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Halocafeteria seosinensis gen. et sp. nov. (Bicosoecida), a halophilic bacterivorous nanoflagellate isolated from a solar saltern

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Abstract Recently, heterotrophic nanoflagellates (HNF) have been reported to actively ingest prokaryotes in high salinity waters. We report the isolation and culture of an HNF from a Korean saltern pond of 300‰ salinity. The organism is biflagellated with an acronematic anterior flagellum and never glides on surfaces. The mitochondria have tubular cristae. Neither transitional helix nor spiral fiber were observed in the transition zones of the flagella. The cell has a cytostome supported by an arc of eight microtubules, suggesting that our isolate is a bicosoecid. Our isolate had neither mastigonemes, lorica, body scales, nor cytopharynx and thus could not be placed in any of the presently described bicosoecid genera. Phylogenetic analysis of 18S rRNA gene sequences from stramenopiles confirmed the bicosoecid affinities of our isolate, but did not place it within any established genus or family. Its closest relatives include *Caecitellus* and *Cafeteria*. The optimal range of growth temperature was 30–35°C. The isolated HNF grew optimally at 150‰ salinity and tolerated up to 363‰ salinity, but it failed to grow below 75‰ salinity, indicating that it could be a borderline extreme halophile. On the basis of its morphological features and position in 18S rRNA trees we propose a novel genus for our isolate; *Halocafeteria*, n. gen. The species name *Halocafeteria seosinensis* sp. nov. is proposed.

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Introduction

For almost a century, heterotrophic nanoflagellates (HNF) have been observed frequently in natural saturated brines and appeared to be feeding on prokaryotes in these habitats (Namyslowski 1913; Ruinen 1938; Post et al. 1983; Patterson and Simpson 1996). Compared to studies on the ecology and diversity of prokaryotes in high salinity waters, including solar salterns, our knowledge of halophilic heterotrophic eukaryotes in hypersaline environments has been limited (Casamayor et al. 2002; Oren 2002). However, molecular approaches have demonstrated a higher diversity of eukaryotes in high salinity waters than previously realized (Casamayor et al. 2002), and it was recently reported that HNF from high salinity waters actively ingest prokaryotes, and that mixed cultures of HNF could be obtained from these habitats (Park et al. 2003). Interestingly, Patterson and Simpson (1996) reported the occurrence of four new HNF species among a total of eight species observed at 150‰ salinity in a hypersaline pond and two new HNF species among a total of five observed in a saturated puddle in Australia, suggesting a high probability of finding new HNF species in hypersaline environments. In the present study, we were curious to discover if an HNF that we isolated from the high salinity waters of a solar saltern was a novel extremely halophilic or halotolerant eukaryote, and if the isolated HNF was active at the high in situ temperatures found in solar salterns. The behavioral and ultrastructural characteristics, growth physiology, and 18S rRNA gene sequence of an isolated HNF from high salinity water are described for the first time. This organism is described here as *Halocafeteria seosinensis* n. gen. n. sp.

Materials and methods

Source of the isolated heterotrophic nanoflagellates

Strain EHF34 of *H. seosinensis* was isolated from high salinity waters (300‰ salinity) collected in May 2002 from multi-pond systems located at Seosin on the west coast of Korea (37°09′36″N, 126°40′44″E). Salinity in the solar saltern was measured by diluting saltern waters with distilled water to fall within the scale of a Temperature/Conductivity/Salinity Instrument (YSI 30, YSI, OH, USA).

Cultivation and light microscopy

For enrichments of HNF from high salinity waters, 1 l water samples in 2 l polycarbonate bottles were amended with autoclaved barley grains in May, 2002 and kept in continuous darkness at 30°C for 6 months. The culture was diluted with fresh FAHS medium (0.2 μm filtered and autoclaved high salinity water) at a probability of one HNF per 10 culture tubes. The media in a culture tube consisted of FAHS medium supplemented with an autoclaved barley grain. The isolation step was further repeated two times. Motile flagellates were observed with phase contrast microscopy using a Zeiss Axiovert 200M microscope equipped with an Axiocam HR digital camera. A permanent slide of protargol-stained cells (USNM slide 1023202) has been deposited in the Protist Type Specimen Slide Collection, National Museum of Natural History, Smithsonian Institution, Washington, DC.

Electron microscopy

For scanning electron microscopy (SEM), cultures were centrifuged at 4,000*g* and fixed at 4°C in 1% v/v glutaraldehyde (electron microscopy grade) in 0.05 M cacodylate buffer (pH 7.8). Fixed cells were transferred to glass coverslips coated with 1% poly-L-lysine. Cells were rinsed with 0.05 M cacodylate buffer and then dehydrated with a graded series of ethanols. Ethanol was replaced by isoamyl acetate before critical-point drying. Fixed cells were coated with gold/platinum with an ion sputter system. Specimens were examined with a field emission scanning electron microscope (JSM-6700F, Japan).

For preparation of negatively stained whole-mounts, a drop of a suspension of living cells was placed on a formvar-coated grid. A drop of 2% glutaraldehyde in 0.2 M cacodylate buffer was added and the grid was left for 5 min to allow the cells to settle on the film. The remaining solution was removed by filter paper, and a drop of 1% uranyl acetate was placed on the grid and removed by filter paper 30 s later. After drying the grid, specimens were examined with a transmission electron microscope (JEOL 2000 EXII, Japan).

For ultrathin sections, cells were grown in 90‰ salinity media. Cells were concentrated by centrifugation and fixed for 30 min at room temperature (4% glutaraldehyde and 30% sucrose in 0.1 M cacodylate at pH 7.4). After rinsing the cells three times with 30% sucrose in 0.1 M cacodylate buffer, cells were fixed for 1 h in a cocktail containing 0.8% OsO_4 and 30% sucrose in 0.1 M cacodylate. Fixed cells were harvested by centrifugation and trapped in 1.5% (w/v) agarose. Agarose blocks were dehydrated with a graded series of ethanols and then embedded in Spurr's resin. Serial sections were cut with a diamond knife on a Leica UC6 ultramicrotome (Leica, UK) and were subsequently stained with saturated uranyl acetate in 50% ethanol and lead citrate. Sections were observed using a Tecnai 12 electron microscope (Philips, ON, Canada). Fixation quality was mediocre, probably due to the high salinity of the growth media.

Heterotrophic nanoflagellates abundance, optimal temperature and salinity

Samples for measurement of HNF abundance were immediately fixed with glutaraldehyde (1% final concentration; Bloem et al. 1986). DAPI-stained HNF were collected on 0.8 μm polycarbonate filters (25 mm in diameter) under a vacuum not exceeding 100 mmHg. Cells were enumerated at 1,000× magnification with UV excitation using an epifluorescence microscope. Varying volumes (0.5–1 ml) of samples were filtered depending on cell abundance. At least ten microscopic fields and a total of 30–102 non-pigmented flagellates were counted.

To determine the optimal growth temperature, culture flasks containing 30 ml of FAHS medium supplemented with an autoclaved barley grain were inoculated with an actively growing culture, and maintained in the dark at temperatures ranging from 15 to 40°C at 5°C intervals (two cultures per temperature). Cell abundance was monitored daily for 11 days, and growth rates at each temperature were calculated during exponential growth.

To determine the effect of salinity on growth, an artificial seawater stock was prepared (AS medium, 325 ± 0.7‰ salinity; 283.2 g NaCl, 7.7 g KCl, 54.4 g $MgCl_2{\cdot}6H_2O$, 59.4 g $MgSO_4{\cdot}7H_2O$, 1.3 g $CaCl_2{\cdot}2H_2O$), and various salinity media were generated by serial dilution with double-distilled water. The measured salinity was slightly lower than expected due to salt precipitation. To prepare heat-killed bacteria, *Idiomarina seosinensis* (Choi and Cho 2005) was grown at 25°C for 2 days in marine broth (Difco, Quebec, Canada), and then incubated at 70°C for 3 h. To confirm the non-viability of the heat-treated bacteria, 100 μl of culture was spread onto the Marine Agar 2216 (Difco) in triplicate, and incubated at 25°C. No bacterial colonies formed even after 7 days. Heat-killed bacteria were centrifuged at 3,000*g* for 5 min, and washed once with 1 ml of PBS solution (0.05 M Na_2HPO_4− 0.85% NaCl,

pH 9). After centrifugation, the prey was resuspended in AS media of the appropriate salinity to avoid salinity changes in the experimental culture. Various salinity AS media (40 ml) were inoculated with actively growing HNF and heat-killed bacteria (up to 34.7×10^7 cells ml^{-1}), and incubated in the dark at 35°C for 10 days, with additional heat-killed bacteria added at 2-day intervals. The growth rates of HNF were determined as described in the temperature experiment.

Nucleic acid preparation, polymerase chain reaction, cloning and sequencing of the 18S rRNA gene

Nucleic acids were extracted and purified using CTAB (hexadecyltrimethyl ammonium bromide) and organic extractions as described by Ausubel et al. (1999). Cells were harvested from 1 ml (2.88×10^5 cells ml^{-1}) of pure culture in 300‰ salinity by centrifugation (10 min at 12,000*g*). The DNA yield was quantified by Picogreen dye (Molecular Probes, OR, USA) according to the manufacturer's instructions.

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 0.3 μM, 75 mM Tris–HCl [pH 9.0], 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 2.5 U of *Taq* DNA polymerase (Biotools, Spain). PCR-amplification was conducted according to the following cycle parameters: an initial denaturation step (5 min, 94°C), was followed by 30 cycles consisting of denaturation (45 s, 94°C), annealing (1 min, 55°C), and extension (3 min, 72°C), with a final 10 min extension step at 72°C at the end. The size of the PCR products (~1.8 kb) was confirmed by agarose gel electrophoresis. Amplified products were purified using a PCR purification kit (Bioneer, Korea) according to the manufacturer's recommendations, then ligated into the prepared vector (pCR 2.1) supplied with a TA cloning kit (Invitrogen, Carlsbad, CA, USA) by following the manufacturer's protocols. Plasmid DNA from putative positive colonies was harvested using a Bioneer plasmid purification kit (Bioneer). Sequencing was performed with an Applied Biosystems automated sequencer (ABI 3730xl) at Macrogen Corp. in Korea. We also performed denaturing gradient gel electrophoresis (DGGE)-sequencing (about 520 bp, Díez et al. 2001) to allow us to design a 18S rRNA forward primer specific to a phylotype from Seosin saltern water, 'EHF0502'. Our primer, EHF370, has the sequence 5′ ACCCCTTAACGA AAGCCATT 3′. PCR-amplification of eukaryotic 18S rRNA genes with EHF370 and EukB primers was conducted as described above. Purified PCR products were cloned and sequenced. The 18S rRNA gene sequences from the uncultured sample and isolate EHF34 have been deposited in GenBank under the accession numbers DQ269469 and DQ269470, respectively.

Phylogenetic analysis

The 18S rRNA gene sequence from the isolated HNF was compared to the sequences in the GenBank database using a BLASTN search. The sequence was manually aligned with those of related taxa obtained from GenBank using 18S rRNA secondary structure information from *Bacillaria paxillifer* (Van de Peer et al. 2000). For phylogenetic analysis, a dataset was constructed that included 18S rRNA gene sequences from all available Bicosoecida except the long branching *Symbiomonas scintillans*, together with selected other stramenopiles, with 1,431 unambiguously aligned sites retained for analysis. We also constructed a dataset including *Symbiomonas* (1,431 sites) and one excluding non-bicosoecid sequences (1,445 sites). These alignments are available on request.

Phylogenetic trees were inferred by maximum likelihood (ML) (Felsenstein 1981), ML distance, and maximum parsimony (MP) (Fitch 1971) methods using PAUP* 4b10 (Swofford 1998), and by Bayesian analysis (BA) using MrBAYES 3.0 (Huelsenbeck and Ronquist 2001). For the analyses except parsimony, the Tamura-Nei + gamma + I model (Tamura and Nei 1993) was used (This model was chosen over similar models by likelihood ratio tests). For the distance and likelihood analyses the parameter values were estimated from a test tree using PAUP*. For each distance analysis, the minimum evolution (ME) tree was found using 20 random additions and tree bisection–reconnection (TBR) branch-swapping and a bootstrap analysis (Felsenstein 1985) was performed with 10,000 replicates (five random additions and TBR). For each ML analysis, the best tree was found using 20 random additions and TBR, and a 500 replicate bootstrap analysis was performed (neighbor-joining starting tree, then TBR; 1,000 replicates performed for the 'bicosoecid only' analyses). Posterior probabilities of phylogenetic trees under the Tamura-Nei + gamma + I model were estimated using MRBAYES 3.0 (Huelsenbeck 2000). Four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations and sampled every 500 generations (burnin 200,000 generations).

Results

Morphological and behavioral features under light and epifluorescence microscopy

The isolated HNF are rounded, bean-shaped or roughly triangular cells 3–5 μm in length (Fig. 1a–e). Cells have two sub-equal flagella, approximately 1.5–2 times body length, which insert subapically at an acute angle

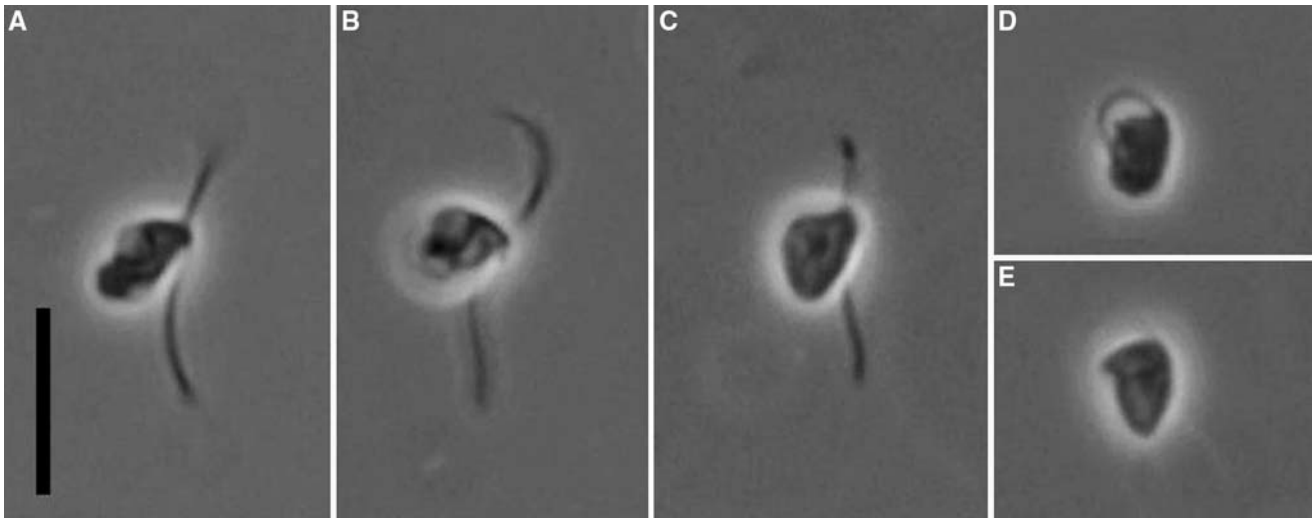

Fig. 1 Phase-contrast micrographs of live *H. seosinensis* n. gen., n. sp. cultured from a solar saltern (isolate EHF34), showing cell size and shape, and position of the flagella. *Scale bar* 10 μm

(Figs. 1a, 2b). Cells often attached to surfaces by the posterior flagellum, in which case the anterior flagellum created a feeding current using an oaring beat. Attached cells sometimes displayed a 'jumping' motion caused by flexure of the posterior flagellum. Unattached cells were most often observed swimming with both flagella beating, but in some cells only the anterior flagellum was beating, in which case the cells would tumble. We did not observe any gliding motility. A nucleus was located in the mid-anterior part of the DAPI-stained cell (data not shown). Occasionally a circular cytostome was observed at the anterior edge of the cell opposite the flagellar insertion.

Fine structure of heterotrophic nanoflagellates

No mastigonemes were observed on either flagellum in whole-mounts, scanning electron micrographs or thin-section transmission electron micrographs (Figs. 2a–d, 3c, e). The anterior flagellum is markedly acronematic (has a hair-tip), while the posterior flagellum is not (Figs. 2a–d, 3b, c). The length of hair-like tip on the anterior flagellum is about 1/4 of the total length of the flagellum and it is supported by the central pair of axonemal microtubules (Fig. 3b, c). Some cells with two additional short flagella were observed, presumably representing pre-division stages (Fig. 2d).

Within the cell body the nucleus, with central nucleolus, is located near the flagellar insertion, as is the single Golgi dictyosome, which has 3–5 cisternae (Figs. 3a, 4a–c). A paranuclear microbody-like organelle was not observed. Mitochondria were always located close to the nucleus and had tubular cristae (Figs. 3a, d, 4a–c). Food vacuoles, some containing intact prokaryotic prey, were observed towards the posterior end of the cell (Figs. 3a, 4a). The main portion of the axoneme has a standard 9 + 2 microtubule structure with no conspicuous paraxonemal structures (Figs. 3e, 4e). The transition zone has a thin transverse plate—no transition helix and no spiral fibers were observed (Fig. 3f). The basal bodies are oriented at 70° to each other (Fig. 4e). No extrusomes, lorica, surface scales or elongate cytopharynx were observed.

The cytostome is readily visible by electron microscopy. It is supported by a C-shaped ridge that originates near the flagellar insertion and curves around the cytostome opening (Fig. 2b, c). This ridge is supported by a row of microtubules that originates in association with the basal bodies (Fig. 4d, f). Tentatively we identify this unit as the R2 microtubular root of bicosoecids, according to the current terminology of Karpov et al. (2001). A conspicuous non-microtubular fiber connects the R2 root to the anterior basal body (Fig. 4f). Near its origin there are around ~11 microtubules in this root and the root has an L-shape in transverse section (Fig. 4g). As the R2 root travels away from the basal bodies a single microtubule diverges from (or near) the interior end of the R2 root (Fig. 4h). As the R2 structure approaches the cytostome there are nine microtubules visible (Fig. 4i). Eight microtubules continue in the ridge surrounding the cytostome (Fig. 4j). A second row of a few microtubules extends away from the anterior basal body and subtends the cell membrane on the opposite side of the cell to the cytostome (Fig. 4c, e). Tentatively, this is identified as an R1 root according to Karpov et al.'s (2001) scheme for bicosoecids. We cannot exclude the existence of other small microtubular roots.

Optimal temperature and salinity

The optimum temperature of the HNF culture at 300‰ salinity was 30–35°C, close to the in situ temperature in

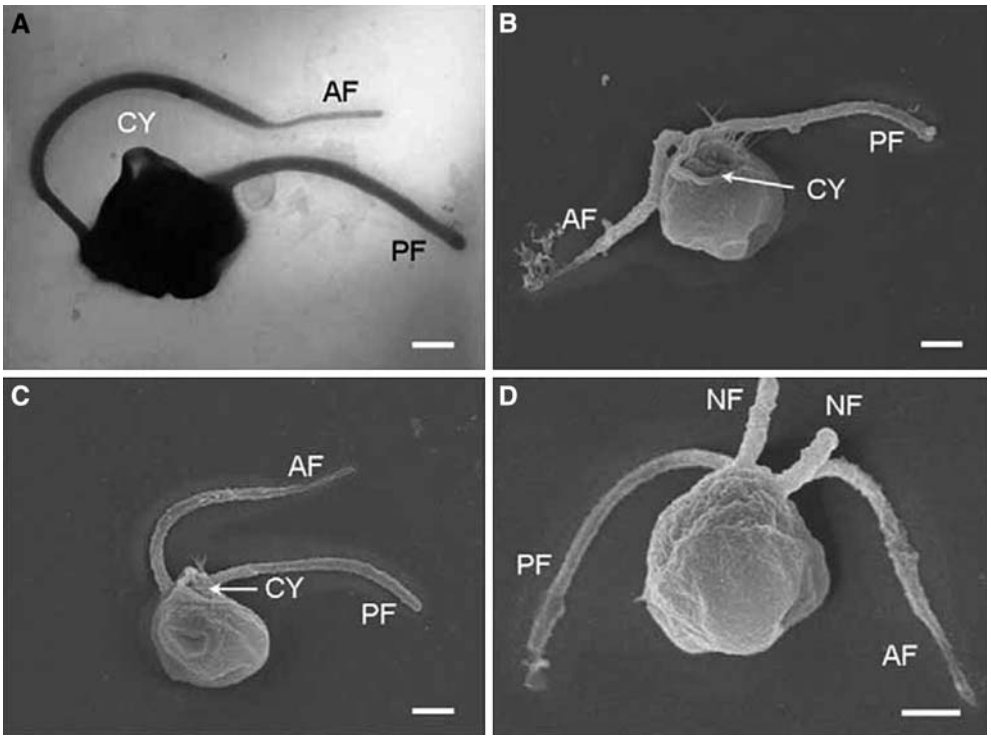

Fig. 2 *Halocafeteria seosinensis*, electron micrographs **a** Transmission electron micrograph. Whole mount: general view of the cell showing the hair-like tip of anterior flagellum (*AF*) and lack of mastigonemes on both flagella. The cytostome (*CY*) is visible on the right side of AF. **b** and **c** Scanning electron micrographs showing the positions of CY, AF, and the posterior flagellum (*PF*). **d** Dividing cell forming two new flagella (*NF*). *Scale bars* 1 μm

summer (Fig. 5a). At optimal temperature, the HNF grew with a doubling time of 18 h (Fig. 5a). The HNF isolate grew at much lower rates below 20°C or above 35°C. The optimum salinity of the HNF for growth at 35°C was 150‰ salinity (Fig. 5b). They could not grow (i.e. decreased in their abundance) below 75‰ salinity, but tolerated up to 363‰ salinity (data not shown). At optimal temperature and salinity (i.e. at 35°C and 150‰ salinity) with an initial bacterial concentration of 4×10^7 cells ml^{-1}, a doubling time of 12 h was recorded.

Molecular phylogenetics

The 18S rRNA gene sequence of the isolate was 1,758 bp long. The most similar sequences returned by a BLASTN search of the Genbank database were *Adriamonas peritocrescens*, *Cafeteria roenbergensis*, *Caecitellus parvulus* and *Siluania monomastiga*, suggesting that our isolate was a member of Bicosoecida. The sequence from our strain was even more similar (98%) to the uncultured sequence 'EHF0502' determined after denaturing gradient gel electrophoresis (DGGE)-sequence analysis. All phylogenetic trees estimated for the dataset excluding *Symbiomonas* showed clearly that *H. seosinensis* is a member of the bicosoecid clade with high bootstrap support or posterior probability (ME 85%, MP 93%, ML 97%, and BA 1; Fig. 6).

Within bicosoecids, *H. seosinensis* strain EHF34 was specifically and strongly related to EHF0502, and these two sequences formed a clade with *Cafeteria* and *Caecitellus* with high ME and ML bootstrap values (ME 85%, ML 90%) and posterior probability 1 (bootstrap support with parsimony was moderate—66%). However, the relationships within this clade were uncertain. *H. seosinensis* was specifically related to *Caecitellus* in our ML and BA trees, but the ML bootstrap support and posterior probability was very low (ML 43%, BA 0.58). Our distance and parsimony analyses united *Cafeteria* and *Caecitellus* as a weak clade (ME 47%, MP 23%) with *Halocafeteria* as their sister group.

As in previous analyses (Karpov et al. 2001), the minimal *Caecitellus–Cafeteria* clade excluded *A. peritocrescens* and *S. monomastiga*, which formed their own clade with high bootstrap support (ME 100%, MP 100%, ML 100%) and posterior probability 1, while *Pseudobodo* is basal within bicosoecids, with moderate support. Thus, both family Siluaniidae (including *Siluania*, *Adriamonas*, and *Caecitellus*) and family Cafeteriidae (including *Cafeteria* and *Pseudobodo*) formed paraphyletic or polyphyletic groups in our phylogenetic tree (Fig. 6). Congruent trees were obtained when the highly divergent *Symbiomonas* sequence was included, and/or when outgroups were excluded, although bootstrap values and posterior probabilities were lower for some clades within Bicosoecida. When included, *Symbiomonas* fell as the sister-group to *Adriamonas* and *Siluania* in the 'full' ML analysis, with negligible ML bootstrap support (24%), but formed the specific sister to *Caecitellus* in the bicosoecid-only analysis, with moderately strong ML bootstrap support (70%, data not shown).

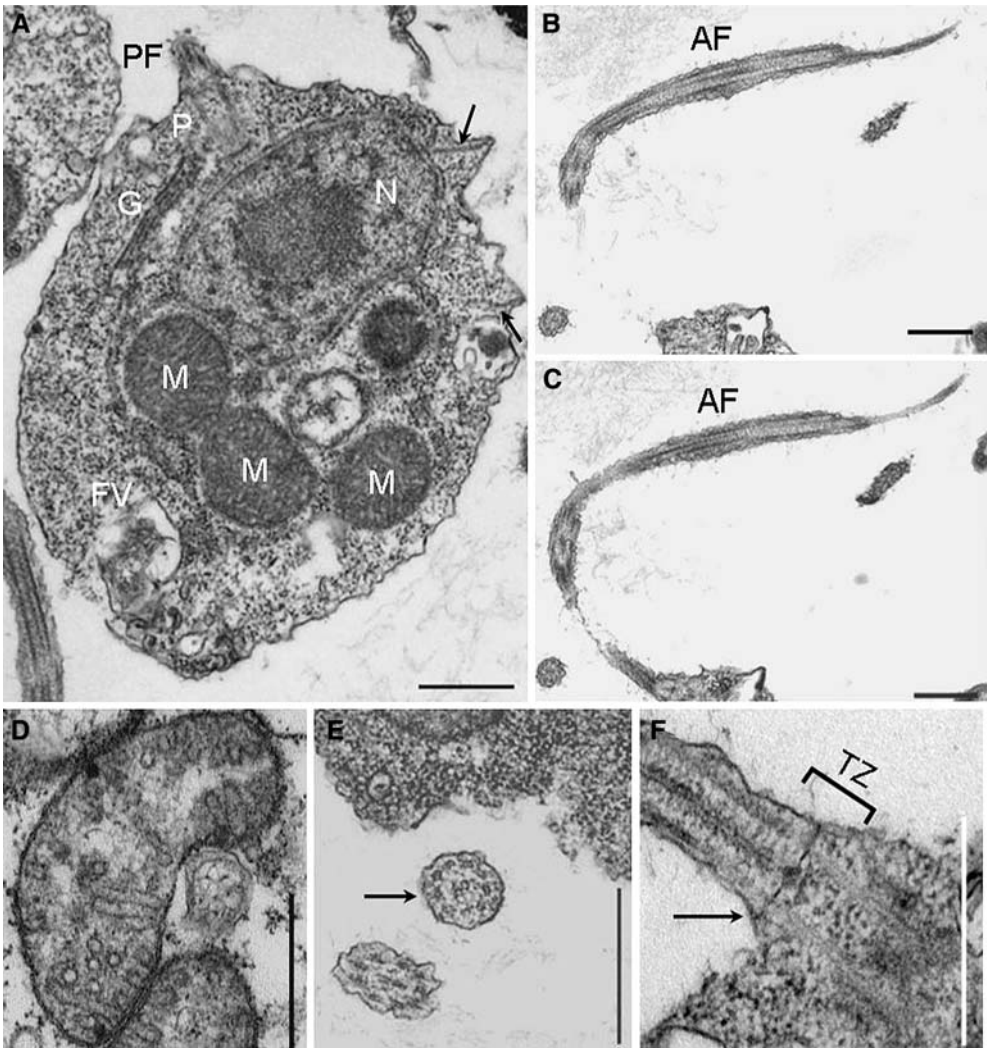

Fig. 3 *Halocafteria seosinensis*, transmission electron micrographs, ultra-thin sections **a** longitudinal section through the cell. *N* Nucleus, *M* mitochondrion, *FV* food vacuole, *G* Golgi body, *PF* posterior flagellum, and *P* basal body of posterior flagellum. Note the microtubular band—R2—associated with the cytostome (*arrows*). **b** and **c** Serial sections through the anterior flagellum (*AF*), showing the hair-like tip. **d** Mitochondrion, showing numerous tubular cristae. **e** Transverse section of the flagellar axoneme (*arrow*). **f** Longitudinal section through the flagellar transition zone (*TZ*), showing the transverse plate (*arrow*). *Scale bars* 500 nm

After review of this paper, Cavalier-Smith and Chao (2006) reported several new 18S rRNA gene sequences from bicocoecids. A preliminary phylogenetic analysis indicates that *Halocafeteria* is not specifically related to any of these new sequences, and our phylogenetic results are not altered by the inclusion of these new sequences (not shown).

Discussion

Various HNF taxa were occasionally observed in natural saturated brines (Namyslowski 1913; Ruinen 1938; Post et al. 1983; Patterson and Simpson 1996), but long-term cultivation of HNF from high salinity waters had been unsuccessful (Post et al. 1983). Since our isolate appears to be intolerant of media at the salinity of normal seawater, and thrives at much higher salinities, we assume that any previous accounts of this organism would also come from highly saline environments. Our isolate is very similar in terms of cell size and shape to Patterson and Simpson's (1996) observations under the name *Bodo saltans* from a natural saturated salt puddle in Western Australia and samples from an artificial highly saline lake in South Australia. The similarity includes the 'jumping' activity of some cells, which resembles a languid version of a characteristic behavior of *B. saltans*—a well-known free-living kinetoplastid. However, in most accounts of *B. saltans* the posterior flagellum is very long (approximately three times longer than the cell body and the anterior flagellum) whereas the posterior flagellum is shorter and a more similar length to the anterior flagellum both in our isolate and in Patterson and Simpson's micrographs of organisms from the natural saturated puddle (Fig. 1e–g in Patterson and Simpson 1996). In addition, the cells we observed by electron microscopy lacked any of the classical features of kinetoplastids, such as a kinetoplast, discoidal mitochondrial cristae, paraxonemal rods, or a flagellar pocket, all of which were documented from *B. saltans* by Brooker (1971). Furthermore, the 18S rRNA gene sequence we determined clearly branches within Bicosoecida, and we did not obtain a PCR product from

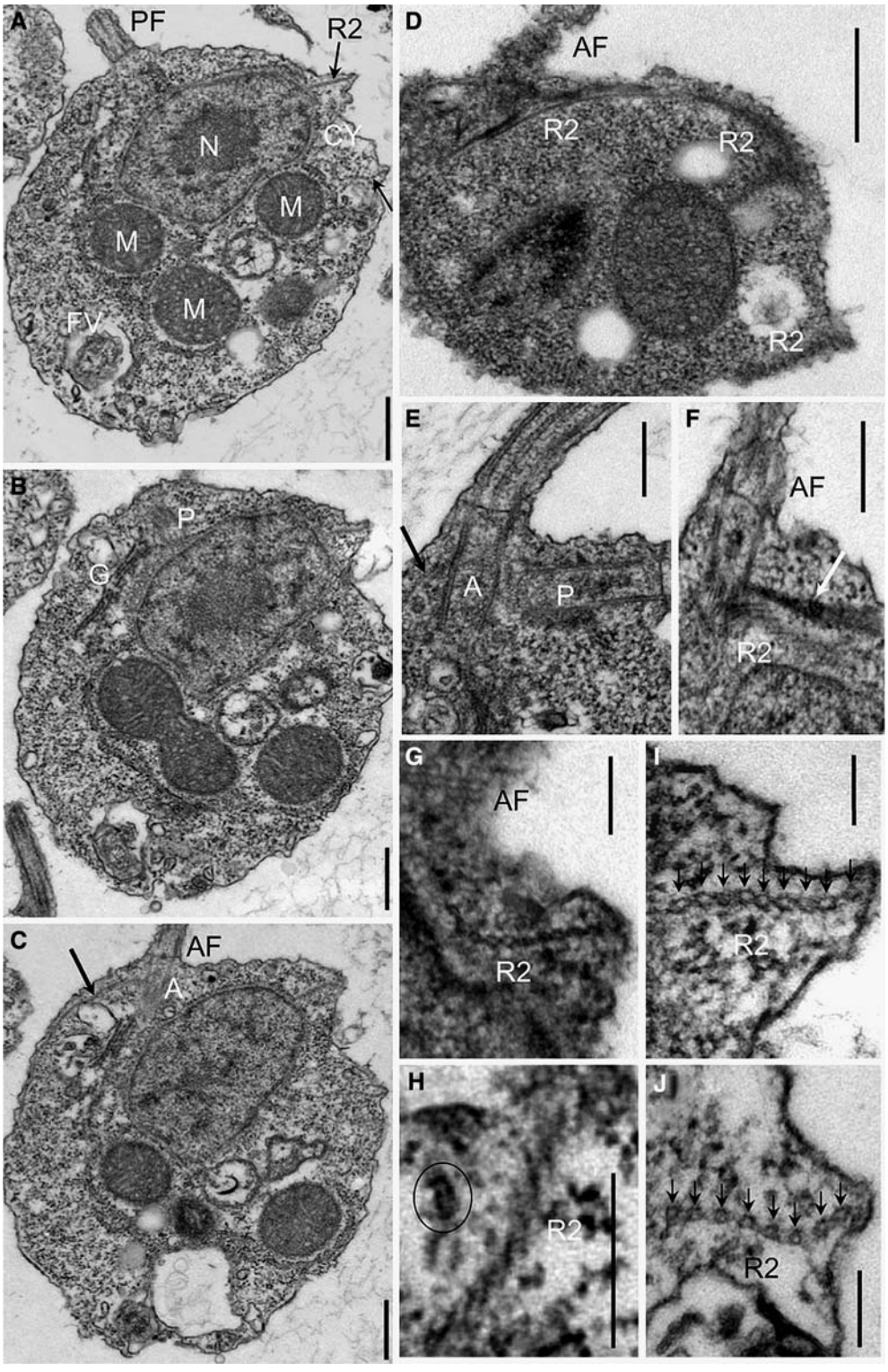

Fig. 4 *Halocafeteria seosinensis*, transmission electron micrographs, ultra-thin sections. **a**–**c** Longitudinal non-consecutive serial sections of the cell. *N* Nucleus, *M* mitochondria, *CY* cytostome, *FV* food vacuole, *G* Golgi body, *AF* anterior flagellum, *PF* posterior flagellum, *A* basal body of AF, *P* basal body of PF, and *R2* R2 microtubular root. The cytostome is supported by a curving row of microtubules—the R2 (*arrows* in **a**). Note second microtubular structure (R1 sensu Karpov et al. 2001) originating near the anterior basal body (*arrow* in **c**). **d** View showing the R2 root traveling between the flagellar region and cytostomal region of the cell. **e** Flagellar apparatus showing the relative orientation of the basal bodies and R1 originating in association with the anterior basal body (*arrow*). **f** Flagellar apparatus showing R2 near its origin and associated dense fiber (*arrow*). **g** R2, showing the L-shape in transverse section and approximately 11 microtubules (**h**) R2 root near the flagellar apparatus showing the diverging single microtubule (within circle). **i** and **j** Microtubules in the ridge surrounding the cytostome (same cell as **a**–**c**); **i** Nine microtubules (*arrows*) present on the side closest to the basal bodies and **j** Eight microtubules (*arrows*) present on the side furthest away from the basal bodies. *Scale bars* for **a**–**d** 500 nm, *Scale bars* for **e**–**j** 250 nm

the whole DNA from the culture using kinetoplastid-specific primers (von der Heyden et al. 2004; data not shown), so it is unlikely that a *B. saltans*-like kinetoplastid exists as a minor contaminant. We suspect that the organisms identified as *B. saltans* by Patterson and Simpson (1996) largely because of their jumping behavior were mis-identified, and actually represent the same morphospecies as our isolate. Irrespective, our isolate clearly cannot be equated with *B. saltans*.

Other than this dubious account of *B. saltans*, the isolate studied here is most similar to several species in the genus *Amphimonas* described by Namyslowski

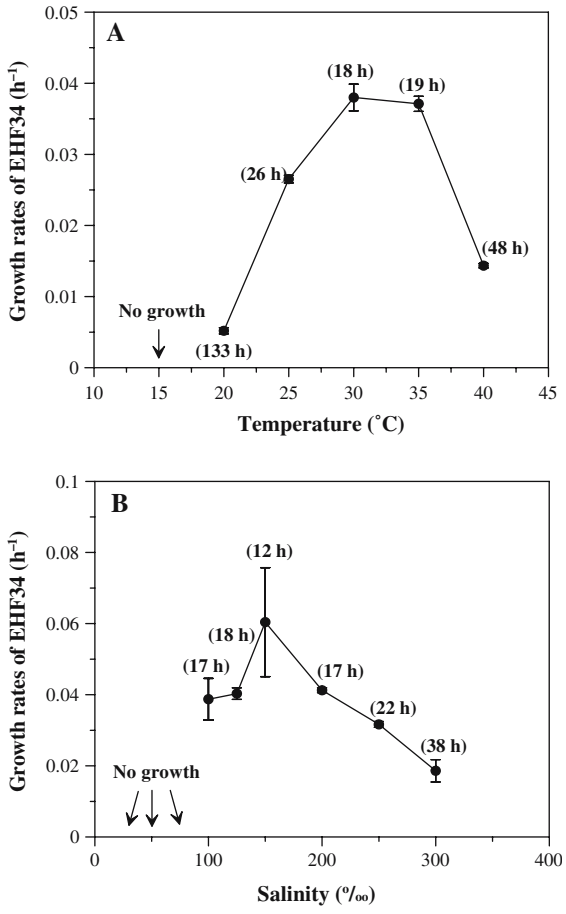

Fig. 5 Specific growth rates of *H. seosinensis* (isolate EHF34) determined for a range of temperatures from 15 to 40°C at 300‰ salinity (**a**) and a range of salinity from 35 to 300‰ salinity at 35°C (**b**). Doubling times of the isolate are represented in *parentheses*. *Error bars* show 1SD

(1913) from the Wieliczka salt mine in Poland. Namyslowski's descriptions of *Amphimonas ankyromonadides*, *A. salinus*, *A. polymorphus*, *A. angulatus*, and *A. rostratus* all refer to cells less than 7 μm in size, with two subequal flagella in the range of 1–3 times the length of the cell and inserted close together at one end of the cell. Unfortunately, Namyslowski's descriptions are illustrated only by low-quality drawings and there is no preserved type material. It is unclear how many distinctly different species entities he actually observed. All of Namyslowski's descriptions differ in some respect from our observations—unlike our isolate, *A. ankyromonadides*, *A. angulatus*, and *A. rostrastus* are all described as having flagella no longer than cell length (*A. ankyromonadides* is also described as S-shaped), while the range of sizes reported for *A. polymorphus* (2–3 μm) suggests smaller cells than our isolate (3–5 μm). In addition, the length of the flagella in *A. salinus* (three times of cell length) is longer than our isolate (~1.5–2 times of cell length). We cannot confidently equate our isolate with any one of Namyslowski's descriptions, and so are left with a subjective decision—either (a) 'lump' and consider our isolate and many of Namyslowski's *Amphimonas* spp. as a single variable species, or (b) hold open the possibility that multiple distinct entities exist, and consider our isolate as a new species. Evidence is emerging that at least some HNF morphospecies consist of multiple genetic lineages with distinct autoecologies and possibly distinct biogeographical distributions (von der Heyden and Cavalier-Smith 2005). Hypersaline sites are extremely sparsely distributed on the globe and may represent very different habitats to microbes in terms of temperature and mineral composition (Javor 1989). For this reason, we consider it most prudent to erect a new species for the isolate from a coastal solar saltern in Korea—a locale that is biogeographically, and perhaps ecologically remote from the subterranean, inland Wieliczka salt mine.

The genus name *Amphimonas* is not appropriate for the new isolate. *Amphimonas* is a genus with no contemporary identity and no established type (Patterson et al. 2002). Since its creation by Dujardin (1841) a wide morphological diversity of species have been described, united by little other than having two flagella of near-equal length. These clearly represent several different groups within eukaryotes. For example, *Amphimonas metabolicus* Namyslowski (1913), a large plastic cell, is likely a diplonemid Euglenozoan, while *Amphimonas divaricans* Kent (1880–1882), a small sessile cell with a stalk, is probably a histionid jakobid. Phylogenetic analyses of 18S rRNA gene sequences, while placing the new isolate within bicosoecids, do not establish a clear relationship with any one genus within this group (see below). The organism is distinct in motility behavior from its closest relatives within Bicosoecida. *Cafeteria* (and *Symbiomonas*), like most stramenopiles, have tubular mastigonemes that reverse the direction of fluid flow along the anterior flagellum during swimming, while *Caecitellus* moves by gliding along surfaces, rather than swimming. Placing the new isolate within any of these genera would be (a) phylogenetically speculative, and (b) result in a significant change to the concept of the existing taxon. Therefore we have described this isolate as a new genus and species: *H. seosinensis* n. gen. n. sp.

Halocafeteria seosinensis was isolated from high salinity (300‰ salinity) waters and maintained for more than 6 months, yet has an optimal salinity of 150‰. This indicates that *H. seosinensis* has been acclimated to high salt concentration. Chlorophyte algae of the genus *Dunaliella* are common in hypersaline environments (Javor 1989). *Dunaliella viridis* has an optimal salinity of 58–89‰ and tolerates up to 232‰ salinity. *Dunaliella salina* grows best in 120‰ and tolerates up to 350‰. Recently, Clavero et al. (2000) reported that 34 diatoms isolated from hypersaline environments cease to grow at salinities above 175‰. Among these isolates, *Amphora*, *Nitzschia*, and *Entomoneis* species grow well in 5–150‰, but *Pleurosigma strigosum* does not grow at salinities below 50‰, indicating that *P. strigosum* represents a true

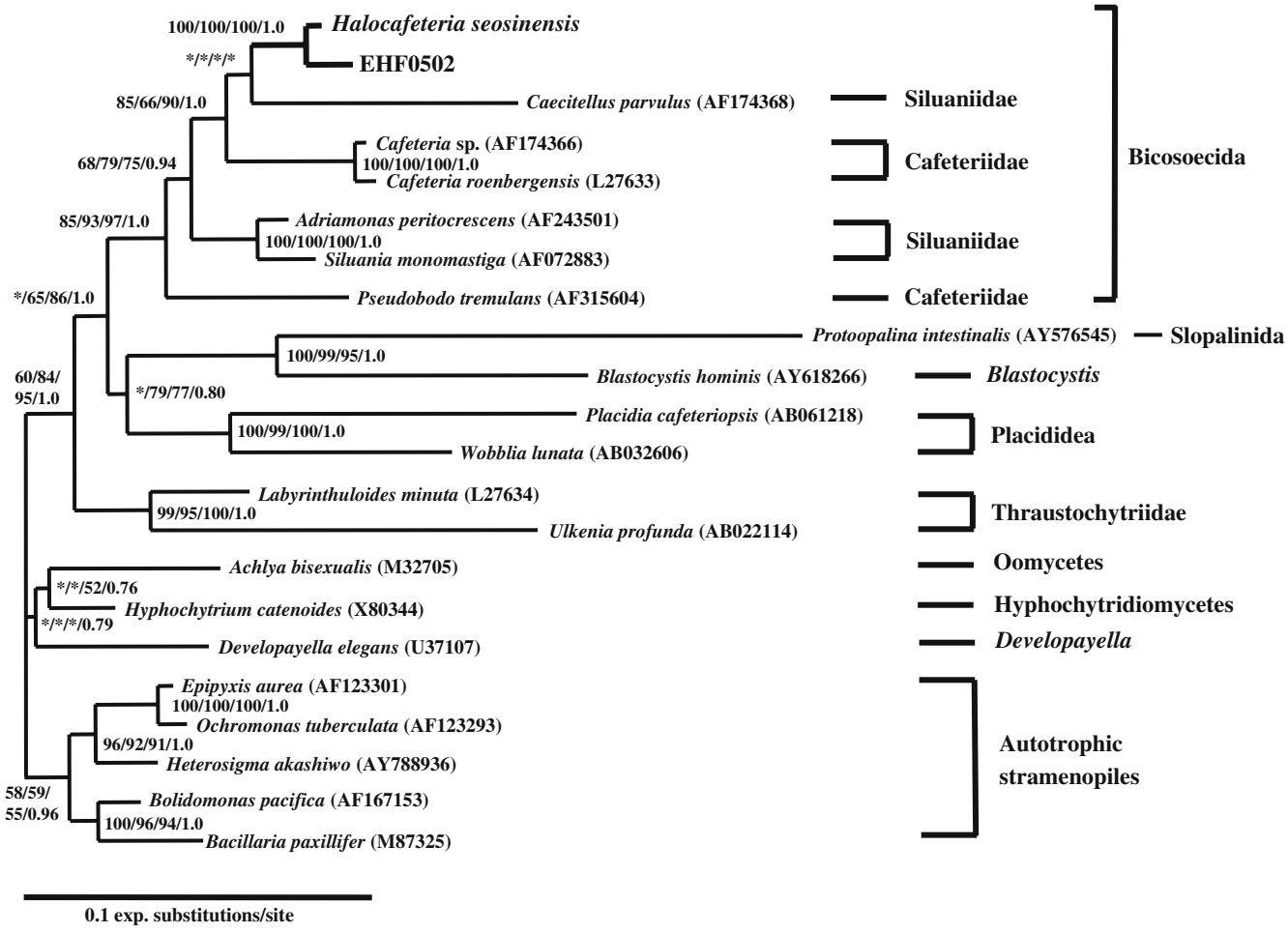

Fig. 6 ML tree of 18S rRNA genes showing the phylogenetic position of *H. seosinensis* (isolate EHF34) and environmental sequence EHF0502 relative to 22 other stramenopiles. Bootstrap values (>50%) from ME (10,000 replicates), MP (10,000 replicates), and ML (500 replicates) analyses and Bayesian posterior probabilities (MB) are shown at the nodes. The bootstrap values are presented in the order ME/MP/ML/MB. When *S. scintillans* is included in the phylogenetic analysis, it falls as the sister group to *Caecitellus* (ME 70%, MP 80%, and MB 0.51) or as sister to *Adriamonas* and *Siluania* (ML tree, with 24% ML bootstrap support). Accession numbers of each taxon are presented in parentheses. *Asterisk* represents a bootstrap value < 50% or posterior probability < 0.7

halophilic diatom. Further, *Picocystis salinarum*, a halophilic chlorophyte isolated from 100‰, grows optimally in 40‰ salinity and tolerates up to 260‰ salinity (Roesler et al. 2002). Thus, the optimal salinity of *H. seosinensis* for growth is the highest among the halophilic and halotolerant protists examined so far. The isolate is a 'borderline extreme halophile' if the same criterion used for halophilic prokaryotes is applied (Kusher 1978). The halophilicity of *H. seosinensis* from high salinity waters is similar to a report that amoebae isolated from Dead Sea muds grew optimally in 150–180‰ salinity and survived in saturated salt conditions (Volcani 1944).

It is likely that *Halocafeteria* is only one of several HNF with high optimal salinities for growth. Certainly a diversity of HNF taxa have been observed in brines above 300‰ salinity: In addition to organisms similar to *Halocafeteria* (see above), Patterson and Simpson (1996) reported *Colpodella pugnax*, *Palustrimonas yorkeensis*, *Pleurostomum flabellatum*, and *P. turgidum* from saturated brine in the Shark Bay region of Western Australia, while Post et al. (1983) observed *Bodo* spp., *Phyllomitus* sp., and *Tetramitus* spp. at salinities up to saturation in a lagoon in Western Australia. Earlier, Namyslowski (1913) and Ruinen (1938) reported about ten different genera in saturated saline samples. Many of the generic assignments are dubious but these accounts clearly represent several different species. It is not yet known to what extent these records represent populations actually capable of growth at extreme salinities. In crude culture *C. pugnax* and *Colpodella turpis* complete their life cycle (which includes feeding on algal prey, then dividing within a specialized cyst) at 265‰ salinity, although at least for *C. pugnax*, the life cycle is completed more quickly at 240‰ salinity (Patterson and Simpson 1996). The principal barrier to exploration of

this issue has been the apparent failure, until now, to produce monoprotistan cultures of HNF from high salinity water (Post et al. 1983). Interestingly, the DGGE band corresponding to phylotype EHF0502 (which is closely related to *H. seosinensis*) was observed from uncultured samples at salinities above 300‰ in June and August 2001 and in May and November 2002 (data not shown). This suggests that phylotype EHF0502 frequently occurs in high salinity waters and is adapted to living there.

Temperature is generally recognized as a regulating factor of HNF growth rates in non-hypersaline environments (Caron 1986). The optimal growth temperature of *H. seosinensis* (30–35°C) is higher than that of other bicosoecids studied so far (10–27°C, Lee and Patterson 2002). The high adaptability of *H. seosinensis* to a wide range of environmental conditions (i.e. 20–40°C, > 75–363‰ salinity) could explain the occurrence of the flagellate in solar salterns. In addition, our data supports the notion that HNF actively graze on prokaryotes in high salinity waters (Park et al. 2003). The doubling times of the HNF isolate at 300‰ salinity differed between FAHS medium and AS medium though incubation conditions were the same (prey abundance, salinity of media, and incubation temperature). The doubling time in FAHS medium (19 h) was faster than that in AS medium (38 h), suggesting that some factors regulating the cell density in FAHS medium might be in a more suitable concentration than those in AS medium. Post et al. (1983) reported that ratio of Mg^{2+} and Ca^{2+} could be of greater importance than salt level in maintaining protozoan communities. *H. seosinensis* has a doubling time of 12 h under ideal conditions (150‰ salinity at 35°C, AS medium) but 38 h in 300‰ salinity at 35°C (AS medium), while maximal abundance at 300‰ salinity and 35°C (mean ± SD of $8.5 \pm 0.9 \times 10^4$ cells ml^{-1}) is 23 times lower than that under ideal conditions (mean ± SD of $2.0 \pm 0.2 \times 10^6$ cells ml^{-1}). By contrast Fenchel (1986) reported that marine and freshwater heterotrophic flagellates have doubling times of 3–5 h under ideal conditions. This difference might reflect the energetic cost of osmoregulation in the high salinity environment. Glycerol, a well-known osmoregulatory substance of the halophilic alga *Dunaliella*, provides a carbon and energy source for growth of halophilic archaea (i.e. Family Halobacteriaceae) in high salinity water (Oren 1995). When we added glycerol (final concentration 1 μM) to *H. seosinensis* culture at 300‰ salinity, the duplicate treated samples showed a slightly faster growth rate than the untreated sample (doubling times of 34 ± 1.5 and 38 ± 2.3 h, respectively) but the difference was not significant (*t*-test; $P = 0.20$).

Traditionally Bicosoecida has been a taxon containing various HNF with tubular mastigonemes, which are absent in *Halocafeteria*. However, detailed flagellar apparatus studies and molecular phylogenies have demonstrated that the bicosoecid clade includes several previously unassigned HNF that lack mastigonemes (Verhagen et al. 1994; O'Kelly and Nerad 1998; Karpov 2000; Karpov et al. 2001). Our 18S rRNA trees clearly show the bicosoecid affinities of *Halocafeteria*. In addition, while we have not attempted a reconstruction of the flagellar apparatus, the morphology of the 'R2' root (composed of approximately 11 microtubules, eight of which curve around the opening of the cytostome) is very similar to that seen in other bicosoecids (O'Kelly and Nerad 1998; Karpov 2000; Karpov et al. 2001). Like *Cafeteria*, *Caecitellus*, and *Symbiomonas* a transitional helix was not observed in our strain (Fenchel and Patterson 1988; O'Kelly and Nerad 1998; Guillou et al. 1999). Our electron microscopy observations are thus consistent with an assignation of *H. seosinensis* to Bicosoecida.

Karpov (2000) subdivided the order Bicosoecida into four families (i.e. Bicosoecidae, Siluaniidae, Cafeteriidae, and Peudodendromonadidae) using three discrete characters based on ultrastructural observations: the presence/absence of a cytopharynx, the presence/absence of a lorica, and the presence/absence of body scales. *Halocafeteria* lacks the lorica characteristic of Bicosoecidae, and lacks the body scales and cytopharynx characteristic of Pseudodendromonadidae (Table 1). Our isolate is similar to the family Cafeteriidae in the absence of lorica, cytopharynx, and body scales according to Karpov's scheme (2000), but unlike Cafeteriidae, our isolate has no mastigonemes, nor microbody nor extrusomes (Table 1). Interestingly, our 18S rRNA trees suggest, albeit weakly, that *Halocafeteria* may be more closely related to *Caecitellus* (family Siluaniidae) than to any member of Cafeteriidae (with the possible exception of *Symbiomonas*). There are some morphological similarities between *H. seosinensis* and *Caecitellus* that are not shared by typical members of Cafeteriidae—both have an acronematic anterior flagellum and lack mastigonemes (O'Kelly and Nerad 1998). However, *Caecitellus* has a distinct cytopharynx (characteristic of Siluaniidae as a whole) and gliding motility, unlike *Halocafeteria*. In fact, our 18S rRNA trees, and those of a previous study (Karpov et al. 2001) demonstrate clearly that neither Cafeteriidae (including *Cafeteria* and *Pseudobodo*) nor Siluaniidae (including *Siluania*, *Adriamonas*, and *Caecitellus*) are monophyletic. Further, the position of *Bicosoeca* (family Bicosoecidae) relative to aloricate bicosoecids was only very recently examined in detail with molecular data, and is not stable (Cavalier-Smith 2000; Cavalier-Smith and Chao 2006). Therefore without a comprehensive revision of the systematics of bicosoecids (which we consider would be premature, considering the incomplete molecular and ultrastructural sampling of the group), there is no appropriate family assignation for *Halocafeteria*. Rather than assign *Halocafeteria* to an existing family in an artificial way, or create a new family for *Halocafeteria* (which may precipitate taxonomic inflation across the Bicosoecida),

Table 1 The ultrastructural features of *H. seosinensis* and other bicosoecids based on Karpov's classification (2000). Note that neither Cafeteriidae nor Siluaniidae are monophyletic

Genera (family)	Habitat	Lorica	Body scales	Cytopharynx	Mastigonemes	Microbody	Extrusomes	R2 (former R3)	Transition zone	
									Spiral fiber	Transitional helix
Bicosoeca (Bicosoecidae)	ma, fr	+	–	–	+	±	±	11	+	–
Siluania, Adriamonas, Caecitellus (Siluaniidae)	ma, so, fr	–	–	+	±	–	±	11 or 4	+	–
Cafeteria, Pseudobodo, Symbiomonas, Acronema, Discocelis (Cafeteriidae)	ma, br	–	–	–	±	±	±	11 or 9	+	–
Pseudodendromonas, Cyathobodo (Pseudodendromonadidae)	fr	±	+	+	Scales	–	±	6 or 7	–	+
H. seosinensis	h	–	–	–	–	–	–	~11	–	–

ma marine, *fr* freshwater, *so* soil, *h* high salinity water, + present, – absent, ± present or absent

we consider *Halocafeteria* as *incertae sedis* within Bicosoecida.

Taxonomic summary

Halocafeteria seosinensis belongs to a heterotrophic stramenopile lineage and is described under the International Code of Zoological Nomenclature.

Halocafeteria n. gen.

Cells with two heterodynamic flagella, moving by swimming rather than gliding, lacking tubular mastigonemes (tripartite flagellar hairs), with cytostome supported by a curving flagellar microtubular root. No cytopharynx, extrusomes, or lorica. Growth only in high salinity habitats (> 75‰ salinity).

Type species

Halocafeteria seosinensis n. sp.

Halocafeteria seosinensis n. sp.

Description

Bean-shaped or triangular cells 3–5 μm in diameter. Flagella of equal length (1.5–2 times the cell length in living cells). Anterior flagellum acronematic.

Hapantotype

A slide of preserved cells from a monoprotistan culture of *H. seosinensis* (EHF34) is deposited in the Protist Type Specimen Slide Collection, US Natural History Museum, Smithsonian Institution, Washington, DC (USNM slide 1023202).

Type locality

A solar saltern at Seosin (37°09′36″N, 126°40′44″E), Republic of Korea, collected from high salinity water (300‰ salinity) in May 2002.

Assignation

Eukaryota; Stramenopiles; Bicosoecida *incertae sedis*

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