Characterization of *Pharyngomonas kirbyi* (= "Macropharyngomonas halophila" nomen nudum), a Very Deep-branching, Obligately Halophilic Heterolobosean Flagellate

Jong Soo Park\(^a\), and Alastair G.B. Simpson\(^b,c,1\)

\(^a\)School of Life Science, Kyungpook National University, Daegu, 702-701, Republic of Korea
\(^b\)Department of Biology, Dalhousie University, Halifax, NS, B3H 4R2, Canada
\(^c\)Canadian Institute for Advanced Research, Program in Integrated Microbial Biodiversity

Submitted October 5, 2010; Accepted February 14, 2011
Monitoring Editor: Barry S. C. Leadbeater

The tetraflagellate *Pharyngomonas* is among the most commonly reported morphotypes of halophilic protozoa. We have established two cultures of *Pharyngomonas kirbyi*, SD1A and AS12B, from 300‰ and 210‰ salinity waters from the USA and Australia, respectively. 18S rRNA gene phylogenies confirm that *Pharyngomonas* is the same entity as 'Macropharyngomonas' (nomen nudum), and represents the deepest branch in the heterolobosean lineage. *Pharyngomonas kirbyi* (Strain SD1A) has flattened/discoidal cristae, and lacks conspicuous Golgi dictyosomes. It also has a heterolobosean 'double bikont' flagellar apparatus, with two right roots, each associated with an 'I' fibre and part of a rhizoplast-like complex. One right root splits shortly after its origin, and supplies most of the microtubules that support both the ventral groove, and the sub-anterior cytopharynx. Interestingly, *Pharyngomonas* has some potentially ancestral features not found in typical Heterolobosea, including elongated left roots associated with multilayered 'C' fibres, orthogonal basal bodies, and a spur structure that might represent a 'B' fibre homolog. Both isolates are obligate halophiles that grow best at 100-200‰ salinity and do not grow below 75‰ salinity. *Pharyngomonas* is therefore of considerable evolutionary importance, both as a deep-branching, plesiomorphic heterolobosean, and a borderline extreme halophile.

© 2011 Elsevier GmbH. All rights reserved.

**Key words:** halophile; Heterolobosea; molecular phylogeny; Percolozoa; protozoa; ultrastructure.

**Introduction**

A wide range of microorganisms live in hypersaline environments. Although there is a steep decrease in diversity in habitats with >200‰ salinity (Benlloch et al. 2002; Javor 1989), some halophiles/extreme halophiles grow well at or above 200‰ salinity. The best known of these are halophilic prokaryotes, principally *Salinibacter* and various Haloarchaea, and certain species of the chlorophyte alga *Dunaliella* (Antón et al. 2000; Javor 1989). Much less recognized is the presence in these environments of predatory microbes, that is, protozoa. There is some evidence that protozoa can be key grazers of prokaryotes in brine (Park et al. 2003), and several heterotrophic protozoa that grow in culture at >200‰ salinity have been recently isolated and characterized. These are 1) the bicosoecid *Halocafeteria seosinensis*...
(Park et al. 2006), 2) a species of the anaerobic ciliate genus Trinymea – Trinymea koreanaum (Cho et al. 2008), 3) the heterolobosean flagellate Pleurostomum flabellatum (Park et al. 2007), 4) the heterolobosean amoeba Tulamoeba peronophora (Park et al. 2009b), and 5) Euplaesiobystra hyper-salinica (Park et al. 2009b), which is also an heterolobosean and has both amoeba and flagellate phases. None of these isolates could grow at <75‰ salinity in laboratory culture, and none of these species have been reported in natural environments at marine salinity. With the exception of T. koreanum they are also assigned to distinct genera on the basis of sub-cellular morphology and molecular phylogeny.

Nonetheless, these forms represent only some of the protozoan diversity that can inhabit highly saline habitats. Park et al. (2009b) recorded at least 25 additional morphologically distinguishable protozoa that had been reported in saturated or near-saturated brines in previous light microscopy-based studies, and speculated that many or most of these were capable of growth at very high salinities. Additional protozoan species have been reported from various hypersaline environments in the 100‰ to 250‰ salinity range (Hauer and Rogerson 2005). Therefore, it is likely that most of the protozoan biota in hypersaline habitats has not yet been examined using modern methods.

One of the more frequently reported morphotypes in hypersaline habitats is the collection of organisms now assigned to the genus Pharyngomonas Cavalier-Smith (see Cavalier-Smith and Nikolaev 2008). These protozoa have four flagella that insert at the head of a ventral groove, and were first described by Entz (1904) as Trichomastix salina, and later observed by Kirby (1932) under the name Tetramitus salinus. The organisms have usually been found at salinities ranging from 110‰ to 150‰ (Kirby 1932; Post et al. 1983), and sometimes occur at 220‰ salinity, or possibly higher (Post et al. 1983). The morphology of this organism was studied in some detail by Gunderson (1981), but no molecular data was obtained and the culture employed is no longer available. The organism known by the nomen nudum ‘Macropharyngomonas halophila’ apparently also corresponds to this morphotype, and was isolated from a ∼140‰ salinity crude culture (Tom Nerad, personal observations, as reported by Cavalier-Smith and Nikolaev 2008). There are no published molecular data on ‘Macropharyngomonas’, but an 18S rRNA gene sequence is available (Genbank accession number AF011465). Phylogenies of 18S rRNA genes, and the absence of the heterolobosean-specific helix 17_1 place ‘Macropharyngomonas’ as the deepest branch within Heterolobosea sensu lato (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004; Park et al. 2007, 2009b), making this an organism of evolutionary interest, in addition to its intriguing status as a probable obligate halophile (note that we here use the taxon name Heterolobosea in a broad sense that includes the Pharyngomonas/ ‘Macropharyngomonas’ lineage; this is identical in composition to the taxon Percolozoa sensu Cavalier-Smith and Nikolaev 2008).

Unfortunately our knowledge of Pharyngomonas/ ‘Macropharyngomonas’ has been restricted, with no direct linkage of published microscopy data and gene sequence data.

We have established two monoeukaryotic cultures of Pharyngomonas, Strain SD1A from 300‰ salinity water from a solar saltern in California, USA, and Strain AS12B from a 210‰ saline puddle in Western Australia. The 18S RNA gene sequences from these isolates are very similar to one another, and also very similar to the sequence from ‘Macropharyngomonas halophila’ (AF011465), confirming that Pharyngomonas and ‘Macropharyngomonas’ are one. The ultrastructural characteristics of Strain SD1A, and basic growth physiology of both isolates are described.

Results

Light Microscopy

Pharyngomonas kirbyi strains SD1A and AS12B were both usually spindle-shaped (Fig. 1A-H), but with the posterior end of the cell more pointed than the anterior end and with the dorsal side more convex than the ventral side (Fig. 1A, D, E, F, H). Strain SD1A (Fig. 1A-D) ranged from 9 to 14 µm long (mean ± SD of 11.6 ± 1.4 µm, n = 20), and was between 3 and 6 µm wide (mean ± SD of 4.6 ± 1.0 µm, n = 20). The average ratio of length to width was 2.6 (range 1.9-4.1). Strain AS12B (Fig. 1E-H) was larger, with length varying from 11 to 18 µm (mean ± SD of 15.0 ± 1.8 µm, n = 20), and width between 3 and 8 µm (mean ± SD of 5.3 ± 1.1 µm, n = 20). The average ratio of length to width was 2.9 (range 1.7-3.8). Two anterior and two posterior flagella inserted within a broad bulge at the anterior end of the cell (Fig. 1A, C, E-H). The two anterior flagella inserted more or less apically, and were directed anteriorly during swimming, and actively beat. The two posterior flagella were directed laterally/posteriorly during swimming, and...
Figure 1. Light micrographs of *Pharyngomonas kirbyi* strains SD1A (A-D) and AS12B (E-H) cultured from 300‰ and 210‰ salinity waters, respectively. A-B. Phase contrast images of Strain SD1A. C-D. Differential interference contrast images of Strain SD1A. E-F. Phase contrast images of Strain AS12B. G-H. Differential interference contrast images of Strain AS12B. Note the slightly pointed posterior end, the anterior nucleus (most visible in H), the fairly subtle ventral groove (D, G), the curving cytopharynx immediately posterior to the nucleus (A, F-H), the four flagella (A, E, H) in two pairs (E, H), with the posterior flagella (especially one of them) tending to be slightly longer than the anterior flagella (A, C, G). Scale bar in A represents 20 μm for all images.

Slowly beat. The two anterior flagella were similar to body length, while the two posterior flagella were slightly longer at ~1.0-1.5 times body length (Fig. 1C, G). Sometimes one posterior flagellum was longer than the other (Fig. 1A, C, H). The cells swam with a slow rotation (~0.5 rotations per second). Cells were also observed attached to substrates by the anterior flagella, while the posterior flagella usually beat for feeding (Fig. 1C, E). The nucleus was visible in the anterior portion of the cell close to the flagellar insertion (Fig. 1E, H). The subtle ventral groove started near the anterior end of the cell and extended for much the length of cell body (Fig. 1A, G). About one-third down the cell (i.e. slightly posterior to the nucleus) a curved cytopharynx extended into the cell (Fig. 1A, D, F-H). Food vacuoles containing prey bacteria were visible in the posterior two-thirds of the cell (Fig. 1D, F). No amoeboid form was observed in liquid culture during this study, and attempts to induce an amoeboid form on plates were unsuccessful.

Transmission Electron Microscopy

Ultrastructural studies were performed on *Pharyngomonas* Strain SD1A. The nucleus was located anteriorly, and was very roughly oval or pear-shaped (Fig. 2A), but with a channel on its ventral side that was aligned to the ventral groove (Fig. 2B, arrowhead). The nucleus had a conspicuous subcentral nucleolus (Fig. 2A), but otherwise was mostly homogenous; small dense condensations were occasionally seen, and there were small peripheral accumulations associated with the nuclear pores that were probably fixation-dependent (Fig. 2B). The cytoplasm contained well-preserved rounded/sausage-shaped mitochondria with cristae that were irregular in...
Figure 2. *Pharyngomonas kirbyi*, Strain SD1A, transmission electron micrographs, ultra-thin sections. A. Longitudinal section through the cell. Scale bar: 2 μm. B. Transverse section showing nucleus with a channel (arrowhead) aligned to the ventral groove. Note the small dense vesicles also in this region (arrow). Scale bar: 500 nm. C-D. Mitochondria, showing shape and flattened cristae. PP in D represents a putative peroxisome. Scale bars: 500 nm. E-F. High magnification views of putative peroxisomes (PP) showing the dense and paracrystalline inclusions. Scale bars: 200 nm. G. Small electron-dense vesicle from vicinity of the groove, with a single bounding membrane. Scale bar: 200 nm. Abbreviations (A-G): FV: food vacuoles, L: lipid droplets, M: mitochondrion, N: nucleus. V: ‘empty’ vacuoles, RR: right root, PP: putative peroxisome.
Figure 3. *Pharyngomonas kirbyi*, Strain SD1A, transmission electron micrographs, ultra-thin sections (A and G are lateral views of the cells, B-F are ventral views). **A.** Longitudinal section of the flagellar apparatus (basal bodies 1 and 2). Scale bar: 500 nm. **B.** Section showing the four basal bodies and the right roots. Double-headed arrow: fine spur. Scale bar: 200 nm. **C.** Transverse section of the anterior basal bodies 3 and 4, showing the connecting fibres (asterisks). Scale bar: 200 nm. **D.** Transverse section of the right root (RR) attached to basal body 1 (in D) and RR' attached to basal body 2 (in E). Note that ‘I’ fibres are associated with each right root, and that the RR' shown in E is of the large type (contrast with B) Arrow: microfilament bundle, Double-headed arrow: fine spur. Scale bar: 200 nm. **F.** General view of the posterior flagellar apparatus, showing the relative positions of the left and right roots and showing the positions of some of the individual microtubules (IMt). The formation of IMt microtubules into extensive ribbons only seen occasionally. Scale bar: 500 nm. **G.** Rhizoplast homolog connected to both right roots, and the termination of RR' in the anterior ventral portion of the cell, near RR. Scale bar: 500 nm. Abbreviations (A-G): 1: posterior basal body/flagellum 1, 2: posterior basal body/flagellum 2, 3: anterior basal body/flagellum 3, 4: anterior basal body/flagellum 4, I: I fibre, IMt: individual microtubules, LR: left root associated with basal body 1, LR': left root associated with basal body 2, N: nucleus, Rh: rhizoplast homolog, RR: right root associated with basal body 1, RR': right root associated with basal body 2.
Figure 4. Pharyngomonas kirbyi, Strain SD1A, transmission electron micrographs, ultra-thin sections. All micrographs are ventral views. A-C. Transverse section of left roots (LR, LR') associated with posterior basal bodies 1 and 2 (B and C are non-consecutive sections from one series). Note that conspicuous ‘C’ fibres are associated with each left root, though micrograph C shows LR immediately distal to the termination of its C fibre. Scale bars: 200 nm. D-E. Non-consecutive sections from one series depicting the left roots (LR, LR') in oblique section, showing their origin, then their extension down the left side of the cell. Scale bars: 500 nm. F. Longitudinal section showing the splitting of the right root (double-ended arrow) close to left root. Scale bar: 500 nm. G-I. Non-consecutive series of longitudinal sections of the ventral region of the cell showing the right side of right root (RR) and the posterior band (PB) that forms from the LR, LR', and the left-most ~ 2 microtubules of RR. Double-ended arrow: point of splitting of the RR. Asterisks (*): Microtubules derived from the RR and associated with the cytopharynx. Scale bars: 1 μm. Abbreviations (A-I): 1: posterior basal body/flagellum 1, 2: posterior basal body/flagellum 2, 3: anterior basal body/flagellum 3, 4: anterior basal body/flagellum 4,
shape, but mostly flattened/discoidal (Fig. 2C-D). The cytoplasm also contained some lipid droplets (Fig. 2A, E). Food vacuoles containing multiple partially digested prokaryotes were commonly observed (Fig. 2A). Numerous empty vacuoles were usually present (Fig. 2A). Most were smaller than food vacuoles (Fig. 2A), but some cells also contained larger empty vacuoles. No discrete Golgi dictyosomes were observed. A type of electron-dense organelle ∼600 nm long and ∼200 nm wide with a single bounding membrane was observed in the general vicinity of the nucleus (Fig. 2E-F). There were several per cell. The matrix of these organelles included a more electron-dense region, and a second region that had a paracrystalline appearance in some sections (Fig. 2E-F). These organelles may be peroxisomes. Many smaller electron-dense organelles ∼150 nm in diameter were located in the vicinity of the groove (arrow in Fig. 2B). These had a single bounding membrane (Fig. 2G) and were probably lysosomes.

The flagellar apparatus was located anteriorly and was closely associated with the nucleus (Figs 2A, 3A-B). The basal bodies of the four flagella were arranged in two pairs, each pair with one anterior basal body and one posterior basal body (Fig. 3B). The pairs lay side-by-side; very close together but at slightly diverging angles; the right pair composed of basal bodies 1 and 4 and the left pair composed of basal bodies 2 and 3, with basal bodies 1 and 2 being posterior, and basal bodies 3 and 4 anterior (Fig. 3B). Within each pair the basal bodies lay at an ∼80° angle to one another, and the base of the posterior basal body lay almost adjacent to the side of the anterior basal body (within 30 nm) but slightly offset rightwards (Fig. 3A-B). The anterior and posterior basal bodies were about 600 nm and 700 nm long, respectively (Fig. 3A), and contained conventional cartwheel structures (Fig. 3B). The transition zones between the axoneme and the basal body were located at the surface of the cell (Fig. 3A), and no remarkable features were observed in the transition region (e.g. the central pair ended in a small dense axosome). Broad striated connectors joined the anterior and posterior basal bodies in each pair, but were offset to the left of the centre of the posterior basal body of each pair (Fig. 3C).

The posterior basal bodies in each pair (i.e. 1 and 2) were each linked to two major microtubular roots, right and left. The roots associated with basal body 1 were labelled RR (right root) and LR (left root), respectively, those associated with basal body 2 were labelled RR’ and LR’ (Fig. 3B, D-F). The RR originated from the right/posterior side of basal body 1 and near its origin was a curved row of ∼25 microtubules (Fig. 3D), while the RR’ originated from the right/posterior side of basal body 2 and usually contained ∼12 microtubules near its origin (Fig. 3B). In some cells the number of the microtubules in RR’ was doubled to ∼25 (Fig. 3E) – this may have been a developmental state preceding cell division (see below). There was a conspicuous element of fibrous material that surrounded the bases of basal bodies 1 and 2, and extended as two lobes along the convex/left sides of the proximal parts of RR and RR’ (Rh in Fig. 3A-B, G). This was presumably the homolog of the heterolobosean rhizoplast, although it was much shorter than is typical, and a striated substructure was not clearly observed (Fig. 3F), either because striations were unusually subtle, or absent altogether, or due to fixation conditions. The concave/right faces of RR and RR’ were each closely associated with an ‘I’ fibre that appeared as a latticed structure in transverse section and was ∼40 nm thick (Fig. 3B, D-F). For each of basal bodies 1 and 2 there was a narrow fibrous element that attached 1-2 triplets clockwise (when looking tip-down) from the point of attachment of RR/RR’, and then connected back to the concave face of the I fibre on RR/RR’ (single arrow in Fig. 3D-E). This might be homologous to the microfilament bundle of certain heterolobosea, but if so, it is very small. There is also a fine sheet-like spur that attaches to the basal body ∼3 triplets clockwise from the point of attachment of RR/RR’ and projects rightward, more or less parallel to the RR/RR’ (double arrow in Fig. 3A, D). No discrete ‘singlet root’ was observed near either RR or RR’.

The LR and LR’ originated near the left/anterior side of the proximal portion of basal bodies 1 and 2, respectively (Fig. 4A-C). At their origins LR and LR’ each ran at a large angle to their attendant basal body (nearly 45°; Fig. 4D-E). The LR was composed of a slightly curved row of ∼6 microtubules (Fig. 4A). The LR’ was similar, but usually larger – we observed between 6 and 9 microtubules, depending on the cell (Fig. 4A-C). Interestingly, the proximal portions of LR and LR’ were each

IMt: individual microtubules, LR: left root associated with basal body 1, LR’: left root associated with basal body 2, N: nucleus, PB: posterior bundle, Rh: rhizoplast homolog, RR: right root associated with basal body 1, RR’: right root associated with basal body 2.
linked to a non-microtubular ‘C’ fibre that was connected to the concave right/posterior face of the root (Fig. 4A). The ‘C’ fibres were about 100 nm thick, and appeared in transverse section as multilayered structures with four layers (Fig. 4A-C). The layers closest to the root itself included spokes connecting to each microtubule (Fig. 4A-C). The length of ‘C’ fibres associated with LR and LR’ was about 200 nm and 330 nm, respectively (Fig. 4C shows the LR shortly after termination of its ‘C’ fibre). Both LR and LR’ extended down the left side of the cell immediately beneath the cell membrane, more or less parallel to one another (Fig. 4D-E), with LR to the right of LR’ and thus closer to RR (Fig. 4F).

In addition to the microtubular roots, there were a large number of individual microtubules that originated from around the flagellar apparatus and extended in different directions both through the cytoplasm and immediately under the cell membrane (Figs 3A, F-G, 5A). In particular these individual microtubules contributed to the support of the right and left margins of the cell (Figs 3F, 5B).

The RR curved down the ventral side of the cell, but soon split into two unequal parts. The smaller left portion quickly divided into individual microtubules. The left-most ~2 microtubules of RR associated with the LR to form one loose band that marked the left side of the groove region (Fig. 4H-I). Further down the cell, LR’ also joined this structure to make one broad ‘posterior band’ of parallel microtubules that continued to the end of the cell (PB in Fig. 4H-I). The posterior band might also have included some internal microtubules. The remaining microtubules derived from the left portion of the RR run interiorly (i.e. no longer subtending the cell membrane), firstly associated with the channel in the nucleus, then curving posterior to the nucleus (asterisk in Figs 4G-H, 5C). Presumably these microtubules were associated with the cytopharynx that was observed by light microscopy. The right-most portion, which constituted the bulk of the right root microtubules, remained as a coherent band and ran down the right-ventral side of the cell (Fig. 4G-I). Individual microtubules curved off from its left-most side, and curved leftwards, supporting the ventral side of the cell between the RR and the posterior band (Fig. 4I). Within the anterior portion of the cell the ‘I’ fibre terminates. Instead, the left-most edge of the right part of the RR has an extra sheet-like element, about 200 nm wide, connected to its ventral side (arrowhead in Fig. 5B). This is positionally similar to the ‘microfibrilar string’ reported by Broers et al. (1990) and Brugerolle and Simpson (2004) from some other heteroloboseids, but we did not determine the origin of this element in our organism.

Some cells in our cultures may have represented pre-division stages. Some cells were observed with 25 microtubules rather than 12 microtubules in the RR’ (mentioned above; Fig. 3E). This is consistent with a scenario wherein RR’ grows the size of the RR prior to transforming into the RR during flagellar apparatus replication. In addition, some cells had an additional non-flagellar basal body at the anterior-right region of basal body 2. Finally, one cell observed had two nuclei adjacent to one another (Fig. 5D).

An interpretative diagram of the flagellar apparatus is shown in Figure 6, and a comparison between Pharyngomonas and related organisms is shown in Table 1.

Molecular Phylogeny

The 18S rRNA gene sequence was 2,944 bp long for Strain SD1A and 3,325 bp long for Strain AS12B. The exonic regions of the sequences were 98% similar to one another, and 97% similar to ‘Macropharyngomonas halophila’ (Genbank sequence AF011465), which was the top hit returned by a BLASTN search of the Genbank non-redundant database. The 18S rRNA gene sequence of Strain SD1A included two group I introns, one of 389 bp (positions 1448 – 1836 of the amplified sequence) and one of 361 bp (position 2153 – 2513). These positions were the same as those of group I introns in ‘Macropharyngomonas’ AF011465 and mapped to helices 34 and 38 of the 18S rRNA gene. The first intron position was also shared with Acrasis rosea and Pleurostomum flabellatum (Park et al. (2007) incorrectly stated that this intron was within the V8 region). The 18S rRNA gene sequence of Strain AS12B contained three group I introns; 388 bp (positions 1449 – 1836), 382 bp (positions 1938 – 2319), and 361 bp (positions 2535 – 2895). The first and second introns in Strain AS12B were in the same positions as the introns in Strain SD1A, but the second intron was in a previously unreported position in the 18S rRNA gene sequences of Heterolobosea. Helix 17_1 was not observed in either new sequence. This structure was thought to be a unique helix found in heterolobosean 18S rRNA gene sequences, except for ‘Macropharyngomonas’ AF011465.

The two strains were identical to each other and to ‘Macropharyngomonas’ AF011465 in the sites retained for phylogenetic analysis. They formed a basal clade in Heterolobosea with maximal support
### Table 1. Comparison of the morphological features of *Pharyngomonas kirbyi* with other Discoba.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Ventral groove</th>
<th>Flagellar vanes</th>
<th>Number of basal bodies (flagella)</th>
<th>Basal body angle</th>
<th>Spilt</th>
<th>RR</th>
<th>RR'</th>
<th>A fibre /Rh</th>
<th>B fibre</th>
<th>MB</th>
<th>I fibre</th>
<th>SR</th>
<th>LR</th>
<th>AR</th>
<th>C fibre</th>
<th>MC</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heterolobosea</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pharyngomonas kirbyi</em></td>
<td>+</td>
<td>−</td>
<td>4 (4)</td>
<td>O</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Rh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Percolomonas cosmopolitus</em></td>
<td>+</td>
<td>−</td>
<td>4 (4)</td>
<td>P</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Rh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Percolomonas sulcatus</em></td>
<td>+</td>
<td>−</td>
<td>4 (4)</td>
<td>P</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lyromonas vulgaris</em></td>
<td>+</td>
<td>−</td>
<td>4 (4)</td>
<td>P</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Rh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Psalteriomonas lanterna</em></td>
<td>+  b</td>
<td>+</td>
<td>4 (4)</td>
<td>P</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Rh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tetramitus rostratus</em></td>
<td>+</td>
<td>−</td>
<td>4 (4)</td>
<td>P</td>
<td>+</td>
<td>+</td>
<td></td>
<td>Rh</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Naegleria gruberi</em></td>
<td>−</td>
<td>−</td>
<td>2 (2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Euglenozoa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>−</td>
<td>2 (2)</td>
<td>O</td>
<td>+</td>
<td>+</td>
<td></td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Jakobids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


* Inferred ancestral states shown for Euglenozoa and jakobids.

b The flagellar vanes of *Psalteriomonas* are on all four flagella, not just the posterior flagellum, and homology with the vanes of ‘typical excavates’ (e.g. jakobids) is questionable.

c per flagellar apparatus. *Psalteriomonas lanterna* has four flagellar apparatuses.

d albeit the rhizoplast homolog of *Pharyngomonas* is unusually short and broad, while clear striations were not seen either *Pharyngomonas* or *Percolomonas cosmopolitus*

e i.e. the fine spur attached to each posterior basal body (see Fig. 3B, 3D).

f *Pharyngomonas kirbyi* also has a second left root (LR) associated with posterior basal body 2.

g Although a true anterior root appears to be absent in jakobids, most other typical excavates have an anterior root, and it is probable therefore that the ancestor of Discoba had an anterior root too.
Figure 5. *Pharyngomonas kirbyi*, Strain SD1A, transmission electron micrographs, ultra-thin sections. **A.** Lateral view showing many microtubules supporting the cell membrane and internal microtubules. Scale bar: 1 μm. **B.** Transverse section showing the right side of the ventral groove after the splitting of the right root (RR), showing the remaining part of the RR, and the individual microtubules supporting the right side of the cell. Note the small sheet-like structure associated with the left side of the RR (arrowhead). Scale bar: 500 nm. **C.** Longitudinal section (ventral-lateral view) through the cell showing the two left roots (LR, LR'), and the cytopharynx microtubules derived from the RR (asterisk) curving inwards posterior to the nucleus. Scale bar: 1 μm. **D.** Cell with two nuclei, possibly a predivision stage. Scale bar: 1 μm. Abbreviations (A-D): IMt: individual microtubules, LR: left root associated with basal body 1, LR': left root associated with basal body 2, M: mitochondrion, N: nucleus, RR: right root associated with basal body 1.

Values supporting this basal position (bootstrap support - ML: 100% and posterior probability 1, Fig. 7), consistent with previous studies (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004; Park et al. 2007, 2009b).

Growth of *Pharyngomonas* in Different Salinities and Temperatures

The two *Pharyngomonas* strains showed slightly different growth responses to various salinity...
Table 2. Qualitative growth response to various salinities (30-300‰) for the two *Pharyngomonas kirbyi* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Salinity (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>SD1A</td>
<td>−−</td>
</tr>
<tr>
<td>AS12B</td>
<td>−−−</td>
</tr>
</tbody>
</table>


media. When feeding on natural prokaryotes Strain SD1A grew at 75-200‰ salinity, while Strain AS12B grew at 100-250‰ salinity (Table 2). Growth was very slow at either 75‰ for SD1A or 250‰ salinity for AS12B. Best growth was observed at 150‰ salinity in both strains (based on qualitative observations). Both strains could survive for 5 days at 50‰ salinity, but did not grow long-term. They seemed to die within 5 days at 30‰ and 300‰ salinities. Neither strain grew on heat-killed *Enterobacter aerogenes* at any examined salinity (1.4 × 10⁷ bacterial cells per ml, 2- or 4-day intervals).

We also examined the effect of temperature on growth in 150‰ salinity media. When feeding on natural prokaryotes Strain SD1A grew at 21–40°C, but failed to grow long-term at 15°C or at 43°C (and above). Growth was more rapid at 37°C than at 21°C or 40°C.

Discussion

Identity of *Pharyngomonas*

The taxon *Pharyngomonas* was introduced by Cavalier-Smith (in Cavalier-Smith and Nikolaev 2008) as a new generic vehicle for organisms originally studied by Entz (1904) and Kirby (1932) under the names *Trichomastix salina* and *Tetramitus salinus* respectively (and subsequently renamed *Percolomonas salina* by Larsen and Patterson 1990). Both original accounts were based on light microscopy data alone, and differed in several minor respects, including a small difference in size, and the report only by Entz (1904) of two contractile vacuoles (Kirby 1932). Kirby (1932) considered his organism to be conspecific with that of Entz (1904). However, based on the differences, Cavalier-Smith and Nikolaev (2008) regarded the two accounts as representing different species. Cavalier-Smith (in Cavalier-Smith and Nikolaev 2008) therefore described a new species, *Pharyngomonas kirbyi*, based on Kirby’s (1932) account, and coined the new combination *Pharyngomonas salina* for the organism described by Entz (1904).

Our light microscopic observations are clearly consistent with the existing morphological data for *Pharyngomonas* flagellates (Entz 1904; Kirby 1932; see also Gunderson, 1981; Post et al. 1983; Ruinen 1938). Both of our isolates have a pointed posterior end to the cell body, two anterior and two posterior flagella that are ∼1.0 to 1.5 times the length of the cell body, a large angle between the anterior and posterior flagella, an anterior location for the nucleus, a ventral groove, and a cytostome located slightly posterior to the nucleus. The organisms also are alike in occurring exclusively, or nearly so, in hypersaline waters (Entz 1904; Gunderson, 1981; Kirby 1932; Post et al. 1983; Ruinen 1938, see below).

*Pharyngomonas kirbyi* (15-19 μm) is smaller than *Pharyngomonas salina* (20-30 μm) and lacks anterior ‘contractile vacuoles’ (Cavalier-Smith and Nikolaev 2008; Kirby 1932). Both of our strains lack structures similar to contractile vacuoles and are smaller than 20 μm, and thus more closely resem-
Figure 7. Maximum likelihood phylogenetic tree (GTR + gamma + I model) showing evolutionary position of Pharyngomonas kirbyi strains SD1A and AS12B relative to 25 heterolobosean taxa as well as four environmental sequences. Outgroups are Euglenozoa (eleven sequences) and Jakobida (four sequences). Bootstrap values (> 55%) from maximum likelihood analysis are shown at the nodes (10,000 replicates). Solid circles indicate a Bayesian posterior probability of 1.
ble Pharyngomonas kirbyi than Pharyngomonas salina. Strain AS12B is particularly similar to P. kirbyi, being 11-18 µm long. The minor difference in size could easily be due to differing growth conditions or ‘strain-level differences’ (see below). Our second strain, SD1A, at 9-14 µm long, is smaller than the original description of P. kirbyi. It is smaller on average than AS12B (though the size ranges overlap substantially) and we saw some difference in the salinity ranges for growth. The 18S rRNA gene sequences of SD1A and AS12B are 98% identical. With only two strains available, we cannot know whether the morphological and physiological differences we observe are characteristic of distinct groupings within Pharyngomonas (that might then be usefully considered separate species on these criteria) or whether these traits vary markedly amongst closely related strains, and don’t map clearly to phylogeny (Lowe et al. 2005; Tarcz et al. 2006; Weisse 2002). Bearing this in mind, we have assigned both isolates to the existing nomenclatural species Pharyngomonas kirbyi.

The 18S rDNA gene sequences from our two strains are 97% identical to Genbank sequence AF001145. The sequences are identical in the sites retained for phylogenetic analysis and (unsurprisingly) form a strongly supported clade. Sequence AF001145 was originally accessed under the nomen nudum ‘Macrophyaryngomonas halophila’ and appears in several phylogenies of Heterolobosea under that name (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004; Park et al. 2007, 2009b). There are no published microscopy data from this organism, and the culture is apparently no longer extant, though data on the other flagellates listed here was not available at that time). The right root is supported by a structure that positionally resembles a rhizoplast (though see below). The anterior placement of the cytostome is similar to some other heterolobosean flagellates that feed (see Cavalier-Smith and Nikolaev 2008). By contrast, other cells with a suspension feeding groove (typical excavates) usually ingest prey at the posterior end of the groove.

Most intriguingly, however, Pharyngomonas has a ‘double bikont’ flagellar apparatus like that seen to a greater or lesser extent in most tetraflagellated Heterolobosea (Brugerolle and Simpson 2004). In this organization, the four flagella and basal bodies form two pairs, each with a similar set of associated microtubular roots. This arrangement is most obvious in Percolomonas, Psalteriomonas and Lyромonas the right root of Pharyngomonas is a very large, broadly curving ribbon (this structure was also reported by Gunderson, 1981, but data on the other flagellates listed here was not available at that time). The right root is supported by a structure that positionally resembles a rhizoplast (though see below). The anterior placement of the cytostome is similar to some other heterolobosean flagellates that feed (see Cavalier-Smith and Nikolaev 2008). By contrast, other cells with a suspension feeding groove (typical excavates) usually ingest prey at the posterior end of the groove.
than RR, and does not directly support the groove (it also originally contains fewer microtubules). The two left roots are very similar, except that LR’ usually has more microtubules than LR, and they follow parallel paths down the cell (note that a left root was not observed in *Pe. sulcatus* by Brugerolle and Simpson 2004). Thus *Pharyngomonas* probably shows the least derived double bikont flagellar apparatus in Heterolobosea, other than that of *Pe. sulcatus*.

*Percolomonas sulcatus* shares some other similarities with *Pharyngomonas*. Both have the flagellar pairs arranged side-by-side, rather than in tandem or orthogonally, as in other tetraflagellated heteroloboseids (see Brugerolle and Simpson 2004), although this might be linked to the double bikont state. Both also have a relatively shallow ventral groove. *Percolomonas sulcatus* was assigned to *Percolomonas* on the basis of light microscopy (Larsen and Patterson 1990), and under a broad concept of this genus that clearly does not represent a monophyletic group (Cavalier-Smith and Nikolaev 2008). In practice, the phylogenetic position of *Pe. sulcatus* within Heterolobosea is unknown. It is possible that *Pe. sulcatus* is related to *Pharyngomonas* (though see below) or represents another deep branch within Heterolobosea. Phylogenetic analyses that included sequence data from *Pe. sulcatus* would be very valuable.

**Unusual Features Including Potential Plesiomorphies**

The likely deep-branching position of *Pharyngomonas* within Heterolobosea opens a window into the early evolution of this group. Of particular interest is whether any of the unusual morphological features of *Pharyngomonas* could be ancestral for Heterolobosea. Characters shared by *Pharyngomonas* and relatives of Heterolobosea are therefore of especial importance. Recent molecular phylogenies support the placement of Heterolobosea within the clades Excavata and Discoba, with the closest relatives of Heterolobosea being Euglenozoa, and then Jakobida (Hampl et al. 2009; Rodríguez-Espeleta et al. 2007) or the recently discovered *Tsukubamonas* (Yabuki et al. 2011). The equivalent root in Euglenozoa is of moderate length, but *Tsukubamonas* lacks a left root (Yabuki et al. 2011). The fact that the left roots of *Pharyngomonas* are elongate, and perform a clear structural role is likely therefore to reflect an inherited ancestral state. By contrast, their extension to the full length of the cell is unusual for an excavate and may be an apomorphy for the *Pharyngomonas* lineage.

In *Pharyngomonas* the basal bodies within each pair lie almost at right angles. This is unlike other flagellated Heterolobosea, which characteristically have parallel basal bodies (Brugerolle and Simpson 2004), but similar to most typical excavates. Cavalier-Smith and Nikolaev (2008) proposed that the angled basal bodies of *Pharyngomonas* were an ancestral state for Heterolobosea. However, Euglenozoa, which are phylogenetic sisters to Heterolobosea, likely had parallel basal bodies ancestrally, since parallel basal bodies are the norm for biflagellated members of every subgroup of

especially interesting. Patterson (1990) reported the ‘C’ fibre first in *Jakoba*. The ‘C’ fibre in jakobids is a multilayered structure associated with the left root (Lara et al. 2006; O’Kelly 1993). Similarly positioned structures with different layering patterns are present in other typical excavates; *Trimastix* and retortamonads (O’Kelly et al. 1999; Simpson et al. 2000), *Carpediemonas* (Simpson and Patterson 1999), *Hicanonectes* (Park et al. 2009a), *Dysnectes* (Yubuki et al. 2007), *Malawimonas* (O’Kelly and Nerd 1999), and *Ergobibamus* (Park et al. 2010).

The highly multilayered nature of the *Pharyngomonas* ‘C’ fibre is similar to that of jakobids. The substructure differs, but is within the range of variability seen in other excavates. Assuming the ‘C’ fibre of *Pharyngomonas* is homologous to that of other excavates, it was apparently still present in the last common ancestor of Heterolobosea, and lost after the divergence of *Pharyngomonas* (in one event or several). ‘C’ fibres are absent in Euglenozoa and the newly discovered *Tsukubamonas* (Table 1, Yabuki et al. 2011). On the evidence of *Pharyngomonas*, these other Discoba appear to have lost the ‘C’ fibre independently of (derived) Heterolobosea.

In other Heterolobosea, the left roots, if present, are very short, and have few microtubules (A left-root-like element in *Percolomonas cosmopolitus* is actually part of the right root - see Simpson 2003). The left roots of typical excavates, including jakobids, usually extend about half the length of the cell as a coherent structure, although separated microtubules derived from the left root can run the entire length of the cell (Simpson and Patterson 1999). The equivalent root in Euglenozoa is of moderate length, but *Tsukubamonas* lacks a left root (Yabuki et al. 2011). The fact that the left roots of *Pharyngomonas* are elongate, and perform a clear structural role is likely therefore to reflect an inherited ancestral state. By contrast, their extension to the full length of the cell is unusual for an excavate and may be an apomorphy for the *Pharyngomonas* lineage.
Euglenozoa, except metakinetoplastids, which are nested within Kinetoplastea (Moreira et al. 2004; Simpson et al. 2004; von der Heyden et al. 2004). Thus there are two equally parsimonious evolutionary scenarios, assuming angled basal bodies is the ancestral state for Excavata; 1) Pharyngomonas retained the ancestral state, and other Heterolobosea and Euglenozoa transformed to the parallel state independently (this is the Cavalier-Smith and Nikolaev scenario), and 2) the common ancestor of Euglenozoa and Heterolobosea had parallel basal bodies, and Pharyngomonas secondarily reverted to the ancestral angled basal body state. In our view these scenarios are more-or-less equally plausible on present data.

The spur on each posterior basal body has not been seen before in Heterolobosea. The spurs resemble the ‘B’ fibre of typical excavates, in position and orientation, and are broadly similar to most ‘B’ fibres in being sheet-like. However the spurs are thinner, much shorter, and do not associate as closely with the right root. We also did not observe lateral striations, which are typical of ‘B’ fibres, though not seen in every study. It was noted previously that the microfibrillar bundle of several Heterolobosea has positional similarity to the ‘B’ fibre, but little structural similarity (Brugerolle and Simpson 2004). For either feature homology is possible, but clearly arguable at present. Further, since the spur and bundle are present together in Pharyngomonas (albeit the bundle is very small, and is identified only tentatively), it is unlikely that both are ‘B’ fibre homologues.

Early reports of Pharyngomonas were of flagellated cells, and the general view was therefore that this organism is a pure flagellate (Cavalier-Smith and Nikolaev 2008; Larsen and Patterson 1990). In fact, it has been proposed that the ability to transform into amoebae evolved after the divergence of Pharyngomonas (Cavalier-Smith and Nikolaev 2008). During the course of this present study we did not observe an amoeboid form in either of our strains. Gunderson (1981) however, reported extensively on an amoeba stage in his strain of Pharyngomonas, in fact it was originally isolated as an amoeba and subsequently was found to transform to the flagellate stage. The existence of amoebae in Pharyngomonas may be of evolutionary and systematic importance, and will be addressed in detail in an upcoming work.

The presumed rhizoplast homolog differs markedly from the classic rhizoplasts of Heterolobosea, which are more rod-like, and almost always conspicuously striated (Brugerolle and Simpson 2004; Outka and Kluss 1967; Park et al. 2007; Simpson and Dingle 1971). The heterolobosean rhizoplast is positionally similar to the non-striated ‘A fibre’ of jakobids and might be homologous, but these are smaller and less distinct than rhizoplasts. The broad and amorphous nature of the rhizoplast homolog might be an apomorphy for Pharyngomonas. Again, more data from other deep-branching Heterolobosea would be valuable, if they are extant.

Pharyngomonas lacks any particular tendency for the endoplasmic reticulum to encircle its mitochondrial organelles, which was one of the characteristics identified originally for Heterolobosea (Page and Blanton 1985). However, this feature is absent from several other Heterolobosea (sensu lato) of diverse phylogenetic position, including Percolomonas cosmopolitius, Stephanopogon spp., Pleurostomum flabellatum, and Lyromonas vulgaris (where the mitochondrial organelles are probably hydrogenosomes, Broers et al. 1993). This character must have a complex evolutionary history.

In summary, Pharyngomonas shows a mixture of heterolobosean characters and features that are absent in most/all other Heterolobosea, but found in typical excavates, the paraphyletic group from which Heterolobosea descend. This pattern is consonant with the deep-branching position of Pharyngomonas in 18S rRNA gene trees, and provides some additional support for this placement. Additional molecular study of Pharyngomonas would be useful to further test the phylogenetic position of this group, and to better understand the early evolution of Heterolobosea, and the larger groups to which it belongs (Discicristata, Discoba).

Physiological Characteristics and Halophilic Heterolobosea

The two strains show a slightly different salinity range for growth (SD1A grew at 75-200‰ salinity, while AS12B grew at 100-250‰ salinity). Overall, however, both strains grew well at 100-200‰ salinity and neither grew at all below 75‰ salinity. Gunderson (1981) saw growth of his single strain of Pharyngomonas at 60‰ salinity in the flagellate form and 50‰ salinity in the amoeba form, but not below, while both strains grew in 250‰ salinity (higher salinities were not tested). These results indicate that Pharyngomonas strains are obligate halophiles, like Halocafeteria seosinensis, Trimyema koreanum, Pleurostomum flabellatum, Tulamoeba peronaphora, and Euplaesiobystra hypersalinica (Cho et al. 2008; Park et al. 2006, 2007, 2009b). Interestingly, Pharyngomonas spp.
were usually observed between 110‰ and 220‰ salinity in previous studies of natural material and crude enrichments (Cavalier-Smith and Nikolaev 2008; Entz 1904; Kirby 1932; Post et al. 1983), which is similar to the salinity ranges for growth observed in the laboratory. There are outliers, however; Ruinen (1938) reported Pharyngomonas in a salinity range of 30‰ to 200‰, while Post et al. (1983) reported Pharyngomonas in a crude enrichment of saturated brine, even though they did not see cells above 223‰ salinity in natural samples. Given the rarity of the observations at very high or low salinities, we suspect that they represent populations persisting beyond the salinities at which they can achieve net growth. Nonetheless, we cannot exclude the possibility that salinity ranges for growth may be affected by factors such as the types of prey available, or ion composition, and thus underestimated by our culturing experiments. There may also be strains of Pharyngomonas that can grow at much higher or lower salinities than others. Still, most of the data suggest that Pharyngomonas spp. are true halophiles, but rather less extraordinary in their degree of halophilicity than some other halophilic protozoa cultured recently (Cho et al. 2008; Park et al. 2006, 2007, 2009b).

There are now three groups in Heterolobosea confirmed to be obligate halophiles: 1) the Pleurotomum-Tulamoeoba clade, 2) Euplaesiobystra, and 3) Pharyngomonas. It is likely that other clades of halophilic heteroloboseids exist. A large fraction (~36%) of the morphospecies observed at ~300‰ salinity are probably heteroloboseids (Park et al. 2009b). Percolomonas cosmopolitis, which is a genetically diverse morphospecies usually found in marine habitats (Nikolaev et al. 2004), has been also recorded in (near-) saturated brines (Ruinen 1938), and we recently isolated a Pe. cosmopolitis-like organism from 200‰ salinity water (Park et al., unpublished). It is possible that Pe. cosmopolitis contains halophilic (or very halotolerant) subclades. We have also isolated a heterolobosean amoeba from 73‰ salinity water that has no close relatives in 18S rRNA gene trees (Park et al., unpublished). These results speak to the probability of finding other halophilic or halotolerant heterolobosea in hypersaline habitats.

From this study and other similar work, it is clear that many lineages of protozoa independently became obligate halophiles. Since almost nothing is known yet about their physiology, the time is ripe for comparative study of halophilic protozoa.

Methods

**Isolation and culturing**: Strain SD1A of Pharyngomonas kirbyi. Cavalier-Smith 2008 was sourced from a water sample collected from a solar salt pan located at San Diego, USA (32° 35' 36" N, 117° 6' 50" W). The salinity of the sample at collection was 300‰. Pharyngomonas kirbyi strain AS12B was sourced from a mixed water/sediment sample from a very shallow hypersaline puddle within a birrida in Shark Bay, Australia (25° 52' 45" S, 113° 32' 34" E). The water phase of this sample had a salinity of 210‰ at collection, although the sediment phase consisted mostly of encrusted salt. Both samples were collected in June 2009 (Boreal early summer; Austral early winter). 0.1 ml of fluid from each sample was used to inoculate crude cultures in 5 ml of 100‰ salinity liquid media, made by dilution of AS medium (300‰, 272 g NaCl, 7.6 g KCl, 17.8 g MgCl2, 1.8 g MgSO4·7H2O, 1.3 g CaCl2·1 H2O) with sterile distilled water, and supplemented by LB broth (final concentration 0.5%) plus autoclaved barley grains to grow prokaryotes. Monoprotistan cultures of each strain of Pharyngomonas kirbyis were then obtained using serial dilution in the same media. Cultures of each strain were maintained in the same 100‰ salinity media, or in the equivalent 150‰ salinity media, incubated at room temperature and subcultured every two weeks. Pharyngomonas kirbyis strains SD1A and AS12B have been deposited in the Culture Collection of Algae and Protozoa (CCAP) under the accession numbers 1955/2 and 1955/1, respectively.

To investigate whether flagellate-to-amoebo transformation could be induced on solid media, 300 μl of liquid culture of each strain was inoculated on CAS-15 agar plates (7.5 g casamino acids, 1.0 g yeast extract, 5.0 g protease peptone, 3.0 g sodium citrate, 20.0 g MgSO4·7H2O, 0.5 g K2HPO4, 2.0 g KCl, 150.0 g NaCl, 20 g BactoAgar per 1 liter distilled water, pH 8.0), and incubated at 22.5 °C for 30 days. The samples on the plates were examined using an inverted microscope every 7-9 days.

**Microscopy**: Live cells of both strains were observed with differential interference and phase contrast light microscopy using a Zeiss Axiosvert 200 M microscope equipped with an AxioCam HR digital camera. Lenghts and widths of free-living cells were determined from digital micrographs using the camera software (Axiovision 4.6).

For transmission electron microscopy (TEM), Strain SD1A was grown in 100‰ salinity media. The cells were centrifuged at 8000 g for 3 min and fixed for 30 min at room temperature in 1 ml of a cocktail containing 0.92 ml of 100‰ salinity media and 0.08 ml of 25% w/v glutaraldehyde (final concentration 2%). After rinsing three times with 100‰ salinity media, cells were post-fixed for 1 hour in 1 ml of a cocktail containing 0.25 ml of 4% w/v OsO4 (final concentration 1%) and 0.75 ml of 100‰ salinity media. After being rinsed free of post-fixative, cells concentrated by centrifugation were trapped in 2% w/v OsO4 (final concentration 1%) and 0.75 ml of 100‰ salinity media. After being rinsed free of post-fixative, cells concentrated by centrifugation were trapped in 2% (w/v) agarose. Agarose blocks were dehydrated by applying a graded series of ethanol, and embedded in Spur’s resin. Serial sections (~70 nm) were cut with a diamond knife on a Leica UC6 ultramicrotome (Leica, UK), then stained with saturated uranyl acetate in 50% ethanol and with lead citrate. Sections were observed using a Tecnal 12 electron microscope (Philips) with a goniometer stage.

**18S rRNA gene sequencing and phylogeny**: Nucleic acids from both strains were prepared using a DNeasy Blood and Tissue Kit (Qiagen, Maryland, USA) according to the supplied protocol. 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, 0.5 μM EuKA and EukB,
2 mM MgCl₂, 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 the PCR products (3–3kb) was determined by agarose gel electrophoresis. Amplicons were cloned into a pGEM-T Easy vector, and four or five positive clones were partially sequenced using vector sequencing primer T7. The 18S rRNA gene sequences from Pharyngomonas strains SD1A and AS12B have been deposited in GenBank as HQ898858 and HQ898857, respectively.

The 18S rRNA gene sequences from our isolates were compared to the sequences in the Genbank database using BLASTN search. The 18S rRNA gene sequences were aligned by eye to those of other heteroloboseans and outgroups, using the alignment of Park et al. (2009b) as a seed. The phylogenetic ingroup was the Heterolobosea and environmental sequences closely related to Heterolobosea, while the outgroups were Euglenozoa and Jakobida. In total, 1014 unambiguously aligned sites were retained for phylogenetic analysis. These alignments are available on request.

Phylogenetic trees were inferred by ML and by Bayesian analysis. The GTR + gamma + I model of sequence evolution was selected using MrModeltest 2.2, and used for both analyses (Nylander 2004). The ML tree was estimated using RAxML- HPC v.7 (Stamatakis 2006), with statistical support estimated using non-parametric bootstrapping with 10,000 replicates. The Bayesian analysis was carried out using MrBAYES 3.2 (Huelsenbeck and Ronquist 2001) with two independent runs, each with four independent chains running for 2.0 × 10⁹ generations (0.2 × 10⁶ generations burn-in), with default heating parameter (0.2) and sampling frequency (0.01).

**Growth response to salinity and temperature:** To estimate the salinity ranges for growth of Pharyngomonas strains SD1A and AS12B, we performed two different experiments using media of 30‰ to 300‰ salinity, made from an artificial seawater stock (AS medium; see above) as reported by Park et al. (2009b), and Park and Simpson (2010). Briefly, to support growth of Pharyngomonas the medium was supplemented in one trial by LB broth (final concentration 0.5%) plus an autoclaved barley grain, and supplemented in a second trial by heat-killed Enterobacter aerogenes at an initial density of 1.4 × 10⁹ cells ml⁻¹ as used by Park and Simpson (2010), with additional heat-killed bacteria (1.4 × 10⁷ cells ml⁻¹) added at 2- to 4-day intervals. All treatments were performed in duplicate. AS media (1 ml) with a range of salinities (30-300‰) were inoculated with 50 µl of actively growing stock cultures (100‰ salinity media with LB broth plus an autoclaved barley grain) and incubated in the dark at 22–25 °C for at least 21 days. Culture viability was assessed weekly by looking for actively moving cells in 100 µl of the culture using phase-contrast microscopy. Active growth in the medium containing LB broth and a barley grain was confirmed by a serial transfer into fresh media with the same salinity (1 ml of media, inoculum of 50 µl), and re-examining the culture weekly for active cells, as above. Heat-killed bacteria trials were stopped after three weeks because no active protists were seen at any salinity.

To examine growth responses of Pharyngomonas to various temperatures, culture tubes containing 1 ml of AS medium (150‰ salinity) supplemented with LB broth (final concentration 0.5%) plus an autoclaved barley grain were each inoculated with an actively growing culture. Two cultures of each strain were incubated at 15, 21, 37, 40, 43, and 46 °C. Cultures were examined by microscopy at seven-day intervals for up to three weeks. Growth was confirmed by subculturing followed by incubation at the same temperature, and microscopy observation as above.

**Acknowledgements**

We thank Ivan Cepicka and Tomas Panek at Charles University, Prague and Akinori Yabuki at the University of Tsukuba for valuable comments on the manuscript. This work was supported by NSERC grant 298366-04, and the Canadian Institute for Advanced Research (CIfAR), Program in Integrated Microbial Biodiversity. JSP was supported by a CGEB (Dalhousie University) post-doctoral fellowship funded by the Tula Foundation.

**References**


Kirby H (1932) Two protozoa from brine. Trans Am Microsoc Soc 51:9–15


Medlin L, Elwood HJ, Stickel S, Sogin ML (1989) The charac-
terization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. Gene 71:491–499


Park JS, Simpson AGB (2010) Characterization of halotoler-
ant Bicosoecida and Placididea (Stramenopila) that are distinct from marine forms, and the phylogenetic pattern of salinity preference in heterotrophic stramenopiles. Environ Microbiol 12:1173–1284

Park JS, Cho BC, Simpson AGB (2006) Halofacetera seosi-
nensis gen. et sp. nov. (Bicosoecida), a halophilic bacterivorous nanoflagellate isolated from a solar saltern. Extremophiles 10:493–504


Park JS, Simpson AGB, Brown S, Cho BC (2009b) Ultrastructure and molecular phylogeny of two heterolobosean amoeboae, Euplaesiobysta hypersalinica gen. et sp. nov. and Tulamoea peronaphora gen. et sp. nov., isolated from an extremely hyper-
saline habitat. Protist 160:265–283


Patterson DJ (1990) Jakoba libera (Ruinen, 1938), a heter-

Post FJ, Borowitza LJ, Borowitza MA, Mackay B, Moul-


Simpson AGB, Patterson DJ (1999) The ultrastructure of Car-
pediemonas membranifera (Eukaryota) with reference to the “excavate hypothesis”. Eur J Protistol 35:353–370

Simpson AGB, Bernard C, Patterson DJ (2000) The ultrastruc-
ture of Trimastix marina Kent, 1880 (Eukaryota), an excavate flagellate. Eur J Protistol 36:229–251


Simpson PA, Dingle AD (1971) Variable periodicity in the rhi-


