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

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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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(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*

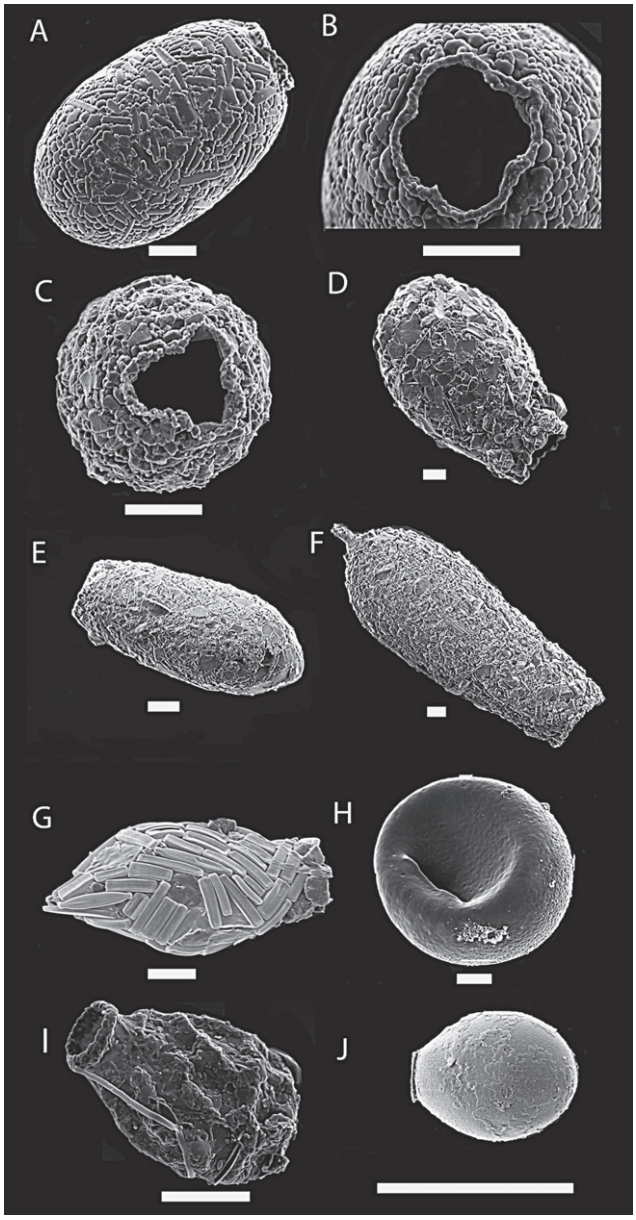

Figure 1. Scanning electron micrographs of tests from species treated in this study. **A)** *Netzelia oviformis*, **B)** Aperture view of *N. oviformis*, **C)** *Difflugia achlora*, **D)** *D. hiraethogii*, **E)** *D. lanceolata*, **F)** *D. acuminata*, **G)** *D. bacillariarum*, **H)** *Bullinulaira gracilis*,, **I)** *Physochila griseola*, **J)** *Cryptodifflugia oviformis.* Species with endogenous siliceous elements (idiosomes) **(A and B)**; agglutinated species **(C, D, E, F, G, H and I)**; and calcified species **(J)**, all scale bars represents (20 μm).

(AY848970). In addition, we found an 85 bp insertion in the *Difflugia achlora* SSU rDNA (between positions 783 to 868).

Phylogenetic Relationships among Taxa

The topologies of phylogenetic trees inferred from maximum likelihood and Bayesian inference were identical (Fig. 2). These show most arcellinid SSU rRNA sequences branching together in a monophyletic clade that receives high support including 84% Expected-Likelihood weights of local rearrangements edge support (LR-ELW; equivalent to approximate bootstraps) (Strimmer and Rambaut 2002) and 0.94 Bayesian inference posterior probability (PP). This large arcellinid clade is further divided into two major clades, referred to here as Clade A and Clade B (Fig. 2).

Clade A includes *Nebela, Apodera, Hyalosphenia, Bullinularia, Centropyxis, Spumochlamys* spp. and some *Difflugia* (namely *D. lanceolata, D. acuminata, D. bacillariarum*, and *D. hiraetogii*). The clade is well supported (94% LR-ELW / 0.95 PP) and composed of two sub-clades, A1 and A2. Sub-clade A1 comprises the group referred to as "Core *Nebelas*" by Lara et al. (2008), and the newly obtained SSU rRNA gene sequence of *Bullinularia gracilis* (Fig. 1H), which clusters with *Bullinularia indica* (Nikolaev et al. 2005) with strong support (95% LR-ELW / 1.00 PP). Sub-clade A2 comprises the four sequences of pyriform-shaped Difflugia (*D. lanceolata, D. acuminata, D. bacillariarum* and *D. hiraethogii*). These branched together with maximal support (100% LR-ELW / 1.00 PP), with the relatively long branched *D. hiraethogii* sequence branching off first. These four sequences also share a deletion of four nucleotides at a position corresponding to nucleotide 1034 in *D. bacillariarum*.

Clade B is moderately well supported (73% LR-ELW / 0.97 PP) and includes *Heleopera rosea*, *Argynnia dentistoma*, *Physochila griseola*, *Difflugia achlora, Netzelia oviformis*, and genus *Arcella.* Within the clade, the newly obtained SSU rRNA gene sequence of *Netzelia oviformis* branches together with the new *D. achlora* sequence with moderate support (70% LR-ELW / 0.97 PP), although both form long branches. Together they appear as the most closely related group to genus *Arcella* (clade B1) (Lahr and Lopes 2009;Tekle et al. 2008) (100% LR-ELW / 0.98 PP). The rest of the clade B taxa, *Physochila griseola, Argynnia dentistoma* and *Heleopera rosea* appear as a series of basal branches, all with good support (respectively 73% LR-ELW / 0.97 PP; 76% LR-ELW / 0.98 PP; and 77% LR-ELW / 0.98 PP).

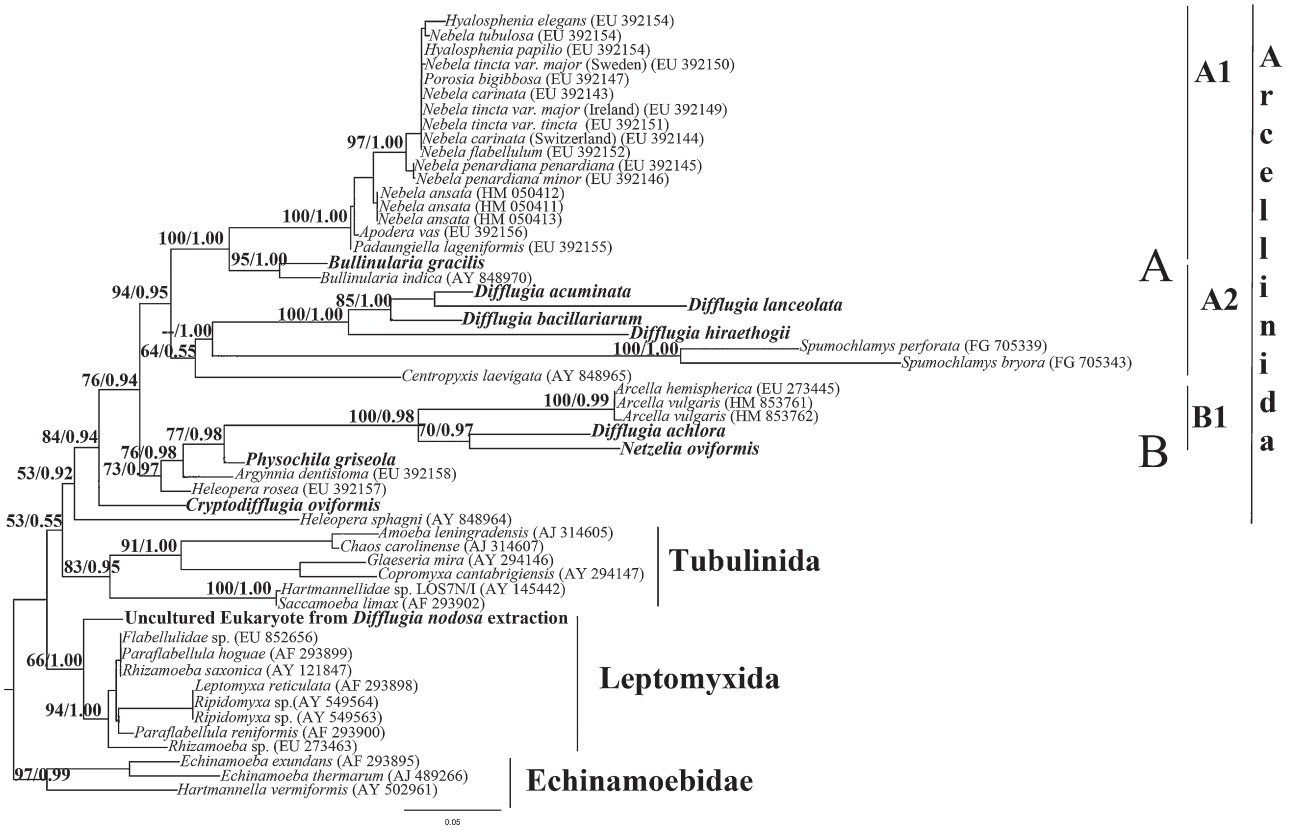

Figure 2. Molecular phylogeny based on small subunit (SSU) rRNA gene sequences of Arcellinida and related Amoebozoa illustrating the paraphyly of genus *Difflugia*. The tree is rooted with Echinamoebidae and includes new sequences from *Bullinularia gracilis, Netzelia oviformis, Physochila griseola* and *Cryptodifflugia oviformis*. The tree was derived by Bayesian Inference using MrBayes, and an identical topology was obtained by maximum likelihood analysis. Numbers at the nodes indicate Expected-Likelihood Weights edge support (approximate bootstrap) (Strimmer and Rambaut 2002) and Bayesian inference posterior probabilities. The scale bar indicates 0.05% sequence divergence.

In all our analyses, *Cryptodifflugia oviformis* branches as one of the most basal taxa of the arcellinid clade with high support (84% LR-ELW / 0.94 PP). In addition, *Heleopera sphagni* (AF848964) branches outside the two clades described (A and B), but with only low support (53% LR-ELW / 0.92 PP; Fig. 2).

Discussion

Structure of the SSU rRNA Gene

The existing data on SSU rRNA genes of Arcellinida reveal a complex pattern of presence or absence of group I introns. An intron of the same size and position as found in the newly sequenced species was previously found in several *Nebela* and related genera, in particular in *Bullinularia indica* and in *Heleopera rosea* (Lara et al. 2008; Nikolaev et al. 2005). However, we found no intron in *Bullinularia gracilis*, which forms an exclusive clade with *B. indica* in our tree (95% LR-ELW / 1.00 PP; Fig. 2). Lara et al. (2008) described a similar case within a single morphospecies of the genus *Nebela*, where an intron was found in *N. tincta* var. *major* from Ireland but was absent in *N. tincta* var. *major* from Sweden. As the sequence of these introns was generally conserved and easy to align between the two *Nebela* species, it is unlikely that these were acquired independently; a more probable explanation points towards multiple independent losses. The two introns found in the sequence of *Difflugia bacillariarum* have possibly another origin, because their size, position and sequence are different from the introns found in the other taxa (i.e *Nebela* spp., *Bullinularia indica* and *Heleopera rosea*).

Table 1. List of sequenced taxa, sampling location and morphometric measurements. n: Number of shells measured.

Taxon	Sampling location	Co-ordinates	Altitude (m)	Shell dimensions (um)			n
				Length	Breadth	Aperture	
Bullinularia gracilis	Arkutino, mosses on the sand beech of Black sea coast (BG)	42°19 N 27°44′E	0	83 ± 2	106 ± 3	41.5 ± 1.13	30
Cryptodifflugia oviformis	Agriculture soil (DE)	56°30′ N 09°37′ E	25	15.2 ± 0.6	11.8 ± 0.2	3.74 ± 0.1	8
Difflugia achlora	Aquatic mosses from the littoral zone of small artificial swamps, Dragichevo Bog, Ljulin Mountain (BG)	42°36′N 23°09′ E	960	58.3 ± 3.5	46.1 ± 2.2	18.3 ± 1.3	30
Difflugia acuminata	Wet *Sphagnum* mosses “Platoto”, Vitosha Mountain (BG)	42°36′N 23°17′E	1850	295.6 ± 42.0	93.7 ± 5.6	50.2 ± 4.0	30
Difflugia bacillariarum	*Sphagnum* Chaux d’ Abel peatland (CH)	47°10′N 06°56′ E	1006	122.3 ± 0.5	58.8 ± 0.6	31.2 ± 0.5	6
Difflugia hiraethogii	Wet *Sphagnum* mosses “Platoto”, Vitosha Mountain (BG)	42°36′N 23°17 E	1850	204.3 ± 18.2	131.6 ± 5.2	58.9 ± 6.3	30
Difflugia lanceolata	Aquatic mosses from the littoral zone of small artificial swamps, Dragichevo Bog, Ljulin Mountain (BG)	42°36′ N 23°09′ E	960	166.2 ± 10.5	69.8 ± 3.4	28.3 ± 1.3	30
Netzelia oviformis	Wet *Sphagnum* mosses “Platoto”, Vitosha Mountain (BG)	42°36′ N 23°17′ E	1850	95.9 ± 6.4	74.2 ± 5.8	24.8 ± 1.2	30
Physochila griseola	*Sphagnum* Les Saignolis peat bog (CH)	47°05′ N 06°45′ E	1257	79.8 ± 4.1	57.0 ± 3.3	21.0 ± 1.3	30

Table 2. List of taxon-specific primers used in our study (mixed-base sites are denoted by the IUB nomenclature).

Primer	Sequence 5′-3′	Specificity
Arcell 1F	GAA AGT GGT GCA TGG CCG TTT	General Arcellinida
Diff2R	AAT CCA ATG TAA CCC GCG TGC	*Difflugia (D. bacillariarum, D. hiraethogii, D. acuminata, D. lanceolata)*
Arc2R	GGC GCG GGY TGR TGA CC	*Netzelia oviformis + Arcella*
Bull1R	GAT CTA KCC CKA TCA CGC	*Bullinularia gracilis + B. indica*
AchloR1	TCA CAG ACC TGT TTT CGC CTC AAG CC	*Difflugia achlora*

General Phylogeny of Arcellinida

The placement of *Cryptodifflugia oviformis* (Fig. 1J) (Order: Phryganellina) within Arcellinida has long been debated. The pointed, branched or even sometimes anastomosing pseudopodia led Hedley et al. (1977) to place this genus outside Arcellinida. Ogden and Hedley (1980) later suggested that these pseudopodia could represent a transitional form between filose (i.e. Rhizaria) and lobose testate amoebae. Meisterfeld (2002) also highlighted the importance of this character, but still placed this genus together with genus *Phryganella* in a separate order within Arcellinida, the Phryganellina. Our results suggest that *Cryptodifflugia* might constitute a new clade, branching at the base of Arcellinida. However, sequences from presumably related genera such as *Wailesella* and *Phryganella* are required in order to test Meisterfeld's Phryganellina hypothesis and to clarify the position of these taxa with respect to other Arcellinida. This also applies to the unstable position of *Heleopera sphagni* (AY848964, Lara et al. 2008), which shows no affinity for *Heleopera rosea* and instead forms a long branch deeper than even *Cryptodifflugia*. Further work including more isolates from genus *Heleopera* is also needed.

Non-monophyly of some Arcellinid Genera

Our data clearly show, with strong support, that the genus *Difflugia* is not monophyletic, since *D. achlora* appears more closely related to *Arcella* spp. than to other *Difflugia* species. In addition, *Argynnia* and *Physochila* (clade B), two taxa with a pyriform shell and previously classified together in genus *Nebela* (clade A) appear to be only distantly related to this genus. This confirms the validity of Jung's (1942) revision of genus *Nebela* (see Kosakyan et al. 2012 for a more detailed analysis of this group). Likewise, the division of arcellinids between those with proteinaceous versus agglutinating tests as proposed by Anderson (1988) is not supported, since these taxa appear mixed in the tree, for example *Arcella* spp. and *Netzelia oviformis* (Fig. 1A) and *Difflugia achlora* (Fig. 1C; Table 3).

Leclerc (1815) first described genus *Difflugia*, which was later defined by its rough agglutinated shells of different shapes (oval, pyriform. etc) but always a terminal aperture and composed of mineral particles or diatom fragments in structured organic cement (Anderson 1988; Meisterfeld 2002). These criteria correspond to a definition by default, which is often problematic in systematics. Indeed, some arcellinids such as *Heleopera rosea* (Lara et al. 2008) also present these characteristics, suggesting that these characters are plesiomorphic in Arcellinida.

Clade A

Clade A consists of two sub-clades. Sub-clade A1 comprises the "Core *Nebelas*" (Lara et al. 2008) and *Bullinularia* spp., and sub-clade A2 includes *Difflugia, Centropyxis* and *Spumochlamys* spp. (Kudryavtsev et al. 2009). Clade A thus comprises organisms characterised by a wide variety of shapes and lifestyles, with no obvious common features. The evolution of a sub-terminal pseudostome has been observed in both arcellinid and euglyphid testate amoebae and in both cases existing molecular evidence suggests that this is a derived character (Lara et al. 2007a, 2008). Interestingly, our extended phylogeny shows that a sub-terminal pseudostome has in fact evolved at least twice within Arcellinida, once in an ancestor of *Bullinularia* and once in an ancestor of *Centropyxis*.

In sub-clade A2, the four *Difflugia* species that branch together robustly are cylindrical but present differences in the ultrastructure of the cement that holds the xenosomes together (Fig. 1D, E, F and G). This feature has previously been considered as a significant taxonomic criterion (Lahr and Lopes 2006; Ogden 1979, 1983; Wanner and Meisterfeld

Table 3. Comparative description of shell structure and morphology in some arcellinid taxa.

Taxon name	Shell shape	Aperture shape and position	Shell composition	Shell building units
Arcella spp.	discoid	terminal, circular	proteinaceous	-
Argynnia dentistoma	pyriform, compressed	terminal, circular	agglutinated, euglyphida plates, mineral grains	xenosomes
Bullinularia spp.	hemisphaerical	subterminal, slit like	agglutinated, fine mineral grains	xenosomes
Centropyxis laevigata	discoid	subterminal, circular	agglutinated, mineral grains	xenosomes
Cryptodifflugia oviformis	pyriform	terminal, circular	proteinaceous, inner layer calcified	-
Difflugia acuminata	pyriform, aboral horn	terminal, circular	agglutinated, mineral grains	xenosomes
Difflugia achlora	globular	terminal, lobed	agglutinated, mineral grains	xenosomes
Difflugia bacillariarum	pyriform, aboral horn	terminal, circular	agglutinated, mineral grains	xenosomes
Difflugia hiraethogii	pyriform	terminal, circular	agglutinated, mineral grains	xenosomes
Difflugia lanceolata	pyriform	terminal, circular	agglutinated, mineral grains	xenosomes
Heleopera rosea	pyriform, compressed	terminal, slit like	agglutinated, mineral grains	xenosomes
Hyalosphenia spp.	pyriform, compressed	terminal, circular	proteinaceous	-
Nebela spp.	pyriform, compressed	terminal, circular	agglutinated, mainly euglyphida plates	xenosomes
Netzelia oviformis	globular	terminal, lobed	self secreted siliceous scales	idiosomes
Padaungiella lageniformis	pyriform, compressed, elongated neck	terminal, circular	agglutinated, mainly euglyphida plates	xenosomes
Physochila griseola	pyriform	terminal, circular	agglutinated, euglyphida plates, mineral grains	xenosomes
Spumochlamys spp.	discoid	terminal, circular	proteinaceous, flexible	-

1994). In spite of the high polymorphism observed within this group of *Difflugia* (Chardez 1974; Mazei and Tsyganov 2006), it appears that general shell shape (i.e. pyriform vs. elongated) (Fig. 1D, E, F and G) might also be an important phylogenetic criterion, as suggested by phylogeny and the unique presence (among arcellinids) of a four nucleotide deletion in their SSU rRNA genes. This needs to be tested by sequencing of additional similar-shaped species such as *D. nodosa*, *D. gigantea,* and *D. bacillifera*.

Clade B

Clade B shows a striking result for *Difflugia achlora* (Fig. 1C). This globular-shaped species is only distantly related to the other *Difflugia* species and instead branches close to *Netzelia oviformis*, which was removed from genus *Difflugia* by Ogden (1979). Ogden (1979) also proposed a new genus "*Netzelia"* to accommodate species of *Difflugia* which secrete endogenous siliceous elements (idiosomes), but which can also use small sand grains as supplementary building material (Fig. 1A) (Netzel 1976). *Difflugia wailesi* and *Difflugia tuberculata* were later transferred to genus *Netzelia* for the same reasons (Meisterfeld 1984; Netzel 1983). However, it has been shown that *N. tuberculata* can also coat foreign particles with a thin layer of biomineralized silica when grown in a low-silica medium (Anderson 1992), and thus possibly represents an intermediate case between self-secreted and agglutinated shells. The existence of a continuum in the material used for shell construction from siliceous (self-secreted or idiosomes) in genus *Netzelia* to agglutinated (xenosomes) in genus *Difflugia* suggests that this is not a useful criterion for deep phylogenetic relationships. However, sequences from other rounded *Difflugia* (such as *D. corona, D. labiosa, D. tuber* etc.) are needed to examine this further.

The basal positions of the pyriform-shaped testate amoebae *Physochila griseola* (Fig. 1I) and *Argynnia dentistoma* are noteworthy, suggesting that this represents an ancestral character for clade B. This morphology is also found in clade A, in the "core Nebelas" (sensu Lara et al. 2008) and some *Difflugia* species in clade A suggesting that it could be ancestral to the Arcellinida as a whole (Table 3). It is interesting to note that pyriform-shaped tests are also hypothesized to be an ancestral character in the euglyphid amoebae (Lara et al. 2007b). This suggests the appealing hypothesis that pyriform shells represent a basal condition in testate amoebae in general, which is congruent with the fact that the oldest testate amoebae fossils (perhaps the oldest true eukaryote fossils, see Berney and Pawlowski 2006), dating back to ca. 740 Mya are also vase-shaped (Porter and Knoll 2000; Porter et al. 2003).

Previous classifications emphasized considerably the importance of shell texture and composition as morphological criteria for separating major arcellinid taxa (Anderson 1988; Meisterfeld 2002). Our results suggest rather that general shell shape is a much more relevant criterion for distinguishing among groups; pyrifom-flattened shapes for core Nebelas, cylindrical for the *Difflugia acuminata* group, rounded for the *Arcella/Netzelia/Difflugia achlora* group, etc. However, there are also convergences between clades A and B. For instance the discoid shell shape of *Spumochlamys* spp. (Kudryavtsev et al. 2009) superficially resembles that of *Arcella* spp. However, the extreme divergence of the *Spumochlamys* SSU rRNA gene sequences suggests that their inclusion in clade A should still be viewed with caution. Sequences from related taxa such as other *Spumochlamys* or possibly *Amphizonella* may help to clarify the position of this group but it is already clear that they do not branch close to genus *Arcella*.

Possible Pitfalls of SSU rRNA Phylogeny

SSU rRNA gene sequences have proven useful for high-level phylogeny of Amoebozoa in general (Cavalier-Smith et al. 2004; Fahrni et al. 2003; Smirnov et al. 2007) and Arcellinida in particular (Lara et al. 2008; Nikolaev et al. 2005). However, there are also potential problems with phylogeny based on a single gene, including SSU rRNA (Pawlowski and Burki 2009). Two potential causes for concern here are contaminated cultures and phylogenetic artefact.

1) Arcellinida cells are relatively large and often host a high number of symbionts and/or epibionts that can be co-amplified in the PCR reaction. In addition, most arcellinids are eukaryote predators and contain undigested prey. Sometimes, these co-amplified eukaryotes can even be closely related to Arcellinida, such as minute lobose naked amoebae. To illustrate this, we include in our tree the sequence of a leptomyxid naked amoeba obtained while amplifying SSU rDNA from *Difflugia nodosa* (Fig. 2). This problem is most likely to occur if single extractions are used. Therefore, most of our newly added sequences are confirmed by two or more independent DNA extractions, thus

minimising the risk of contamination or others pitfalls. Future work will include using the wider sequence sampling now available to design specific primers for the different arcellinid subgroups. This is also required to verify whether the existing sequence of *Heleopera sphagni* is correct and thus suggests a fast evolving taxon, or if it is a contamination.

2) Under-sampling and/or fast-evolving sequences can sometimes produce major artefacts in tree reconstruction (Philippe and Germot 2000a; Philippe et al. 2000b). The Arcellinida combine both problems. For example, the two *Spumochlamys* species for which molecular data are available (Kudryavtsev et al. 2009) have extremely divergent sequences. However, including these sequences in our tree did not affect the general topology of the tree and had very little effect on the support values of the nodes. Yet, increasing sampling effort is required within this group.

Methods

Sample collection and documentation: Amoebae were obtained from *Sphagnum,* other mosses, fresh water sediment, and agricultural soil (Table 1). *Cryptodifflugia oviformis* was isolated from a soil sample and cultured in flasks containing 1:300 tryptone soy broth-enriched amoeba saline. For each *C. oviformis* extraction, tens of cells were extracted. For the other species, 5 to 15 individuals were extracted separately and placed in different tubes following previously described protocols (Lara et al. 2008; Nikolaev et al. 2005). Shells were documented using scanning electron microscopy (SEM) as described previously (Todorov and Golemansky 2007), and the following measurements were taken: length and width of the shell and pseudostome opening (Table 1 and Fig. 1).

DNA isolation, PCR amplification and sequencing: DNA was extracted using guanidine thiocyanate protocol (Chomczynski and Sacchi 1987). SSU rRNA sequence were obtained in two steps. A first amplification was performed using universal eukaryotic primers EK555F (AGTCTGGTGCCAGCAGCCGC) or EK 42F (CTCAARGAYTAAGCCATGCA) and EK1498R (CACCTACGGAAACCTTGTTA) in a total volume of 30 μl with amplification profile consisting of (3 minutes at 95 °C followed by 40 cycles of 30 sec at 94 °C, 30 sec at 58 °C and 1 min 30 sec at 72 °C with a final elongation of 10 min at 72 °C). The obtained product served as template for the second amplification using designed taxon-specific primers (Table 2) in a total volume of 30 μl with amplification profile consisting of (3 minutes at 95 °C followed by 40 cycles 30 sec at 94 °C, 30 sec at 60 °C and 1 min 30 sec at 72 °C with a final elongation of 10 min at 72 °C).

The PCR products were screened by gel electrophoresis and the positive amplifications at the expected size were purified with the NucleoFasts 96 PCR Clean Up kit from Macherey-Nagel (Düren, Germany) and sequenced with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Genève, Switzerland) using a BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequences are deposited in GenBank with accession numbers (*Bullinularia gracilis* JQ366061; *Cryptodifflugia oviformis* JQ366062; *Difflugia achlora* JQ366063; *Difflugia acuminata* JQ366064; *Difflugia bacillariarum* JQ366065; *Difflugia hiraethogii* JQ366066; *Difflugia lanceolata* JQ366067; *Netzelia oviformis* JQ366068 and *Physochila griseola* JQ366069).

Alignment and phylogenetic analysis: The SSU rRNA gene sequences obtained in this study were aligned manually using the BioEdit software (Hall 1999), starting from the alignment used by Lara et al. (2008). Introns, insertions and variable regions in the SSU rRNA alignment that could not be aligned unambiguously were removed. The phylogenetic trees were reconstructed using Maximum Likelihood and Bayesian approaches with an alignment length of 640 bp. The group of *Echinamoeba*/"*Hartmanella*" *vermiformis* is considered as the basal-most clade in the Tubulinea (Nikolaev et al. 2005) and was therefore used to root all the trees. We did not include *Trigonopyxis arcula* GeneBank (AY848967) in our alignment. A ca. 300 bp unpublished sequence from two independent isolates of this taxon revealed that the published sequence was most likely a contamination from *Bullinularia indica* (Lara, unpublished results). The two sequences are indeed almost identical in the common part of the sequences. Likewise, the published *Arcella artocrea* (AY848969) sequence is almost identical to *Centropyxis laevigata* (AY848965), and very different from other *Arcella* species, and we suspect therefore also a contamination, these findings were also supported by the results of Tekle et al. in 2008.

The maximum likelihood tree was built using the software TREEFINDER (Jobb et al. 2004) with the GTR+I+G model of nucleotide substitution. The reliability of internal nodes was estimated by Expected-Likelihood Weights of local rearrangements edge support = approximate bootstraps (1000 replicates) (Strimmer and Rambaut 2002). The resulting tree was compared to the one obtained by Bayesian analysis which was obtained using the software MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). We performed two simultaneous MCMC chains, and 500,000 generations. The generations were added until standard deviation of split frequencies fell below 0.01 according to the manual of MrBayes 3.1. For every 1,000th generation, the tree with the best likelihood score was saved, resulting in 10,000 trees. The burn in value was set to 25%. Trees were viewed using FigTree (a program distributed as part of the BEAST package).

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