Published in Protist 166, issue 1, 122-130, 2015
which should be used for any reference to this work

Morphological and Molecular Diversification of Asian Endemic *Difflugia tuberspinifera* (Amoebozoa, Arcellinida): A Case of Fast Morphological Evolution in Protists?

Fatma Gomaa[a,b,1,2], **Jun Yang**[c,1,2], **Edward A.D. Mitchell**[a,d], **Wen-Jing Zhang**[e], **Zheng Yu**[c,f], **Milcho Todorov**[g], and **Enrique Lara**[a]

[a]Laboratory of Soil Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland
[b]Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt
[c]Aquatic Ecohealth Group, Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[d]Jardin Botanique de Neuchâtel, Chemin du Perthuis-du-Sault 58, CH-2000 Neuchâtel, Switzerland
[e]Marine Biodiversity and Global Change Center, College of Ocean and Earth Sciences, Xiamen University, Xiamen 361102, China
[f]Graduate University of Chinese Academy of Sciences, Beijing 100049, China
[g]Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin St., 1113 Sofia, Bulgaria

Planktonic arcellinid testate amoebae exhibit a broad-range of morphological variability but it is currently unclear to what extent this variability represents phenotypic plasticity or if it is genetically determined. We investigated the morphology and phylogenetic relationships of three endemic east-asian *Difflugia* taxa 1) the vase-shaped *D. mulanensis*, 2) and a spinose and a spineless morphotypes of *D. tuberspinifera* using scanning electron microscopy and two ribosomal genetic markers (SSU rDNA and ITS sequences). Our phylogenetic analyses shows that all three taxa are genetically distinct and closely related to *D. achlora* and *Netzelia oviformis*. The genetic variations between the spineless and spinose morphotypes of *D. tuberspinifera* were low at the SSU rRNA level (0.4%), but ten times higher

---

[1] Both authors contributed equally to this work. [2] Corresponding authors;
e-mail fatma.gomaa@gmail.com (F. Gomaa), jyang@iue.ac.cn (J. Yang).

at the ITS level (4.5-6%). Our data suggest that the two forms of *D. tuberspinifera* are sufficiently differentiated in terms of morphology and genetic characteristics to constitute two separate entities and that the presence of spines does not result from phenotypic plasticity due to environmental selective pressure. However further observational and experimental data are needed to determine if these two forms constitute different biological species.

Key words: Molecular phylogeny; SSU rRNA gene; ITS; *Difflugia tuberspinifera*; morphological variation; fast evolution; China.

Introduction

Recent molecular phylogenetic studies have considerably expanded our knowledge of the phylogenetic relationships and diversity of microbial eukaryotes (Cuvelier et al. 2008; Lara et al. 2011; López-García et al. 2001; Nikolaev et al. 2004). However, diversification and speciation processes in free-living protists remain major questions in evolutionary biology (Coyne and Orr 2004; Lahr et al. 2011b; Logares et al. 2007, 2008). In particular, the pace at which new morphologies appear has remained largely understudied, which may, for example, be a serious problem when interpreting fossil records.

Protist phenotypes are believed to be extremely old, because of a supposed stabilizing selection that acts on large asexual populations (Civetta and Singh 1999; Fenchel and Finlay 2006). In line with this, Arcellinid morphotypes are thought to remain very stable in time, as illustrated by the resemblance observed between extant forms and 742 Mya old vase-shaped microfossils. For example, the fossil *Palaeoarcella athanata* shares a striking resemblance with the extant genus *Arcella* (Porter et al. 2003). This conservatism contrasts with the derived position of this genus in molecular phylogenies of genus *Arcella* with respect to other Arcellinida (Gomaa et al. 2012; Lahr et al. 2013) and would thus suggest that Arcellinida as a whole are much older than 742 Mya (Fiz-Palacios et al. 2013).

However, such morphological conservatism is not the rule in all protist groups. Recent speciation events have been demonstrated in coccolithophorids, where the morphs *Calcidiscus leptoporus* ssp. *leptoporus* and *quadriperforatus* diverged only 0.3 Mya ago as demonstrated both by the fossil record and molecular clock approaches (Saez et al. 2003). Instances of rapid evolutionary radiations can also be expected in ciliates inhabiting tank bromeliads in the neotropics, where large predatory forms and closely related diminutive bacterivores share the same habitat and har-bour almost identical SSU rRNA gene sequences despite their extremely different sizes, morphologies and lifestyles (Foissner et al. 2003). Similarly the dinoflagellates *Scripsiella hangoei* and *Peridinium aciculatum* were described as separate genera based on their morphology, but their ribosomal DNA sequences were shown to be identical (Logares et al. 2008). Likewise, the ITS sequence of the tank bromeliad-inhabiting aplanosporic oomycete *Geolegnia helicoides* appeared to be nested within the flagellate genus *Leptolegnia*, showing an incipient loss of the whole flagellar apparatus (Steciow et al. 2013).

However, understanding trait evolution can be impaired by phenotypic plasticity, which has been demonstrated or suggested in Arcellinida, especially among members of genus *Difflugia*, leading to much confusion in species identification and differentiation (Chardez 1974; Lahr and Lopes 2006; Medioli et al. 1987; Meisterfeld 2002; Ogden 1983; Ogden and Meisterfeld 1989; Todorov and Golemansky 2007). The current systematic scheme of genus *Difflugia* is inconsistent and unsatisfactory due to the lack of good diagnos-tic characters, often inadequate descriptions and a general lack of molecular data (Gomaa et al. 2012). It is thus difficult to determine which traits are genetically fixed and which represent variable characters, and this makes the evaluation of their stability throughout evolutionary times even more challenging. Given the paleontological significance of Arcellinida it is crucial to better understand the relationships between morphological and molecular diversity within this group, both overall and within groups of closely-related taxa.

To address these questions, we studied the spinose and spineless morphotypes of *Difflugia tuberspinifera*, a freshwater planktonic raptorial species described from China (Han et al. 2008; Yang et al. 2004). *D. tuberspinifera* was described as a spherical agglutinated shell, ornamented by two to eight conical hollow spines at the upper equatorial region with a short collar and an aperture ornamented with 6-11 tooth-like structures. Spineless individuals that were otherwise highly similar in shape and structure to *D. tuberspinifera* were also often observed in the same bodies of water (Yu et al. 2014). It remained unclear if the absence of spines was genetically determined, and, more generally, if the presence or absence of spines is a valid taxonomical criterion for Arcellinid testate amoebae in general (e.g. including also other genera such as *Centropyxis* or *Argynnia*) or if it is just an expression of phenotypic plasticity as was suggested in the case of the *Centropyxis aculeata* (but with no molecular data existing for that group of taxa) (Lahr et al. 2008). If this difference was genetically determined, its high variability would suggest that this is a fast-evolving charac-ter. If no genetical difference was found this would rather suggest the existence of different life-phases, such as the benthic-planktonic cycle reported for *Difflugia limnetica* (Schönborn 1962), or an envi-ronmental control on morphology (Wanner 1999). We amplified and sequenced the SSU rRNA gene of both spinose and spineless morphotypes of *D. tuberspinifera* and placed these sequences on a large Arcellinid tree together with another Eastern Asian species, *D. mulanensis*. In addition, we also sequenced the internal transcribed spacer region (ITS1/5.8S/ITS2) of the two *D. tuberspinifera* forms and of *D. labiosa*.

Results

SSU rRNA Gene Analysis and Phylogenetic Relationships within Arcellinida

We obtained nine partial SSU rRNA gene sequences from three independent extractions of each *Difflugia tuberspinifera* morphotype, and three isolates of *D. mulanensis* (Fig. 1). The amplified SSU rRNA fragment length was 2192 bp in *D. tuberspinifera* spinose morphotype, 1697 bp in *D. tuberspinifera* spineless morphotype, and 1134 bp in *D. mulanensis*. An identical intron of 433 bp between positions 434 and 867, and identical insertions of 95 bp between positions 1241 and 1336 were found in both morphotypes of *D. tuber-spinifera*. An additional intron of 432 bp was found between positions 1698 and 2130 in the SSU rRNA gene of *D. tuberspinifera* spinose morpho-type, but not in the spineless morphotype. No intron was found in *D. mulanensis*, but an insertion of 196 bp was found starting at the same position as the *D. tuberspinifera* insertion, but differing in both sequence and length.

We observed no intra-morphotype sequence polymorphism in the sequenced portion of the SSU rRNA gene among the *D. tuberspinifera* sequences. The sequences of the SSU rRNA exon regions in spinose and spineless morphotypes of *D. tuberspinifera* were highly similar to each other (99.6% identity).

The general topologies of the SSU rRNA gene phylogenetic trees inferred from maximum likelihood and Bayesian inference were similar (Fig. 2) and correspond to previously published data by Gomaa et al. (2012). Most members of Arcellinida branch together in a single well supported clade that receives 93% bootstrap support (BS), 94% expected-likelihood weights of local rear-rangements edge support (LR-ELW; equivalent to approximate bootstraps) (Strimmer and Rambaut 2002) and 1.00 Bayesian inference posterior prob-ability (PP). This main clade divides into two major groups; the first group comprises Hyalospheniidae, *Bullinularia* spp., *Centropyxis laevigata* and the cylindrical/elongated *Difflugia* spp. (*D. acuminata*, *D. lanceolata*, *D. bacillariarum*, *D. hiraethogii*) and receives moderate support (BS=60%, ELW=85%, PP=0.80). The second group comprises genus *Arcella*, the globular/spherical species *Difflugia achlora*, *Netzelia oviformis*, and the newly obtained sequences of *D. mulanensis* and *D. tuberspinifera* (both morphotypes). *Physochila griseola*, *Argyn-nia dentistoma* and *Heleopera rosea* branch in distant positions at the base of this clade. This clade receives high support (BS=82%, ELW=87%, PP=0.98). *Pyxidicula operculata* branches distantly from the rest of Arcellinida with moderate sup-port (BS=53%, ELW= 87%,PP=0.98), while both *Cryptodifflugia operculata* (Lahr et al. 2011a) and *Cryptodifflugia oviformis* (Gomaa et al. 2012; Lahr et al. 2011a) have an uncertain position with respect to other Arcellinida. *Heleopera sphagni* (AF 848965) (Nikolaev et al. 2005) branches outside the main Arcellinida clade with an uncertain phyloge-netic position within tubulinida as shown in previous phylogenies (Gomaa et al. 2012; Lahr et al. 2011a).

ITS Sequence Analysis of the Two Morphotypes of *Difflugia tuberspinifera*

We successfully obtained twelve ITS sequences from twelve independent extractions: eight from the spinose morphotype of *D. tuberspinifera*, three from the spineless morphotype and one from *D. labiosa*. The sequenced fragments of ITS1 + 5.8S + ITS2 were 716 bp long in *D. tuberspinifera* spineless

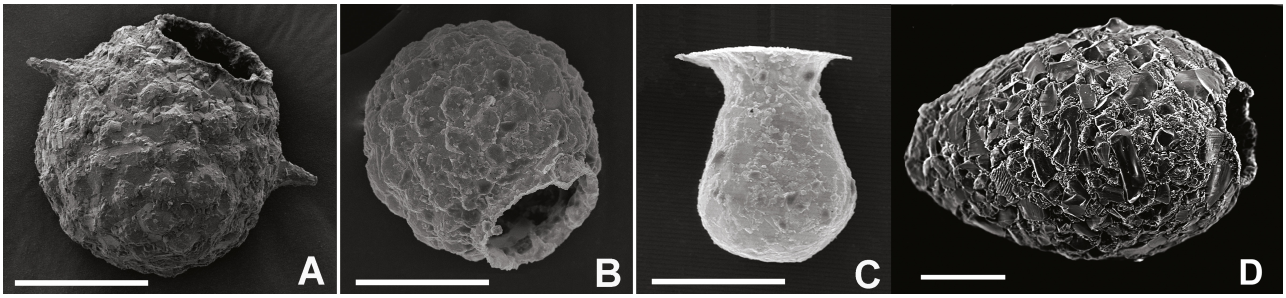

Figure 1. Scanning electron micrographs of *Difflugia* species analyzed in this study. *Difflugia tuberspinifera* spinose morphotype (**A**); *D. tuberspinifera* spineless morphotype (**B**); *D. mulanensis* (**C**); *D. labiosa* (**D**). Scale bars = 50 μm.

morphotype, 300 bp in *D. tuberspinifera* spinose morphotype, and 463 bp in *D. labiosa.* At the ITS level, both spinose and spineless morphotypes of *D. tuberspinifera* were clearly discriminated, the levels of inter-morphotype sequence divergence ranged from 4.5 to 6%, while the intra-morphotype divergence within the spinose morphotype of *D. tuberspinifera* ranged from 0.4% to 1.4%. The ITS trees showed two clades of *D. tuberspinifera* morphotypes where spinose form a single clade excluding all spineless sequences, which form an unresolved cluster. This suggests the possibility

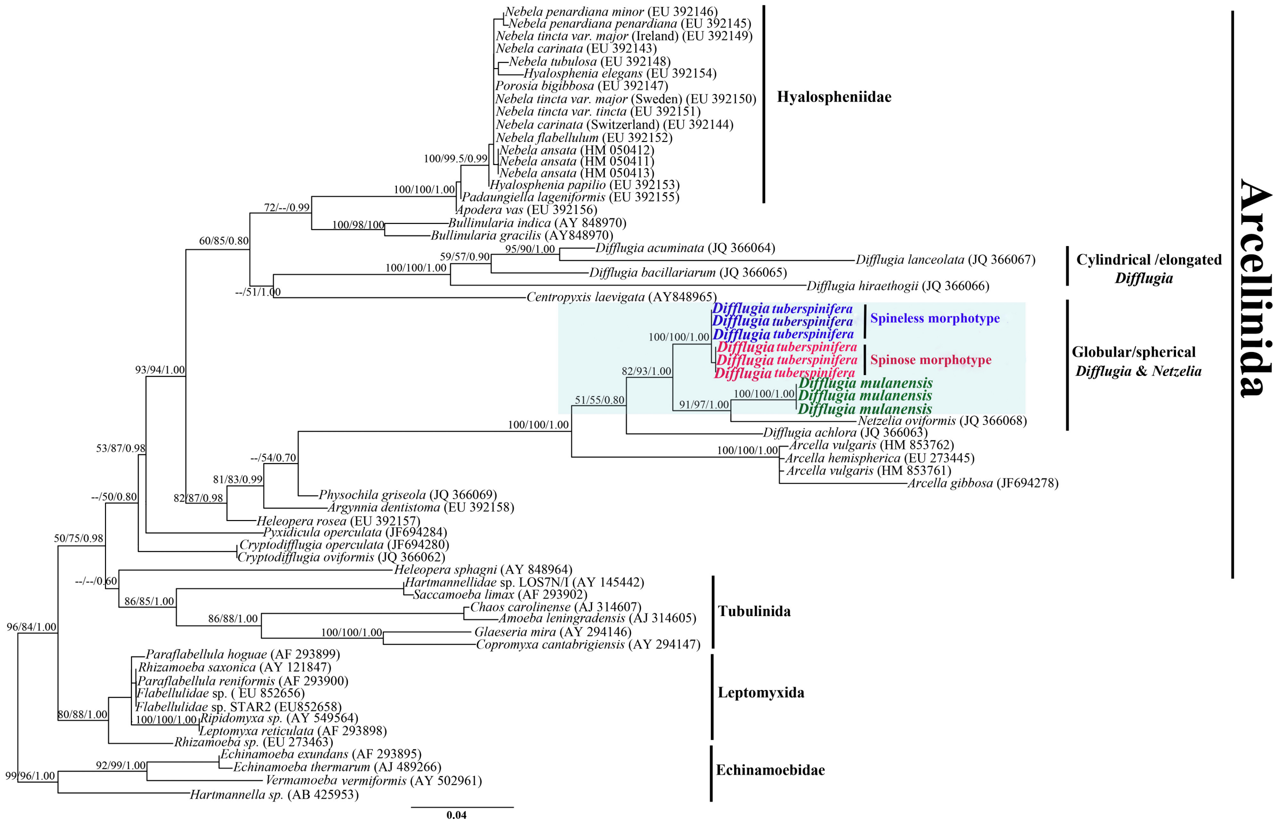

Figure 2. Molecular phylogenetic tree based on SSU rRNA gene sequences illustrating the phylogenetic position of Asian *Difflugia* spp. (*Difflugia tuberspinifera* the spined and the spineless morphospecies and *D. mulanensis*). The tree is rooted with Echinamoebidae. The tree was derived by Bayesian inference using MrBayes, and an identical topology was obtained by maximum likelihood analysis. Numbers at the nodes represent Bootstrap values, Expected-Likelihood Weights edge support (approximate bootstrap) and Bayesian inference posterior probabilities respectively. The scale bar indicates 0.04 substitutions/site.

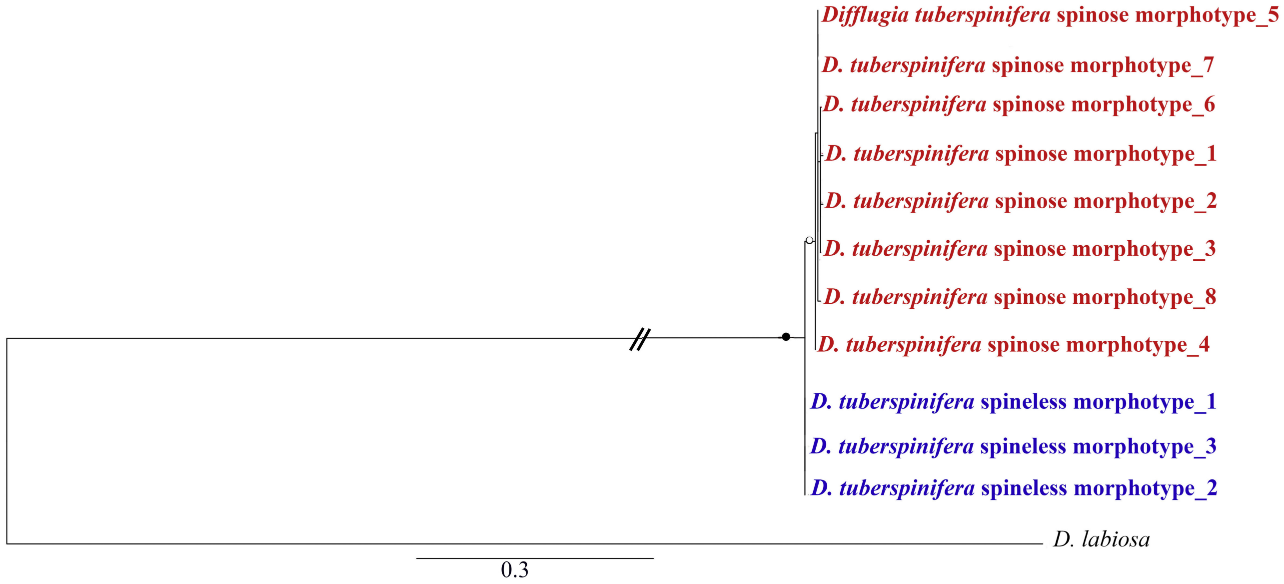

Figure 3. Molecular phylogenetic tree based on ITS sequences of the spinose and spineless morphotypes of *D. tuberspinifera*. The tree is rooted with *D. labiosa*. The tree was derived by Bayesian inference using MrBayes, and an identical topology was obtained by maximum likelihood analysis. Black circle represents high bootstrap support. The main clade trimmed for display purposes. The scale bar indicates 0.3 substitutions/site.

that the spinose clade was derived from spineless morphs (Fig. 3).

Discussion

Phylogenetic Position of *Difflugia tuberspinifera* and *D. mulanensis*

Both spinose and spineless morphotypes of *D. tuberspinifera* are included within a clade (called clade B in Gomaa et al. 2012) that also contains *Netzelia oviformis* and other globose taxa such as *Difflugia achlora* and the newly barcoded species *D. mulanensis*. This clade also includes *Lesquereusia modesta* as determined with other genes (Gomaa et al. 2012; Lahr et al. 2013). There is also evi-dence that species with a more elongated shell may belong in this group, such as *Difflugia bryophila* (Lahr et al. 2013). More data are required to deter-mine if a globose shape can be used as a deep phylogenetic criterion. If this is the case, then the elongated species *D. bryophila* would be an excep-tion. The alternative explanation would be that general shape can change rapidly within this major group of Amoebozoa.

Further, our results suggest that the presence or absence of an apertural collar or necklace (a small raised rim of organic layer surrounding the shell aperture) might constitute a reliable criterion for phylogenetic relationships for some arcellinid genera (*Arcella*, *Difflugia* and *Netzelia*). One possibility is that this character may relate to a key function such as cell division and the ontogenesis of a daughter cell (Ogden and Meisterfeld 1989). So far, five morpho-taxa with a collar or necklace (*Net-zelia oviformis, D. achlora, D. mulanensis* and both morphotypes of *D. tuberspinifera*) branch together robustly in the Arcellinida tree (Fig. 2). *Difflugia tuberspinifera* has a circular aperture with a distinct collar, *D. mulanensis* has a widely projecting aper-tural collar, and *Netzelia oviformis* and *D. achlora* both have a thick organic rim or necklace around the aperture (Ogden and Hedley 1980; Ogden and Meisterfeld 1989; Yang et al. 2004, 2005; Yu et al. 2014). Furthermore, both *Arcella vulgaris* and *A. hemisphaerica*, which lie within the same clade, are also characterized by a “necklace” because their aperture is circular and bordered by a small lip (Meisterfeld 2002; Ogden and Hedley 1980). In contrast, none of the four pyriform-shaped *Difflugia* species have any distinct collar or necklace.

Recently, the new genus *Mediolus* has been established by Patterson (2014) in order to accommodate members of genus *Difflugia* that branched in clade B, thus solving the problem of paraphyly (Gomaa et al. 2012). *D. corona* has been given as the type species for the new genus. This genus is defined by its distinctive tooth-like inward oriented apertural crenulations and tests generally characterized by a variable number of hollow basal spines (Patterson 2014), which are characters that are shared with *D. tuberspinifera*. However, until a SSU rRNA gene sequence from *Mediolus* (*Difflugia*) *corona* is made available, we keep the two morphotypes of *D. tuberspinifera* provisionally within *Difflugia*.

Genetic Variation Between the Two Morphotypes of *Difflugia tuberspinifera*

Our data show that the two *D. tuberspinifera* morphotypes are genetically distinct, but very closely related. The insertion of a class I intron, as seen in the ribosomal operon of *D. tuberspinifera*, is common among Arcellinida. The appearance and disappearance of these elements has been documented within a single species complex in Arcellinida (Lara et al. 2008). Our phylogenetic data analyses based on the ITS sequences clearly showed that all inter-specific variations were greater than intra-specific variations, and a clear gap existed between both *D. tuberspinifera* morphotypes. The fact that the ITS sequences of the spinose morphs form a clade, whereas the spineless morphs do not, suggests that the spinose morphotypes might have evolved from spineless ancestors.

Our results demonstrate that the ITS region provides much better resolution than the SSU rRNA gene for discriminating closely related species within genus *Difflugia* (Fig. 3) and potentially can be used as a genetic barcoding marker for assessing the diversity and inferring the phylogenetic relation-ships within Arcellinida.

Previous work has shown that spines can appear quickly in evolution, for instance as a result of preda-tion pressure. For example, in planktonic organisms such as *Daphnia*, and monogonont rotifers (e.g. genus *Keratella*), spines have been shown to have a protective function (Marinone and Zagarese 1998; Riessen 2012). Interestingly, the development of spines in cladocerans originally arose as a prod-uct of phenotypic plasticity in the presence of predators (Lüning 1992). A similar mechanism has been documented in protists, specifically in a vari-ety of ciliates (Wicklow 1997). It was shown that the spinose morphotype of *D. tuberspinifera* has a wider distribution than the spineless morphotype in China (Yu et al. 2014), a remarkable fact if the spinose morphotype was derived from a spineless ancestor as we suggest here. Indeed, high popu-lation densities of spinose *D. tuberspinifera* were observed in many artificial reservoirs that were con-structed after the Second World War (Liu et al. 2010). This may indicate that the spinose mor-photype of *D. tuberspinifera* has a competitive advantage in these reservoirs and is more adapted to local ecosystem conditions. Future studies are needed to investigate the possible role of environmental selective pressure (i.e. predation, food sources) on the differentiation of both forms of *D. tuberspinifera.*

Our study shows that both *D. tuberspinifera* morphotypes are genetically closely related, yet nevertheless distinct. Very little is currently known about the extent and structure of genetic diversity within and among populations of Arcellinida in general and genus *Difflugia* in particular. Therefore, a more comprehensive sampling and the analysis of additional genetic markers are necessary to ascertain if the two morphotypes of *D. tuberspinifera* indeed represent two different species and what the biological relevance of such a difference might be.

Methods

Sample collection and documentation: The two morphospecies of *Difflugia tuberspinifera* (i.e. spinose and spineless morphotypes) were collected from Hubian Reservoir, Xiamen, southeast China (24°30′N, 118°10′E) in September and October 2010; *D. mulanensis* was collected from Tingxi Reservoir, Xiamen, southeast China (24°48′N, 118°08′E) in September and October 2010, while *D. labiosa* was collected from Lake Pancharevo, Sofia, Bulgaria (42°35′N, 23°24′E) in May 2010. The day after sampling, the individual cells were identified under inverted microscope and morphologically identical cells from each morphotype were isolated and washed several times with ddH$_2$O. We prepared several independent DNA extrac-tions form each morphotype, each extraction contained from one to twelve cells. Single cell extractions did not work well and this might be due to low DNA concentration or the fact that the opacity of the *Difflugia* shell made it difficult to recognize if the organism was alive or dead. Specimens for scanning elec-tron microscopy (HITACHI S-4800, Japan) observations were prepared following Yang et al. (2004).

DNA extraction, PCR amplification and sequencing: DNA was extracted using the guanidine thiocyanate proto-col (Chomczynski and Sacchi 1987). The SSU rRNA gene sequences of both *D. tuberspinifera* morphotypes and *D. mulanensis* were obtained in two steps. A first amplifica-tion was performed using universal SSU rRNA eukaryotic primers EK555F (AGTCTGGTGCCAGCAGCCGC) or EK 42F (CTCAARGAYTAAGCCATGCA) and EK1498R (CACCTACGGAAACCTTGTTA) in a total volume of 30 μl with an amplification profile consisting of 4 min 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 resulting product served as template for the second amplification using the universal eukaryotic forward primer (Marande et al. 2009) and taxon-specific reverse primers, TuberR (GCATCACAGACCTGTTTTCGCCTCGCG) for both spinose and spineless morphotypes of *D. tuberspinifera* and MulR (GCATCACAGAC-CTGTTTTCGCCTCA) for *D. mulanensis*. Amplification was carried out in a total volume of 30 μl with an amplification pro-file consisting of 4 min 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 internal transcribed

Table 1. List of sequenced taxa and their GenBank accession numbers in this study.

Taxon	**GenBank accession number**	
	SSU rRNA	ITS
Difflugia tuberspinifera spinose morphotype_1	KM975699	KM975684
D. tuberspinifera spinose morphotype_2	KM975700	KM975685
D. tuberspinifera spinose morphotype_3	KM975701	KM975686
D. tuberspinifera spinose morphotype_4	-	KM975687
D. tuberspinifera spinose morphotype_5	-	KM975688
D. tuberspinifera spinose morphotype_6	-	KM975689
D. tuberspinifera spinose morphotype_7	-	KM975690
D. tuberspinifera spinose morphotype_8	-	KM975691
D. tuberspinifera spineless morphotype_1	KM975702	KM975692
D. tuberspinifera spineless morphotype_2	KM975703	KM975693
D. tuberspinifera spineless morphotype_3	KM975704	KM975694
*D. mulanensis*_1	KM975696	-
*D. mulanensis*_2	KM975697	-
*D. mulanensis*_3	KM975698	-
D. labiosa	-	KM975695

spacer (ITS1, 5.8S and ITS2) sequences of the *D. tuberspinifera* the spinose and the spineless morphotypes and *D. labiosa*, were obtained in two steps. A first amplification was performed using universal SSU rRNA eukaryotic primer EK 42F (CTCAARGAYTAAGCCATGCA) and universal LSU rRNA eukaryotic primer 803R (ACTTCGGAGGGAACCAGCTA) or ITS4R (TCCTCCGCTTATTGATATGC) in a total volume of 30 μl with amplification profile consisting of (4 min at 95 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °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 taxon-specific forward primer SSUTUBERF (TGTACACACCGCCCGTCGCT) and universal eukaryotic LSU reverse primers 803R or ITS4R, (Marande et al. 2009) in a total volume of 30 μl with amplification profile consisting of 4 min 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. Extractions that contained five or more cells were amplified two to four times.

The PCR products were screened by gel electrophoresis and amplification products 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 the accession numbers given in Table 1.

Alignment and phylogenetic analysis: The SSU rRNA gene and the ITS sequences were aligned manually using the BioEdit software (Hall 1999). Introns, insertions and variable regions in the SSU rRNA alignment that could not be aligned unambiguously were removed from the phylogenetic analysis. The SSU rRNA phylogenetic analysis data set contained 55 amoeba taxa including 37 Arcellinida, 6 Tubulinida, 8 Leptomyxida, and 4 Echinamoebidae that were used as outgroups; a total of 700 characters were kept for phylogenetic anaylsis. For ITS analysis, we used *D. tuberspinifera* from both morphotypes and *D. labiosa* as outgroup. *D. labiosa* is a closely related species but morphologically and genetically distinct from both *D. tuberspinifera* morphotypes.

Maximum likelihood trees were built using the RaxML version 7.2.8 algorithm (Stamatakis et al. 2005) as proposed on the Black Box portal (http://phylobench.vital-it.ch/raxml-bb/) using the GTR+Γ+I model. The reliability 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 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). Bayesian inference was per-formed using the software MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). With two independent sets of four simulta-neous MCMC chains, for 500,000 generations until standard deviation of split frequencies fell below 0.01 as recommended by the manual. Results were saved every 1,000th genera-tion. A burn in of 25% was discarded before summing the results. Trees were viewed using FigTree in the BEAST package (http://tree.bio.ed.ac.uk/software/figtree/). The percent identity between sequences was calculated using the program BioEdit (Sequence Identity Matrix) (Hall 1999).

Acknowledgements

This study was supported by the National Basic Research Program of China (2012CB956103), the National Natural Science Foundation of China (31172114 and 30800097), the Natural Science Foundation for Distinguished Young Scholars of Fujian Province (2012J06009), the International Science and Technology Cooperation Program of China (2011DFB91710), and Swiss NSF grant n° PZ00P2_122042 (Ambizione fellowship for EL). We thank Sandra Baldauf and anonymous reviewers for constructive comments on the manuscript.

References

Chardez D (1974) Note sur la Morphologie des Thécamoebiens. 8 p

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

Civetta AS, Singh RS (1999) Broad-sense sexual selection, sex gene pool evolution, and speciation. Genome **42**:1033–1041

Coyne JA, Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA, USA, 545 p

Cuvelier ML, Ortiz A, Kim E, Moehlig H, Richardson DE, Heidelberg JF, Archibald JM, Worden AZ (2008) Widespread distribution of a unique marine protistan lineage. Environ Microbiol **10**:1621–1634

Fenchel T, Finlay B (2006) The diversity of microbes: resurgence of the phenotype. Philos Trans R Soc B **361**:1965-1973

Fiz-Palacios O, Romeralo M, Ahmadzadeh A, Weststrand S, Ahlberg PE, et al. (2013) Did terrestrial diversification of amoebas (Amoebozoa) occur in synchrony with land plants? PLoS ONE **8(9)**:e74374

Foissner W, Strüder-Kypke M, van der Staay G, Moon-van der Staay S, Hackstein J (2003) Endemic ciliates (Protozoa, Ciliophora) from tank bromeliads (Bromeliaceae): a combined morphological, molecular, and ecological study. Eur J Protistol **39**:365–372

Gomaa F, Todorov M, Heger TJ, Mitchell EAD, Lara E (2012) SSU rRNA phylogeny of Arcellinida (Amoebozoa) reveals that the largest Arcellinid genus, *Difflugia* Leclerc 1815, is not mono-phyletic. Protist **163**:389–399

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

Han BP, Wang T, Lin QQ, Dumont HJ (2008) Carnivory and active hunting by the planktonic testate amoebae *Difflugia tuberspinifera*. Hydrobiologia **596**:197–201

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17**:754–755

Jobb G, Von Haeseler A, Strimmer K (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol **28**:4–18

Lahr DJ, Lopes SG (2006) Morphology, biometry, ecology and biogeography of five species of *Difflugia* Leclerc, 1815 (Arcellinida: Difflugiidae), from Tiete River, Brazil. Acta Protozool **45**:77–90

Lahr DJ, Bergmann PJ, Lopes SG (2008) Taxonomic identity in microbial eukaryotes: A practical approach using the testate amoeba *Centropyxis* to resolve conflicts between old and new taxonomic descriptions. J Eukaryot Microbiol **55**:409–416

Lahr DJ, Grant JR, Katz LA (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

Lahr DJ, Grant J, Nguyen T, Lin JH, Katz LA (2011a) Comprehensive phylogenetic reconstruction of amoebozoa based on concatenated analyses of SSU-rDNA and actin genes. PLoS One **6**:e22780

Lahr DJG, Wegener PL, Mitchell EAD, Katz LA, Lara E (2011b) The chastity of amoebae: re-evaluating evidence for sex in amoeboid organisms. Philos Trans R Soc B **278**:2081–2090

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

Lara E, Mitchell EAD, Moreira D, López García P (2011) Highly diverse and seasonally dynamic protist community in a pristine peat bog. Protist **162**:14–32

Liu LM, Yang J, Zhang WJ, Yu Z (2010) Morphometric analysis of six natural populations of *Difflugia tuberspinifera*. Zool Res **31**:435–443

Logares R, Daugbjerg N, Boltovskoy A, Kremp A, Laybourn-Parry J, Rengefors K (2008) Recent evolution-ary diversification of a protist lineage. Environ Microbiol **10**:1231–1243

Logares R, Rengefors K, Kremp A, Shalchian-Tabrizi A, Boltovskoy A, Tengs T, Shurtleff A, Klaveness D (2007) Phenotypically different microalgal morphospecies with identical ribosomal DNA: A case of rapid adaptive evolution? Microb Ecol **53**:549–561

López-García P, Rodríguez-Valera F, Pedrós-Alió C, Moreira D (2001) Unexpected diversity of small eukaryotes indeep-sea Antarctic plankton. Nature **409**:603–607

Lüning J (1992) Phenotypic plasticity of *Daphnia pulex* in the presence of invertebrate predators: morphological and life history responses. Oecologia **92**:383–390

Marinone MC, Zagarese HE (1998) A field and laboratory study on factors affecting polymorphism in the rotifer *Keratella tropica*. Oecologia **86**:372–377

Marande W, López-García P, Moreira D (2009) Eukaryotic diversity and phylogeny using small- and large-subunit ribosomal RNA genes from environmental samples. Environ Microbiol **12**:3179–3188

Medioli FS, Scott DB, Abbott BH (1987) A case study of protozoan intraclonal variability: Taxonomic implications. J Foramin Res **17**:28–47

Meisterfeld R (2002) Order Arcellinida Kent, 1880. In Lee JJ, Leedale GF, Bradbury P (eds) The Illustrated Guide to the Protozoa. vol 2, Second edition, Society of Protozoologists, Lawrence, Kansas, USA, pp 827–860

Nikolaev SI, Mitchell EAD, Petrov NB, Berney C, Fahrni J, Pawlowski J (2005) The testate lobose amoebae (order Arcellinida Kent, 1880) finally find their home within Amoebozoa. Protist **156**:191–202

Nikolaev SI, Berney C, Fahrni J, Bolivar I, Polet S, Mylnikov AP, Aleshin VV, Petrov NB, Pawlowski J (2004) The twilight of Heliozoa and rise of Rhizaria: a molecular revolution in the phylogeny of amoeboid protists. Proc Nati Acad Sci USA **101**:8066–8071

Ogden CG (1983) Observations on the systematics of the genus *Difflugia* in Britain (Rhizopoda, Protozoa). Bull Br Mus Nat Hist Zool **44**:1–73

Ogden CG, Hedley RH (1980) An Atlas of Freshwater Testate Amoebae. Oxford University Press, London

Ogden CG, 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 RT (2014) *Mediolus*, a new genus of Arcellacea (Tes-tate Lobose Amoebae). Palaeontol Electron **17**:28A

Porter SM, Meisterfeld R, Knoll AH (2003) Vase-shaped microfossils from the neoproterozoic Chuar group, grand canyon: A classification guided by modern testate amoebae. J Paleontol **77**:409–429

Riessen HP (2012) Costs of predator-induced morphological defences in *Daphnia*. Freshwater Biol **57**:1422–1433

Saez AG, Probert I, Geisen M, Quinn P, Young JR, Medlin LK (2003) Pseudo-cryptic speciation in coccolithophores. Proc Natl Acad Sci USA **100**:7163–7168

Schönborn W (1962) Über Planktismus und Zyklomorphose bei *Difflugia limnetica* (LEVANDER) PENARD. Limnologica **1**:21–34

Stamatakis A, Ludwig T, Meier H (2005) RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics **21**:456–463

Steciow MM, Lara E, Pillonel A, Pelizza SA, Lestani EA, Rossi GC, Belbahri L (2013) Incipient loss of flagella in the genus *Geolegnia*: the emergence of a new clade within Leptolegnia? IMA Fungus **4**:196–175

Strimmer K, Rambaut A (2002) Inferring confidence sets of possibly misspecified gene trees. Proc Roy Soc Lond B **269**:137–142

Todorov M, Golemansky V (2007) Morphological variability of *Difflugia urceolata* CARTER, 1864 (Testacealobosia: Difflugi-idae) 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

Wanner M (1999) A review on the variability of testate amoebae: Methodological approaches, environmental influences and taxonomical implications. Acta Protozool **38**:15–29

Wicklow BJ (1997) Signal-induced defensive phenotypic changes in ciliated protists: Morphological and ecological implications for predator and prey. J Eukaryot Microbiol **44**: 176–188

Yang J, Beyens L, Shen YF, Feng WS (2004) Redescription of *Difflugia tuberspinifera* Hu, Shen, Gu et Gong, 1997 (Protozoa: Rhizopoda: Arcellinida: Difflugiidae) from China. Acta Protozool **43**:281–289

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

Yu Z, Zhang WJ, Liu LM, Yang J (2014) Evidence for two different morphotypes of *Difflugia tuberspinifera* from China. Eur J Protistol **50**:205–211