Published in European Journal of Protistology, Vol. 59, 2017, p. 99-107
which should be used for any reference to this work

A contribution to the phylogeny of agglutinating Arcellinida (Amoebozoa) based on SSU rRNA gene sequences

Fatma Gomaa[a,b,*], Daniel J.G. Lahr[c], Milcho Todorov[d], Jingchun Li[a], Enrique Lara[e]

[a]*Department of Organismic and Evolutionary Biology, Biological Laboratory, Harvard University, Cambridge, Massachusetts, USA*
[b]*Ain Shams University, Faculty of Science, Zoology Department, Cairo, Egypt*
[c]*Department of Zoology, University of Sao Paulo, Sao Paulo, Brazil*
[d]*Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin St., 1113 Sofia, Bulgaria*
[e]*Laboratory of Soil Biodiversity, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland*

Abstract

Arcellinid testate amoebae include a wide variety of amoeboid organisms whose test (shell) varies in shape, composition and size. A decade ago, we initiated molecular phylogenetic analyses based on SSU rRNA gene sequences and a taxonomic revision of Arcellinida. However, many lineages within Arcellinida still lack molecular data, and the phylogeny of this group is largely incomplete. In this study, we obtained SSU rRNA gene sequences from seven taxa, of which six have agglutinated shell (*Difflugia oblonga*, *D. labiosa*, *D. gramen*, *Mediolus corona*, *Netzelia wailesi*, and *N. tuberculata*), and one has an entirely proteinaceous shell (*Arcella intermedia*). All species but *Difflugia oblonga* branched within the recently erected suborder Sphaerothecina, confirming the synapomorphic value of an oviform or discoid shell. Thus, we propose that species with an oviform or discoid shell currently classified within genus *Difflugia* must be transferred to other genera, thus continuing the process of taxonomic revision of genus *Difflugia*, the largest Arcellinida genus. We therefore transferred the current and the previously sequenced oviform *Difflugia* spp. to *Netzelia* spp., based on the shared globular/oviform shell shape and their monophyly. Another species, *D. labiosa*, formed an independent lineage that branched as a sister clade to *Arcella* spp.; based on the shell morphology and their phylogenetic position, we considered *D. labiosa* as *incertae sedis*.

Keywords: Agglutinated shell; Classification; *Difflugia*; Morphology; Sphaerothecina; SSU rRNA gene sequences

Introduction

The Arcellinida are a diverse group of protists distinguished by a shell (test) with a single aperture from which lobose pseudopodia protrude during feeding and locomotion (Kosakyan et al. 2016a; Meisterfeld 2002). These amoebae occur in a wide array of aquatic and terrestrial environments, distributed from the tropics to the poles (Dalby et al. 2000). They are very ancient: the fossil records suggest that some of the extant genera may have been present in the Cretaceous (Schmidt et al. 2010; Van Hengstum et al. 2007). The oldest putative records date back to the Neoproterozoic period, 750

*Corresponding author. Present address: Dept. of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA. Fax: +1 617 496 6933.
E-mail address: fatmagomaa@fas.harvard.edu (F. Gomaa).

MYA ago, and are often considered the oldest unambiguous eukaryotic fossils (Porter et al. 2003; Porter 2016).

Several taxonomic classifications have been proposed for the Arcellinida. These were traditionally based on shell size and composition, as well as aperture shape and size (Anderson 1988; Meisterfeld 2002; Ogden and Hedley 1980; Ogden and Meisterfeld 1989). However, like in many other protist groups, the number of morphological characteristics to be used is limited, and may vary in response to changes in environmental condition (Schönborn, 1992; Schlegel and Meisterfeld 2003; Wanner and Meisterfeld 1994). For these reasons, molecular phylogenetics is essential in defining the taxonomic framework for the whole clade. The last five years saw a considerable increase of knowledge on Arcellinid evolutionary patterns. Fine-level (i.e. species level) phylogeny, mostly based on the mitochondrial cytochrome oxidase (COI) marker considerably improved our knowledge of phylogenetic relationships within family Hyalospheniidae (Kosakyan et al. 2012, 2016b). In addition, this approach revealed a considerable diversity of species that could be differentiated from each other only by small differences in the general outline of the shell, inflating previous diversity estimates (Kosakyan et al. 2013; Singer et al. 2015).

Deep phylogenetic relationships have been investigated mostly using the nuclear SSU rRNA gene (Gomaa et al. 2012, 2015; Kudryavtsev et al. 2009; Lara et al. 2008; Lahr et al. 2013). From these studies, it appeared that the two best known and most species-rich genera *Nebela* and *Difflugia* were paraphyletic, thus challenging traditional taxonomy. Altogether, phylogenetic reconstructions have demonstrated that a more reasonable approach is to rely on shell morphology, rather than shell composition (Kosakyan et al. 2016a).

However, even in the most recent tree reconstructions, many Arcellinida species present long branches, which are best exemplified with members of genus *Spumochlamys* (Kudryavtsev et al. 2009). This suggests that parts of the Arcellinida tree may be undersampled. In this study, we aimed at increasing the number of taxa in species building agglutinated shells. Among them, the largest arcellinid genus, *Difflugia* has been established by Leclerc (1815). It includes nowadays ca. 500 species and subspecies (Meisterfeld 2002). The taxonomy of this genus was recently reassessed based on morphological criteria using both Penard's permanent slides and Ogden's mounted stubs collections at the Natural History Museum of London (Mazei and Warren 2012, 2014, 2015). Members of genus *Difflugia* have been classically defined by their shells that are always composed of mineral particles and diatoms embedded in sheet-like organic cement secreted by the amoebae (Meisterfeld 2002). Based on molecular SSU rRNA gene data, Gomaa and coworkers (Gomaa et al. 2012, 2015) showed the non-monophyly of the genus. All species with an elongated/cylindrical shell branched together, while those with a globular shell formed a strongly supported clade where they were intermixed with members of genus *Netzelia*, which was originally separated from *Difflugia* based on their capacity to build agglutinated shells with self-secreted particles (Netzel 1976). Deeper investigations in the literature demonstrated that this capacity had been misinterpreted, and is in fact pervasive among *Difflugia* with globular/oviform shells. All those species were classified into a redefined genus *Netzelia* (Kosakyan et al. 2016a). An independent investigation transferred *Difflugia corona* to a newly erected genus *Mediolus* based on its general shape, indentations around the pseudostome and the presence of variable number of spines extending outward on the shell (Patterson 2014).

Because of the large diversity of shapes and lifestyles, Arcellinids with an agglutinated shell (i.e. *Difflugia sensu lato*) are expected to be very diverse. Therefore, we hypothesized that a larger sampling effort focused on *Difflugia* and *Netzelia* should considerably help in stabilizing the phylogeny of Arcellinida as a whole. In the present study, we present a new systematic revision of genus *Difflugia* based on a newly constructed phylogeny and previous findings.

Material and Methods

Sample collection and documentation

Amoebae were obtained from *Sphagnum* and other mosses as well as fresh water sediment (Table 1). Five to 15 individuals were isolated and placed in separate tubes following previously described protocols (Lara et al. 2008). 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 aperture opening (Table 2, Fig. 1).

DNA isolation, PCR amplification and sequencing

DNA was extracted using a guanidine thiocyanate protocol (Chomczynski and Sacchi 1987). SSU rRNA sequences were obtained in two steps. A first amplification was performed using universal eukaryotic primers EK555F (5′-AGTCTGGTGCCAGCAGCCGC-3′) or EK 42F (5′-CTCAARGAYTAAGCCATGCA -3′) and EK1498R (5′-CACCTACGGAAACCTTGTTA-3′). The obtained products served as template for the second amplification using designed taxon-specific reverse primers and universal eukaryotic forward primers (Table 3). The specific primers were designed based on a preliminary short segment (∼300 bp) of the SSU rRNA gene sequence obtained from each taxon using universal eukaryotic primer and Arcellinida-specific forward primer Arcell 1F (GAAAGTGGTGCATGGCCGTTT) (Gomaa et al. 2012). The PCR products were screened by gel electrophoresis and positive amplifications were purified with 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

Table 1. List of sequenced species and sampling locations.

Taxa	Sampling site	Coordinates	GenBank accession number
Arcella intermedia	Aquatic mosses from littoral zone of small swamp (Bulgaria)	42°39′N, 23°18′E	KY273245
Difflugia oblonga	Ljulin Mountains, Dragichevsko Bog (Bulgaria)	42°39′N 23°09′E	KY273249
"Difflugia" labiosa	Lake Pancharevo (Bulgaria)	42°36′N 23°24′E	KY273246–KY273248
Netzelia corona comb. nov.	Lake Pancharevo (Bulgaria)	42°36′N 23°24′E	KY273250–KY273252
Netzelia gramen comb. nov.	Aquatic mosses from the littoral zone of small swamp, near Sofia (Bulgaria)	42°39′N, 23°18′E	KY273253–KY273255
Netzelia tuberculata	Aquatic mosses from littoral zone of small swamp near Sofia (Bulgaria)	42°39′N, 23°18′E	KY273256–KY273261
Netzelia wailesi	Aquatic mosses from the littoral zone of small swamp, near Sofia (Bulgaria)	42°39′N, 23°18′E	KY273262–KY273263

Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Sequences are deposited in GenBank (Table 1).

Alignment and phylogenetic analysis

The SSU rRNA gene sequences were aligned using the MUSCLE software (Edgar 2004). The SSU rRNA phylogenetic analysis data set contained 90 Amoebozoa taxa including 59 Arcellinida, 19 Tubulinida, 8 Leptomyxida, and 4 Echinamoebidae that were used as outgroups; a total of 900 characters were kept for phylogenetic analysis. Maximum likelihood trees were built using the RAxML version 7.2.8 algorithm (Stamatakis 2006) as proposed on the RAxML BlackBox portal (http://phylobench.vital-it.ch/raxml-bb/) using the GTR + G+ I model. The robustness of internal nodes was estimated by bootstrapping (1000 replicates). Model parameters were estimated in RAxML over the duration of the tree search. 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 1,000,000 generations. The standard deviation of split frequencies between the two chains were below 0.01 at the end of the run. For every 1000th generation, the tree with the best likelihood score was saved, resulting in 10,000 trees. The two chains were combined and a majority-rule consensus tree was generated after removing 25% of samples as a burn-in. Trees were viewed using FigTree (a program distributed as part of the BEAST package). The sequences identity percentages were calculated using BioEdit v7.1.9 (Hall 1999).

Results

SSU rRNA gene sequences analysis and phylogenetic relationships within the Arcellinida

We obtained twenty partial SSU rRNA gene sequences from seven Arcellinid taxa (*D. oblonga*, *D. gramen*, *D. labiosa*, *M. corona*, *Netzelia wailesi*, *N. tuberculata*, and *Arcella intermedia*). The length of the amplified SSU rRNA fragment for all taxa was around 1200 bp. No introns were found among the studied taxa, but insertions of approximately 110 bp were found in *Difflugia gramen*, *D. labiosa*, *M. corona*, *Netzelia wailesi*, and *N. tuberculata*. These insertions start at the same positions as in *D. tuberspinifera*, *D. achlora* and *N. oviformis* (Gomaa et al. 2012, 2015), but differ in length and sequence.

Multiple SSU rRNA gene sequences from independent DNA extractions, each of them containing between two to five cells, were obtained from all the studied taxa, except for *D. oblonga*. We observed clear intra-morphospecies genetic diversity: the sequences divergence was of 2.5% among the three sequences of *D. gramen*, 5% among the three sequences of *M. corona*, 2.1% among the three sequences of *D. labiosa*, 1.2% among the six sequences of *N. tuberculata*, 1.1% among the two sequences of *N. wailesi*, and 1.2% among the two sequences of *A. intermedia*.

The topologies of phylogenetic trees inferred from maximum likelihood and Bayesian inference were congruent with previous analyses (Fig. 2) (Gomaa et al. 2012, 2015; Lahr et al. 2013). Most of the Arcellinida sequences clustered together in a moderately supported clade, with the exception of *Heleopera sphagni*, *Cryptodifflugia operculata*, *C. oviformis*, and *Pyxidicula operculata* (Fig. 2). The new sequences of the agglutinated shell arcellinids obtained here were distributed within two main lineages, the cylindrical/elongated *Difflugia* lineage, and the globular/oviform "*Difflugia*", *Netzelia*, *Arcella* lineages. The newly obtained sequence of *D. oblonga* branched within the first lineage together with other cylindrical/elongated *Difflugia* spp. (*D. acuminata*, *D. lanceolata*, *D. bacillariarum*, and *D. hiraethogi*); this group received maximum bootstrap support (Fig. 2). The other newly obtained globular "*Difflugia*" spp. (*D. gramen*, *M. corona* and *D. labiosa*), *Netzelia* spp. (*N. tuberculata* and *N. wailesi*) and *Arcella intermedia* branched within Sphaerothecina (Fig. 2).

Table 2. Biometric characterization of the investigated species. **M**—median; **SD**—standard deviation; **SE**—standard error of the mean; **CV**—coefficient of variation in %; **Min**—minimum; **Max**—maximum; **n**—number of examined individuals (measurements in μ).

Character	Mean	M	SD	SE	CV	Min	Max	N
Arcella intermedia								
Diameter of shell (D)	61.9	61.5	3.13	0.57	5.06	57	69	30
Depth of shell (H)	33.7	34.0	2.25	0.41	6.68	29	39	30
Diameter of aperture (d)	15.8	16.0	0.86	0.16	5.44	14	18	30
H/D ratio	0.54	0.55	0.02	0.004	3.70	0.50	0.59	30
Mediolus corona (*Netzelia corona* comb. nov.)								
Length of shell (L)	154.8	152.0	8.46	1.54	5.46	141	173	30
Breadth of shell (B)	151.6	150.0	7.49	1.37	4.94	138	168	30
Diameter of aperture (d)	75.5	78.0	6.82	1.24	9.03	61	83	30
B/L ratio	0.98	0.98	0.01	0.002	1.02	0.96	1.0	30
Horn	32.0	32.0	1.34	0.24	4.18	29	34	30
"Difflugia" labiosa								
Length of shell (L)	191.6	191.0	9.62	1.76	5.02	177	208	30
Breadth of shell (B)	122.6	122.5	8.22	1.50	6.70	110	134	30
Diameter of aperture (d)	47.8	48.5	3.43	0.63	7.17	41	55	30
Neck	8.3	8.0	0.64	0.12	7.71	7	9	30
B/L ratio	0.64	0.66	0.06	0.01	9.37	0.55	0.72	30
Difflugia gramen (*Netzelia gramen* comb. nov.)								
Length of shell (L)	95.9	94.5	6.21	1.13	6.48	85	110	30
Breadth of shell (B)	88.6	88.5	4.24	0.77	4.78	80	98	30
Diameter of aperture (d)	37.5	37.0	1.14	0.21	3.04	36	40	30
B/L ratio	0.92	0.92	0.02	0.005	2.17	0.88	0.97	30
Difflugia oblonga (small morphotype)								
Length of shell (L)	157.1	156.0	12.62	2.30	8.03	137	185	30
Breadth of shell (B)	80.1	79.5	6.01	1.09	7.50	70	93	30
Diameter of aperture (d)	23.4	23.0	1.59	0.29	6.79	21	28	30
B/L ratio	0.51	0.51	0.01	0.001	1.96	0.49	0.53	30
Netzelia tuberculata								
Length of shell (L)	117.1	115.5	5.12	0.93	4.37	111	129	30
Breadth of shell (B)	105.5	104.0	5.01	0.91	4.75	99	119	30
Diameter of aperture (d)	31.3	31.0	1.42	0.26	4.54	29	34	30
B/L ratio	0.89	0.90	0.03	0.005	3.37	0.83	0.94	30
Netzelia wailesi								
Length of shell (L)	106.7	105.5	6.88	1.26	6.45	98	120	30
Breadth of shell (B)	84.7	84.0	4.77	0.87	5.63	78	93	30
Diameter of aperture (d)	32.0	32.0	2.07	0.38	6.47	29	36	30
B/L ratio	0.80	0.79	0.01	0.001	1.25	0.78	0.81	30

Table 3. List of taxon-specific primers used in our study.

Primer	Sequence 5′-3′	Specificity
NETZWAILR	GAG GCT TGT TGT TCG TGT CAC T	*N. wailesi* and *N. tuberculata*
LIMNETZR	AGC TCG TTG CCC GTG TCA CTG T	*N. gramen* and *Netzelia* spp.
AchloR1	GCTAGTTGACGACGAACCGC	*"Difflugia" labiosa*
CORONAR	AAC GGT CCG TCC CCA CCG CG	*N. corona*
OBLONGAR	TCC CTA GCA TTT TCA TGC AAG GAC	*D. oblonga*

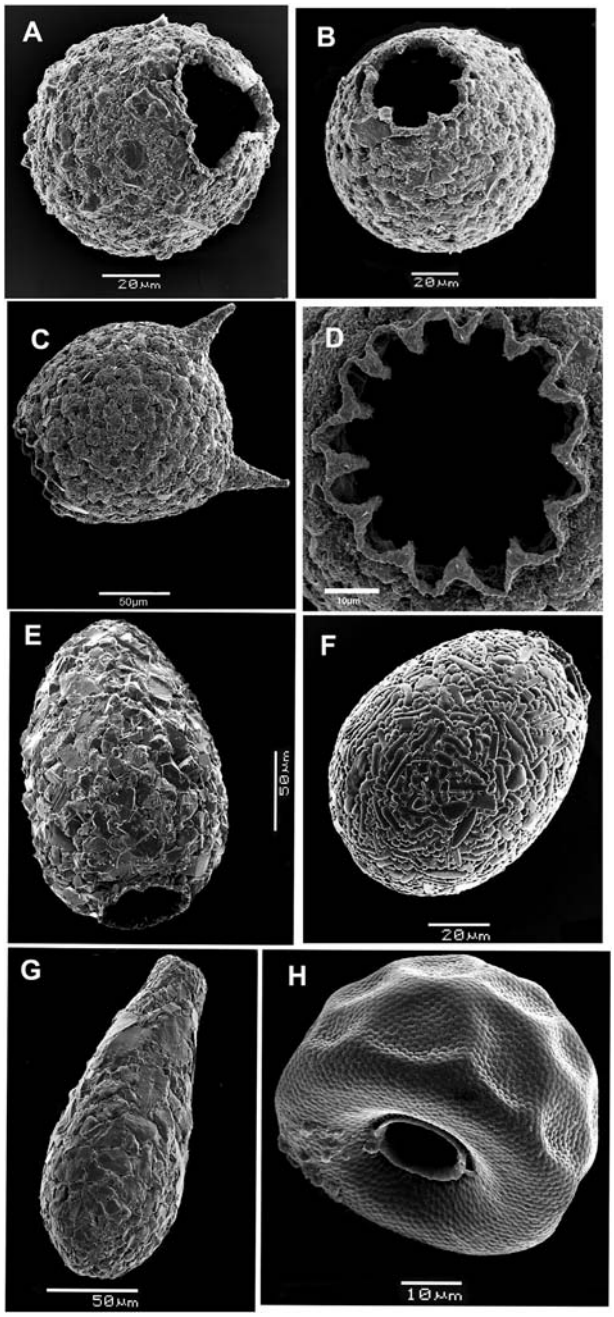

Fig. 1. Scanning electron micrographs of tests from the studied species: *Netzelia gramen* comb. nov. (A); *Netzelia tuberculata* comb. nov. (B); *Netzelia corona* comb. nov. later view (C); *Netzelia corona* comb. nov. aperture (D); *Difflugia* incertae sedis (E); *Netzelia wailesi* comb. nov. (F); *Difflugia oblonga* (small morphotype) (G); and *Arcella intermedia* (H).

Discussion

Phylogenetic relationships among Arcellinida taxa

The presented phylogenetic reconstruction illustrates the taxonomic position of seven new Arcellinid taxa, thus expanding the Arcellinida sequences dataset. Our addition of new taxa belongs to the oviform *Difflugia* spp. (*D. gramen*, *D. labiosa* and *M. corona*) and *Netzelia* spp. (*N. tuberculata*, *N. wailesi*) confirmed that *Difflugia* is non-monophyletic (Fig. 2) (Gomaa et al. 2012, 2015). The SSU rRNA sequences divergences among different species were much higher than between different isolates of the same species. Except for *M. corona*, all sequence divergences among the isolates of the same species fell within the 3.2% threshold proposed by Nassonova et al. (2010) for naked lobose amoebae intra-specific discrimination based on the SSU rRNA gene sequences. The robust grouping of oviform "*Difflugia* spp." and *Netzelia* spp. corroborated the recently established family Netzelliidae Kosakyan et al. (2016a). Originally, genus *Netzelia* was established to include some oviform *Difflugia* spp. (*D. oviformis*, *D. tuberculata* and *D. wailesi*) that had been found able to reinforce their shells with self-secreted mineral elements (idiosomes) alone or in conjunction with gathered mineral particles (xenosomes) (Meisterfeld 1984; Netzel 1976, 1983; Ogden 1979, 1983). However, other species were recognized later to have the capacity to build their own shell out of organic material, siliceous elements, or a combination of both. Indeed, experiments performed on clonal cultures of the similar looking *D. geosphaira* showed that this species was also able to secrete organic shells mixed with self-secreted siliceous particles (idiosomes) in the absence of mineral grains or other xenosomes (Ogden 1991). Other members of *Difflugia* spp. such as *D. lobostoma* were also observed to alter the composition of their shell from agglutinated to entirely organic shell if maintained in clonal cultures in absence of mineral grains (Figs 1–4 in Ogden 1988). Similar observations were made for *M. corona*, a species that constructs shells out of mineral grains and diatom frustules, but was sometimes observed with a tuberculated shell made out of siliceous elements. This shell composition appears very similar to *Netzelia tuberculata*, or *D. urceolata*, which build proteinaceous shells in absence of mineral grains (when growing on *Sphagnum* mosses for instance; Ferry Siemensma, personal communication; see also http://www.arcella.nl/difflugia-corona, http://www.arcella.nl/difflugia-urceolata). Altogether, these observations together with our results suggest that most, if not all ovoid *Difflugia* have the capacity of building shells without using any recycled foreign material, which was considered as the synapomorphy for genus *Netzelia* (Netzel 1976, 1983).

In a recent study, Patterson (2014) erected a new genus for *Difflugia corona* and *D. tuberspinifera*, *Mediolus*, based on their distinctive "tooth-like, inward oriented apertural crenulations, and a typical test with variable number of spines extending outward on the test". However, Gomaa et al. (2015) demonstrated that *D. tuberspinifera* comprises two genetically closely-related forms a spinose (with variable number of spines) and a spineless morphotypes, which suggests that the presence of spines is a labile trait in evolution and might have appeared, for instance, in response to predation. In the

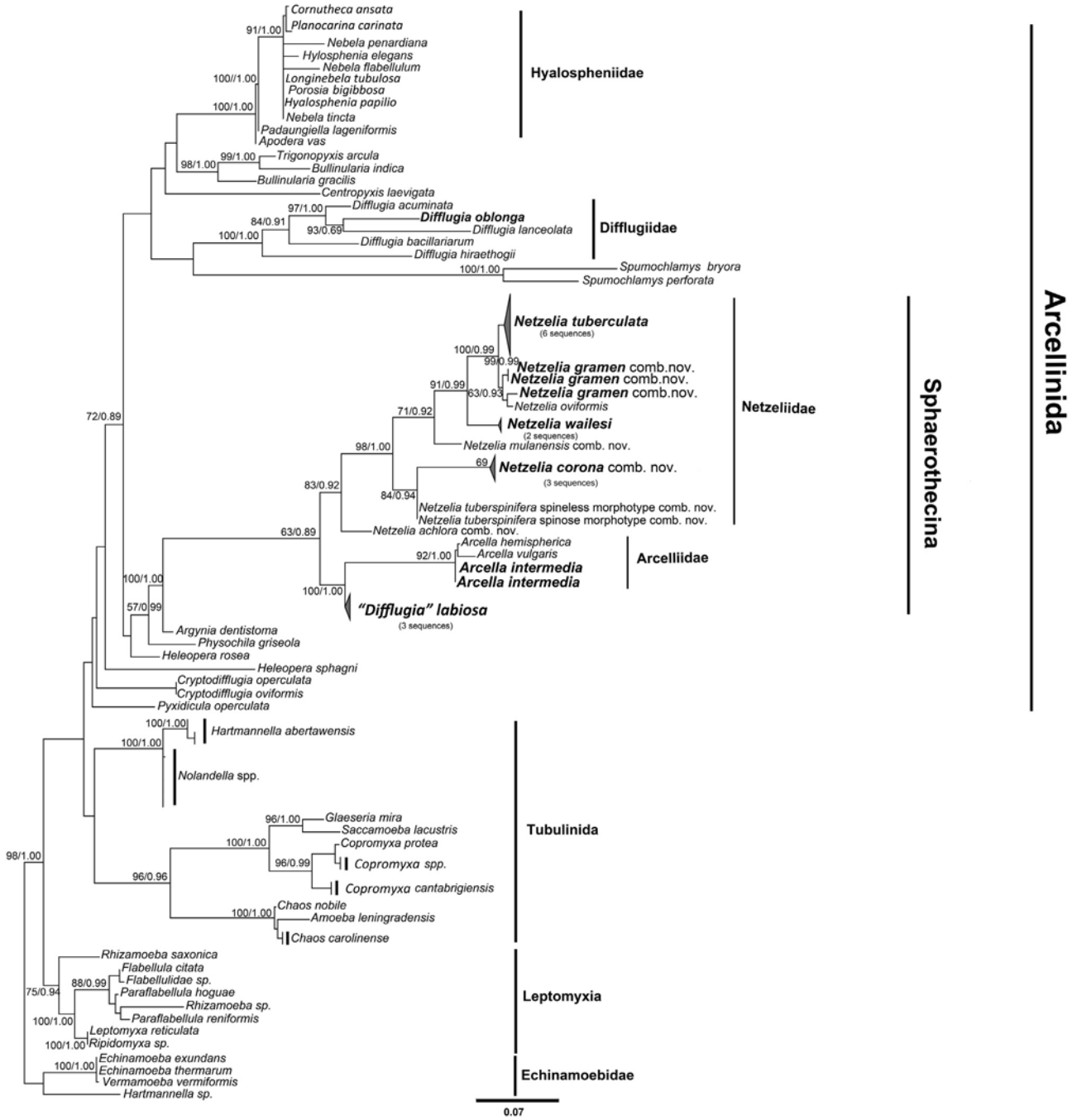

Fig. 2. Molecular phylogeny based on small subunit (SSU) rRNA gene sequences illustrating the relationships within Arcellinida and related Amoebozoa. The tree is rooted with Echinamoebidae. Taxa in bold are novel data. The tree was obtained by maximum likelihood analysis and a topology was obtained by Bayesian inference using MrBayes. Numbers at the nodes indicate bootstrap values and Bayesian inference posterior probabilities. The scale bar indicates 0.07% sequence divergence.

phylogeny presented here, the presence of spines does not appear as the synapomorphy of a given group and therefore, cannot be used to define a genus. Therefore, we transferred *Mediolus tuberspinifera* and *M. corona* to genus *Netzelia*.

Furthermore, our phylogenetic analyses show that *Difflugia gramen* and *Netzelia oviformis* form together a group where sequences are intermixed, suggesting that both forms should be lumped, or at least that diagnostic criteria should be redefined. It has been shown that the distinction between *D. gramen* and four other related species, *D. limnetica*, *D. lobostoma*, *D. pseudolimnetica* and *D. hydrostatica* can be difficult because of the insufficient morphological differences among those species (Ogden and Meisterfeld 1989; Ogden 1980). Shell size and the presence of a collar around the pseudostome are considered to be the main criteria to discriminate those species. However, polymorphism in the shell size has

been reported in at least three of those species, including the *D. gramen* (Ogden and Meisterfeld 1989; Ogden 1980 and references therein).

The features of our species fits best to *D. gramen* because of its agglutinated shell constructed out of smooth mineral grains. The aperture is tri-lobed with a pronounced organic necklace embedded with small grains. Furthermore, it does not host any symbiotic algae in the cytoplasm. Remarkably, these features may fall as well within the variation found in *N. oviformis* (Netzel 1976). Clearly, more data are needed to decide whether both forms are just the product of phenotypic plasticity, as it has been already observed for other arcellinids (Lahr and Lopes 2009). As SSU rRNA is not a good phylogenetic marker for closely-related taxa in arcellinids (Lara et al. 2008), another more variable genetic marker such as COI (Kosakyan et al. 2012) would help considerably in resolving the relationships in this group.

The phylogenetic placement of *D. labiosa* Wailes 1919 (synonym *D. amphora* Penard 1902) as a sister lineage to *Arcella* spp. and not with the other globular/oviform *Difflugia* spp. and *Netzelia* spp. is noteworthy. *D. labiosa* is characterized by a pyriform or egg-shaped agglutinated shell, and a raised collar with 8–9 lobes, the collar is often recessed into the body of the shell (Ogden 1980, 1983; Penard 1902). The shell of *D. labiosa* is characterized by its tapered aboral end, unlike the other oviform species in Netzeliidae (Figs 1, 2). Indeed Ogden (1983) illustrated that other species such *D. amphoralis*, *D. mamillaris* and *D. microclaviformis* have similar shell outlines to that of *D. labiosa*. Future studies with ribosomal gene sequences of these species will definitely clarify whether the pointed pyriform shell shape is a phylogenetic criterion for this group or not. Therefore, for now we prefer not to take any taxonomic action for *D. labiosa* and considered it as *incertae sedis*.

Elongated/cylindrical *Difflugia* spp. belong to another branch the Arcellinida tree, and the newly added sequence of *D. oblonga* clusters with these organisms. This confirms that the cylindrical/elongated shell shape and the lack of collar are reliable phylogenetic criteria for this group as suggested by Gomaa et al. (2012, 2015). *D. oblonga* as it is considered nowadays has a great range of variation in shell length (80–300 μm). In several publications, Ogden (see Mazei and Warren 2014) separated and redescribed the smaller morphotypes (131–224 μm) of *D. oblonga* to *D. parva*. Detailed morphometric and SEM analyses by Mazei and Warren (2014) showed that *D. parva* is a junior synonym of *D. oblonga.* All five sequenced species in this clade, including the newly sequenced *D. oblonga*, share a deletion of four nucleotides at a position corresponding to nucleotide 1034 in *D. bacillariarum*. All species so far are each represented by a single SSU rRNA gene sequence and species within this clade are known to be highly polymorphic and include species-complex such as *D. acuminata*, *D. lanceolata* and *D. oblonga* (Mazei and Warren 2012, 2014). It is therefore too early to draw any conclusion about trait evolution within this group. To the best of our knowledge, members of this group were never observed with organic or chitinous shells. They use a wide variety of materials to construct their agglutinated shell (sand grains, diatoms, quartz). *D. bacillariarum* for example constructs the entire shell with elongated diatom frustules.

Our new sequences from Arcellinids with an agglutinated shell illustrate well the diversity in this ancient and poorly studied group. They reveal ancient relationships, but also probable recent radiations (like the *Netzelia oviformis*/*N. gramen*, or even the whole genus *Arcella*), whose specific diversity still remains to be evaluated, preferentially with fast-evolving markers. We may then expect diversity estimations to be multiplied, as it has been shown for another relatively recent Arcellinida radiation, family Hyalospheniidae (Kosakyan et al. 2012). The genetic, but also physiological and ecological diversity of Arcellinida still remains an open field for discovery.

Taxonomic actions

The following taxonomic actions are taken in accordance to the International Code of Zoological Nomenclature:

Order: Arcellinida Kent 1880
Suborder: Sphaerothecina Kosakyan et al. 2016a
Family: Netzeliidae Kosakyan et al., 2016a
Genus: *Netzelia* Ogden 1979

---

1) Newly added taxa:

***Netzelia achlora* (Penard 1902) comb. nov.**

Original name: *Difflugia achlora* Penard 1902 (Tab. II–Fig. 3)

Updated description: shell ovoid to elongated-ovoid, composed of a mixture of small pieces of quartz and siliceous particles (like diatoms frustules), the particles are bound together with an organic cement. The aperture is tri-lobed and surrounded by a collar. The shell of *N. achlora* is very similar to *N. gramen*, but smaller in size; dimensions (Length: 58 μm, Breadth: 46 μm, Aperture diameter: 18 μm).

***Netzelia gramen* (Penard 1902) comb. nov.**

Original name: *Difflugia gramen* Penard 1902

Updated description: shell ovoid or spherical. The shell surface is rough and composed of a mixture of small to medium pieces of quartz, the particles are bound together with an organic cement. Shells with siliceous plates were also reported by Cash et al. (1919). The aperture is tri-lobed and is surrounded by slightly raised collar of small particles which are cemented together. Shell dimensions are given in Table 2.

***Netzelia corona* (Wallich 1864) comb. nov.**

Original name: *Difflugia corona* Wallich 1864

Junior synonym: *Mediolus corona* Patterson 2014

Updated description: shell spherical to sub-spherical. The shell wall is composed of mineral grains, quartz and diatoms frustules, which are agglutinated together by an organic cement. The shell is ornamented by conical hollow spines at the posterior third. The aperture is circular, surrounded by a variable number of inward-oriented angular crenulations (tooth-like structures). Shells ornated with tuberculate structures similar to *Netzelia tuberculata* have been observed by F. Siemensma personal communication; see also http://www.arcella.nl/difflugia-corona, http://www.arcella.nl/difflugia-urceolata). Shell dimensions are given in Table 2.

***Netzelia tuberspinifera* (Hu, Shen and Gong 1997) comb. nov.**

Original name: *Difflugia tuberspinifera* Hu, Shen and Gong 1997

Junior synonym: *Mediolus tuberspinifera* Patterson 2014

Updated description: shell spherical to sub-spherical, mulberry-shaped, composed of fine sand grains, muddy particles and flattish pieces of quartz. The shell surface has many protuberances similar to *N. tuberculata.* Shells ornamented with two to eight conical hollow spines at the upper equator region. Spineless morphotypes have been also described (Gomaa et al. 2015; Yu et al. 2014). The aperture is circular with a short collar and bordered by a variable number of tooth-like structures.

***Netzelia mulanensis* (Yang, Meisterfeld, Zhang, Shen 2005) comb. nov.**

Original name: *Difflugia mulanensis* Yang, Meisterfeld, Zhang, Shen 2005

Description: see Yang et al. (2005).

2) Sphaerothecina *Incertae sedis*:

***"Difflugia" labiosa* Wailes, 1919**

Original name*: Difflugia labiosa* Wailes, 1919

Expanded diagnosis: shell pyriform to egg-shaped, agglutinated and composed of mineral elements such as quartz; aperture circular with 6–8 undulating lobes. Collar often recessed in the shell body. Can be differentiated from other similar species by its ovoid-conical shape and by the number of lobes surrounding the aperture.

Acknowledgements

This work was funded by Science and Technology Cooperation Program Switzerland—Russia grant IZLR Z3_128338, Swiss NSF grants n○ 310003A_143960 and PZ00P2_122042 (Ambizione fellowship to E. Lara), IB73A0-111064/1 (SCOPES), and 205321- 109709/1 and 205321-109709/2. D. Lahr is funded by a FAPESP Young Investigator Award (#2013/04585-3).

References

Anderson, O.R., 1988. Testate amoebae (classes: Lobosea and Filosea). In: Anderson, O.R. (Ed.), Comparative Protozoology: Ecology, Physiology, Life History. Springer-Verlag, Berlin, New York, pp. 63–65.

Cash, J., Wailes, G.H., Hopkinson, J., 1919. The British Freshwater Rhizopoda and Heliozoa, vol. 4. Ray Society, London, pp. 1–71.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Dalby, A.P., Kumar, A., Moore, J.M., Patterson, R.T., 2000. Preliminary survey of arcellaceans (thecamoebians) as limnological indicators in tropical Lake Sentani, Irian Jaya, Indonesia. J. Foram. Res. 30, 135–142.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Gomaa, F., Todorov, M., Heger, T.J., Mitchell, E.A.D., Lara, E., 2012. SSU rRNA phylogeny of Arcellinida (Amoebozoa) reveals that the largest Arcellinid genus, *Difflugia* Leclerc 1815, is not monophyletic. Protist 163, 389–399.

Gomaa, F., Yang, J., Mitchell, E.A.D., Zhang, W.J., Yu, Z., Todorov, M., Lara, E., 2015. Morphological and molecular diversification of Asian endemic *Difflugia tuberspinifera* (Amoebozoa, Arcellinida): a case of fast morphological evolution in protists? Protist 166, 122–130.

Hu, D.L., Shen, Y.F., Gu, M.R., Gong, X.J., 1997. New species and new records of protozoa from Wuling Mountains Area. In: Song, D.-X. (Ed.), Invertebrates of Wuling Mountains Area, Southwestern China. Science Press, Bejing, China, pp. 40–72 (in Chinese).

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Kosakyan, A., Heger, T.J., Leander, B.S., Todorov, M., Mitchell, E.A.D., Lara, E., 2012. COI barcoding of Nebelid testate amoebae (Amoebozoa: Arcellinida): extensive cryptic diversity and redefinition of family Hyalospheniidae Schultze. Protist 163, 415–434.

Kosakyan, A., Gomaa, F., Mitchell, E.A.D., Heger, T.J., Lara, E., 2013. Using DNA-barcoding for sorting out protist species complexes: a case study of the *Nebela tincta–collaris–bohemica* group (Amoebozoa; Arcellinida, Hyalospheniidae). Eur. J. Protistol. 49, 222–237.

Kosakyan, A., Gomaa, F., Lara, E., Lahr, D.J., 2016a. Current and future perspectives on the systematics, taxonomy and nomenclature of testate amoebae. Eur. J. Protistol. 55, 105–117.

Kosakyan, A., Lahr, D.J., Mulot, M., Meisterfeld, R., Mitchell, E.A.D., Lara, E., 2016b. Phylogenetic reconstruction based on COI reshuffles the taxonomy of hyalosphenid shelled testate amoebae and reveals the convoluted evolution of shell plate shapes. Cladistics 32, 606–623.

Kudryavtsev, A., Pawlowski, J., Hausmann, K., 2009. Description and phylogenetic relationships of *Spumochlamys perforate* n. sp and *Spumochlamys bryora* n. sp (Amoebozoa, Arcellinida). J. Eukaryot. Microbiol. 56, 495–503.

Lahr, D.J.G., Lopes, S.G.B.C., 2009. Evaluating the taxonomic identity in four species of the lobose testate amoebae genus *Arcella* Ehrenberg, 1832. Acta Protozool. 48, 127–142.

Lahr, D.J., Grant, J.R., Katz, L.A., 2013. Multigene phylogenetic reconstruction of the Tubulinea (Amoebozoa) corroborates four of the six major lineages, while additionally revealing that shell composition does not predict phylogeny in the Arcellinida. Protist 164, 323–339.

Lara, E., Heger, T.J., Ekelund, F., Lamentowicz, M., Mitchell, E.A.D., 2008. Ribosomal RNA genes challenge the monophyly of the Hyalospheniidae (Amoebozoa: Arcellinida). Protist 159, 165–176.

Leclerc, M., 1815. Notes sur la Difflugie, nouveau genre de polype amorphe. Mem. Mus. Hist. Nat. (Paris) 2, 474–478.

Mazei, Y., Warren, A., 2012. A survey of the testate amoeba genus *Difflugia* Leclerc, 1815 based on specimens in the E. Penard and CG Ogden collections of the Natural History Museum, London. Part 1: species with shells that are pointed aborally and/or have aboral protuberances. Protistology 7, 121–171.

Mazei, Y., Warren, A., 2014. A survey of the testate amoeba genus *Difflugia* Leclerc, 1815 based on specimens in the E. Penard and CG Ogden collections of the Natural History Museum, London. Part 2: species with shells that are pyriform or elongate. Protistology 8, 133–171.

Mazei, Y., Warren, A., 2015. A survey of the testate amoeba genus *Difflugia* Leclerc, 1815 based on specimens in the E. Penard and CG Ogden collections of the Natural History Museum, London. Part 2: species with shells that are spherical or ovoid. Protistology 9, 3–49.

Meisterfeld, R., 1984. Taxonomic problems in *Difflugia* species with lobed aperture-biometry and impact of supplied building material. J. Protozool. 31 (Suppl. (62A)), 226.

Meisterfeld, R., 2002. Order Arcellinida Kent, 1880. In: Lee, J.J., Leedale, G.F., Bradbury, P. (Eds.), The Illustrated Guide to the Protozoa, vol. 2, second edition. Society of Protozoologists, Lawrence, Kansas, USA, pp. 827–860.

Netzel, H., 1976. Die Ultrastruktur der Schale von *Difflugia oviformis* (Rhizopoda, Testacea). Arch. Protistenkd. 118, 321–339.

Netzel, H., 1983. Gehäusewandbildung durch mehrphasige Sekretion bei der Thekamöbe *Netzelia oviformis* (Rhizopoda, Testacea). Arch. Protistenkd. 127, 351–381.

Nassonova, E., Smirnov, A., Fahrni, J., Pawlowski, J., 2010. Barcoding amoebae: comparison of SSU, ITS and COI genes as tools for molecular identification of naked lobose amoebae. Protist 161, 102–115.

Ogden, C.G., 1979. Siliceous structures secreted by members of the subclass lobosia (Rhizopodea: Protozoa). Bull. Br. Mus. Nat. Hist. (Zool.) 36, 203–207.

Ogden, C.G., 1980. Notes on some Difflugiidae from Norfolk (Rhizopodea, Protozoa). Bull. Brit. Mus. Nat. Hist. (Zool.) 39, 125–138.

Ogden, C.G., 1983. Observations on the systematics of the genus *Difflugia* in Britain (Rhizopoda, Protozoa). Bull. Br. Mus. Nat. Hist. (Zool.) 44, 1–173.

Ogden, C.G., 1988. Morphology of the organic shell matrix of *Difflugia* (Rhizopoda) in culture, including modification by the addition of agglutinate particles. Arch. Protistenkd. 136, 365–376.

Ogden, C.G., 1991. The biology and ultrastructure of an agglutinate testate amoeba *Difflugia geosphaira* sp. nov. (Protozoa, Rhizopoda). Arch. Protistenkd. 140, 141–150.

Ogden, C.G., Hedley, R.H., 1980. An Atlas of Freshwater Testate Amoebae. British Museum (Natural History) and Oxford University Press, London and Oxford, 222 p.

Ogden, C.G., Meisterfeld, R., 1989. The taxonomy and systematics of some species of *Cucurbitella*, *Difflugia* and *Netzelia* (Protozoa: Rhizopoda); with an evaluation of diagnostic characters. Eur. J. Protistol. 25, 109–128.

Patterson, R.T., 2014. *Mediolus*, a new genus of arcellacea (testate lobose amoebae). Palaeontol. Electron. 17, 1–8.

Penard, E., 1902. Fauna rhizopodique du bassin du Léman. Henry Kündig, Genève, pp. 1–714.

Porter, S.M., 2016. Tiny vampires in ancient seas: evidence for predation via perforation in fossils from the 780–740 million-year-old Chuar Group, Grand Canyon, USA. Proc. R. Soc. B 283, 1831.

Porter, S.M., Meisterfeld, R., Knoll, A.H., 2003. Vase-shaped microfossils from the Neoproterozoic Chuar Group, Grand Canyon: a classification guided by modern testate amoebae. J. Paleontol. 77, 409–429.

Schlegel, M., Meisterfeld, R., 2003. The species problem in protozoa revisited. Eur. J. Protistol. 39, 349–355.

Schönborn, W., 1992. Adaptive polymorphism in soil-inhabiting testate amoebae (Rhizopoda): its importance for delimitation and evolution of asexual species. Arch. Protistenkd. 142, 139–155.

Schmidt, A.R., Girard, V., Perrichot, V., Schönborn, W., 2010. Testate amoebae from a Cretaceous forest floor microbiocoenosis of France. J. Euk. Microbiol. 57, 245–248.

Singer, D., Kosakyan, A., Pillonel, A., Mitchell, E.A.D., Lara, E., 2015. Eight species in the *Nebela collaris* complex: *Nebela gimlii* (Arcellinida, Hyalospheniidae), a new species described from a Swiss raised bog. Eur. J. Protistol. 51, 79–85.

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.

Todorov, M., Golemansky, V., 2007. Morphological variability of *Difflugia urceolata* CARTER, 1864 (Testacealobosia: Difflugiidae) and taxonomical status of its varieties *D. urceolata* var. *olla* LEIDY, 1879, and *D. urceolata* var. *sphaerica* PLAYFAIR, 1917. Acta Zool. Bulg. 59, 3–10.

Van Hengstum, P.J., Reinhardt, E.G., Medioli, F.S., Gröcke, D.R., 2007. Exceptionally preserved late albian (Cretaceous) arcellaceans (thecamoebians) from the Dakota formation near Lincoln Nebraska, USA. J. Foram. Res. 37, 300–308.

Wallich, G.C., 1864. On the extent, and some of the principal causes, of structural variation among the Difflugian Rhizopods. J. Nutr. Hist. 13, 215–245.

Wanner, M., Meisterfeld, R., 1994. Effects of some environmental factors on the shell morphology of testate amoebae (Rhizopoda, Protozoa). Eur. J. Protistol. 30, 191–195.

Yang, J., Meisterfeld, R., Zhang, W., Shen, Y., 2005. *Difflugia mulanensis* nov. spec., a freshwater testate amoeba from Lake Mulan, China. Eur. J. Protistol. 41, 269–276.

Yu, Z., Zhang, W., Liu, L., Yang, J., 2014. Evidence for two different morphotypes of *Difflugia tuberspinifera* from China. Eur. J. Protistol. 50, 205–211.