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

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

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

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Introduction

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

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

Pleurostomum has not been placed in any higher taxon that is in contemporary usage, and is usually considered as one of several dozen unclassified genera of free-living heterotrophic flagellates (Patterson 1999; Patterson et al. 2000b).

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

Results

Light Microscopy and Scanning Electron Microscopy

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

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