to 19 heterolobosean taxa as well as four environmental sequences. Outgroups are *Diplonema ambulator* and *Euglena gracilis*. Bootstrap values (>50%) from maximum likelihood (ML; 500 replicates), maximum parsimony (MP; 10,000 replicates), and minimum evolution (ME; 10,000 replicates) analyses are shown at the nodes. The bootstrap values are presented in the order ML/MP/ME. Solid circles indicate a Bayesian posterior probability of 0.95 or greater. Genbank accession numbers for each taxon are presented in parentheses. *: Bootstrap value of <50%. Circled 'A's denote species with an amoeba phase; Squared 'F's denote species with flagellate phases (sequences for which there are no related morphological data are left blank). Note that while the morphology of *Stachyamoeba* is described there are no published morphological data specifically for *Stachyamoeba* sp. ATCC 50324. Boxed groups are extreme or near-extreme halophiles (the dashed box around '*Macropharyngimonas halophila*' indicates that the halophilicity of this organism is not known directly, and may be lower than the other species considered here — see Discussion).

Table 1. Qualitative growth response to various salinities (30—300‰) for *Tulamoeba peronaphora* and *Euplasiobystra hypersalinica*.

	Salinity (‰)							
Species	30	50	75	100	150	200	250	300
Tulamoeba peronaphora	−	−	+	+	++	++	+	−
Euplaesiobystra hypersalinica	−	−	−	+	++	++	+	+

−: No observation of moving trophozoites, +: relatively low density of moving trophozoites, ++: relatively high density of moving trophozoites (after 14 days incubation).

Rosculus is small, but is flabellate not limax (see Patterson et al. 2000). In our phylogenetic trees, *T. peronaphora* was not specifically related to any of the non-flagellate-forming species for which there are molecular data. Its closest relative is actually *Pleurostomum*, which is a flagellate with no described amoeboid phase (Namyslowski 1913; Park et al. 2007; Ruinen 1938b), negating most ultrastructural or morphological comparisons. In our molecular phylogenies the closest amoeboid relatives of *T. peronaphora* are *Naegleria* and *Willaertia*. It is possible that, in common with most species of these genera, *T. peronaphora* can form flagellates, however it lacks the layer of refractile granules around the nucleus which seems to be characteristic of both *Naegleria* and *Willaertia* species (Page 1988). In summary, we cannot assign *Tulamoeba peronaphora* to a previously described genus of heterolobosean amoebae. We have therefore proposed a new genus to accommodate this isolate.

The isolate that we have named *Euplaesiobystra hypersalinica* is 99.5% identical to Genbank sequence AF011459, which is assigned the name '*Plaesiobystra hypersalinica*', and which has appeared under this name in some phylogenetic studies (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004; Park et al. 2007). This sequence is from an amoeba that was isolated from water samples of ~140‰ salinity from Portugal, and it forms cysts with plugged pores (T.A. Nerad, pers. comm.). We provisionally suppose it to be of the same species as our isolate. Unfortunately, '*Plaesiobystra hypersalinica*' is a nomen nudum — there is no formal description of this organism to accompany publication of the name. This genus name and genus—species binomial are therefore technically 'unavailable' as formal names.

Euplaesiobystra hypersalinica shows the classic three forms of a heterolobosean; amoebae, cysts and flagellates. Previously described Heterolobosea with both amoeboid and flagellate phases include *Heteramoeba*, *Naegleria*, *Willaertia*, *Tetramitus, Psalteriomonas, Tetramastigamoeba* (which may be *Willaertia*, Page 1988), and *Trimastigamoeba* (Patterson et al. 2000), noting that the last two taxa have not been examined using contemporary methods. Many of these have flagellates with three or four flagella per flagellar apparatus (*Tetramastigamoeba*, most *Tetramitus*, *Psalteriomonas*, *Trimastigameba*, and *Willaertia*), rather than two as in *Euplaesiobystra. Psalteriomonas* also has four flagellar apparatuses and is an anaerobe (Broers et al. 1990). Unlike *E. hypersalinica*, most *Tetramitus* species lack cyst pores, including biflagellate forms originally described under the genus names *Paratetramitus* and *Didascalus* (Brown and De Jonckheere 1999; Patterson et al. 2000). *Tetramitus horticolus* (formerly *Singhamoeba horticola*), *T. angularis*, and *T. parangularis* have conspicuously protruding cyst pores (Robinson et al. 2007; Sawyer et al. 1992). *Euplaesiobystra* is arguably most similar to *Naegleria*, with commonalities including: (1) pores in the cyst wall, (2) round-shaped cyst with a double wall, (3) usually two flagella in the flagellated stage, and (4) lack of a cytostome in the flagellated stage (De Jonckheere, 2002; De Jonckheere and Brown 2005; Page 1988; Patterson et al. 2000; Pernin and De Jonckheere 1996). However the flagellate is rather different in shape (rounded vs ovoid), and the morphology of the cyst pores is different (see below). Our molecular phylogenies show clearly that *Euplaesiobystra* is not closely related to *Naegleria.* In our phylogenetic tree *E. hypersalinica* is closely related to *Heteramoeba clara,* the type species of *Heteramoeba.* This organism is a marine amoeba that, like *Euplaesiobystra*, forms fairly rounded biflagellated flagellates (Droop 1962). However, the cysts of *H. clara* have smooth walls and lack pores (Droop 1962), also the flagellate stage of *H. clara* has a conspicuous cytostome, and is probably able to divide (Droop 1962; Patterson et al. 2000). Likewise, the nucleolus in *H. clara* is scattered in large peripheral patches

(Droop 1962), whereas *Euplaesiobystra* has a discrete central nucleolus. Assigning our isolate to *Heteramoeba* would change substantially the concept of *Heteramoeba*. We consider it preferable to describe our isolate as a new genus.

Many heterolobose amoebae have been observed in hypersaline environments over the last >100 years (Hamburger 1905; Hauer and Rogerson 2005b; Namyslowski 1913; Post et al. 1983; Rogerson and Hauer 2002; Ruinen and Baas Becking 1938; Volcani 1943, 1945), including a few that have salinity tolerances similar to those seen in our isolates. All previous accounts are based on light microscopy. The accounts that most closely resemble *E. hypersalinica* are by Volcani (1943, 1945) and Post et al. (1983), neither of whom assigned formal species names to their observations, and those of Ruinen and Baas Becking (1938) under the names *Amoeba salina* Hamburger and *Hyalodiscus limax* Dujardin. In the latter case, both names seem inappropriate, since Hamburger's (1905) *Amoeba salina* (now *Vahlkampfia salina* — see below) is several times smaller than Ruinen and Baas Becking's account, while the original account of *Amoeba (Amiba) limax* (the presumptive basionym of '*Hyalodiscus limax* Dujardin'), is several times larger than Ruinen and Baas Becking's account of *Hyalodiscus limax*, and is from freshwater (Dujardin 1841). The organism referred to as *Amoeba salina* by Ruinen and Baas Becking (1938) seems to be distinguishable from *E. hypersalinica*, since it is more elongate than *E. hypersalinica* (length:width; 3—6 vs 1.6—3.5), is somewhat larger (40—60 μm vs 19—41 μm) and always appeared to have uroidal filaments at the posterior end (Ruinen and Baas Becking 1938), while those structures were observed only sometimes in *E. hypersalinica*. The organism referred to as *Hyalodiscus limax* by Ruinen and Baas Becking 1938, is not well described, but is similar to *E. hypersalinica* in general shape, and in terms of the following available data: (1) length (~30 μm vs 19—41 μm, average 30.5 μm), (2) the ratio of length and width (~2 vs 1.6—3.5, average 2.6), (3) in having eruptive movement, and (4) in having a nucleus with central nucleolus. It is possible that this organism is the same species as our *E. hypersalinica*. Volcani (1945) reported that '*Dimastigamoeba*' isolated from the Dead Sea grew optimally at a salinity of 150—180‰, similar to the apparent optimal salinity for growth of *E. hypersalinica* (i.e. 150—200‰ salinity). The amoeba from the Dead Sea has cyst, flagellate, and amoeboid stages, and varies in size from 17—80 μm by 7—54 μm, overlapping with *E. hypersalinica* (Volcani 1945). However, Volcani's amoeba has a deep cytostome in the flagellate stage, indicating that it differs from *E. hypersalinica*. Later, Volcani's amoeba was regarded as belonging to the genus *Heteramoeba* (Droop 1962; Post et al. 1983). The '*Naegleria* sp.' described by Post et al. (1983) from samples of 150—320‰ salinity is very similar to *E. hypersalinica* under light microscopic observation. The *Naegleria* sp. has at least one plugged pore in the cyst wall, and cysts are 10—23 μm in diameter. Also, it forms non-dividing, and non-feeding flagellates that are 15—20 μm in length and have two flagella. Thus, it is possible that Post et al.'s '*Naegleria* sp.' is in fact *E. hypersalinica. Tulamoeba peronaphora* is most similar to *Vahlkampfia salina* (formerly *Amoeba salina*, Hamburger 1905) as observed at Salton Sea, California (~44‰ salinity; Rogerson and Hauer 2002). The length of the trophozoite of *V. salina* (8—17 μm, mean: 11 μm) is similar to that of *T. peronaphora* (6—17 μm, mean: 8.8 μm). In addition, both amoebae have posterior uroidal filaments and a nucleus with a central nucleolus. However, Hamburger (1905), and illustrations by Rogerson and Hauer (2002) suggest that *V. salina* produces more extensive uroidal filaments, and has a greater tendency to be multipodial than our isolate. Rogerson and Hauer (2002) did not observe any cysts. Possible cysts were illustrated by Hamburger (1905), although the nature of these structures has been questioned (Page 1983; Rogerson and Hauer 2002). If these structures are cysts then they are bipolar rather than rounded, reportedly have two distinct walls (Hamburger 1905), and seem to lack the large inclusion seen in cysts of our isolate. The *V. salina* observed by Rogerson and Hauer (2002) could grow at, and below 44‰ salinity, while our *T. peronaphora* could not grow at 50‰ salinity. Overall, we consider it unlikely that *Tulamoeba peronaphora* is the same species as those seen by Rogerson and Hauer (2002) or Hamburger (1905). In summary, while *E. hypersalinica* at least might have been observed and reported previously, we cannot equate either of our isolates with any formally described species, and consider them both as new species, in a nomenclatural sense.

Cyst Morphology

The morphology of the cyst has been considered a useful character for the classification of the Heterolobosea (Page 1988; Patterson et al. 2000; Schuster 1975), and the presence or absence of

cyst pores has often been used to help distinguish genera (although the genus *Tetramitus* comprises species with and without cyst pores; see Robinson et al. 2007). When the present study is included there are currently eight genera (i.e. *Naegleria*, *Willaertia*, *Tetramitus, Trimastigamoeba*, *Pernina, Monopylocystis*, *Euplaesiobystra*, and *Tulamoeba*) in Heterolobosea that are known to include species which form cyst pores (Page 1988; Patterson et al. 2000; Robinson et al. 2007, this study). There are previous studies by TEM for *Naegleria* (Schuster 1975), *Willaertia* (Michel et al. 1987), *Pernina* (Kadiri et al. 1992) and *Monopylocystis* (O'Kelly et al. 2003). The pores of *Naegleria*, *Willaertia* and *Pernina* are similar in that there are usually multiple pores per cyst, the pores penetrate both the endocyst and ectocyst, and the plugs have a basal plaque of cyst wall material that closely contacts the encysted cell (Kadiri et al. 1992; Michel et al. 1987; Schuster 1975). *Euplaesiobystra hypersalinica* is somewhat similar in having multiple pores within a cyst wall that has an endocyst and an ectocyst. However, the pores of *E. hypersalinica* do not penetrate the endocyst wall, and there is no distinct plaque at the base of the plugs separate from the ectocyst material. Thus, the morphology of the pores of *E. hypersalinica* is only passingly similar to the *Naegleria*/*Willaertia*/*Pernina* type. The cyst pores of *Tulamoeba peronaphora* are very different from those discussed above. *Tulamoeba peronaphora* resembles *Monopylocystis* (O'Kelly et al. 2003) in that both have a single cyst pore that penetrates a cyst wall with no distinct ectocyst and endocyst, and the pore has a mucoid plug with no basal plaque. In both taxa the plug has a distinct electron-lucent cap. There is one apparent difference — the plug in *Monopylocystis* protrudes markedly from the cyst, while that of *T. peronaphora* is intrusive, although Figure 6 of O'Kelly et al. (2003) might indicate that the plug of *Monopylocystis* is both protruding and intrusive. Clearly cyst wall evolution in the Heterolobosea is complex — there are at least three distinct cyst pore types, and these do not assort simply on a phylogenetic tree, since, for example, *Tulamoeba* is more closely related to *Naegleria* and *Willaertia* than to *Monopylocystis*. Cyst pores may well have evolved more than once within the Heterolobosea.

Halophilic Protozoa

Interestingly, *T. peronaphora* grew in culture in a salinity range of 75—250‰, while *E. hypersalinica* grew at salinities of 100—300‰. In our qualitative observations the most rapid growth of the two amoebae occurred at 150—200‰ salinity, but both amoebae failed to grow below 75‰ salinity. Those observations suggest strongly that both amoebae are 'extremely halophilic' eukaryotes if the same criterion is applied as in Park et al. (2006a, 2007) and Cho et al. (2008), namely that the optimal salinity for growth is at least 150‰. More experimental data quantifying their optimal growth salinity would be required to confirm their 'extreme halophile' status.

To date, morphology, physiological characteristics, and 18S rRNA gene sequences have been reported for three extremely halophilic heterotrophic eukaryotes (Cho et al. 2008; Park et al. 2006a, 2007). *Halocafeteria seosinensis* is a bicosoecid flagellate with an optimal salinity for growth of 150‰, *Trimyema koreanum*, a ciliate, has an optimal salinity for growth of 225‰, and *Pleurostomum flabellatum*, a heterolobosean flagellate, has an optimal salinity for growth of 300‰. Like the two amoebae reported here the studied isolates of these three species fail to tolerate seawater or even moderately hypersaline habitats, and all can grow in media of at least 250‰ salinity. In light of the demonstrated abilities of this range of cultured organisms to grow, and often thrive in extremely hypersaline media, we think it increasingly reasonable to assume that organisms observed in natural extremely saline habitats are, in fact, growing actively in these environments. There is a substantial diversity of such species. For example, Namyslowski (1913) reported 16 protozoan species from saturated brines. Ruinen (1938a, b) reported 15 flagellate species, and seven ciliate species in saturated brines, while Post et al. (1983) described eight protozoan species at $>$300‰ salinity, and Patterson and Simpson (1996) observed five flagellate species in saturated brine. Many of the previous species- and genus-level identifications are suspect and/or uninformative. Nonetheless by a conservative estimate they seem to refer to at least 25 distinguishable morphospecies in addition to the morphospecies represented already by cultures (Supplementary Table 1).

The high representation of heteroloboseans in this list is noteworthy (Supplementary Table 1). Some 36% of the total nominal species recorded from high salinity waters are assignable to the Heterolobosea. The several nominal species of *Pleurostomum* are all reported from extremely hypersaline habitats (Namyslowski 1913; Park et al. 2007; Patterson and Simpson 1996; Ruinen 1938b). Ruinen (1938b) and Post et al. (1983)

also reported that the heterolobosean flagellate morphospecies *Percolomonas cosmopolitus* (basionym *Tetramitus cosmopolitus*) occurred in saturated and near-saturated brines, although the same morphospecies also occurs in seawater and seawater-strength saltwater (Fenchel and Patterson 1986; Ruinen 1938b), and appears to be molecularly diverse (Nikolaev et al. 2004). Assuming that adaptation to extremely hypersaline habitats is a derived condition, our current 18S rRNA gene phylogenies (e.g. Fig. 7) indicate that these organisms represent at least three different invasions of this extreme environment by heterolobosean (note that there are no sequences yet for *Percolomonas cosmopolitus* from hypersaline sites). Only *Tulamoeba* and *Pleurostomum* are specifically related to each other, and thus may stem from a common extremely halophilic ancestor. In addition, the organisms represented by Entz's (1904) descriptions under the name *Trichomastix salina*, and observations by Kirby (1932) and Ruinen (1938b) under the name *Tetramitus salinus*, are likely to represent heterolobosean (see Larsen and Patterson 1990), and may be similar to, or the same as the organism referred to by the nomen nudum '*Macropharyngomonas halophila*' (Cavalier-Smith and Nikolaev 2008). If so, these organisms are not closely related to the species mentioned above, and represent another putative invasion of markedly hypersaline habitats by a heterolobosean. It must be noted, however, that this last species (or species cluster) has apparently not been observed at $>220‰$ salinity, but has been observed at $\sim30‰$ salinity (Post et al. 1983; Ruinen 1938b), and therefore might not be an extreme halophile.

How do extremely halophilic eukaryotes colonize new high salinity habitats separated by large geographic distances? Cysts may be a plausible means for dispersal of halophilic microbial eukaryotes that cannot tolerate normal salinity seawater for long periods in an active state. Protist cysts may remain viable for long periods of time, over 15 years in some cases (Hausmann et al. 2003), and long-range aerosol transport of desiccation-resistant cysts has been observed or inferred (Fenchel and Finlay 2004). Cho (2005) proposed that the cysts of halophilic protozoa may be transported by either aerosols or by marine currents. Here we have found that both *T. peronaphora* and *E. hypersalinca* were able to encyst and these cysts remained viable for at least 60 days at 30‰ salinity. This indicates that the cysts may survive in natural seawater for a long time, and that transport through seawater in cyst form is a plausible long-range dispersal mechanism for these extremely halophilic organisms.

Pedrós-Alió et al. (2000) proposed that there were no grazers on prokaryotes in environments with salinities above 250‰. However, Park et al. (2003) reported contradictory results. More recently, the flagellates *Halocafeteria seosinesis* and *Pleurostomum flabellatum*, and the ciliate *Trimyema koreanum* have been shown to feed on prokaryotes in extremely high salinity waters (Cho et al. 2008; Park et al. 2006a, 2007). Both *T. peronaphora* and *E. hypersalinica* have been observed with food vacuoles containing prokaryotes, indicating that they are also bacterivores (*E. hypersalinica*, at least, also eats larger particles). While the species-level diversity of heterotrophic eukaryotes in extremely hypersaline habitats is clearly lower than in seawater, it is equally clear that the major ecological types of prokaryote grazers (flagellates, ciliates, and amoebae) may all be present nonetheless. It appears that at least some extremely hypersaline ecosystems are complex, include active predation, and may have a functionally diverse microbial eukaryote component.

Taxonomic Summary

Tulamoeba n. gen.

Heterolobosean limax amoeba without known flagellate phase, but forming cysts. Cyst with a simple cyst wall (no ectocyst) and with a single plugged pore. A single nucleus with a central nucleolus. Restricted to hypersaline habitats. Type species *Tulamoeba peronaphora* n. sp.

Tulamoeba peronaphora n. sp.

Trophozoites: 6—17 μm (average: 8.8 μm) and 2—6 μm (average: 4.1 μm) in length and width, respectively, during locomotion; average *L*/*B* ratio = 2.2; sometimes with fine uroidal filaments; a single nucleus with a single nucleolus.

Cysts: Typically spherical or slightly ellipsoidal; 6—10 μm (average: 7.9 μm) in diameter; with a single plugged pore; pore plug intrusive; single very large inclusion with a granular consistency; no ectocyst.

Growth in salinities ranging from 75‰ to 250‰. No flagellate stage observed. Type culture (see below) isolated from a solar saltern (300‰ salinity) located at Seosin on the west coast of Korea (37° 09′ 36″ N, 126° 40′ 44″ E).

Type Material

A culture has been deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession number CCAP 1578/1. This culture is considered the hapantotype (name-bearing type) of the species (see Art. 73.3 of the International Code for Zoological nomenclature, 4th Edition).

Etymology

The genus name refers to the bolt-like or peg-like shape of the cyst pore plug, which is elongate and penetrates into the interior of the cyst ('tulos' means 'wooden bolt' or 'trenail' etc. in Greek). The name also refers to the Tula Foundation, in recognition of their generous support of microbial biodiversity research in Canada. The species epithet '*peronaphora*' means 'rivet-bearer' (Greek), and also refers to the cyst pore plug.

Assignation

Eukaryota; Discicristata: Heterolobosea

Euplaesiobystra n. gen.

Heterolobosean limax amoeba with flagellate phase and capable of forming cysts. Flagellate with two flagella and no obvious cytostome. Cysts form a distinct ectocyst with plugged pores penetrating only the ectocyst. A single nucleus with a central nucleolus. Restricted to hypersaline habitats. Type species *Euplaesiobystra hypersalinica* n. sp.

Euplaesiobystra hypersalinica n. sp.

Trophozoites: 19—41 μm (average: 30.5 μm) and 9—16 μm (average: 12.0 μm) in length and width, respectively, during locomotion; average *L/B* ratio = 2.6; sometimes with fine uroidal filaments; a single nucleus with a single nucleolus.

Flagellate: Rounded, roughly 20 μm in diameter; with two flagella of equal length; no cytostome visible.

Cyst: Approximately spherical; 14—20 μm (average: 16.8 μm) in diameter; 2—4 plugged pores; wall 1—2 μm in depth, consisting of a thick endocyst and a thin ectocyst, which is sometimes separated from the endocyst.

Growth in salinities ranging from 100‰ to 300‰. Type culture (see below) isolated from a solar saltern (293‰ salinity) located at Seosin on the west coast of Korea (37° 09′ 36″ N, 126° 40′ 44″ E).

Type Material

This culture has been deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession number CCAP 1528/1. This culture is considered the hapantotype (name-bearing type) of the species (see Art. 73.3 of the International Code for Zoological Nomenclature, 4th Edition).

Etymology

Modified from '*Plaesiobystra hypersalinica*', an unavailable name that was introduced as a nomen nudum for a strain that is ~99.5% identical in 18S rRNA gene sequence to our strain, and which we therefore regard as conspecific. The construction '*-plaesiobystra*' means 'oblong plug' (Greek), and refers to the distinctive shape of the cyst pores (T.A. Nerad., pers comm.).

Assignation

Eukaryota; Discicristata: Heterolobosea.

Methods

Isolation and cultivation: *Tulamoeba peronaphora* (isolate A1) and *Euplaesiobystra hypersalinica* (isolate A2) were isolated from 300‰ and 293‰ salinity waters collected in May 2002 and June 2001 respectively, from a solar saltern located at Seosin on the west coast of Korea (37° 09′ 36″N, 126° 40′ 44″E). The saltern commenced operation around 1950 and was composed of 72 salt ponds, with a total area of about 50,000 m^2 when full (Park et al. 2003). The average water depth of the ponds was about 10 cm, and the saltern was eutrophic (Park et al. 2003, 2006b). The temperature of the high salinity (~300‰) water varied between 18 and 40 °C from April to October in 1998—2001 (Park et al. 2006b). Monoprotistan cultures of amoebae were obtained using a slightly modified version of the isolation method described by Nerad and Daggett (1992). Briefly, plates of CAS-20 agar media (7.5 g casamino acids, 1.0 g yeast extract, 5.0 g protease peptone, 3.0 g sodium citrate, 20.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 2.0 g KCl, 200.0 g NaCl, 20 g BactoAgar l^{-1} distilled water, pH 8.0) were inoculated with 100 μl of the high salinity water sample that had been passed through 1.2 μm pore-sized filters to exclude eukaryotes, and incubated at 37 °C for 7 days to produce a prokaryotic lawn. Then 200 μl of unfiltered high salinity water was dispensed onto one plate (without spreading), and the plate was incubated at 37 °C for 1 day. Several agar blocks (~5 mm^2) containing trophozoites or cysts were then cut from the plate, and placed upside-down on a new CAS-20 prokaryotic lawn plate, which was incubated in a sealed plastic bag with a wet paper towel. This procedure was performed two times, and then a small sample from a cleared portion of the plate was transferred to

liquid media. Both isolates were maintained in liquid media consisting of sterile 200‰ artificial salinity seawater (AS medium; 181.3 g NaCl, 5.1 g KCl, 11.9 g $MgCl_2$, 1.2 g $MgSO_4 \cdot 7H_2O$, 0.9 g $CaCl_2$ l^{-1} water) plus autoclaved barley grains. The cultures were incubated at 37 °C and subculturing was performed every 2—3 weeks. Cultures of *E. hypersalinica* and *T. peronaphora* have been deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession numbers CCAP 1528/1 and CCAP 1578/1, respectively.

Light microscopy: Live *E. hypersalinica* and *T. peronaphora* cells were observed with differential interference microscopy using a Zeiss Axiovert 200M microscope equipped with an Axiocam HR digital camera. Lengths and widths of amoebae and cysts were determined from digital micrographs using the camera software (Axiovision 4.6). Only actively moving amoebae that were not compressed by the coverslip were used for these measurements.

Scanning electron microscopy: For scanning electron microscopy (SEM), cultures were fixed at 4 °C in 1% v/v electron microscopy grade glutaraldehyde (Sigma) in 0.05 M cacodylate buffer (pH 7.8). Fixed cells were transferred to glass coverslips coated with 0.1% poly-L-lysine. Other steps are the same as described by Park et al. (2006a). Cells were examined with a scanning electron microscope (HITACHI S-4700, Japan).

Transmission electron microscopy: For transmission electron microscopy (TEM), cells were grown in 200‰ salinity media. The cells were centrifuged at 8000*g* for 3 min and fixed for 30 min at room temperature in a cocktail containing 4% v/v glutaraldehyde, 19.2% w/v NaCl and 0.1 M cacodylate buffer (pH 7.4). After rinsing the cells three times with 19.2% w/v NaCl in 0.1 M cacodylate buffer, cells were post-fixed for 1 h in 0.8% w/v OsO_4 and 18% 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 ethanols, and then embedded in Spurr's resin. Serial sections (~70 nm) 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).

18S rRNA gene sequencing: Nucleic acids from *E. hypersalinica* and *T. peronaphora* cultures were prepared as per Park et al. (2006a, 2007). 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 annealing temperature was 55 °C. The reaction was cycled 40 times. The size of each PCR product (~1.9 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. To help confirm whether each culture was monoprotistan, we independently directly sequenced PCR products (i.e. with no cloning) for the two cultures. The 18S rRNA gene sequences from *Euplaesiobystra hypersalinica* and *Tulamoeba peronaphora* have been deposited in GenBank under the accession numbers FJ222604 and FJ222603, respectively.

Phylogenetic analysis: The 18S rRNA gene sequences from *E. hypersalinica and T. peronaphora* were compared to the sequences in the GenBank database using a BLASTN search. Sequences were aligned by eye with those from the Heterolobosea and environmental sequences closely related to the Heterolobosea, using the alignment of Park et al. (2007) as a seed. A total of 1198 of unambiguously aligned sites was retained for phylogenetic analysis. 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 all 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 taxon addition sequences 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 (default heating parameter) were run for 1,000,000 generations and sampled every 500 generations (burn-in 200,000 generations).

Salinity and temperature tolerance: To determine the salinities at which cultures would grow, an artificial seawater stock (AS medium; see above) was used. AS media (30 ml) with a range of salinities were inoculated with 1.5 ml of actively growing *Euplaesiobystra hypersalinica* and *Tulamoeba peronaphora* stock cultures (200‰ salinity media) and incubated in the dark at 37 °C for at least 14 days. Activity of the culture was assessed weekly by examining 100 μl of the culture using phase contrast light microscopy, and looking for actively moving amoebae. Active growth at a particular salinity was confirmed by transferring into fresh media with the same salinity (30 ml media, inoculum of 1.5 ml) once more, and re-examining the culture for actively moving cells, as above. To reduce osmotic shock, salinity growth experiments at salinities of 30—100‰ were repeated using inoculum sources with lower salinity than the stock culture (100‰ salinity for *T. peronaphora*; 150‰ salinity for *E. hypersalinica*). Similarly growth experiments at 300‰ salinity were repeated using 250‰ salinity inoculum sources (both species). All treatments were performed in duplicate.

To examine the resistance of cysts to seawater, 200‰ salinity cultures (two per species) that contained both amoebae and cysts were centrifuged at 8000*g* for 5 min, and then washed three times with sterile seawater (32‰ salinity). Samples were then incubated in sterile seawater at 37 °C for 60 days. Only cysts were found in the two samples at 20, 40, and 60 days incubation. For subsequent excystment, the samples were centrifuged, and then supernatants were discarded. The cysts were washed two times with sterile 200‰ artificial salinity seawater. The prepared cysts were then inoculated into sterile 200‰ artificial salinity seawater (30 ml) with five autoclaved barley grains. After 7—10 days incubation, numerous trophozoites were observed in each sample.

To determine the maximum temperature tolerated, culture tubes containing 5 ml of FAHS medium (i.e. 200‰ salinity) supplemented with an autoclaved barley grain were each inoculated with an actively growing culture, and incubated at

different temperatures. Cultures were examined by microscopy at 7-day intervals for up to three weeks. Two cultures of each species were incubated at 40, 43, 46, and 50 °C (the 50 °C cultures were inoculated from actively growing cultures at 43 °C). In addition two cultures of *Euplaesiobystra hypersalinica* were incubated at 55 °C (inoculation from cultures grown at 40 °C and 50 °C).

Acknowledgements

The authors thank A. Heiss (Dalhousie University) for assistance with scanning electron microscopy, and H. Tarrant (University of Newcastle, Australia) for advice on ancient Greek. The present study was supported by NSERC grant 298366-04 to AGBS. Computational resources were funded by the "Prokaryotic Genome Evolution and Diversity" Genome Atlantic/Genome Canada large-scale project. JSP is supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, No. KRF-2007-357-C00119) and NSERC Grant 298366-04 (AGB Simpson). AGBS thanks the Canadian Institute for Advanced Research (CIfAR), programs in Evolutionary Biology and Integrated Microbial Biodiversity for support as a 'scholar'. BCC was in part supported by the Brain Korea (BK) 21 project of the Korean government.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.protis.2008.10.002.

References

Antón J, Rosselló-Mora R, Rodríguez-Valera F, Amann R (2000) Extremely halophilic bacteria in crystallizer ponds from solar salterns. Appl Environ Microbiol **66**: 3052—3057

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

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

Brown S, De Jonckheere JF (2004) Isolation of a new vahlkampfiid amoeba from soil: *Paravahlkampfia lenta* n. sp. Europ J Protistol **40**: 289—294

Cavalier-Smith T, Nikolaev S (2008) The zooflagellates *Stephanopogon* and *Percolomonas* are a clade (Class Percolatea: Phylum Percolozoa). J Eukaryot Microbiol (in press). Published online 24 July 2008, doi:10.1111/J.1550-7408.2008.00356.X

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, Berlin, Heidelberg, New York, pp 543—549

Cho BC, Park JS, Xu K, Choi JK (2008) Morphology and molecular phylogeny of *Trimyema koreanum* n. sp., a ciliate from the hypersaline water of a solar saltern. J Eukaryot Microbiol **55**: 417—426

De Jonckheere JF (2002) A century of research on the amoeboflagellate genus *Naegleria*. Acta Protozool **41**: 309—342

De Jonckheere JF, Brown S (2005) Description of a new species with a remarkable cyst structure in the genus *Naegleria*: *Naegleria angularis* sp. n. Acta Protozool **45**: 61—65

Droop MR (1962) *Heteramoeba clara* n. gen., n. sp., a sexual biphasic amoeba. Archiv Mikrobiol **42**: 254—266

Dujardin F (1841) Histoire naturelle des zoophytes. Infusoires comprenant la physiologic et la classification de ces animaux, et la manière de les étudier à l'aide du microscope. Paris

Entz GS (1904) Die Fauna der kontinentalen Kochsalzwässer. Math Naturwiss Ber Ungarn **19**: 89—124

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, Finlay BJ (2004) The ubiquity of small species; patterns of local and global diversity. Biosience **54**: 777—784

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

Garstecki T, Brown S, De Jonckheere JF (2005) Description of *Vahlkampfia signyensis* n. sp. (Heterolobosea), based on morphological, ultrastructural and molecular characteristics. Europ J Protistol **41**: 119—127

Guixa-Boixareu N, Calderón-Paz JI, Heldal M, Bratbak G, Pedrós-Alió C (1996) Viral lysis and bacterivory as prokaryotic loss factors along a salinity gradient. Aquat Microb Ecol **11**: 215—227

Hamburger C (1905) Zur Kenntnis der *Dunaliella salina* und einer Amöbe aus Salinenwasser von Cagliari. Arch Protistenkd **6**: 111—130

Hauer G, Rogerson A (2005a) Remarkable salinity tolerance of seven species of naked amoebae (Gymnamoebae). Hydrobiologia **549**: 33—42

Hauer G, Rogerson A (2005b) Heterotrophic Protozoa from Hypersaline Environments. In Gunde-Cimerman N, Oren A, Plemenitaš A (eds) Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya. Springer, Berlin, Heidelberg, New York, pp 521—539

Hauer G, Rogerson A, Anderson OR (2001) *Platyamoeba pseudovannellida* n. sp., a naked amoeba with wide salt

tolerance isolated from the Salton Sea, California. J Eukaryot Microbiol **48**: 663—669

Hausmann K, Hülsmann N, Radek R (2003) Protistology. E. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany pp 182—184

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

Javor B (1989) Hypersaline Environments: Microbiology and Biogeochemistry. Springer, New York pp 5—204

Kadiri GE, Joyon L, Pussard M (1992) *Pernina chaumonti*, n.g., n.sp., a new marine amoeba (Rhizopoda, Heterolobosea). Europ J Protistol **28**: 43—50

Kirby H (1932) Two protozoa from brine. Trans Am Microsc Soc **51**: 8—15

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

Michel R, Raether W, Schupp E (1987) Ultrastructure of the amoebo-flagellate *Protonaegleria westphali*. Parasitol Res **74**: 23—29

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

Nerad TA, Daggett PM (1992) Cultivation of Bacterivorous Marine Amebae. In Lee JJ, Soldo AT (eds) Protocols in Protozoology. Society of Protozoologists, Allen Press, Lawrence, Kansas, pp A-30.1—A-30.3

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 C, 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 *Hartmannella* and 3 of *Vahlkampfia*. J Protozool **14**: 499—521

Page FC (1983) Marine Gymnamoebae. Cambridge, Institute of Terrestrial Ecology p. 54

Page FC (1988) A New Key to Freshwater and Soil Gymnamoebae. Culture Collection of Algae and Protozoa, Freshwater Biological Association, Ambleside, Cumbria, pp 31—47

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, Simpson AGB, Lee WJ, Cho BC (2007) Ultrastructure and phylogenetic placement within Heterolobosea of the previously unclassified, extremely halophilic heterotrophic flagellate *Pleurostomum flabellatum* (Ruinen 1938). Protist **158**: 397—413

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, 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 (2000) Class Heterolobosea. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa. 2nd ed. Society of Protozoologists, Lawrence, Kansas, pp 1104—1111

Pedrós-Alió C, Calderón-Paz JI, MacLean MH, Medina G, Marrasé C, Gasol JM, Guixa-Boixereu N (2000) The microbial food web along salinity gradients. FEMS Microb Ecol **32**: 143—155

Pernin P, De Jonckheere JF (1996) *Naegleria pussardi*, a new *Naegleria* species phylogentically related to the high temperature tolerant species at the molecular level. Europ J Protistol **32**: 403—411

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

Robinson BS, De Jonckheere JF, Dobson PJ (2007) Two new *Tetramitus* species (Heterolobosea, Vahlkampfiidae) from cold aquatic environments. Europ J Protistol **43**: 1—7

Rogerson A, Hauer G (2002) Naked amoebae (Protozoa) of the Salton Sea, California. Hydrobiologia **473**: 161—177

Ruinen J (1938a) Notizen über Ciliaten aus konzentrierten Salzgewässern. Zool Meded **20**: 243—256

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

Ruinen J, Baas Becking LGM (1938) Rhizopods living in unusual environments. Arch Néerl Zool **3**: 183—198

Sawyer TK, Nerad TA, Munson DA (1992) *Singhamoeba horticola* (Singh & Hanumaiah, 1979) n. comb., type species of *Singhamoeba* n. g. J Protozool **39**: 107—109

Schuster FL (1975) Ultrastructure of cysts of *Naegleria* spp: A comparative study. J Protozool **22**: 352—359

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 Bio Evol **10**: 512—526

Volcani BE (1943) A *Dimastigamoeba* in the bed of the Dead Sea. Nature **152**: 301—302

Volcani BE (1945) The Microorganisms of the Dead Sea. In Papers Collected to Commemorate the 70th Anniversary of Dr. Chaim Weizmann. Daniel Sieff Research Institute, Rehovoth, pp 71—85

Yubuki N, Leander BS (2008) Ultrastructure and molecular phylogeny of Stephanopogon minuta: An enigmatic microeukaryote from marine interstitial environments. Europ J Protistol **44**: 241—253