Protist, Vol. 163, 389–399, May 2012
http://www.elsevier.de/protis
Published online date 9 February 2012

Protist

ORIGINAL PAPER

SSU rRNA Phylogeny of Arcellinida (Amoebozoa) Reveals that the Largest Arcellinid Genus, *Difflugia* Leclerc 1815, is not Monophyletic

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Submitted March 25, 2011; Accepted December 11, 2011
Monitoring Editor: Sandra L. Baldauf

The systematics of lobose testate amoebae (Arcellinida), a diverse group of shelled free-living unicellular eukaryotes, is still mostly based on morphological criteria such as shell shape and composition. Few molecular phylogenetic studies have been performed on these organisms to date, and their phylogeny suffers from typical under-sampling artefacts, resulting in a still mostly unresolved tree. In order to clarify the phylogenetic relationships among arcellinid testate amoebae at the inter-generic and inter-specific level, and to evaluate the validity of the criteria used for taxonomy, we amplified and sequenced the SSU rRNA gene of nine taxa - *Difflugia bacillariarum, D. hiraethogii, D. acuminata, D. lanceolata, D. achlora, Bullinularia gracilis, Netzelia oviformis, Physochila griseola* and *Cryptodifflugia oviformis*. Our results, combined with existing data demonstrate the following: 1) Most arcellinids are divided into two major clades, 2) the genus *Difflugia* is not monophyletic, and the genera *Netzelia* and *Arcella* are closely related, and 3) *Cryptodifflugia* branches at the base of the Arcellinida clade. These results contradict the traditional taxonomy based on shell composition, and emphasize the importance of general shell shape in the taxonomy of arcellinid testate amoebae.

Key words: Arcellinida; phylogeny; Amoebozoa; SSUrRNA; *Difflugia*.

Introduction

Testate lobose amoebae (Order: Arcellinida Kent, 1880) are abundant in soils, mosses, and freshwater and are more rarely found in marine environments. They are considered as reliable bioindicators and biomonitors of environmental gradients, changes or pollution in terrestrial, (Mitchell et al. 2008), moss (Nguyen-Viet et al. 2008) and limnetic habitats (Schönborn 1973; Wall et al. 2010). As their shells are well preserved over time in lake sediments and peat, they are commonly used for quantitative palaeoecological reconstruction (Charman 2001). Yet, an accurate taxonomy is a prerequisite to the efficient use of any organism for bioindication purposes (Birks 2003). Arcellinid systematics is presently based almost exclusively on the morphology and composition of their shell

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doi:10.1016/j.protis.2011.12.001

(test). However, one of the major problems in systematics is a hierarchical evaluation of the relative importance of the morphological criteria retained for taxon discrimination (Schlegel and Meisterfeld 2003). One way to evaluate the taxonomic validity of different criteria is to build a phylogenetic tree based on molecular data obtained from a suitable genetic marker that is not too much influenced by directional selection, and to compare this phylogeny with predictions based on morphology. The most commonly used gene for amoebozoan higher-level phylogeny and taxonomy is the gene coding for the ribosome small subunit RNA, SSU rRNA (Nassonova et al. 2010). This marker was also previously shown to separate species and even infra-specific taxa within Arcellinida (Lara et al. 2008).

Anderson (1988) categorized the lobose testate amoebae into three broad groups based on the composition of their shell: 1) shell composed of proteinaceous subunits either smooth in texture (*Arcella*) or with additional agglutinated particles (*Centropyxis*); 2) shell arenaceous (i.e., agglutinated) composed of mineral grains of various shapes (oval, irregular, rod-like, etc.) glued together with an organic cement, as in *Difflugia,* or using the shell plates obtained from smaller testate amoeba prey (typically Euglyphida; Rhizaria), as in *Nebela* spp.; and 3) shell siliceous and composed of numerous self-secreted smooth, curved, siliceous rods or plates held together by organic cement plaques (e.g. *Lesquereusia*, *Quadrullela*). More recently, Meisterfeld (2002) added another category (Order Phryganellina), which produces a two-layered test: an inner, calcified layer and an outer layer made of organic material, in some cases also with agglutinated mineral particles. Members of this group also differ from other Arcellinida by the presence of conical, pointed pseudopods (e.g. *Cryptodifflugia*).

However, a growing body of evidence suggests that shell composition might not be a valid character for deep taxonomy in the Arcellinida. Indeed, *Hyalosphenia papilio*, a species with a proteinaceous test has been shown to be genetically closely related to *Nebela*, a genus that uses small particles (usually recycled euglyphid scales) to build its test (Lara et al. 2008; Nikolaev et al. 2005). Moreover, some agglutinating species such as *Nebela collaris* are able to form entirely organic tests in the absence of prey (MacKinlay 1936). In a recent phylogenetic study, Kosakyan et al. (2012) showed that *Quadrulella symmetrica*, a species that builds its test with idiosomes, branches within the *Nebela* group.

The application of molecular systematics to the phylogeny of Arcellinida is relatively recent. A first study by Nikolaev et al. (2005) placed representatives of several arcellinid genera together as a monophyletic taxon within the eukaryotic superclass Amoebozoa. Other molecular studies, based on the SSU rRNA gene, focused on the phylogeny of particular groups within the Arcellinida, such as the Hyalospheniidae (Lara et al. 2008), or the genera *Spumochlamys* (Kudryavtsev et al. 2009) or *Arcella* (Lahr et al. 2011; Tekle et al. 2008). However, although hundreds of arcellinid taxa have been described and identified morphologically, very few taxa have been sampled for molecular analysis (Kudryavtsev et al. 2009). Notably, no sequence of *Difflugia*, the largest genus in Arcellinida, is yet available in GenBank. Therefore, including members of this genus is critical to resolving the general phylogeny of the Arcellinida. We therefore conducted a SSU rRNA gene analysis to investigate the phylogenetic placement of nine unclassified taxa from representative genera of arcellinid testate amoebae (*Difflugia*, *Netzelia*, *Physochila* and *Cryptodifflugia*) for which no molecular data are currently available, thus clarifying the backbone of the Arcellinida phylogeny.

Results

We obtained partial SSU rRNA gene sequences and scanning electron micrographs from nine representative taxa of lobose testate amoebae - *Difflugia bacillariarum, D. hiraethogii, D. acuminata, D. lanceolata, D. achlora, Bullinularia gracilis, Netzelia oviformis, Physochila griseola* and *Cryptodifflugia oviformis* (Fig. 1). This sampling includes Difflugina and Phryganellina, representatives from the two major Arcellinida suborders recognised by Meisterfeld (2002).

Structure of the SSU rRNA Gene

The sequenced fragment of the SSU rRNA gene of *Difflugia bacillariarum, D. acuminata*, *D. hiraethogii* and *D. lanceolata* was between 1750 and 2110 bp long. This fragment is considerably longer than its counterpart in more conventional SSU rRNA genes (e.g. 1300 bp in *Saccharomyces cerevisiae* Z75578). This was due to the presence of introns and insertions. We found group 1 introns in two different locations in the SSU rRNA gene of *D. bacilliariarum (*position 432 to 933 and 1581 to 2008) and in one location in the gene of *D. acuminata* (position 634 to 1117). No intron was found in our sequence of *Bullinularia gracilis*, in contrast to the previously published sequence of *B. indica*