Protist, Vol. 164, 75–88, January 2013
http://www.elsevier.de/protis

Protist

ORIGINAL PAPER

Rigifila ramosa n. gen., n. sp., a Filose Apusozoan with a Distinctive Pellicle, is Related to *Micronuclearia*

Akinori Yabuki[a], **Ken-Ichiro Ishida**[c], **and Thomas Cavalier-Smith**[b,1]

[a]Japan Agency for Marine-Earth and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka, Kanagawa 237-0061, Japan
[b]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK
[c]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan

Submitted December 9, 2011; Accepted April 21, 2012
Monitoring Editor: Michael Melkonian

We report the ultrastructure, 18S and 28S rDNA sequences, and phylogenetic position of a distinctive free-living heterotrophic filose protist, *Rigifila ramosa* n. gen., n. sp., from a freshwater paddyfield. *Rigifila* lacks cilia and has a semi-rigid, radially symmetric, well-rounded, partially microtubule-supported, dorsal pellicle, and flat mitochodrial cristae. From a central aperture in a ventral depression emerges a protoplasmic stem that branches into several branching filopodia that draw bacteria to it. Electron microscopy reveals a general cell structure similar to *Micronuclearia*, the only non-flagellate previously known in Apusozoa; the large basal vacuole is probably an unusual giant contractile vacuole. Phylogenetic analysis of concatenated rDNA sequences groups *Rigifila* and *Micronuclearia* as sisters with maximal statistical support. However, novel morphological differences unique to *Rigifila*, notably a double (not single) proteinaceous layer beneath the cell membrane, and cortical microtubules, lead us to place it in a new family Rigifilidae. Our morphological and molecular analyses show that *Rigifila* is the closest known relative of *Micronuclearia*. Therefore we group Micronucleariidae and Rigifilidae as a new order Rigifilida within the existing class Hilomonadea, which now excludes planomonads. Rigifilida groups weakly with *Collodictyon* (Diphyllatea). We discuss the possible relationships of Rigifilida to other Apusozoa and Diphyllatea.

Key words: Apusozoa; Diphyllatea; *Micronuclearia*; Molecular phylogeny; Rigifilida; Ultrastructure.

Introduction

Apusozoa is a small protozoan phylum comprising five orders: Apusomonadida, Planomonadida (=Ancyromonadida), Mantamonadida, Discocelida, and Micronucleariida (Cavalier-Smith 1997; Cavalier-Smith et al. 2008a; Glücksman et al. 2011; Karpov and Mylnikov 1989). Presence of a semi-rigid dorsal pellicle supported by proteinaceous layer(s) beneath the cell membrane was proposed as their synapomorphy (Cavalier-Smith 2002), but it is already known that there is structural diversity in the proteinaceous layer within Apusozoa. All members of the first four orders (i.e., Apusomonadida, Mantamonadida, Planomonadida, and Discocelida) are biciliates that glide on surfaces using their posterior cilia (= flagella) but do not swim,

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[1]Corresponding author; fax +44 1865 281310
e-mail tom.cavalier-smith@zoo.ox.ac.uk (T. Cavalier-Smith).

http://dx.doi.org/10.1016/j.protis.2012.04.005

though other morphological characteristics are distinctive for each. For example, the mitochondrial cristae of planomonads and *Micronuclearia* are flat, whereas apusomonads and discocelids possess tubular ones (Cavalier-Smith et al. 2008a, 2010; Heiss et al. 2011; Karpov and Mylnikov 1989; Mikrjukov and Mylnikov 2001; Vørs 1988). Cell plasticity is also different; cells of apusomonads and mantamonads are more flexible and changeable than the rigid planomonad and discocelid cells, being often able to protrude various kinds pseudopodia ventrally or posteriorly (Cavalier-Smith and Chao 2010; Glücksman et al. 2011).

In contrast to the four orders of gliding apusozoan flagellates, Micronucleariida has only one species (*Micronuclearia podoventralis*), which is an unusual tiny freshwater protist with extremely tenuous ventral filopodia, a dorsal pellicle, and no cilia (Cavalier-Smith et al. 2008a; Mikrjukov and Mylnikov 2001). *Micronuclearia podoventralis* was originally thought to be related to *Nuclearia*, an opisthokont genus of large naked amoebae with flat mitochondrial cristae and prominent filopodia but no rigid pellicle (Mikrjukov and Mylnikov 2001). Phylogenetic analysis using 18S rDNA and actin sequences showed *Micronuclearia* to be unrelated to *Nuclearia* (Cavalier-Smith et al. 2008a), not an opisthokont but nearer to Apusozoa and Amoebozoa, though in an unresolved position. *Micronuclearia* was placed in Apusozoa and grouped with *Planomonas* in a class Hilomonadea because both have a single pellicular proteinaceous layer and flat mitochondrial cristae (Cavalier-Smith et al. 2008a). Since then the phylogenetic position of *Micronuclearia* has been analyzed using combined 18S and 28S rRNA gene sequences (Glücksman et al. 2011); it grouped very weakly with *Breviata anathema*, an anaerobic uniciliate, naked amoeba that belongs to Amoebozoa (Cavalier-Smith et al. 2004; Minge et al. 2009; Zhao et al. 2012), but with insufficient statistical support to contradict its pellicular resemblance to Apusozoa.

The apusomonad *Thecamonas* is phylogenetically sister to opisthokonts according to multigene trees (Derelle and Lang 2012; Torruella et al. 2012) in agreement with earlier studies using one or a few genes (Cavalier-Smith and Chao 1995; Kim et al. 2006). However other apusozoans remain to be analyzed using a large number of gene sequences; single- and two-gene trees though showing that all are related to apusomonads, opisthokonts, and Amoebozoa (and are thus unikonts) do not convincingly establish their relative branching order. Thus it is currently unclear whether Apusozoa are a clade or we consider more likely an ancestral (paraphyletic) group at the base of unikont eukaryotes from which opisthokonts and Amoebozoa might independently have evolved (Glücksman et al. 2011).

This paper throws substantial new light on the previously unispecific *Micronuclearia* lineage by describing a new freshwater filose amoeba isolated from an Indian paddyfield water sample. Phylogenetic analysis using 18S and 28S rRNA gene sequences shows that our new strain (YPF923) forms a tight but deep clade with *Micronuclearia podoventralis*. Several morphological characteristics of this protist observed under both light and electron microscopes were clearly different from *M. podoventralis*, so we formally describe YPF923 as *Rigifila ramosa* n. gen., n. sp., and place it in the new family Rigifilidae. Furthermore we infer the possibility of a novel role of the large basal vacuole in rigifilids as a giant contractile vacuole.

Results

Light Microscopy

Cells possessed well branching filopodia, which emerge from their ventral face and then radiate in all directions and are attached to the bottom of culture vessels (Fig. 1A). Filopodia looked sticky and we frequently observed bacterial cells being captured and carried to the ventral aperture by shortening of the filopodia (Fig. 1A). No filopodial anastomosis was observed, though filopodial overlap was recognized (Fig. 1A). When viewed from the top, a cell appears spherical with a single aperture in the central region of its ventral side (Fig. 1B). When viewed from the side the cell resembles a bloated crescent (Fig. 1D). Its diameter was 5.5–12 μm (mean ± SD = 7.9 ± 1.3 μm, n = 30). Each cell has a single nucleus situated on the dorsal side (Fig. 1C). Thin and well-branched filopodia emerged from the aperture. The filopodia did not possess any additional structures (e.g. granules). In older cultures, there were some floating cells devoid of filopodia. No cilia were ever observed.

Electron Microscopy

Cells sectioned longitudinally had a crescent shape; a single dorsal nucleus with a conspicuous nucleolus was situated at the cell apex or somewhat to its side (Figs 2A, 3A, 4A). The cell outer surface was not decorated by external structures, e.g. scales or lorica (Figs 2A, 3A, 4A). The ventral portion of the cell was abundantly occupied by the