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Molecular Phylogeny and Taxonomy of Testate Amoebae (Protist) and Host-Symbiont Evolutionary Relationships within Mixotrophic Taxa

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Neuchâtel, le 30 avril 2013

Le Doyen, Prof. P. Kropf

“Travel through the earth and find how I originated creation”

(El Ainkabout, 20 Quran 610CE)

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Abstract

Molecular phylogenetic studies have considerably advanced our understanding of the relationships among eukaryotes. In recent classification schemes, amoeboid protists appeared scattered in more than 30 lineages within Amoebozoa, Rhizaria, Stramenopiles, Opisthokonta, and Excavata. Amongst these, some branches tended to develop a test or shell, often ornamented and conspicuous, which has been used for more than 150 years as a diagnostic character to describe more than 2000 species. Testate amoebae are characterized by lobose or filose pseudopodia and one chamber shell that can be agglutinated, proteinaceous, calcareous or siliceous. The acquisition of the shell happened several times independently in the course of evolution. Furthermore, and in spite of the long taxonomic tradition in testate amoebae research, the relationships between the different taxa remained largely unresolved, some genera remaining still without known phylogenetic affiliation.

In this thesis, we aimed at constructing a reliable phylogeny of the largest testate amoebae order, the Arcellinida, using SSU rRNA gene sequences and scanning electron microscopy analyses (chapters 2 and 5). Our results revealed drastic contradictions with traditional taxonomy. Genus *Difflugia*, the largest Arcellinid genus appeared not monophyletic, and divided in two major and distantly related clades that grouped respectively the elongated/pyriform and the globular species. Genus *Netzelia* was phylogenetically very closely related to the globular *Difflugia* despite the inconsistencies in their shell structure.

In addition, Arcellinida tended to show an important morphological conservatism, and closely related morphologies can possibly hide important genetic

distances. We also demonstrated that fast morphological evolution could also be possible in this group. *Difflugia tuberspinifera*, an Asian endemic species had two morphotypes (spiny and spineless) which shared highly similar SSU rRNA gene sequences (99.8%) and identical introns and insertions, but could be nevertheless discriminated on the base of their sequences. This result suggested a recent morphological evolution, presumably due to some differing ecological factors that still need to be clarified.

We determined also the phylogenetic position of two well known *incertae sedis* genera of family Amphitremida, *Amphitrema* and *Archerella* (chapter 3), which appeared surprisingly to be related to Labyrinthulomycetes (Stramenopiles), thus forming a new clade of testate amoebae independent from others (i.e Amoebozoa, Rhizaria). This study also illustrated that accurate taxonomy and phylogeny of protists in general is of crucial important for understanding the evolution and diversity of eukaryotes.

Testate amoebae have been also often found in association with some photosynthetic organisms whose identity remained unknown. We identified the symbionts of four different testate amoeba species using the chloroplastic gene *rbcL* (ribulose-1, 5-diphosphate carboxylase/oxygenase large subunit) as a barcoding gene. The majority of testate amoeba symbionts formed a consistent group with very few sequence diversity that could be reasonably associated to a single species, in spite of the fact that host species were taxonomically distantly related. Interestingly, testate amoebae *Chlorella* symbionts were very closely related to *Chlorella variabilis* and to *Paramecium bursaria Chlorella* symbionts. In the light of these results, we proposed a general evolutionary scenario for association between heterotrophic hosts and their photosynthetic symbionts.

Overall, my thesis illustrated that the reliable phylogeny of testate amoebae based on molecular and morphological approaches is not only essential prerequisite for understanding their evolution, but it also will contribute in resolving debates concerning their diversity and biogeography, and in general will increase their utility as a model group of organisms for applied ecological research.

Keywords: Testate amoebae, Arcellinida, *Difflugia*, Amoebozoa, Rhizaria, Stramenopiles, Protist, symbionts, *Chlorella*, Phylogeny, evolution, classification, SSU rRNA gene, COI gene, *rbcL* gene.

Résumé

Les recherches en phylogénie moléculaire on considérablement avancé notre compréhension des relations entre eucaryotes. Les classifications récentes placent les protistes amoeboides dans plus de 30 lignées au sein des Amoebozoa, Rhizaria, Stramenopiles, Opisthokonta, et Excavata. Parmi celles-ci, certaines branches ont développé des thèques ou coquilles, souvent ornementées et caractéristiques qui ont été utilisées depuis plus de 150 ans comme caractère diagnostique pour décrire plus de 2000 espèces. Les thécamoebiens sont caractérisés par des pseudopodes lobés ou filamenteux et une thèque à une chambre pouvant être agglutinée, protéinique, calcaire ou siliceuse. L'acquisition de la thèque s'est faite plusieurs fois de manière indépendante au cours de l'évolution. De plus, et malgré la logue tradition de recherche en taxonomie sur les thécamoebiens, les relations entre les différents taxons demeurent largement non-résolue, l'affiliation phylogénétique de certains genres restant inconnue.

Le but de cette thèse était de construire une phylogénie fiable du plus grand ordre d'amibes, les Arcellinida, en utilisant des séquences du gène SSU rRNA et des analyses par microscopie électronique (chapitres 2 et 5). Les résultats révèlent des contradictions drastiques avec la taxonomie traditionnelle. Le genre *Difflugia*, le plus grand genre des Arcellinida, n'est pas monophylétique et est divisé en deux clades bien distincts regroupant respectivement les espèces allongées/pyriformes et les espèces globulaires. Le genre *Netzelia* est phylogénétiquement proche des *Difflugia* globulaires malgré les différences de structures de leur thèque.

Par ailleurs, les Arcellinida démontrent un conservatisme morphologique marqué ; les types morphologiques similaires correspondant possiblement à des taxons génétiquement très distants. Nous démontrons la possibilité d'une évolution morphologique rapide an sein de ce groupe. *Difflugia tuberspinifera*, une espèce endémique d'Asie possède deux morpho-types (avec et sans cornes) possédant des séquences similaire du gène SSU rRNA gene (99.8%) et des introns et insertions identiques, mais mouvant toutefois être discriminés sur la base de leur séquences. Ceci suggère une évolution morphologique récente, possiblement liée à des facteurs écologiques à déterminer.

Nous avons déterminé la position phylogénétique des deux genres *incertae sedis* bien connus de la famille des Amphitrematidae, *Amphitrema* et *Archerella* (chapitre 3), qui de manière surprenante sont apparentés Labyrinthulomycetes (Stramenopiles), formant ainsi un nouveau clade de thécamoebiens indépendants des autres (c.à.d. Amoebozoa & Rhizaria). Cette édute illustre également que la taxonomie et la phylogénie des protistes en général est d'une importance cruciale pour comprendre l'évolution de la diversité des eucaryotes.

Les thécamoebiens forment souvent des associations avec les organismes photosynthétiques dont l'identité demeure toutefois inconnue. Nous avons identifié les symbiontes de quatre thécamoebiens différents sur la base du gène chloroplastique rbcL (ribulose-1, 5-diphosphate carboxylase/oxygénase grande sub-unité) utilisé comme gène de barcoding. La majorité des symbiontes de thécamoebiens ont pu être raisonnablement associés à une seule espèce, malgré le fait que leurs hôtes étaient

taxonomiquement très distants. Fait intéressant, les Chlorelles symbiontes des thécamoebiens étaient très proches de *Chlorella variabilis* ainsi que des symbiontes de *Paramecium bursaria*. A la lumière de ces résultats, nous proposons un scénario d'évolution de l'association entre hôtes hétérotrophes et leur symbiontes photosynthétiques.

De manière générale, ma thèse illustre qu'une phylogénie fiable des thécamobiens basée sur les approches morphologiques et moléculaires est non-seulement un prérequis essentiel pour comprendre leur évolution, mais contribuera aussi à résoudre des débats concernant leur diversité et leur biogéographie, et en augmentera en général leur utilisé comme groupe modèle d'organismes pour les recherches en écologie appliquée.

Mots-clés: Thécamoebiens, Arcellinida, *Difflugia*, Amoebozoa, Rhizaria, Stramenopiles, protiste, symbiontes, *Chlorella*, phylogénie, évolution, classification, gène du SSU rRNA, gène COI, gène *rbcL*.

1

Introduction

1. Phylogeny and diversity of eukaryotes, amoeboid protists and testate amoebae

1.1. The eukaryotes phylogeny and diversity

Eukaryotes are one of the three domains of life, along with Bacteria and Archaea (Baldauf, 2008; Woese et al., 1990). Although most of our understanding of eukaryote biology is related to the study of animals, land plants, and fungi, these three lineages only represent a small part of the full extension of eukaryotic diversity (Patterson, 1999; Tekle et al., 2009, Katz et al., 2012). Indeed, the majority of eukaryotes are unicellular organisms that are free living, photosynthetic, pathogenic or symbiotic, and usually referred to as protists. Molecular phylogenetic studies demonstrated that protists are the major constituents of the tree of eukaryotes and that they are present among the major currently recognized supergroups in the tree of eukaryotes: Opisthokonta, Amoebozoa, Plantae, Rhizaria, Excavata, Stramenopiles and Alveolata (Baldauf, 2003; Keeling et al., 2005) (Figure 1.1). However, the phylogenetic relationships among these major groups are still poorly resolved, and the stability of some of them is still questionable. This is mainly due to the fact that the true diversity of eukaryotes remains largely unknown and several groups remain unexplored by molecular approaches (Bass and Cavalier-Smith, 2004; Parfrey et al., 2006).

Recent molecular phylogenetic studies unveiled novel major groups of eukaryotes that provided a fundamental change in our understanding of eukaryotes diversity, phylogeny and evolutionary history. A study by Burki et al. (2008) using a multigene data set and over 65 species and 135 genes strongly suggested that Rhizaria, stramenopiles and alveolates form a monophyletic megagroup (SAR assemblage) within the tree of eukaryotes (Burki et al., 2008). In addition, a novel eukaryotic

supergroup, the Hacrobia, that comprises haptophytes, cryptophytes, katablepharids, and telonemids, has recently been proposed (Okamoto et al., 2009). However, the phylogenetic relationships within the Hacrobia is still questionable (Okamoto et al., 2009).

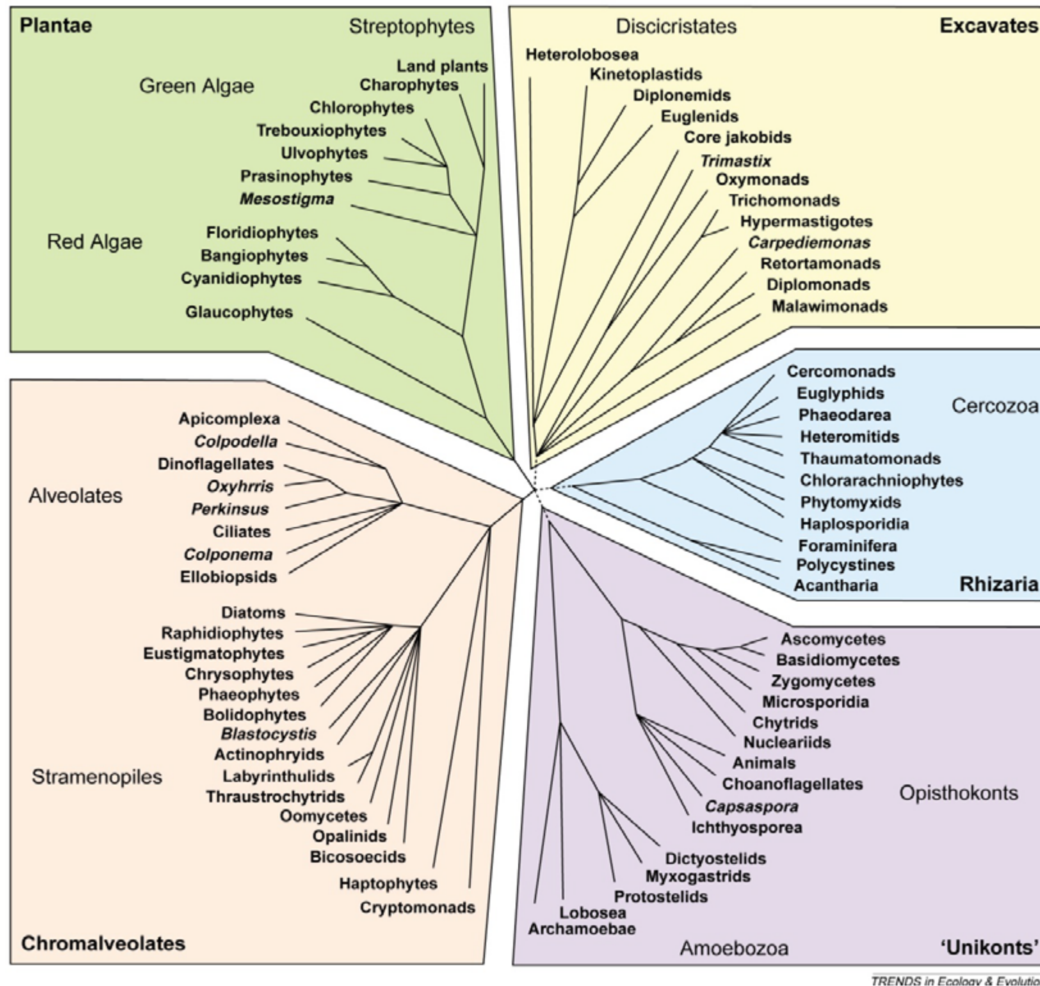

Figure 1.1: A tree of eukaryotes by Keeling et al., (2005), showing the major supergroups, most of the groups are unicellular protists and algae. Note that Rhizaria are now placed together with Alveolata and Stramenopiles in supergroup SAR.

1.2. Phylogenetic position of amoeboid protists

The amoebae or amoeboid protists are considered one of the largest and most diverse assemblages in the tree of eukaryotes (Pawlowski, 2008; Pawlowski and Burki, 2009). Previously, all amoeboid protists were placed within Sarcodina. In the current eukaryotic tree, Sarcodina is a polyphyletic group with Amoebozoa and Rhizaria containing the highest number of species (Adl et al., 2005 ; Cavalier-Smith, 1998; Cavalier-Smith, 2002; Pawlowski and Burki, 2009). Other major eukaryotic clades contain amoeboid protists. For instance, the naked amoeba forms *Vahlkampfia*, *Naegleria* and *Acrasis* belong to Heterolobosea, characterized by the presence of amoeboid stage, and were traditionally considered as Amoebozoa. However, the SSU rRNA and protein sequence data confirmed their phylogenetic position within excavates (Page and & Blanton, 1985; Simpson, 2003). Genus *Nuclearia* (Nucleariids) are filose amoeba traditionally classified within Filosea (i.e. Rhizaria) (Page, 1991). The SSU rRNA and multigene analyses confirmed that Nucleariid amoebae branch within Opisthokonts at the base of fungi (Amaral-Zettler et al., 2001; Steenkamp et al., 2006). It was shown that order Actinophryida (ex-Heliozoa) branch within Stramenopiles (Nikolaev et al., 2004). Class Synchromophyceae includes marine amoeboid algae that have a sessile amoeboid stage and a non-sessile free floating life stage, while order Chrysamoebales includes Chrysophytes that have amoeboid “Rhizopodial” vegetative cells during the greater part of their life cycle. Ribosomal rRNA gene sequences placed both *Chrysamoeba* and *Synchroma* within the Stramenopiles (Cavalier-Smith and Chao, 2006; Patil et al., 2009). Members of family Amphitremidae are currently considered as *incertae sedis* testate amoebae within filosea (Adl et al., 2005).

Other amoeboid eukaryotes still remain “homeless” or have not yet been unambiguously assigned to any major lineage. This includes the Breviatea, a group of free-living amoeboflagelates. Therefore, amoeboid eukaryotes are scattered through all eukaryotic lineages except the plants (Pawlowski, 2008).

1.3. What are the testate amoebae?

Testate amoebae are free living single-celled eukaryotes, highly diverse and abundant in a wide range of habitats such as wetlands, soil, mosses, freshwater, and even marine environments. Testate amoebae are distinguishable from other shelled amoeboid eukaryotes such as foraminifera, radiolarians and heliozoans by 1) their one chambered shell that can be categorized into four main types: agglutinated (species which include extraneous mineral particles in their shell, so-called “xenosomes”), proteinaceous (species with flexible or rigid shell), calcareous or siliceous (secrete their own regular siliceous platelets, so-called “idiosomes”, or produces shell of mixed layers of both xenosomes and idiosomes (Meisterfeld, 2002a; Meisterfeld, 2002b; Wanner, 1999) (Figure 1.2 and 1.3), and 2) their pseudopodia that can be lobose or filose.

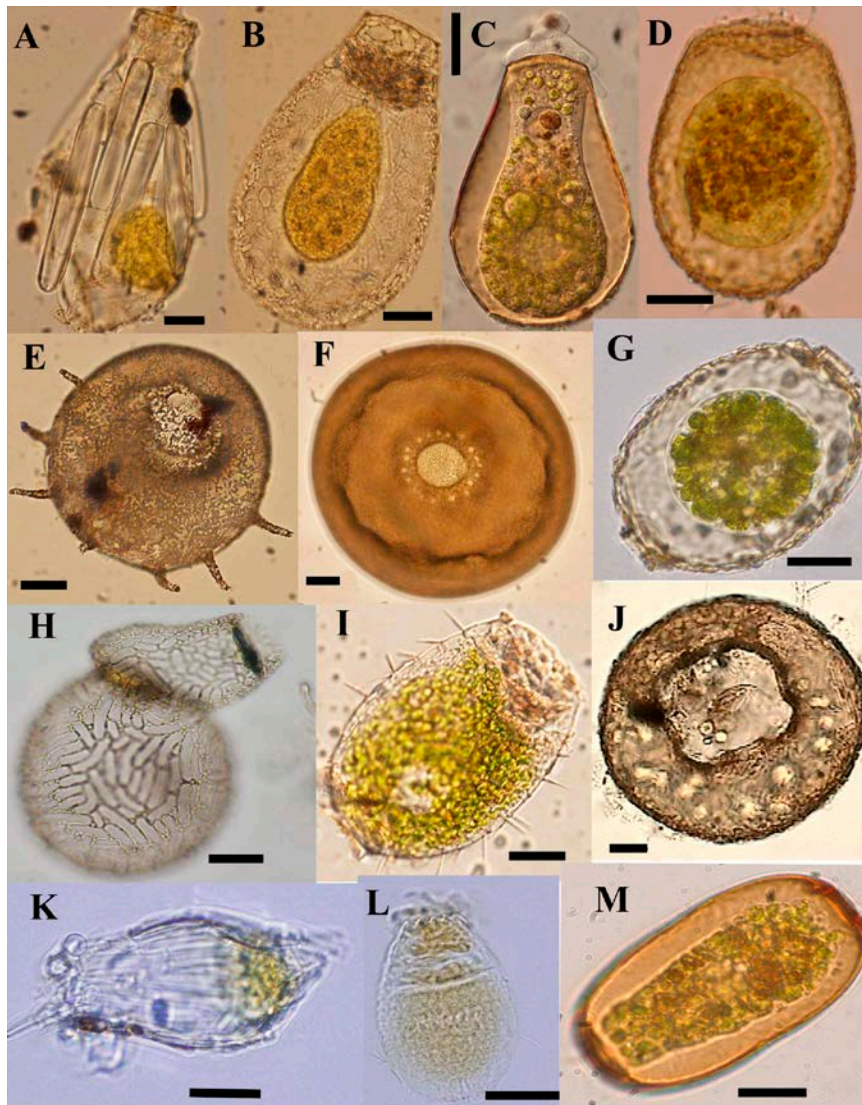

Figure 1.2: Light micrographs of some testate amoebae illustrating their morphological variability. A) *Difflugia bacillifera*, B) *Nebela marginata* C) *Hyalosphenia papilio*, D) *Heleopera sphagni*, E) *Centropyxis aculeata*, F) *Arcella arenaria*, G) *Amphitrema wrightianum*, H) *Lesquereusia epistomium*, I) *Placocista spinosa*, J) *Centropyxis ecornis*, K) *Difflugia bacillariarum*, L) *Euglypha compressa*, M) *Archerella flavum*. Scale bars indicate 20µm. All images by F. Gomaa except (H) E. Mitchell, and (J) M. Lamentowicz.

2. Evolution, phylogeny and physiology of testate amoebae

2.1. Fossil record & phylogeny

Vase-shaped microfossils resembling extant arcellinid testate amoebae have been found in marine deposits, and are considered among the oldest heterotrophic eukaryotes with fossils, dating back to ca. 740 Mya in the Cryogenian period (Porter and Knoll, 2000; Porter et al., 2003). The age of these fossils has been corroborated by a molecular clock study (Berney and Pawlowski, 2006). Molecular phylogenetic studies based on ribosomal RNA and proteins gene sequences revealed that testate amoebae are a polyphyletic assemblage of at least two major groups, the supergroup Amoebozoa comprises Arcellinid testate amoebae (Nikolaev et al., 2005; Gomaa et al., 2012), and supergroup Rhizaria comprises Euglyphida, Chlamydophryidae “Rhizaspididae”, Tectofilosida, and Gromiidae (Bhattacharya et al., 1995; Cavalier-Smith, 1998; Nikolaev et al., 2003; Lara et al., 2007; Howe et al., 2011).

In addition to these groups, some taxa have not yet been placed in the tree of eukaryotes. One of these is family Amphitremidae that are characterized by the presence of a shell with two pseudostomes at the opposite ends of the shell. It includes three genera, *Amphitrema, Archerella* and *Paramphitrema*. The first two genera comprises organisms that possess filamentous anastomosing pseudopodia, and most species harbor endosymbiotic zoochlorellae (Figure 1.2), while *Paramphitrema* lives on marine and freshwater plants and algae, and is characterized by linear pseudopodia. In addition, families Psammonobiotidae (Golemansky, 1979) and Volutellidae (Sudzuki, 1979a), both characterized by filose pseudopodia and organic and/or agglutinated shells. These organisms inhabit the supralittoral zone mostly in marine interstitial and freshwater environments (Sudzuki, 1979b). It has

often been found that eukaryotes with uncertain phylogenetic affinities can represent key lineages, and information on these species can be very important for understanding the eukaryotic evolution (Nikolaev et al., 2004; Yabuki et al., 2010).

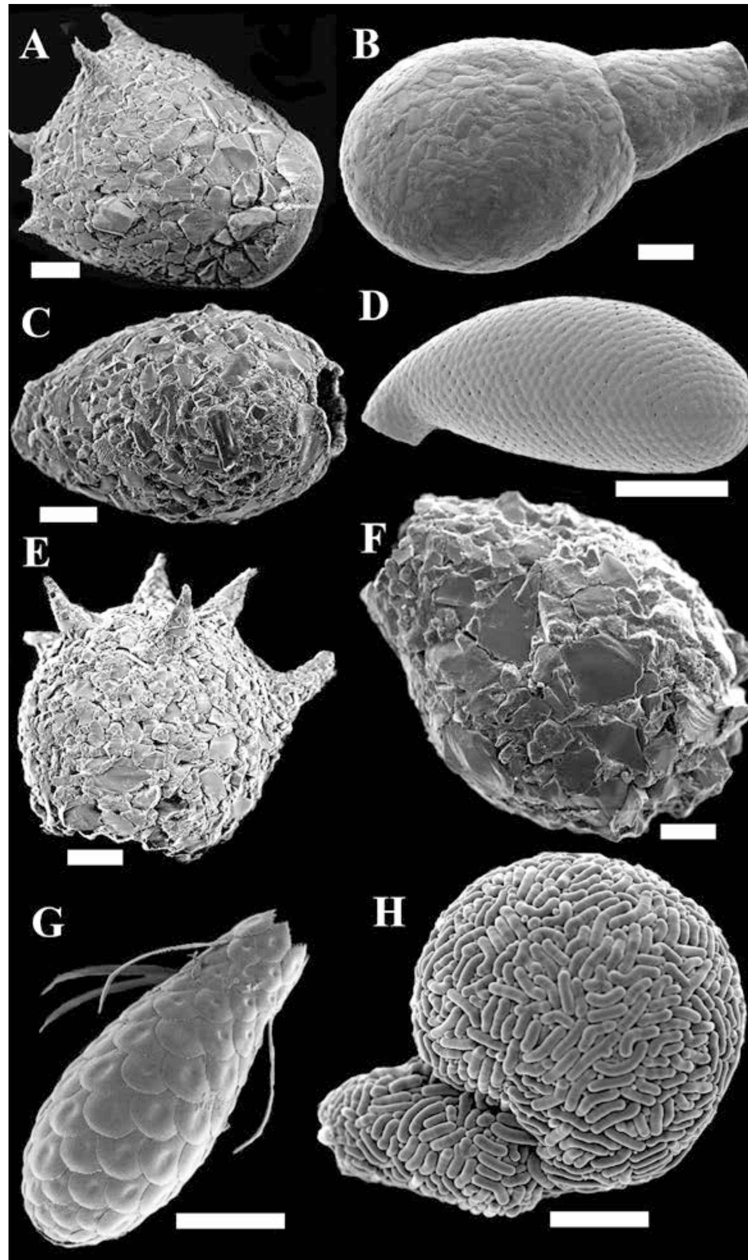

Figure1.3: Scanning electron micrograph of some testate amoebae illustrating the variability of the shell shape and composition. A) *Centropyxis marsupiformis*, B) *Apodera vas*, C) *Difflugia labiosa*, D) *Cyphoderia ampulla*, E) *Difflugia corona*, F) *Zivkovicia compressa*, G) *Euglypha brachiata* H) *Lesquereusia epistomium*. Scale bars indicate 20µm. Images: M. Todorov (A, C, E, F and H) and T. Heger (B, D and G).

2.2. Physiology: the special case of mixotrophy

Many heterotrophic species of protists are capable of building a symbiotic relationship with phototrophic organisms. They can host either viable algal endosymbionts or sequestered plastids (i.e. kleptoplastidy) from their algal prey that remain functional for several days or even months, using by-products of their photosynthesis (Johnson, 2011; Pillet et al., 2011). Such mixotrophic organisms combine the advantages of both phototrophy and heterotrophy (Summerer et al., 2007). Acquired phototrophy is generally more prevalent in nutrient-poor environments, such as bogs, coral reefs and pelagic environment (Margulis and Fester, 1992). The relationship between the host and their associated algal symbionts is considered as a mutualistic relationship. The host usually supplies the algal cells with nitrogen and CO_2. In return, the algae supply the host with photosynthetic products such as maltose and oxygen (Esteban et al., 2010; Summerer et al., 2007). Generally, the mixotrophic individuals have higher growth rate in comparison to the symbiotic-free individuals of the same species (Karakashian, 1963; Stabell et al., 2002). In addition, it has been shown that the symbiotic algae have a photo-protective role for their *Paramecium bursaria* host against UV damage besides their nutritional benefits to their host (Summerer et al., 2009). Recently, the nature of algal symbiosis in protists and invertebrates has attracted considerably scientific interest (Esteban et al., 2010; Summerer et al., 2007; Hoshina and Imamura, 2008; Garcia-Cuetos et al., 2005). However, most of our knowledge about the identity, diversity and host-symbiont phylogenetic relationships concerns marine dinoflagellates of genus *Symbiodinium*, the most prevalent symbiotic algae in Foraminifera, Radiolaria, Acantharea and coral reefs (Garcia-Cuetos et al., 2005; Gast and Caron, 1996 and 2001; Santos et al., 2004). On the freshwater and terrestrial side, the most common association between a

heterotrophic host and a phototrophic symbiont is found in lichens. In addition, the ciliate *Paramecium bursaria* and its chlorophyte symbionts have become a model system for studying host-symbiont specificity and evolutionary relationships (Hoshina and Imamura, 2008; Summerer et al., 2008)

Several testate amoebae species are considered as obligate mixotrophs and are always observed with their green-colored symbionts. These species are distributed among three major eukaryotic clades (1) *Hyalosphenia papilio* and *Heleopera sphagni* (Amoebozoa: Arcellinida) (Nikolaev et al., 2005), 2) *Placocista spinosa* (Rhizaria: Euglyphida) (Bhattacharya 1995, and Cavalier-Smith, 1997), and 3) *Archerella flavum*, *Amphitrema wrightianum* and *Amphitrema stenostoma* (*incertae* sedis). Early experimental field study by Schönborn (1965) showed that *Amphitrema flavum* (= *Archerella*) and *Hyalosphenia papilio* could not survive for long periods in the dark. His study also demonstrated that *Amphitrema flavum* depend entirely on their green symbiotic algae for their nutrition requirements, while *H. papilio* acquired up to 40% of their nutrients from photosynthetic products released by algae and the rest through phagotrophy (Schönborn, 1965). However, to date, the identity of their symbionts and the nature of host-symbiont relationships remain unknown. We believe that two factors have mainly hindered the early research on testate amoeba mixotrophy. The first is the difficultly to establish and maintain cultures for both host and the endosymbionts. The second is the impossibility of identifying species of green algae (*Chlorella*-like) using only morphological criteria. Therefore, we performed the first molecular phylogenetic study based on COI and *rbcL* gene sequences for both host and their endosymbionts respectively. We identified the *in hospite* symbionts in four mixotrophic testate amoebae species. We also assessed the symbiont diversity within a single amoeba cell, and investigated the degree of host-symbiont specificity in

Hyalosphenia papilio. Our study is based on samples collected from *Sphagnum* peatlands (see chapter 4).

3. Testate amoebae in ecological and biogeographical research

3.1. Role of testate amoebae in nutrient cycling

Testate amoebae are mostly phagotrophic organisms, with a few exceptions of mixotrophic (see chapter 4) and one phototrophic species (*Paulinella chromatophora*) (Gilbert et al., 2000; Wilkinson, 2008; Yoon et al., 2006). The phagotrophic testate amoeba species usually graze bacteria, fungi and algae, thus playing an important role in soil nutrient recycling (Wilkinson, 2008). They can also be a food source for other invertebrates such as nematodes, earthworms, collembolans, and predaceous mites (Schroeter, 2001), thus re-entering the classical food chain (Bamforth and Lousier, 1995; Wilkinson and Mitchell, 2010). Some testate amoebae species can predate upon other protozoa and metazoan organisms such as nematodes and rotifers (Yeates and Foissner, 1995; Gilbert et al., 2000). It was also shown that testate amoebae colonized with mutualistic fungi are good source for nutrients particularly nitrogen components for plants in nutrient-poor soil (Figure 1.4) (Vohnîk et al., 2009).

In addition, the testate amoebae with silica rich shells may also form an important part of silica cycle and thus increasing the silica mineralization in soil (Aoki et al., 2007; Wilkinson and Mitchell, 2010). Until recently our knowledge on testate amoebae feeding habits was very limited due to the limitation in the methodologies that used to identify both testate amoebae and their preys (Gilbert et al., 2000; Gilbert et al., 2003). Jassey et al. (2012) succeeded to determine the feeding behavior of some testate amoebae taxa using C and N stable isotope analysis. This study showed that the vertical micro-distribution of the testate amoebae and the abundance of their

identified preys along the *Sphagnum* shoots are probably the main factors influence their feeding behavior (Jassey et al., 2012). However, much remains to be done in order to better assess testate amoebae functional role in the microbial trophic network.

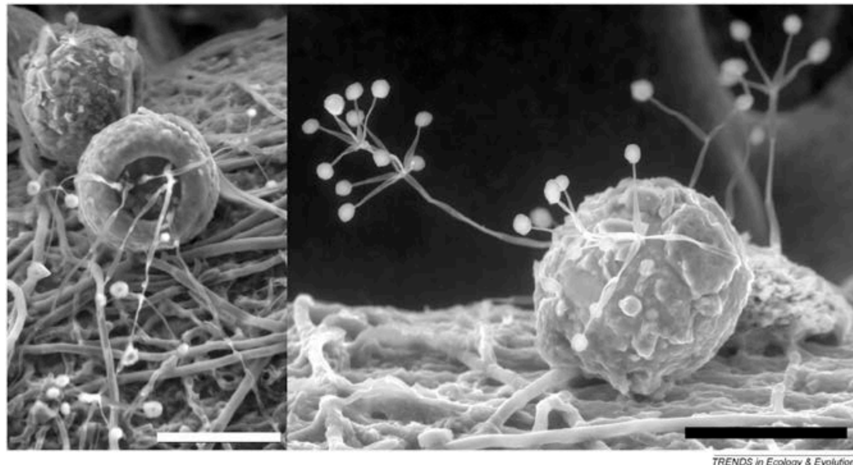

Figure 1.4: *Phryganella acropodia* colonized by fungal hyphae. Laboratory culture by Martin Vohník, Wilkinson (2008).

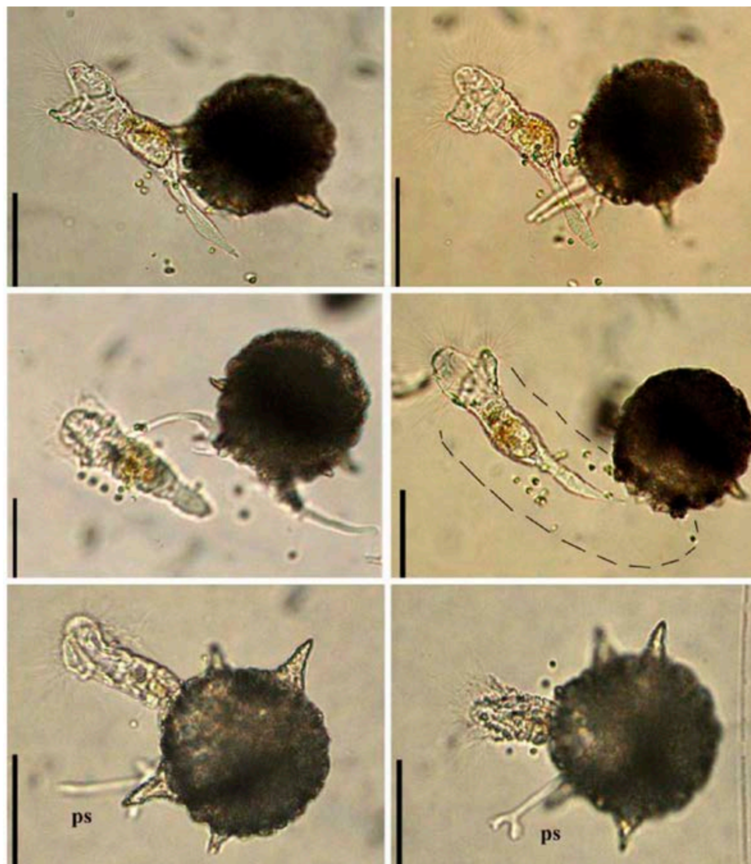

Figure 1.5: Predation phases of *Difflugia tuberspinifera* on a rotifer (Han et al., 2008).

3.2. Bioindicators and biomonitors

Testate amoebae are considered as reliable bioindicators and biomonitors for ecological and palaeoecological studies, in particular as proxies for hydrological change, and therefore for paleoclimate reconstruction in peatlands (Charman and Warner, 1997; Mitchell et al., 2000). Testate amoebae are also very sensitive to other ecological gradients such as water table, dryness, pollutions and human activities (e.g. deforestations or damming) (Booth, 2007; Payne et al., 2012). Thus making them valuables biomonitors for current environmental health (Nguyen-Viet et al., 2008). After death, their shells are very well preserved over time, therefore they are considered as excellent microfossils useful for palaeoenvironmental reconstructions (Charman et al., 2001; Mitchell et al., 2008).

3.3. Testate amoebae as a model to study microbial biogeography

Microorganisms have believed to be cosmopolitan because of their relatively small size, high dispersal rates, and high reproduction rates (Finlay, 2002; Finlay et al., 2004). However, this classical cosmopolitan view or as it was proposed by Beijerinck (1913) that “everything is everywhere, but, the environment selects’ ” has changed during the late of the 20th century. Several studies illustrated that some microbial organisms present a very characteristic morphology that cannot be confused with any others, have restricted geographical distributions, and usually referred to it as “flagship species” (Foissner, 2006; Smith et al., 2008). Some species of testate amoebae are among the most striking examples of microorganisms that present biogeographical patterns in their global distribution. For instance *Apodera vas* and *Alocodera cockayni* and genus *Certesella* are restricted to the south of the Tropic of Cancer desert belt (Smith et al., 2008; Smith and Wilkinson, 2007). Other species are

thought to be endemic to Asia, such as *Difflugia biwae* (Kawamura, 1918) (in China and Japan), *Difflugia tuberspinifera* (Yang et al., 2004) (in China), *Difflugia mulanensis* (Yang et al., 2005) (in China), and *Pentagonia zhangduensis* (Qin et al., 2008) (in China).

3.4. Current limitations to the use of testate amoebae in ecological research

Testate amoebae are a problematic group of protists with respect to their taxonomy and the identification and delimitation of species. This is mainly due to the fact that their taxonomy is largely based on morphological characteristics, such as the shell shape, size and composition, with only a limited contribution from molecular data (Meisterfeld 2002a, b). As a result of this taxonomic confusion and limitations 1) paleoecological studies generally underestimate their diversity and may group together unrelated taxa. This may lead to errors in reconstructed environmental values (see Payne et al., 2011), 2) taxonomic uncertainties also undermine biogeographical studies of testate amoebae (i.e. cosmopolitanism versus endemism) (see Heger et al., 2009; Mitchell and Meisterfeld, 2005), 3) their ecological and functional roles in the environments remains unclear (Gilbert et al., 2000; Gilbert et al., 2003). It was recently discovered that several morphospecies of testate amoebae hide cryptic diversity. These cryptic species might possibly have either restricted or cosmopolitan distributions, as well as different ecological roles in the ecosystem (see Heger et al., 2009; Heger et al., 2010; Heger et al., 2011; Kosakyan et al., 2012). Thus to improve the utility of testate amoebae in different ecological research fields, their taxonomic scheme should be revised based on morphological, ecological and genetic data.

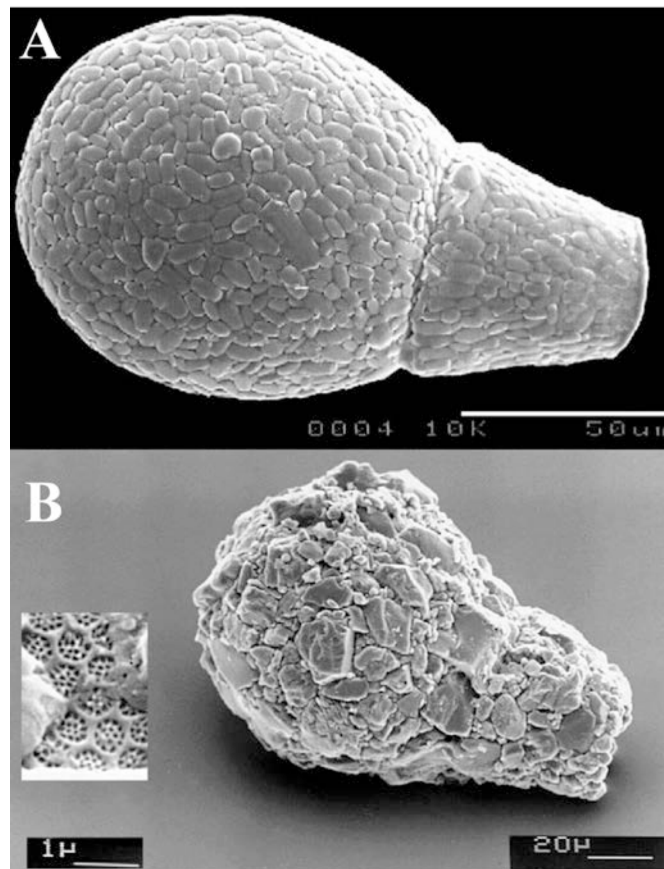

Figure 1.6: Scanning electron micrograph of A) *Apodera vas* and B) *Lagenodifflugia vas*, illustrating that the relative morphological similarity among testate amoebae species blurs the debate on their cosmopolitanism versus local endemism (Mitchell and Meisterfeld, 2005).

4. Arcellinida (Lobosea) testate amoebae

4.1. Arcellinida taxonomy and diversity

Molecular phylogenetic studies have established the phylogenetic position of several testate amoeba taxa in the tree of eukaryotes (Bhattacharya et al., 1995; Cavalier-Smith, 1997; Cavalier-Smith, 1998; Howe et al., 2011; Nikolaev et al., 2005 , Gomaa et al., submitted). However, large phylogenetic gaps remain within these

groups and the proper assessment of their species diversity is lacking (Gomaa et al., 2012; Howe et al., 2011; Kudryavtsev et al., 2009). This is mainly due to the lack of gene sequences on some major taxa. Arcellinid testate amoebae are considered the largest and the most diverse group of testate amoebae containing about three quarters of all known species (Beyens and Meisterfeld, 2001; Meisterfeld, 2002a). Meisterfeld divided arcellinids into three main orders; Arcellinina (characterised by a membranous test; three families), Difflugiina (agglutinated test, twelve families) and Phryganellina (pointed pseudopodia; two families) (Meisterfeld, 2002a). Although about 1100 species have been described in Arcellinida based on morphological criteria (Meisterfeld, 2002a), Adl et al., (2007) estimated that the true diversity of Arcellinida could reach up to 10 000 species.

4.1. Current problems and limitations in Arcellinida taxonomy

The identification and classification of Arcellinida at genus and species level is mainly based on shell dimensions, composition and shape; and the associated morphological features with the shell such as the presence or absence of spines and pores, or with the aperture such as the presence of collar, diaphragm, lobes, and teeth (Beyens and Meisterfeld, 2001; Meisterfeld, 2002a; Wanner, 1999).

Several studies emphasized that the shell morphological features are sometimes not sufficient to warrant species diagnoses particularly in the agglutinated taxa like those in families Difflugiidae and Centropyxidae (Meisterfeld, 2002a; Ogden, 1983; Ogden and Hedley, 1980; Ogden and Meisterfeld, 1989). These species have high diversity in shell composition due to the diverse type of the extraneous material incorporated in the shell matrix; also their basic shell outline might be obscured by the

addition of these extraneous materials (Ogden, 1983). These morphological variations can cause a considerable taxonomic confusion at species or even at genus level in many taxa. Indeed, several authors described new species based on minor variations on shell composition and shape. However, some of these newly described taxa are actually not represent valid species and more likely fall within the natural morphological variability within given species.

There are mounting evidences suggesting that the environmental factors such as moisture, food source, temperature, the availability of extraneous material and pH have a direct influence upon their morphology (Bobrov et al., 2004; Wanner, 1999). Therefore, minor differences in the shell shape, composition could possibly be response by amoeba towards the environmental factors and their combination and probably are not genetically fixed characters.

This interpretation has been supported in several studies for example a biometric data analyses on 32 natural populations of 24 species by Bobrov and Mazei (2004) revealed a significant degree of morphological variability within local population. A morphological, biometrical and ecological study on 2210 *Centropyxis* individuals by Lahr et al., (2008) revealed a continuity in the morphospecies of *C. aculeata* and *C. discoides*, and suggested that both species are actually the same taxon that exhibit a highly morphological polymorphism in shell and aperture shape and number of spines. A review on the morphological variability of testate amoebae by Wanner (1999) emphasized that the effect of ecological factors on the shell morphology should be taken into account, in order to estimate the range of genetic and non-genetic variations within a given taxon.

4.2. The molecular phylogeny of Arcellinida

The application of DNA-based techniques to the study of Arcellinida systematics is relatively recent. A first study by Nikolaev et al. (2005) placed representatives of several Arcellinida genera together as a monophyletic group within the eukaryotic superclass Amoebozoa (Nikolaev et al., 2005). Other molecular studies, based on the SSU rRNA gene, were focused on the phylogeny of particular groups within the Arcellinida, such as the Hyalospheniidae (Lara et al., 2008), and the genera *Spumochlamys* (Kudryavtsev et al., 2009) and *Arcella* (Lahr et al., 2011; Tekle et al., 2008). However, the phylogenetic tree of Arcellinida remains unresolved, because very few species were characterized by molecular methods to date.

The main reasons explaining the limited number of Arcellinida sequences is probably due to the following:

1) The lack of suitable sets of PCR primers that can be generally used for the whole arcellinid group and /or taxon-specific primers. Arcellinids are a very old taxa (Porter and Knoll, 2000) and the genetic distances among taxa are very large, making the design of PCR primers particularly challenging. In addition, contamination is a major problem that scientist encounter when sequencing testate amoebae using general eukaryotes primers set. There are two main source of such contamination A) these microorganisms are free living and are generally isolated from a mixed pool of living and non-living materials such as of soil, organic matters. Other protists some of them quite small even pico-sized can easily be present (unnoticed) in the isolated specimen (i.e. contamination with DNA derived from diverse living material). B) Arcellinida cells are relatively large <300µm, and often host a high number of symbionts and/or

epibionts or even contain undigested protist and metazoan prey. Therefore such contamination can easily be co-amplified in the PCR reaction. Sometimes, these co-amplified eukaryotes can even be closely related to Arcellinida, such as minute lobose naked amoebae.

2) The majority of arcellinid taxa are difficult or even impossible to maintain in cultures.
3) The shell of many Arcellinida, such as members of genera *Difflugia, Trigonopyxis, Bullinularia,* etc are opaque or dark in color and therefore, it is difficult to recognize if the organism alive or dead.

5. Thesis Objectives

My PhD research focuses on the molecular phylogeny and taxonomy of testate amoebae and host-symbiont evolutionary relationships within mixotrophic taxa. In this thesis, I had three main objectives to work on, in order to achieve a better understanding on phylogeny and evolution of testate amoebae:

The first aim is to revise and redefine the systematic of some arcellinid testate amoebae taxa particularly those agglutinated species, based on both fine morphological data using SEM and molecular data based on gene sequences mainly the small subunit ribosomal RNA (SSU rRNA).

The second aim is to illustrate the phylogenetic position of some *incertae sedis* testate amoebae taxa within the tree of eukaryotes.

The third aim is to investigate and evaluate the host-symbiont specificity and evolutionary relationships in some mixotrophic testate amoebae taxa by obtaining genetic data from both host and their *in hospite* symbionts.

These objectives are addressed in four main chapters:

In chapter 2, we performed the first step toward resolving the Arcellinida phylogeny and taxonomy in general and in genus *Difflugia* in particular. We illustrated that genus *Difflugia* (the largest arcellinid genus) is not monopyletic, by developing a new set of SSU rRNA specific primers. We also stressed the major pitfalls that can be encountered in the study of arcellinid phylogeny.

In chapter 3, we determined the phylogenetic position of two testate amoebae of unknown affiliation, *Amphitrema wrightianum* and *Archerella flavum.* These organisms are well-known indicators in palaeoecological studies of *Sphagnum* peatlands. Our molecular data based on SSU rRNA gene sequences showed that they belong to labyrinthulomycete clade, a group of Stramenopiles that were hitherto mostly known as marine osmotrophic organisms. In addition, we described a new clade we named Amphitremida. The new clade is highly diverse genetically, ecologically and physiologically.

In chapter 4, we explored the nature of the endosymbionts in four mixotrophic testate amoebae taxa (*Hyalosphenia papilio*, *Heleopera sphagni*, *Placocista spinosa* and *Archerella flavum*); we also used *Hyalosphenia papilio* as a model to investigate in details the host-symbiont specificity and evolutionary relationships. Our results revealed a new lineage of symbiotic *Chlorella* that comprised, to date, only testate amoebae endobiotic *Chlorella*.

In Chapter 5, we presented an example of fast morphological evolution within Arcellinida, in endemic Chinese *Difflugia tuberspinifera* the spiny and spineless morphospecies. Our SSU rRNA and ITS gene sequences showed limited genetic divergence between both morphospecies. These results suggested that Asian

endemic *D. tuberspinifera* morphospecies have evolved recently from the same ancestor (i.e. evolutionary very close related to each other) and the spines appeared as a result of a strong selective pressure.

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2

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

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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*

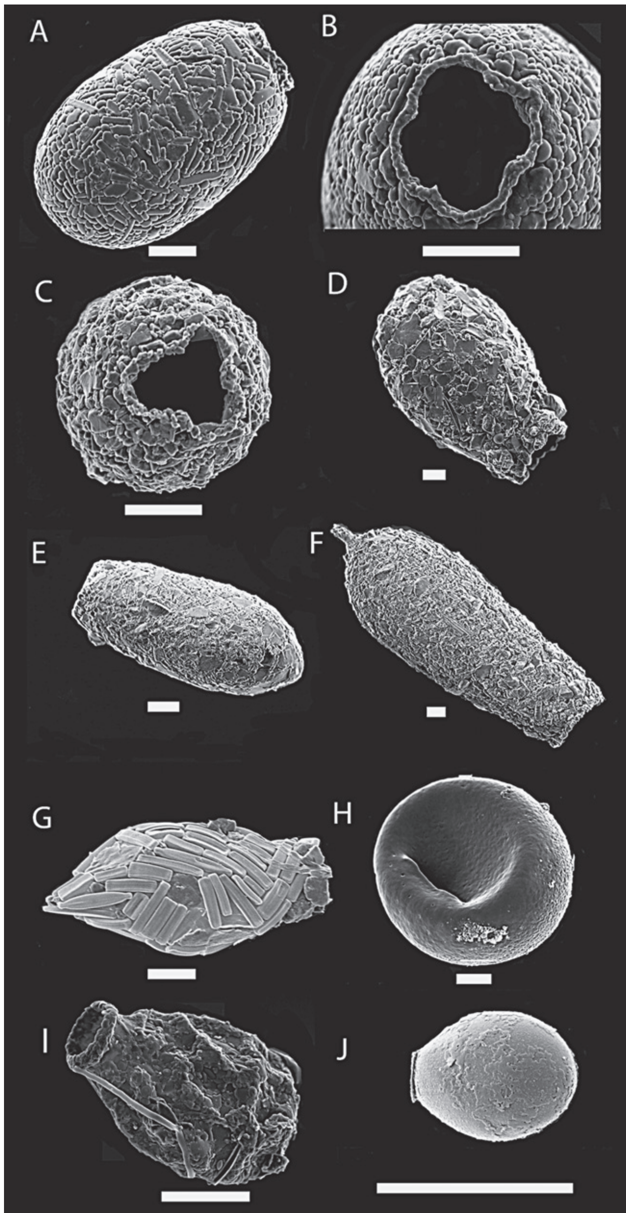

Figure 1. Scanning electron micrographs of tests from species treated in this study. **A)** *Netzelia oviformis*, **B)** Aperture view of *N. oviformis*, **C)** *Difflugia achlora*, **D)** *D. hiraethogii*, **E)** *D. lanceolata*, **F)** *D. acuminata*, **G)** *D. bacillariarum*, **H)** *Bullinulaira 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).

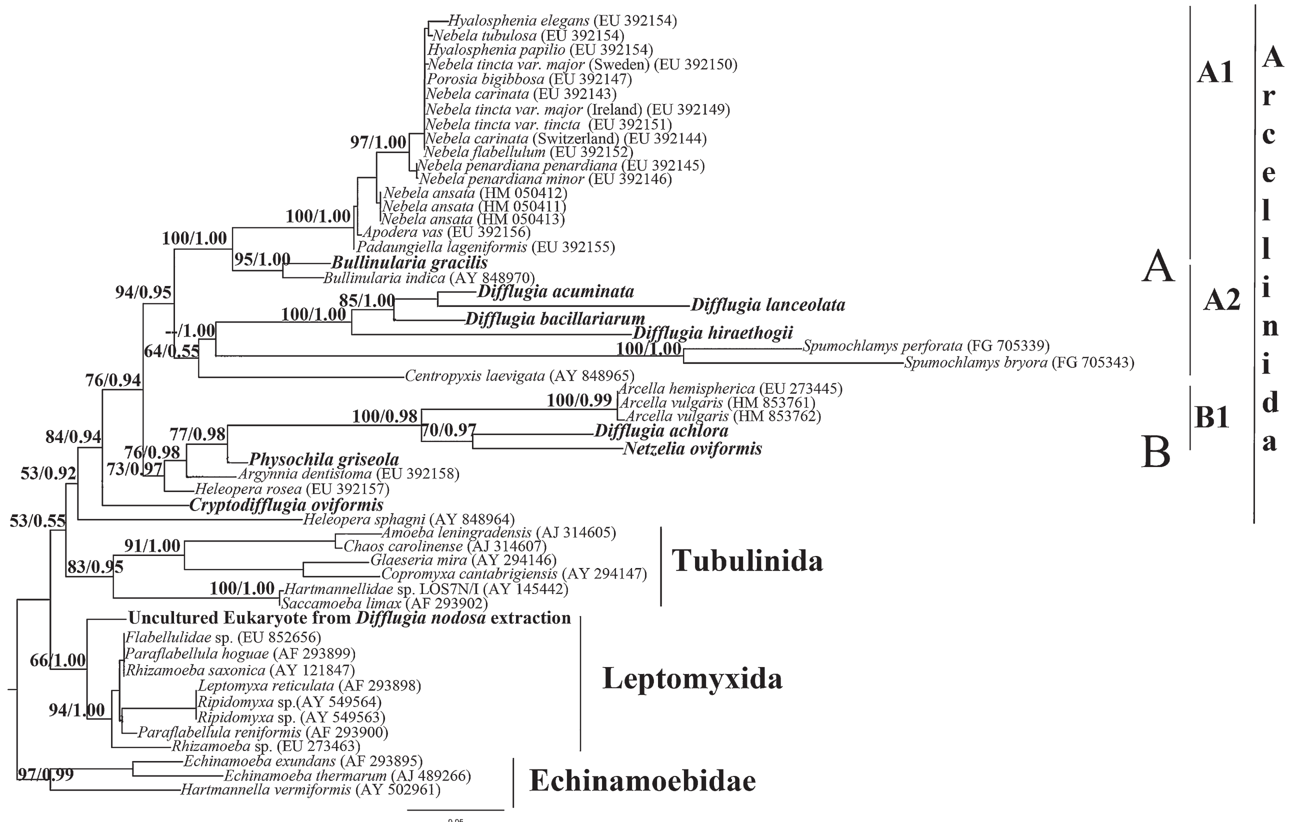

Figure 2. Molecular phylogeny based on small subunit (SSU) rRNA gene sequences of Arcellinida and related Amoebozoa illustrating the paraphyly of genus *Difflugia*. The tree is rooted with Echinamoebidae and includes new sequences from *Bullinularia gracilis, Netzelia oviformis, Physochila griseola* 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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3

Amphitremida (Poche, 1913) is a New Major, Ubiquitous Labyrinthulomycete Clade

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Amphitremida (Poche, 1913) Is a New Major, Ubiquitous Labyrinthulomycete Clade

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Abstract

Micro-eukaryotic diversity is poorly documented at all taxonomic levels and the phylogenetic affiliation of many taxa – including many well-known and common organisms - remains unknown. Among these *incertae sedis* taxa are *Archerella flavum* (Loeblich and Tappan, 1961) and *Amphitrema wrightianum* (Archer, 1869) (Amphitremidae), two filose testate amoebae commonly found in *Sphagnum* peatlands. To clarify their phylogenetic position, we amplified and sequenced the SSU rRNA gene obtained from four independent DNA extractions of *A. flavum* and three independent DNA extractions of *A. wrightianum.* Our molecular data demonstrate that genera *Archerella* and *Amphitrema* form a fully supported deep-branching clade within the Labyrinthulomycetes (Stramenopiles), together with *Diplophrys* sp. (ATCC50360) and several environmental clones obtained from a wide range of environments. This newly described clade we named Amphitremida is diverse genetically, ecologically and physiologically. Our phylogenetic analysis suggests that osmotrophic species evolved most likely from phagotrophic ancestors and that the bothrosome, an organelle that produces cytoplasmic networks used for attachment to the substratum and to absorb nutrients from the environments, appeared lately in labyrithulomycete evolution.

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Introduction

Molecular phylogenetic studies have revealed a tremendous diversity within unicellular eukaryotes, and the existence of ca. 55 major eukaryotic lineages [1,2]. Furthermore, recent environmental DNA studies are continuously revealing novel clades, often comprising pico-sized <2–3 μm microorganisms lacking conspicuous morphological features [3,4]. However, the proper assessment of eukaryotic diversity and the accurate reconstruction of the eukaryote phylogeny are hindered by the unresolved phylogenetic position of many taxa, including abundant and morphologically easily identifiable ones [5,6]. These organisms, referred to as "*incertae sedis*" include several amoeboid eukaryotic groups, among which unusual testate amoebae belonging to family Amphitremidae [7].

Amphitremidae are single-celled eukaryotes characterized by the presence of a shell (test) with two apertures (pseudostomes) at the opposite ends of the shell. It includes the genera *Amphitrema, Archerella* and *Paramphitrema* [8] (Table 1). The first two genera include organisms that possess filamentous and sometimes anastomosing pseudopodia, and harbor endosymbiotic zoochlorellae (Figure 1), while *Paramphitrema* lives on marine and freshwater plants and algae, and is has linear pseudopodia; its classification within Amphitremidae is debatable [8]. *Amphitrema* and *Archerella* are found primarily in *Sphagnum* peatlands and are considered as excellent bioindicators of surface moisture and water chemistry [9,10]. These taxa are also frequently recovered as microfossils from peat deposits and are therefore useful for palaeoenvironmental reconstructions [11,12].

Taxonomical placement of genera *Amphitrema* and *Archerella* has always been problematic. Penard [13] included genus *Archerella* within *Amphitrema* and described *Amphitrema flavum* as a "Thecamoebidae" with a filamentous pseudopodia and rich with *zoochlorellae* endosymbiont. Later, Wailes [14] created a new clade for filamentous amoebae with two apertures on the test, that he called Amphistomina, and that comprised genera *Amphitrema* and *Diplophrys* [15], but doubted on the validity of this taxon, where members shared only the double aperture as common feature. This view was however supported by De Sandeleer [16] who placed all these organisms within Granuloreticulosea (roughly equal to Foraminifera sensu Adl et al. [17]) based on their branched and anastomosing pseudopodia (Table 1). However, later analyses revealed that true foraminiferans are characterized by the presence of granular pseudopods also called granuloreticulopodia [18] that exhibit a typical bidirectional protoplasmic streaming [19]. Bonnet et al., [20] described the ultrastructure of *Amphitrema* (= *Archerella*) *flavum* and its tubulocristate mitochondria; such structures are repeatedly found within "core Cercozoa" [2], suggesting a relationship with filose amoebae such as for instance the Euglyphida. However, similar structures were also found in stramenopiles [17] or in totally unrelated organisms such as jakobids [21]. In the recent literature, *Amphitrema* and *Archerella* are considered as forming part of a single family, the Amphitremidae, together perhaps with the enigmatic *Paramphitrema.* They have been placed as testate amoebae with filopodia *incertae sedis* [22]. Their position remained unsolved by the time of the publication of Adl et al's revision of all micro-eukaryotic taxonomy [17], genus

Amphitrema remained amongst the protist genera with uncertain affiliation.

In order clarify their phylogenetic position within the tree of eukaryote; we performed the first molecular study based on SSU rRNA gene sequences in the two most common genera of Amphitremidae, *Archerella* and *Amphitrema*. In a second step, we performed a search in GenBank to assess the environmental diversity of this clade and the variety of environments colonized.

Materials and Methods

Samples Collection and Documentation

We sampled *Archerella flavum* and *Amphitrema wrightianum* (Figure 1) from wet *Sphagnum* mosses collected from the west shore of Duffey Lake, South Central British Columbia, Canada (50°23′ N 122°27′ W) and Praz-Rodet bog in the Jura Mountains of Switzerland (46°34′ N 6°10′ E). An authorization (No 1449) was delivered by the "Service forêts de la faune et de la nature du canton de Vaud" (state office for nature conservation) for sampling in Praz-Rodet for 2011 and 2012 (January 2011 to end of summer 2012). The Duffey Lake *Sphagnum* sample was not collected within the Provincial Park and therefore, no permits were required. Cells were extracted from *Sphagnum* mosses through serial of filtrations, and then were washed 3 to 4 times with distilled water [23,24]. We prepared seven independent extractions, four from *Archerella flavum* and three from *Amphitrema wrightianum*, each of these extractions contained between 50 to 70 cells. Both species were documented using light microscopy (Figure 1).

DNA Extraction, PCR Amplification and Sequencing

DNA was extracted using a guanidine thiocyanate-based protocol [25]. Seven SSU rRNA sequences (four from *Archerella flavum* and three from *Amphitrema wrightianum*) were obtained by two amplifications. The first amplification was performed using universal eukaryotic primers, 1EKF (CTGGTTGATCCTGC-CAG) and 1498R (CACCTA CGGAAACCTTGTTA) or 1520R (CYGCAGGTTCACCTA), in a total volume of 30 µl with amplification profile consisting of (5 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 positive products were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *E. coli* TOP10' One Shot cells (Invitrogen) according to the manufacturer's instructions. Clone inserts were amplified with vector T7 and SP6 primers. The expected size clones from PCR amplifications 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). We also designed the following primers for internal sequencing: Archer1F (GTAAATTACCCAATCCYAAMTCG), Archer1R (AAACATTTTGCTTTCGC), and Archer2R (TTTGTCCTGCCCTGCT). The positive products were cloned; and two to five clones from each extraction of *Archerella flavum* and *Amphitrema wrightianum* were sequenced. Sequences are deposited in GenBank with the Accession Numbers: *Amphitrema wrightianum* PR-1 (KC245091); *Amphitrema wrightianum* PR-2 (KC245092); *Amphitrema wrightianum* PR-2 (KC245093); *Archerella flavum* BC-1 (KC245094); *Archerella flavum* BC-2 (KC245095); *Archerella flavum* BC-3 (KC245096) and *Archerella flavum* BC-4 (KC245097).

Alignment and Phylogenetic Analysis

All SSU rRNA gene sequences were submitted to BLAST [26] in order to check their similarity with other available data in Genbank. Related sequences together with our sequences were added to a recent dataset [27] and aligned using the BioEdit software [28]. Introns, insertions and variable regions in the SSU rRNA alignment that could not be aligned unambiguously were removed from the analyses. Phylogenetic trees were reconstructed using both Maximum Likelihood and Bayesian approaches based on 800 bp alignment using some sequences of Rhizaria as outgroup.

The maximum likelihood tree was built using RAxML version 7.2.8 algorithm [29] as proposed on the Black Box portal (http://phylobench.vital-it.ch/raxml-bb/) using the GTR+Γ+I model. Model parameters were estimated in RAxML over the duration of the tree search. The obtained tree was compared to the one that built by Bayesian analysis using the software MrBayes v. 3.1.2 [30]. 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.

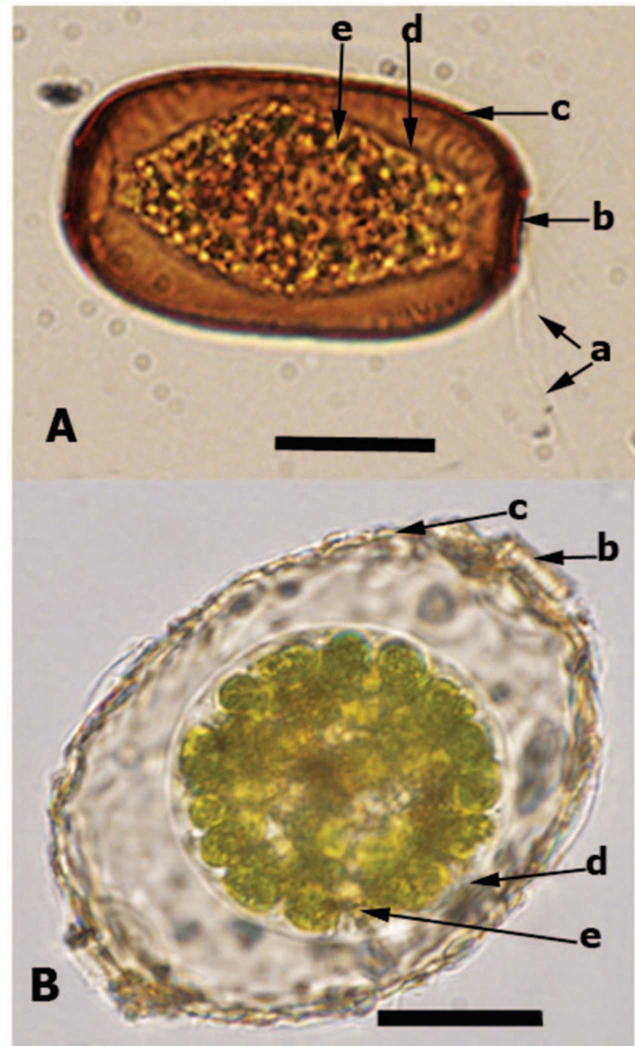

Figure 1. Light microscopy photograph for *Archerella flavum* (A) and *Amphitrema wrightianum* (B): the arrows indicate a) filose pseudopodia; b) pseudostome (shell aperture); c) shell (test); d) cell membrane; e) endosymbioitic green algae. Scale bar = 20 µm.
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Table 1. General characteristics of the four genera of Amphitremida.

	Amphitrema wrightianum (Archer, 1869)	*Archerella flavum* (Loeblich and Tappan, 1961)	*Diplophrys* sp. (Barker, 1868)	*Paramphitrema* sp. (Lauterborn, 1895)
Shell (test) shape	Test elliptical or lemon like shape with convex sides and two pseudostome at the opposite sides	Test elliptical, rigid, and compressed with parallel sides and two pseudostome at the opposite sides	Spherical shape and thin with two pseudostomes at the opposite sides	Test elliptical, compressed, convex sides and two pseudostome at the opposite sides
Shell structure	Inner organic layer and outer agglutinated (xenosomes) layer	Organic 3 layers, no xenosomes	Organic	Agglutinated (xenosomes)
Types of filopodia	Several thin branched filopodia	Several thin branched filopodia	Numerous long radiating, very thin branched filopodia	Two different pseudopodia (at one side one long thick unbranched and at the opposite side thin and branched
Zoochlorella	Present	Present	Absent	Absent
Habitat	Wet to submerged *Sphagnum* mosses, in peat bog pools	Moist to wet *Sphagnum* mosses, in peat bog hollows and wet lawns	Submerged marine and freshwater plants and algae	Submerged marine and freshwater plants and algae

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The burn-in value was set to 25%. Trees were viewed using FigTree (a program distributed as part of the BEAST package). In addition, we performed approximately unbiased (AU) tests [31] to evaluate the likelihood of different alternative topologies to the obtained tree (see Results section).

Results

We obtained seven SSU rRNA gene sequences, four from *Archerella flavum* and three from *Amphitrema wrightianum* with SSU rRNA gene length of 1287 bp and 1351 bp, respectively. The most similar SSU rRNA gene sequences to ours as revealed by BLAST were members of the labyrinthulida and thraustochytrida (Labyrinthulomycetes; Stramenopiles), plus the amoeboid *Diplophrys* and some environmental sequences. Therefore, we built an alignment that included some of the available SSU rRNA gene sequences of Labyrinthulomycetes/Labyrinthulea and other related taxa. Our constructed phylogenetic trees inferred from both RAxML and Bayesian analyses had the same topology (Figure 2). Labyrinthulomycetes receive a moderate support values (BS = 70%, PP = 0.90) and appeared divided into three major groups: (1) labyrinthulida+thraustochytrida, (2) a group comprising thraustochytrida+Amphifilidae (*Amphifila marina* and several freshwater environmental sequences) and (3) the group formed by *Archerella flavum* and *Amphitrema wrightianum* together with other related taxa: *Diplophrys* sp. ATCC50360(AF304465), and several environmental sequences, including PR3_4E_52 (GQ330589) from a peat bog, 528-O7 (EF586082) from freshwater, plus fourteen sequences from anoxic/micro-oxic deep-sea environment. As the position of *Paramphitrema* remains dubious [8], and because many possibly divergent organisms will be included in that clade, we name this third group Amphitremida, keeping Amphitremidae for the group comprising both *Amphitrema* and *Archerella*. As members of this group have traditionally been treated under the ICZN (= Zoological nomenclatural code), this code will continue to being applied to it regardless of current or future phylogenetic placement [32]. As a result, we consider that family Diplophryidae, which has been described by Anderson and Cavalier Smith [33] cannot be valid, since it includes the environmental clone PR3_4E_52 (GQ330589), a sequence that belonged actually to *Amphitrema*. Amphifilidae and Amphitremida both received maximal support values (BS = 100%, PP = 1.00) (Figure 2). *Amphitrema wrightianum* and *Archerella flavum* plus clone PR3_4E_52 (i.e. Amphitremidae) formed together a moderately supported clade (BS = 77%, PP = 0.90). All the four obtained *A. flavum* sequences were exactly identical, while the obtained sequences of *A. wrightianum* PR-2, showed two nucleotides substitution at position 595 bp and 854 bp in comparison of both *A. wrightianum* PR-1 and PR-3 sequences. Our results have been confirmed by cloning the SSU rRNA fragments from each taxa.

The environmental peat bog clone PR3_4E_52 was also very closely related to these sequences and showed 99% similarity to *A. wrightianum* sequences. The freshwater environmental sequence 528-O7 (EF586082) had a basal position with respect to *Archerella flavum* and *Amphitrema wrightianum*. Fourteen SSU rRNA sequences from marine anoxic and micro-oxic water column branched as a sister clade to the peat bog+freshwater clade.

Following up on these results, we performed approximately unbiased (AU) test [31] to test the following alternative hypotheses: a) the monophyly of ((Amphitremida+Amphifilidae)+(thraustochytrida+labyrinthulida)), b) the monophyly of ((thraustochytrida+labyrinthulida+Amphifilidae)+(Amphitremida)). The tests did not reject any of these hypotheses (with p-values = 0.49 and 0.48 respectively).

Discussion

1. Phylogenetic Position of Genera *Amphitrema* and *Archerella*, and Evolution of the Labyrinthulomycetes

This study demonstrates that the genera *Archerella* and *Amphitrema* belong to the Labyrinthulomycetes/Labyrinthulea (Stramenopiles) (Figure 2), rather than to other filose testate amoeba such as Euglyphida, *Pseudodifflugia* (Cercozoa), or the Foraminifera. Filose pseudopodia therefore appeared several times in eukaryotic evolution, not only within Rhizaria but also in some Opisthokonts (Nuclearia; see [34]) and Heterokonts (*Leukarachnion*; see [35]) and now, Amphitremida.

The labyrinthulida and thraustochytrida are characterized by the presence of a bothrosome or a 'sagenetosome' (sometimes also called 'sagenogen'), an organelle that produces cytoplasmic networks (extensions of the plasma membrane) to absorb the nutrients from the surrounding environments, similar in that to fungi [36,37]. Other genera such as *Labyrinthula* and *Aplanochytrium* also use these cytoplasmic networks for gliding [38]. The Labyrinthulomycetes are mainly osmotrophic protists. They are extremely common in marine environments, are often associated with decaying plants such as mangrove leaves [39], and less

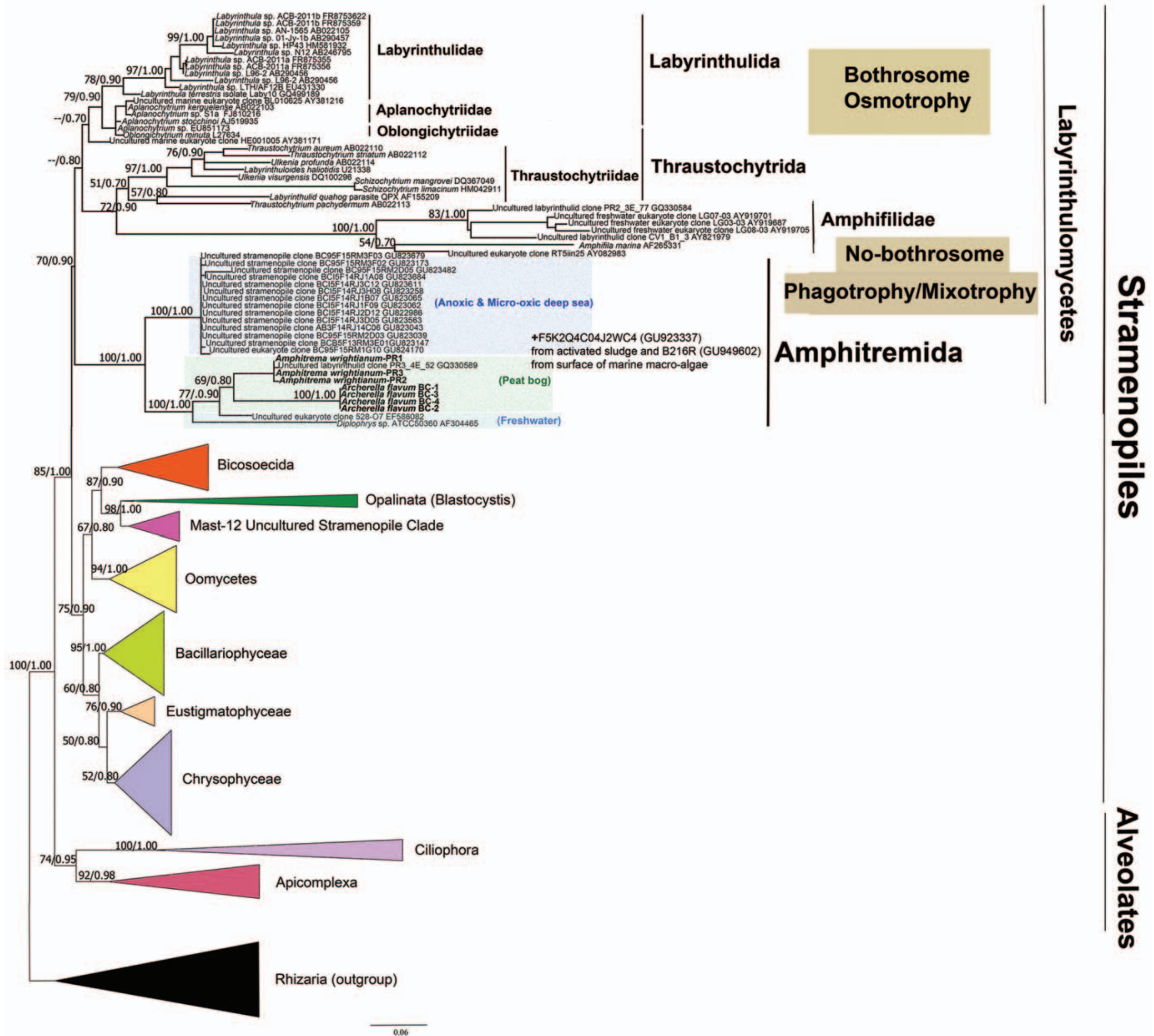

Figure 2. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on small subunit (SSU) rRNA gene sequences and illustrating that the genera *Archerella* and *Amphitrema* (Amphitremida) belong to Labyrinthulomycetes (Stramenopiles). Numbers at nodes indicate the bootstrap values/posterior probabilities. Only values above 50/0.50 are shown. The tree was rooted with the group of Rhizaria. The scale bar indicates 0.06% sequence divergence.
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frequently parasitic [40]. The labyrinthulids and thraustochytrids exhibit a typical dimorphic life cycle with a vegetative absorptive stage and a flagellated zoosporic stage. Although genera *Archerella*, *Amphitrema*, *Diplophrys* and *Amphifila* move also by filose ectoplasmic extensions they do not possess a true bothrosome, and biflagellated stage have not yet been observed [14] (Table 1).

Our phylogenetic analysis suggests that the typical organisation with two symmetrical pseudopodial tufts found in *Amphitrema, Archerella, Diplophrys* and *Amphifila marina* might be ancestral to both Amphitremida and Amphifilidae. The AU test does not reject the existence of one clade grouping both Amphitremida and Amphifilidae and a second clade uniting the thraustochytrida together with labyrinthulida, an evolutionary pathway that appears the most parsimonious because it implies a single appearance of the bothrosome, a unique feature of thraustochytrida and labyrinthulida (Figure 2), and a simultaneous loss of pseudopodia and phagotrophy [38]. Under this evolutionary hypothesis, the bilateral symmetry of the cells would be a synapomorphy of Amphitremida+Amphifilidae, a character shared by all known members. Because osmotrophic state is not likely to be reversed back into a phagotrophic state (this would require regaining structures necessary for phagocytosis), we can hypothesize that the ancestral Labyrithulomycetes were phagotrophic and amoeboid organisms, possibly with a bilateral symmetry. In support to this interpretation, the basal-branching *Schizochytrium mangrovei* and *Thraustochytrium striatum* can shift from osmotrophic vegetative stage to phagotrophic amoeboid stage ingest through pseudopodia, if kept in culture together with

bacteria, illustrating the dual nature of these organisms [41]. *Diplophrys* sp and *Amphifila marina* are both phagotrophic, and recent studies based on stable isotope ratios suggest that *Archerella flavum* is bacterivorous (Vincent Jassey, unpublished data), in contrast to earlier suppositions [20].

Another possible candidate for the assignment to both Amphitremida and Amphifilidae is *Sorodiplophrys stercorea*, an organism isolated from cow and horse dung with bilateral symmetry and filose pseudopodia, and devoid of bothrosome; interestingly, it relies entirely on osmotrophy [42].

2. Environmental Diversity of the Amphitremida

Our tree analysis including environmental clones revealed an unexpected diversity of organisms branching within Amphitremida that derived from a very wide range of environments (Figure 2). These include a freshwater biofilm clone from New Zealand, 528-O7 [43] that showed more than 98% similarity to our *Archerella flavum* sequences, and also, surprisingly, fourteen sequences obtained from anoxic and micro-oxic water column from the Cariaco Basin in the Caribbean Sea [44]. Their pervasive presence suggests that they are either genuine members of planktonic communities, or that they are associated to sinking debris in the water column. In addition, one pyro-tag from activated sludge F5K2Q4C04J2WC4 (GU923337) and one environmental sequence B216R from the surface of marine macro-algae (GU949602), both unpublished data from GenBank have been found to have high similarity with *Archerella* and *Amphitrema* respectively, but were not included in our analysis due to their short length. Nevertheless, these sequences further illustrate the diversity of habitats colonized by the hitherto unrecognised clade of Amphitremida.

The environmental sequence PR3_4E_52 (GQ330589) obtained by Lara et al., [27] from a eukaryotic diversity survey of the central pool in a pristine peat bog in the Swiss Jura Mountains branched together with our *Amphitrema wrightianum* sequences, from which it differs by three nucleotides at most. The different extractions of *A. wrightianum* also showed small differences in their sequences (up to two nucleotides). This diversity suggests the presence of several genotypes within the morpho-species *A. wrightianum* and/or the existence of several closely related taxa (cryptic species). Indeed, confusion with the very similar-looking peat bog species *A. stenostoma* [45] cannot be excluded. The two other described *Amphitrema* species (*A. lemanense* [46] and *A. congolense* [47]) do not possess endosymbiotic algae, and their phylogenetic position within the genus still needs to be determined. In contrast, our analyses did not reveal any intra-species genetic variability within *Archerella flavum*.

The Amphitremida represents a novel major clade within the Labyrinthulomycetes. This clade has colonised environments as divergent as peat bogs, freshwater and the oceans, ranging from nitrogen-depleted environments to eutrophic (sludge); their metabolisms vary from aerobic to anaerobic/micro-aerophilic, they can be phagotrophic or mixotrophic and have a planktonic or a benthic lifestyle. This illustrates the immense versatility of this group that certainly encompasses an even larger environmental genetic diversity than currently known. The true magnitude of this diversity will most probably be revealed by future massive environmental sequencing studies.

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Author Contributions

Conceived and designed the experiments: FG EL EADM. Performed the experiments: FG. Analyzed the data: FG EL. Contributed reagents/materials/analysis tools: EADM EL. Wrote the paper: FG EL EADM.

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4

One alga to rule them all: Unrelated Mixotrophic Testate Amoebae (Amoebozoa, Rhizaria and Stramenopiles) Share the Same Symbiont (Trebouxiophyceae)

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One alga to rule them all: Unrelated Mixotrophic Testate Amoebae (Amoebozoa, Rhizaria and Stramenopiles) Share the Same Symbiont (Trebouxiophyceae)

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Abstract

Endosymbiosis is a central and much studied process in the evolution of eukaryotes. While plastid evolution in eukaryotic algae has been extensively studied, much less is known about the evolution of mixotrophy in amoeboid protists which has been found in three of the five super groups of Eukaryotes.

We identified the endosymbionts in four obligate mixotrophic testate amoeba species belonging to three major eukaryotic clades (1) *Hyalosphenia papilio* and *Heleopera sphagni* (Amoebozoa: Arcellinida), 2) *Placocista spinosa* (Rhizaria: Euglyphida), and 3) *Archerella flavum* (Stramenopiles: Labyrinthulomycetes) based on *rbcL* gene sequences. We further assessed the symbiont diversity within single *H. papilio* cells and the degree of host-symbiont specificity and evolutionary relationships by amplifying two genes: the first unit of the mitochondrial cytochrome oxidase (COI) from the testate amoeba host, and *rbcL* from the endosymbiont.

Results show that all studied endosymbionts belong to genus *Chlorella sensu stricto*, closely related to *Paramecium bursaria Chlorella* symbionts, some lichen symbionts and, also, free-living taxa. Most *rbcL* gene sequences derived from symbionts from all testate amoebae species and all lineages formed were almost identical and were assigned to a new Trebouxiophyceae taxon we named TACS (Testate Amoeba *Chlorella* Symbionts).

This “one alga fits all mixotrophic testate amoebae“ pattern suggests that photosynthetic symbionts have pre-adaptations to endosymbiosis and colonise diverse hosts from a free-living stage.

Keywords: Mixotrophy; testate amoeba *Chlorella* symbionts (TACS); *Hyalosphenia papilio*; *rbcL* gene; COI gene; molecular phylogeny.

Introduction

Endosymbiosis with photosynthetic organisms has played a major role in the evolution of life and represents an evolutionary strategy for many eukaryotic organisms to acquire new biochemical and metabolic functions (Nowack& Melkonian 2010 ; Raven *et al.* 2009). The most conspicuous endosymbiotic relationships encountered in the domain Eukaryota is the primary endosymbiosis that happened between a phototrophic cyanobacterium and a heterotrophic organism, giving rise to plants, green, red and glaucophytes algae in the Eukaryotic supergroup Archaeplastida, and the unusual testate amoeba *Paulinella chromatophora* in the Rhizaria (Keeling 2010; Sagan 1967; Yoon *et al.* 2006). Further associations between some of these organisms and phagotrophic protists eventually gave rise to secondary and tertiary plastid acquisition in all major Eukaryotic supergroups (Archibald 2009; Keeling 2009). These photo-heterotrophic consortia appeared several times separately in the course of evolution of eukaryotes, giving rise the rest of the diverse array of algae found in the modern ecosystems (Johnson 2011). The degree of metabolic integration and dependency of the symbiont within the host that acquired phototrophs varies widely, from the facultative mid-term storage of algae within vacuoles prior to later digestion (Esteban *et al.* 2010; Johnson 2011) to obligatory symbiosis, that can be sometimes combined with gene transfers from the endosymbiont to the heterotrophic partner's (Figueroa *et al.* 2009; Nowack& Melkonian 2010).

A first step in this hetero-phototrophic association (usually referred as mixotrophy) can be found in some species of marine dinoflagellates such as *Noctiluca scintillans* and freshwater ciliates such as *Paramecium bursaria* where the

host can switch to heterotrophic nutrition if exposed to prolonged darkness or if the environment lacks symbionts. This association, albeit facultative, increases the growth rate of the host (Stoecker *et al.* 2009). Furthermore, host selectivity and specificity to symbiont species have been demonstrated experimentally in such facultative symbiosis (e.g. in *P. bursaria,* (Summerer *et al.* 2007; Summerer *et al.* 2008)).

In a further step, these consortia can become obligatory for the host at least, which can no longer survive without its symbionts; this type of association is commonly encountered in diverse groups of organisms including corals, lichens, ciliates, planktonic and benthic foraminifera, polycystines and acantharea (Esteban *et al.* 2006; Stoecker *et al.* 2009). Overall, these organisms harbor a broad range of algae such as dinoflagellates, diatoms, chlorophytes, chrysophytes, and prymnesiophytes.

Host cells are supposed to acquire their endosymbionts by two different mechanisms 1) vertical transmission (i.e. inherited) when cells divide as observed in ciliates, lichens and some foraminifers which have a predominantly asexual life cycle (Garcia-Cuetos *et al.* 2005) or 2) acquisition from the surrounding environments with a highly selective recognition mechanism such as in most Foraminiferans, Radiozoa and Hexacorallia (Gast& Caron 1996; Gast& Caron 2001; Gast *et al.* 2000; Santos *et al.* 2004; Zoller 2003). The difference between these two levels of integration in the host-symbiont relationship is not always clear-cut. Soritid foraminifera can acquire symbionts both maternally and horizontally from the environment (Garcia-Cuetos *et al.* 2005). When the katablepharid cryptophyte *Hatena arenicola* divides, only one of the resulting cells inherits maternally the single symbiont, while the other one has to

acquire it from the environments through a highly selective recognition mechanism (Okamoto& Inouye 2006). Although the host cell still retains the ability of heterotrophic nutrition mode, it was reported that most of these mixotrophic organisms cannot survive for long time if the endosymbiont has been lost or could not be acquired at a certain life stage (Caron *et al.* 1982; Stoecker *et al.* 2009).

The most stable type of endosymbiotic association occurs in some species of dinoflagellates that host diatoms endosymbiont such as *Kryptoperidinium foliaceum*, and the marine ciliates *Myrionecta rubra* (= *Mesodinium rubrum*), which host cryptomonads. Here both host cell and endosymbiont are highly integrated, with a synchronized cell cycle, and endosymbiotic gene transfer was even reported from the symbiont genome to their host (dinoflagellates) nucleus (Figueroa *et al.* 2009; Nowack& Melkonian 2010 ; Stoecker *et al.* 2009).

Despite the fact that many studies have succeeded in identifying the symbionts and documented the modalities of endosymbiotic associations in protists, the nature of this symbiosis remains unknown for certain groups including testate amoebae. Testate amoebae are a polyphyletic group of free-living unicellular eukaryotes, characterized by the presence of shell that can be agglutinated, proteinaceous, siliceous, or calcareous (Meisterfeld 2002a; Meisterfeld 2002b). They are distributed in three major groups of Eukaryotes, Amoebozoa (Arcellinida: (Nikolaev *et al.* 2005)), Rhizaria (Euglyphida: (Bhattacharya *et al.* 1995; Cavalier-Smith 1997; Lara *et al.* 2007)) and, as discovered recently, Stramenopiles [31]. As with other groups of protists, some species do harbor green-colored symbionts, especially those that live in nutrient-poor environments (with the exception of *Difflugia pyriformis* var. *venusta*, a limnetic form that has been found in mesotrophic

habitats (Penard 1902)). Because many testate amoebae are impossible to maintain in cultures, there is very little information on the modalities of the symbiosis that they have established with their phototrophic partners.

In this study, we have identified the *in hospite* symbionts in four mixotrophic testate amoeba species belonging to three different major eukaryotic clades; *Hyalosphenia papilio, Heleopera sphagni* (Amoebozoa: Arcellinida) (Nikolaev *et al.* 2005), *Placocista spinosa* (Rhizaria: Euglyphida) (Bhattacharya *et al.* 1995; Cavalier-Smith 1997; Cavalier-Smith 1998), and *Archerella flavum* (Labyrinthulomycetes: Stramenopiles) (Gomaa *et al.* Submitted) (figure 1). In addition, we focused on the particular case of *H. papilio* to explore more in-depth the modalities of the association by 1) assessing the symbiont diversity within single amoeba cells, and 2) investigating the degree of host-symbiont specificity. We used two barcoding genes to resolve the phylogenetic affiliation to the species level, the chloroplastic *rbcL* (ribulose-1,5-diphosphate carboxylase/oxygenase large subunit) marker for the endosymbionts and the mitochondrial COI (cytochrome oxidase first subunit) marker for *H. papilio* (host). The chloroplast-encoded *rbc*L marker has been used to assess the diversity among green algae (i.e chlorophytes) (Ghosh& Love 2011; Novis *et al.* 2009) and, more specifically, trebouxiophyceae (Rindi *et al.* 2007; Sherwood *et al.* 2000). It was shown to have both the highest universality and genetic variation at the species level, and thus constitutes a promising DNA barcode (Fucikova *et al.* 2011). The COI marker is a commonly used DNA barcode for species identification in eukaryotes including arcellinids and euglyphid testate amoebae (Heger *et al.* 2011; Kosakyan *et al.* 2012).

Materials and Methods

Samples collections and documentation

Four species of mixotrophic testate amoebae (*Hyalosphenia papilio*, *Heleopera sphagni, Placocista spinosa* and *Archerella flavum*) (figure 1) were collected from their natural environments in *Sphagnum* peatland (table 1). Cells were extracted from *Sphagnum* mosses through serial filtrations, then washed several times with ddH_2O and sorted using a fine capillary pipette under an inverted microscope. We prepared independent extractions; two from *Placocista spinosa*, four from *Heleopera sphagni* and three from *Archerella flavum*, each of these extractions contained from 4 to 10 different cells for *Heleopera sphagni* and *Placocista spinosa*, and 50 to 70 cells for *Archerella flavum*. We used single cells for *Hyalosphenia papilio* in forty different extractions.

DNA Extraction, Amplification, Cloning and Sequencing

DNA was extracted using guanidine thiocyanate protocol (Chomczynski& Sacchi 1987). The extracted DNA was pelleted, and re-suspended in 15 µL of Tris buffer (pH 8.5). The *rbcL* gene was amplified in two steps, using general green algae primers that were designed based on sequences retrieved from GenBank. A first amplification was conducted with primers RBF1 (CGGGCAGAKTGCA) and RB800r (TGTRAAACCACCWGTTAAG), and the second amplification with RBF2 (CTCCACAAACTGAAACTARAG) and RB800r. The temperature profile for both reactions consisted of 5 minutes at 95 ^{0}C, followed by 45 cycles of 1 min at 94 ^{0}C, 1 min at 55 ^{0}C and 2min at 72 ^{0}C, with a final elongation of 10 min at 72^{0}C. For *Hyalosphenia papilio*, we used half of the re-suspended DNA from the single cell for *rbcL* amplification (symbionts) as detailed previously and half for mitochondrial

cytochrome oxydase subunit 1 (COI) of the host. The COI fragments were amplified in two steps. The first amplification was performed using universal COI primers LCO and HCO designed by Folmer et al., (1994) following the original protocol. The obtained product served as template for the second amplification using *H. papilio*-specific primers HPCOIF (GTTATTGTTACTGCTCATGCC) and HPCOIR (ATACAAAATAGGATCACCTCCACC) in a total volume of 30 µl with amplification profile consisting of (5 minutes at 95 ºC followed by 40 cycles 15 sec at 94 ºC, 15 sec at 55 ºC and 1min 30 sec at 72 ºC with a final elongation of 10 min at 72ºC). Nineteen out of 49 *rbcL* products were sequenced directly, while the remaining 30 *rbcL* products (24 out of 40 from *H. papilio*, 2 from *Placocista spinosa*, 2 out of 4 from *Heleopera sphagni* and 2 out of 3 from *Archerella flavum*) (table 1) were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *E.coli* TOP10' One Shot cells (Invitrogen) according to the manufacturer's instructions. Clone inserts were amplified with vector T7 and SP6 primers. From 5 to 10 clones 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).

Alignment and phylogenetic analysis

Both COI and *rbcL* sequences were aligned manually using the BioEdit software (Blundell *et al.* 2008) together with sequences retrieved from GenBank that are highly similar to our obtained sequences. Our alignments had respectively 480 bp for COI and 790 by for *rbcL*, and we used respectively members of the *Nebela*

tincta-collaris-bohemica complex (Kosakyan *et al.* 2012) as outgroup for the COI alignment and members of Ulvophyceae for the *rbcL* gene alignment.

The maximum likelihood tree was built using 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. Model parameters were estimated in RAxML over the duration of the tree search. The obtained tree was compared to the one that built by Bayesian analysis using the software MrBayes v. 3.1.2 (Tekle *et al.* 2008). 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 100th generation, the tree with the best likelihood score was saved, resulting in 5000 trees. The burn in value was set to 25%. Trees were viewed using FigTree (a program distributed as part of the BEAST package http://tree.bio.ed.ac.uk/software/figtree/). The divergences between sequences were calculated using the program BioEdit software (Hall 1999).

Table 1. List of sequenced taxa and sampling locations.

Taxa	Sampling site	Country	Coordinates	No. of *rbcL* clones	GenBank accession number	
					rbcL	COI
1- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	_		
2- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	6		
3- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	8		
4- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	5		
5- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	_		
6- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	5		
7- ***Hyalosphenia papilio*** **(PR)**	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 06°10`E	_		
8- ***Hyalosphenia papilio*** **(BD-A)**	Submerged *Sphagnum*, Bois-des-lattes bog	Switzerland	46°58'N 06°42'E	8		
9- ***Hyalosphenia papilio*** **(BD-A)**	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	_		
10- ***Hyalosphenia papilio*** **(BD-A)**	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	6		
11- ***Hyalosphenia papilio*** **(BD-A)**	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	5		
12- ***Hyalosphenia papilio*** **(BD-B)**	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	6		

13- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	5
14- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	_
15- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	6
16- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58'N 06°42'E	_
17- *Hyalosphenia papilio* (LC)	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47°05'N 06°04`E	10
18- *Hyalosphenia papilio* (LC)	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47°05'N 06°04`E	10
19- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	5
20- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
21- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N; 45°24`E	5
22- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	7
23- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
24- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
25- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
26- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	7

27- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	6
28- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	6
29- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
30- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
31- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24`E	_
32- *Hyalosphenia papilio* (AK-A)	*Sphagnum*, rich fen near Chena river on side of road, higher topographic position, near fen-bog transition, Alaska	USA	64°51'N 147°24'W	7
33- *Hyalosphenia papilio* (AK-B)	Brown mosses, rich fen on side of road - short vegetation, small hummock, Alaska	USA	61°24'N 143°03'W	_
34- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	_
35- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	6
36- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	_
37- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	8
38- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	6

39- *Hyalosphenia papilio* (BC)	Wet *Sphagnum*, Allan Creek, South central British Columbia	Canada	52°32'N 119°06'W	6
40- *Hyalosphenia papilio* (BC)	Wet *Sphagnum*, Allan Creek, South central British Columbia	Canada	52°32'N 119°06'W	5
41- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 6°10`E	6
42- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 6°10`E	5
43- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 6°10`E	_
44- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34'N 6°10`E	_
45- *Placocista spinosa* (PT)	*Sphagnum* mosses, poor fen on the side of Pechora River	Russia	61°55'N 57°54'E	7
46- *Placocista spinosa* (PT)	*Sphagnum* mosses, poor fen on the side of Pechora River	Russia	61°55'N 57°54'E	5
47- *Archerella flavum* (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23'N 122°27'W	4
48- *Archerella flavum* (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23'N 122°27'W	7
49- *Archerella flavum* (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23'N, 122°27'W	_

"-" Indicates that sequences have been obtained from direct PCR.

Results

Phylogenetic affinity and molecular identification of the endosymbionts

Our phylogenetic data analysis based on *rbcL* gene sequences identified all examined endosymbionts as members of the genus *Chlorella sensu stricto* (class Trebouxiophyceae, family Chlorellaceae) (figure 2). Forty-six out of 49 (all the *rbcL* gene sequences obtained from *Heleopera sphagni, Placocista spinosa, Archerella flavum* and 37 of *Hyalosphenia papilio*) clustered together in a single clade with a maximum support values including 100% Bootstrap values (BS) and 1.00 Bayesian inference posterior probability (PP). This clade is closely related to other symbiotic Chlorellaceae, such as *Paramecium bursaria Chlorella* symbionts, lichens associated-*Trebouxia*, as well as free-living related strains such as *Chlorella variabilis, C. vulgaris* and *C. sorokiniana, C. pyrenoidosa*, and *Auxenochlorella protothecoides* (for the corresponding GenBank accession numbers see figure 2). Our newly identified clade comprised, to our knowledge, only testate amoebae endobiotic *Chlorella*; we refer to this clade as TACS clade for "Testate amoeba *Chlorella* symbionts" (figure 2). All the *rbcL* gene sequences from *Heleopera sphagni* (Amoebozoa) and *Placocista spinosa* (Rhizaria) symbionts were strictly identical to each other and to 25 *rbcL* sequences obtained from symbionts of single cells of *Hyalosphenia papilio* (Amoebozoa) (figure 2). The three *rbcL* gene sequences of *Archerella flavum* (Stramenopile) symbionts were identical to each other and branch off first from the TACS clade. The genetic diversity among the rest of sequences in this clade (i.e. 12 sequences of *Hyalosphenia papilio*) was low <0.3% (1 to 3 nucleotides out of 780 bp) and mutations were exclusively observed at the third position of the codon (i.e. silent mutations).

The three remaining sequences did not belong to TACS; *H. papilio* symbionts 10/BD-A/6 and 12/BD-B/6 could be identified with *Paramecium bursaria* symbionts and free-living *Chlorella variabilis*, and diverged from the TACS clade by 2.7% (figure 2). The *H. papilio* symbiont 18/LC/10 was more distantly related to the rest of testate amoeba associated *Chlorella* spp., and had 7.3 % genetic divergence with TACS (including mutations on all codon positions).

We also investigated the symbiotic diversity within 24 individual cells *H. papilio* (from the 40 above-mentioned cells) by cloning the *rbcL* amplified fragment. The recovered sequences of the symbionts (5-10 in each cell) were strictly identical within each host cell.

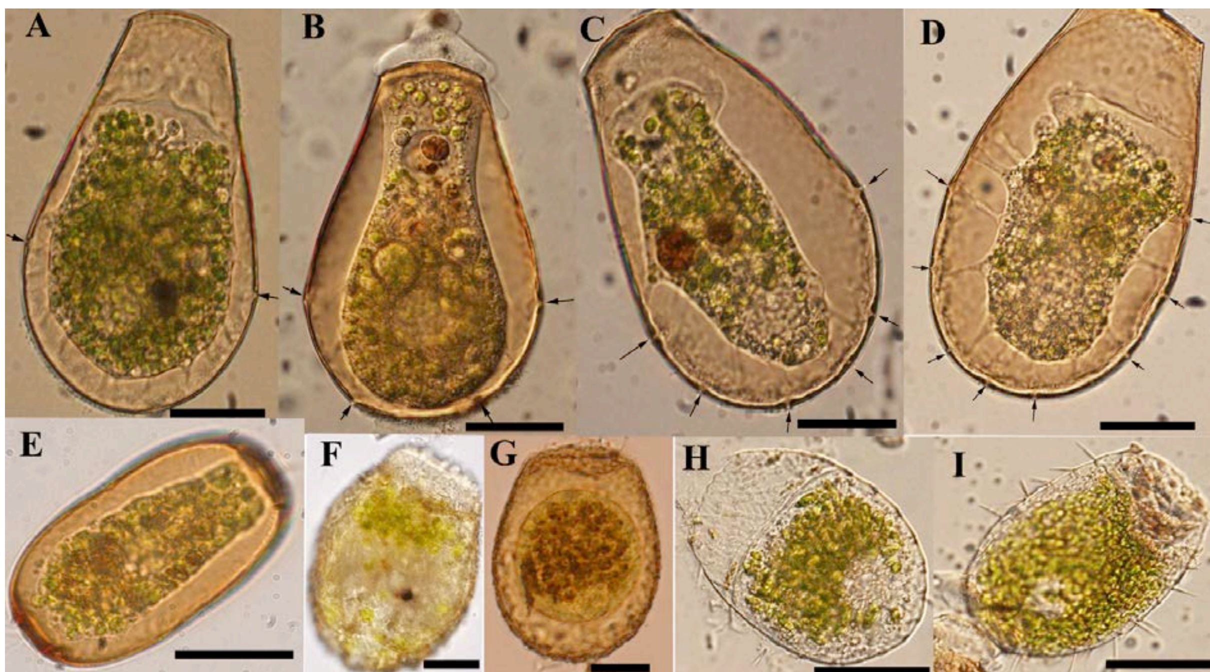

Figure 1. Light microscopy photographs of (A-D) *Hyalosphenia papilio*, arrows indicate the number of pores present at the shell edge, (E) *Archerella flavum,* (F, G) *Heleopera sphagni* and (H, I) *Placocista spinosa*. For all photographs scale bar is 20 µm.

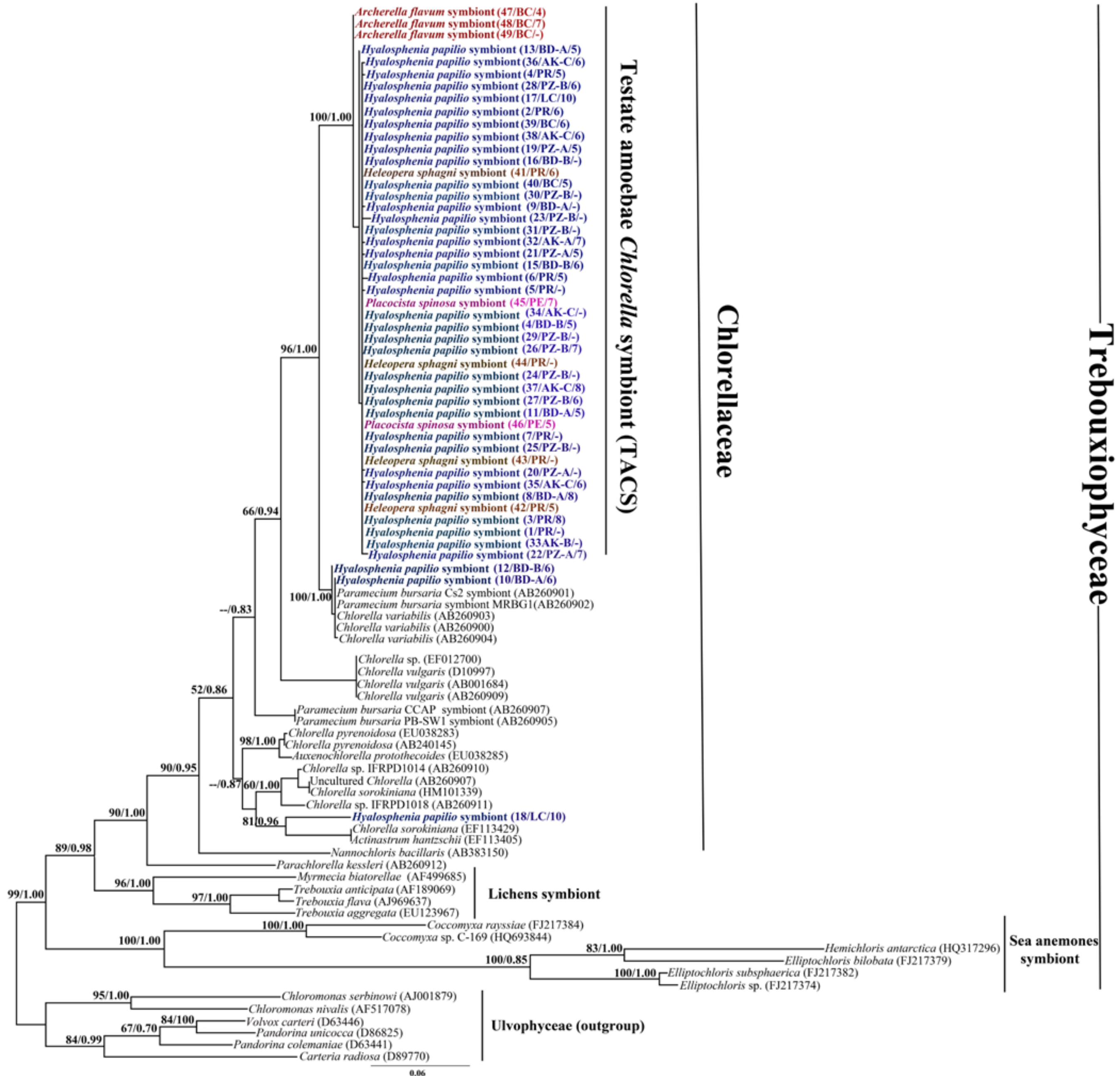

Figure 2. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on the large subunit of the ribulose-bisphosphate carboxylase (*rbcL*) gene sequences obtained from the endosymbionts of four mixotrophic testate amoebae species *Hyalosphenia papilio* (blue), *Archerella flavum* (red), *Heleopera sphagni* (brown) and *Placocista spinosa* (violet). For each sequence we indicated the (number of taxa / geographical origin - code of the *Sphagnum* samples where applicable / number of clones). The tree illustrating the phylogenetic position of the Testate Amoeba *Chlorella* Symbionts (TACS) belong to class Trebouxiophyceae. Numbers at nodes indicate the bootstrap values / posterior probabilities. Only values above 50/0.50 are shown. The tree was rooted with the group of Ulvophyceae. The scale bar indicates 0.06% sequence divergence.

Genetic characterization of one host: *Hyalosphenia papilio*

In contrast to the algal symbiont, we observed a clear intraspecific genetic diversity in the studied amoeba host (*Hyalosphenia papilio*): the 40 analyzed cells formed five different lineages (here designated as A, B, C, D and E) (figure 3). Inter-clade genetic variability ranged between 2.5 and 7%, and the intra-clade variability was below 1%. We did not detect any morphological feature concomitant with assignation to one or another clade; the number of pores in the shells, for instance (figure 1), varied independently from genetic affiliation. However, the phylogenetic tree suggested a possible pattern of geographical origin (figure 3).

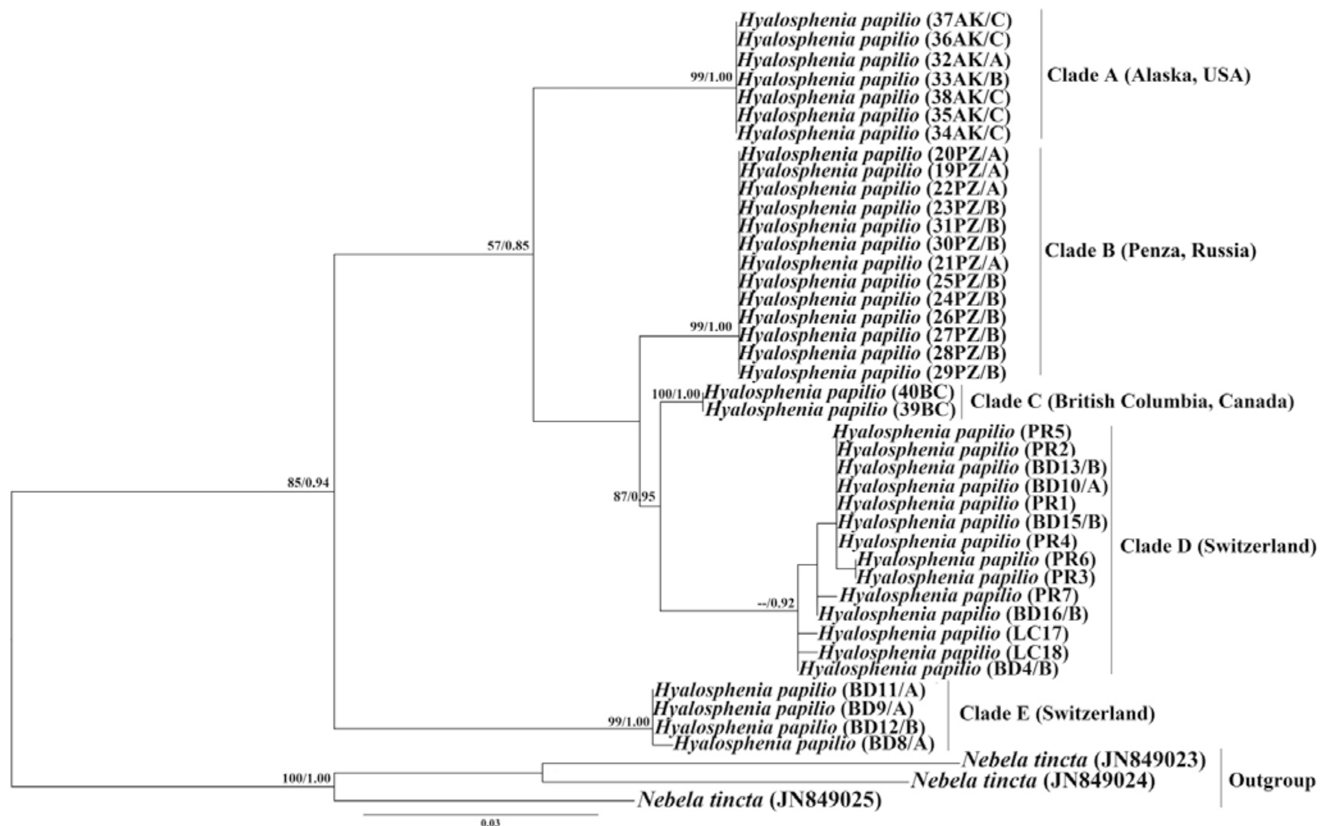

Figure 3. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on mitochondrial cytochrome oxydase first subunit (COI) gene sequences obtained from 40 individual cells of *Hyalosphenia papilio*. The numbers along the branches represent respectively the bootstraps and the posterior probabilities. Only values above 50/0.50 are shown. The tree was rooted with the outgroup *Nebela tincta*. The scale bar indicates 0.03% sequence divergence.

Discussion

Our results based on the chloroplast-encoded *rbcL* gene sequence identified the *in hospite* symbionts of four taxonomically distantly related mixotrophic testate amoebae taxa (*Hyalosphenia papilio*, *Heleopera sphagni, Placocista spinosa* and *Archerella flavum*) (figure 1) as members of one single genus, *Chlorella sensu stricto* (Pröschold *et al.* 2011). Our sequences are therefore included within a larger clade containing many endosymbiotic forms such as *Paramecium bursaria*- and lichen symbionts, as well as free-living forms, such as *Chlorella variabilis*, *C. vulgaris*, *C. pyrenoidosa* and *Auxenochlorella protothecoides* (figure 2). *Chlorella* belongs to class Trebouxiophytes, which contain most known green algal endosymbionts, living in lichens, unicellular eukaryotes (e.g. ciliates, foraminifera etc.), plants (e.g. *Ginkgo*), animals (e.g. cnidarians, mussels, flatworms, etc.), and even parasites such as some *Coccomyxa* species (Lewis& Muller-Parker 2004; Rodríguez *et al.* 2008; Trémouillaux-Guiller& Huss 2007).

Almost all symbiont's *rbcL* sequences fall within the TACS (Testate Amoeba *Chlorella* Symbionts) clade (figure 2). Diversity within TACS (0.3%) is far below the level of genetic divergence usually observed between plant species (i.e. a 1-2% threshold is generally considered for specific discrimination in higher plants (CBOL Plant Working Group 2009; Burgess *et al.* 2011)). This suggests that TACS actually correspond to a single species. In contrast, testate amoebae hosts belong to three different eukaryotic major clades (Amoebozoa, Rhizaria and Stramenopiles) whose roots dive deeply into the origins of eukaryotes (Burki *et al.* 2008; Gomaa *et al.* 2013; Howe *et al.* 2011; Lara *et al.* 2007; Nikolaev *et al.* 2005). Our data clearly show that

symbiosis occurred relatively recently and independently in the different lineages and not once in the common ancestor of these organisms.

Furthermore, by contrast to the low diversity observed in the algal symbionts derived from different geographical locations, we found a large genetic diversity within *Hyalosphenia papilio* at the COI level. This is consistent with previous results on *H. papilio* and other Hyalospheniidae (Kosakyan *et al.* 2012), and has been interpreted as cryptic and/or intra-species genetic diversity. Indeed, the *H. papilio* sequences branch in five different groups that can be discriminated by their geographic origin, suggesting possible allopatric speciation events. This pattern clearly differs from the *rbcL* data; most *rbcL* sequences from the symbionts belong to the same (TACS) group, regardless of their geographic origin. Some *rbcL* sequences have been obtained from cells collected in sites as distant as British Columbia, Switzerland, Alaska and Russia, and are indeed exactly identical. Although COI and *rbcL* have different evolutionary histories (and possible different evolutionary rates), the almost total lack of diversity within the TACS group corroborates further the hypothesis that these organisms evolved independently from their hosts.

The lack of evidence for co-evolutionary patterns in turn suggests the existence of a pool of symbionts in the bog environment that are used by the amoeba hosts from all phylogenetic origins. This implies that the algal symbionts probably have an independent life-stage, as in the case of *Chlorella variabilis*, which can live independently from *Paramecium bursaria* (Blanc *et al.* 2010; Summerer *et al.* 2007). Thus, the symbionts are not strictly “maternally” transferred along the amoeba lineages, even within individual amoeba species. Symbionts, in turn, belong

to a limited number of species, which suggests that only those photosynthetic organisms that have pre-adaptations to intra-vacuolar life will be kept. Indeed, the nuclear genome of *C. variabilis* contains genes coding for many protein involved in protein-protein interaction and adhesion domains, a trait that appears commonly in endosymbiotic eukaryotes and bacteria (Blanc *et al.* 2010). In addition, recent experimental study showed that the digestive vacuoles of *Paramecium bursaria* have the ability to recognize and differentiate symbiotic algae from other ingested food particles based on their size and shape, allowing the *Chlorella* symbiont to escape from the digestive vacuole by budding from the vacuole membrane (Kodama& Fujishima 2012). The ability of *Chlorohydra* and some marine invertebrates to recognize symbiotic algae is believed to depend on algal cell wall associated proteins / proteoglycans (Huss *et al.* 1993; Robert 1989). It is therefore most likely that the TACS clade also have pre-adaptations for endosymbiotic life; a pre adaptation of testate amoebae symbionts would agree with Law's (1985) hypothesis that "*through natural selection genotypes of symbionts are produced that are so accommodating they could be transferred even among unrelated hosts*" (Piercey-Normore& Depriest 2001). The presence of *C. variabilis* in two *Hyalosphenia papilio* cells (10/BD-A/6 and 12/BD-B/6) corroborates further this hypothesis. The predominance of TACS as symbionts rather than, for instance, *C. variabilis* suggests that it is the most frequent *Chlorella* species in *Sphagnum* peatlands. This environment, characterized by low pH and nutrient availability indeed hosts very specific protistan communities (Lara *et al.* 2011) and it is likely that this also applies to *Chlorella* species. We can hypothesize that *C. variabilis* is more common in environments with a different water chemistry. Furthermore, the existence of the distantly related 18/LC/10 suggests that other Trebouxiophyceae strains can bear

similar adaptations. It cannot be ruled out however that these rare cases correspond to undigested prey and not to genuine symbionts.

In contrast to *Paramecium bursaria*, *H. papilio* has never been observed alive without endosymbionts since its description over 130 years ago (Leidy, 1879). Although *H. papilio* was shown to prey on various microorganisms (microalgae, protozoa, micro-metazoa and fungi) (Jassey *et al.* 2012), it appears not to survive without its algal symbionts. The situation is likely to be similar with some other mixotrophic testate amoebae, such as *Archerella flavum.* Although stable isotopic signals suggest that *A. flavum* feeds on bacteria (Jassey *et al.*), it seems that it is not able to survive for long periods (i.e. 3 months in Schönborn's *in-situ* experiment) in the absence of light (Schönborn 1965). Half-digested *Chlorella* cells were observed in the cytoplasm of *A. flavum*, suggesting that it use its algae both as food and as photobionts (Bonnet *et al.* 1981).

In the process of association between a phototrophic symbiont and its heterotrophic host, the mixotrophic testate amoebae have reached the stage where they rely on their symbionts for survival. This close and obligate association contrasts with the symbionts of *Paramecium bursaria*. Such associations can be considered as an intermediate step of integration possibly announcing a specialization of the symbiont for intracellular life, gene transfers to the host and further processes of "algal enslavement" as observed in chlorarachniophytes and cryptophytes. If and when this happens the algal symbionts will finally follow their independent evolutionary paths and the diversity of the TACS will "explode".

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5

Difflugia tuberspinifera (Amoebozoa, Arcellinida): A case of Fast Morphological Evolution in Protists

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Difflugia tuberspinifera (Amoebozoa, Arcellinida): A Case of Fast Morphological Evolution in Protists

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Abstract

It is generally believed that protist morphospecies undergo a strong stabilizing pressure, and therefore are conserved across very long periods of time. In this work, we present an example of fast morphological evolution within Arcellinida, a group that in acknowledged to contain the oldest undisputable eukaryotes ever found. We investigated the phylogenetic relationships of three endemic East-Asian *Difflugia* species with a conspicuous morphology: 1) *D. mulanensis*, 2) a morph of *Difflugia tuberspinifera* that present long spines and 3) a spineless morph of *Difflugia tuberspinifera* with other Arcellinids based on SSU rRNA gene sequences. Our phylogenetic analysis shows that the three taxa are closely related to *D. achlora* and *Netzelia oviformis*. The SSU rRNA gene sequences of the spiny and the spineless morphs of *D. tuberspinifera* differed by only two nucleotides and shared identical introns and insertions, indicating that they diverged only recently. ITS sequences showed also a limited genetic divergence between the two forms. Interestingly, a class I intron was inserted exclusively in ITS gene sequences from the spiny morph of *D. tuberspinifera*, whereas no intron were found in the spineless morphs. The intron sequences showed intraspecific diversity and thus it could potentially be used as a phylogenetic marker for population genetic studies.

Key Words: Arcellinida, molecular phylogeny, SSU rRNA, ITS, *Difflugia tuberspinifera*, *Difflugia mulanensis*, morphological stasis, fast evolution, China.

Introduction

Recent molecular phylogenetic studies have considerably expanded our knowledge of the phylogenetic relationships and the diversity of microbial eukaryotes (Cuvelier et al., 2008; Lara et al., 2011; López-García, 2001; Nikolaev et al., 2004). However, diversification and speciation processes in free-living protists remains a major question in evolutionary biology (Coyne and Orr, 2004; Logares et al., 2008; Logares et al., 2007). In order to understand the pace at which new forms appear, it is of capital importance to evaluate the rate of morphological evolution, as only morphology is preserved in the fossil record.

Protist phenotypes are believed to be extremely old, because of a supposed stabilizing selection that acts on huge sized populations (Civetta, 1999; Fenchel and and Finlay, 2006). Arcellinid morphotypes are thought to remain very stable in time, as illustrated by the resemblance between extant forms and 742 Mya old vase-shaped microfossils; from these, *Palaeoarcella athanata* shares also apparently many traits with the extant genus *Arcella* (Porter et al., 2003) (Figure 1), a suprising result if one considers the derived position of this genus in molecular phylogenies (Gomaa et al., 2012; Lahr et al., 2012; see Figure 3). However, these conservative morphologies are not the rule in all protist groups. Recent speciation events have been evidenced in the coccolithophorids, where the morphs *Calcidiscus leptoporus* ssp. *leptoporus* and *quadriperforatus* separated only 0.3 Mya ago as demonstrated both with 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 close related diminutive bacterivores share the same habitat and harbour almost identical SSU rRNA sequences despite of their extremely different sizes, morphologies and lifestyles (Foissner et al., 2003). Likewise, the ITS sequence of the aplanosporic oomycete *Geolegnia helicoides* appeared to be nested within flagellated genus *Leptolegnia*, showing an incipient loss of the whole flagellar apparatus (Steciow et al., in press); interestingly, this species had been also isolated from tank bromeliads.

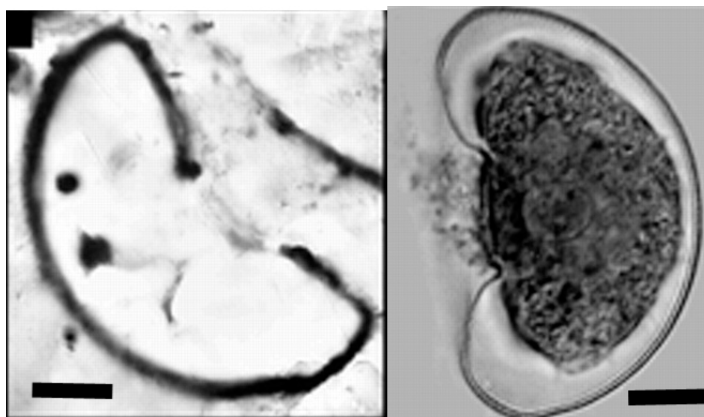

Figure 1: The vas-shaped microfossil *Palaeoarcella athanata* (left) and the modern testate amoabe *Arcella hemisphaerica* (right) analogs. Sacle bar 20 μm, (Porter et al., 2003)

In addition, it has been widely demonstrated that phenotypic plasticity is very common in Arcellinida. Members of genus *Difflugia* have been found to be very much prone to phenotypic variation that sometimes brought 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 diagnostic characters, often inadequate descriptions and the lack of molecular data in support (Gomaa et al., 2012). Thus, traits that are genetically fixed and variable characters are often difficult to tell apart, rendering even more difficult the evaluation of their stability throughout evolutionary times.

In this work, we have chosen a clear-cut example given by the spiny and spineless morphs of *Difflugia tuberspinifera*, a 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 8-10 tooth-like structure. Spineless individuals, but otherwise highly similar in shape and structure to *D. tuberspinifera*, were also observed. 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 in Arcellinid testate amoebae in general (e.g. including also other genera such as *Centropyxis* or *Argynnia*) or it is just an expression of phenotypic plasticity as suggested in the case of *Centropyxis* (Lahr et al., 2008). In case that it was genetically determined, its high variability suggests that it had to be a fast-evolving character. To address this question we sequenced the SSU rRNA gene of both spiny and spineless sequences of *D. tuberspinifera* and we placed these sequences on a large Arcellinid tree together with another Eastern Asian species, *D. mulanensis*. In addition, we sequenced the the internal transcribed

spacer region (ITS1/5.8S/ITS2) of the two forms, plus the related *D. labiosa*.

Materials and Methods

Sample collection and documentation

The two morphospecies of *Difflugia tuberspinifera* (i.e. spiny and spineless) were collected from Hubian Reservoir, Xiamen, southeast China (24°30′N, 118°10′E) in October 2010; *D. mulanensis* was collected from Tingxi Reservoir, Xiamen, southeast China (24°48′N, 118°08′E) in September 2010, while *D. labiosa* was collected from Lake Pancharevo, Sofia, Bulgaria (42°35'N, 23°24'E) in May 2010. The specimens were identified under dissecting microscope and isolated using a glass micropipette. Specimens for scanning electron microscopy (HITACHI S-4800, Japan) observations were prepared following the procedures of Yang et al. (2004) (Figure 2).

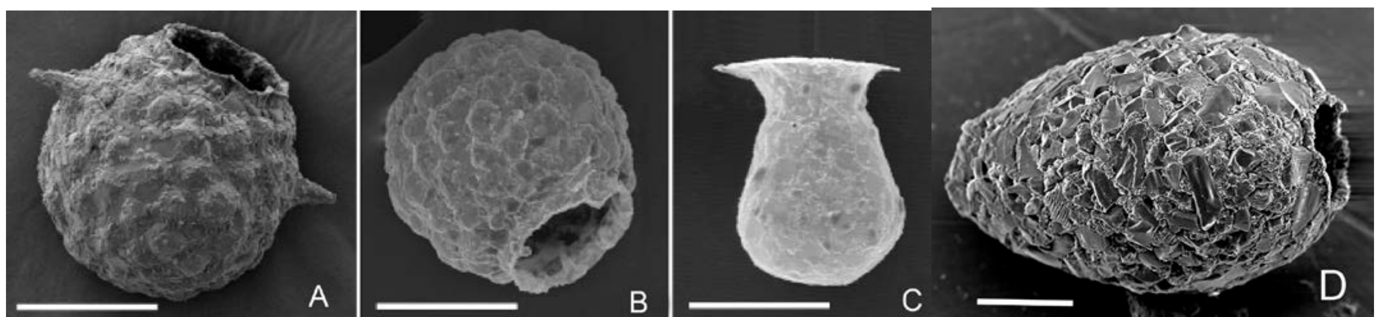

Figure 2. Scanning electron micrographs of Asian endemic *Difflugia* species analyzed in this study. *Difflugia tuberspinifera* spiny type (A); *D. tuberspinifera* spineless type (B); *D. mulanensis* (C); *D. labiosa* (D). Scale bars = 50 μm.

DNA extraction, PCR amplification and sequencing

DNA was extracted using guanidine thiocyanate protocol (Chomczynski and Sacchi, 1987). The SSU rRNA gene sequences of both *D. tuberspinifera* morphs and *D. mulanensis* were obtained in two steps. A first amplification 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 amplification profile consisting of (4 min at 95 ºC followed by 40 cycles of 30 sec at 94 ºC, 30 sec at 58 ºC and 1 min 30 sec at 72 ºC with a final elongation of 10 min at 72ºC). The obtained product served as template for the second amplification using universal eukaryotic forward primers and taxon-specific reverse primers, TuberR (GCATCACAGACCTGTTTTCGCCTCGCG) for both spiny and spineless morphs of *D. tuberspinifera* and MulR (GCATCACAGACCTGTTTTCGCCTCA) for *D. mulanensis*, in a total volume of 30 µl with amplification profile consisting of (4 min at 95 ºC followed by 40 cycles 30 sec at 94 ºC, 30 sec at 60 ºC and 1 min 30 sec at 72 ºC with a final elongation of 10 min at 72ºC). The internal transcribed spacer ITS1, 5.8S and ITS2 gene sequences of the *D. tuberspinifera* the spiny and the spineless morphospecies 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 ºC followed by 40

cycles of 1 min at 94 ºC, 1 min at 55 ºC and 1 min 30 sec at 72 ºC with a final elongation of 10 min at 72ºC). 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, in a total volume of 30 µl with amplification profile consisting of (4 min at 95 ºC followed by 40 cycles 30 sec at 94 ºC, 30 sec at 60 ºC and 1 min 30 sec at 72 ºC with a final elongation of 10 min at 72ºC).

The PCR products were screened by gel electrophoresis and the positive amplification 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).

Alignment and phylogenetic analysis

Both the SSU rRNA and the ITS gene sequences obtained in this study 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 analysis. The SSU rRNA analysis data set contained 60 amoeba taxa including 41 Arcellinida, 6 Tubulinida, 9 Leptomyxida, and 4 Echinamoebidae that were kept as outgroups; a total of 700 characters were kept for phylogenetic anaylses. For ITS analysis, we used *D. tuberspinifera* from both morphs and *D. labiosa* as outgroup; a total of 450 were used for phylogenetic anaylses.

The maximum likelihood tree was built using likelihood 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. Model parameters were estimated in RAxML over the duration of the tree search. The resulting trees 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, which is a program distributed as part of the BEAST package. The divergences between sequences were calculated using the program BioEdit software (Hall, 1999).

The secondary structure model of the group I intron found in the ITS gene sequences of *D. tuberspinifera* was generated with the online program MFOLD (Zuker, 2003) (http://www.bioinfo.rpi.edu/applications/mfold/).

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* morph, and three *D. mulanensis* (Figure 2).

The amplified SSU rRNA fragment length was 1714 bp in *Difflugia tuberspinifera* spiny morph, 1712 bp in *Difflugia tuberspinifera* spineless morph, and 1134 bp in *D. mulanensis*. There was no intra-taxon sequence polymorphism in the considered part of the SSU rRNA gene. *Difflugia tuberspinifera* spiny and spineless morphs differred only in two sites in the whole examined SSU fragment, respectively at positions 616 and 745 corresponding to spineless type of *D. tuberspinifera* resulting in a similarity of 99.8%. Both morphospecies had introns sharing identical sequences of 433 bp between positions 434 and 867, and also an insertion of 95 bp at between positions 1241 and 1336. No intron was found in *D. mulanensis,* but an insertion of 196 bp starting at the same position of both *D. tuberspinifera* morphs insertions, but differing in both sequence and length.

The topologies of phylogenetic trees inferred from maximum likelihood and Bayesian inference were similar (Figure 3) and corresponded to the previously published data by Gomaa et al., (2012). Most members of Arcellinida branched together in a single clade that receives high support values 93% bootstrap value (BS), 94% Expected-Likelihood weights of local rearrangements edge support (LR-ELW; equivalent to approximate bootstraps) (Strimmer and Rambaut, 2002) and 1.00 Bayesian inference posterior probability (PP). This main clade divides into two major groups; the first group comprises Hyaloospheniidae, *Bullinularia* spp., *Centropyxis laevigata* and the cylindrical/elongated *Difflugia* spp. (*D. acuminata*, *D. lanceolata*, *D. bacillariarum*, *D. hiraethogi*) and received moderate support values (BS=60%/ ELW=85%/ PP=0.80). The second group comprised genus *Arcella*, the

globular/spherical species *Difflugia achlora*, *Netzelia oviformis*, and the newly obtained sequences of *D. mulanensis* and *D. tuberspinifera* (both morphs). *Physochila griseola*, *Argynnia dentistoma* and *Heleopera rosea* were basal to that group. This clade receives high support values (BS=82% / ELW=87%/ PP=0.98). *Pyxidicula operculata* branch as the most basal taxa of the Arcellinida with a moderate support value (BS=53% / ELW= 87% /PP=0.98) (Lahr et al., 2011), while both *Cryptodifflugia operculata* and *Cryptodifflugia oviformis* (Gomaa et al., 2012; Lahr et al., 2011) have an uncertain position with respect to other Arcellinida. *Heleopera sphagni* (AF 848965) (Nikolaev et al., 2005) branches outside the main Arcellinida with an uncertain taxonomic position within tubulinida as shown in previous phylogenies (Gomaa et al., 2012; Lahr et al., 2011)

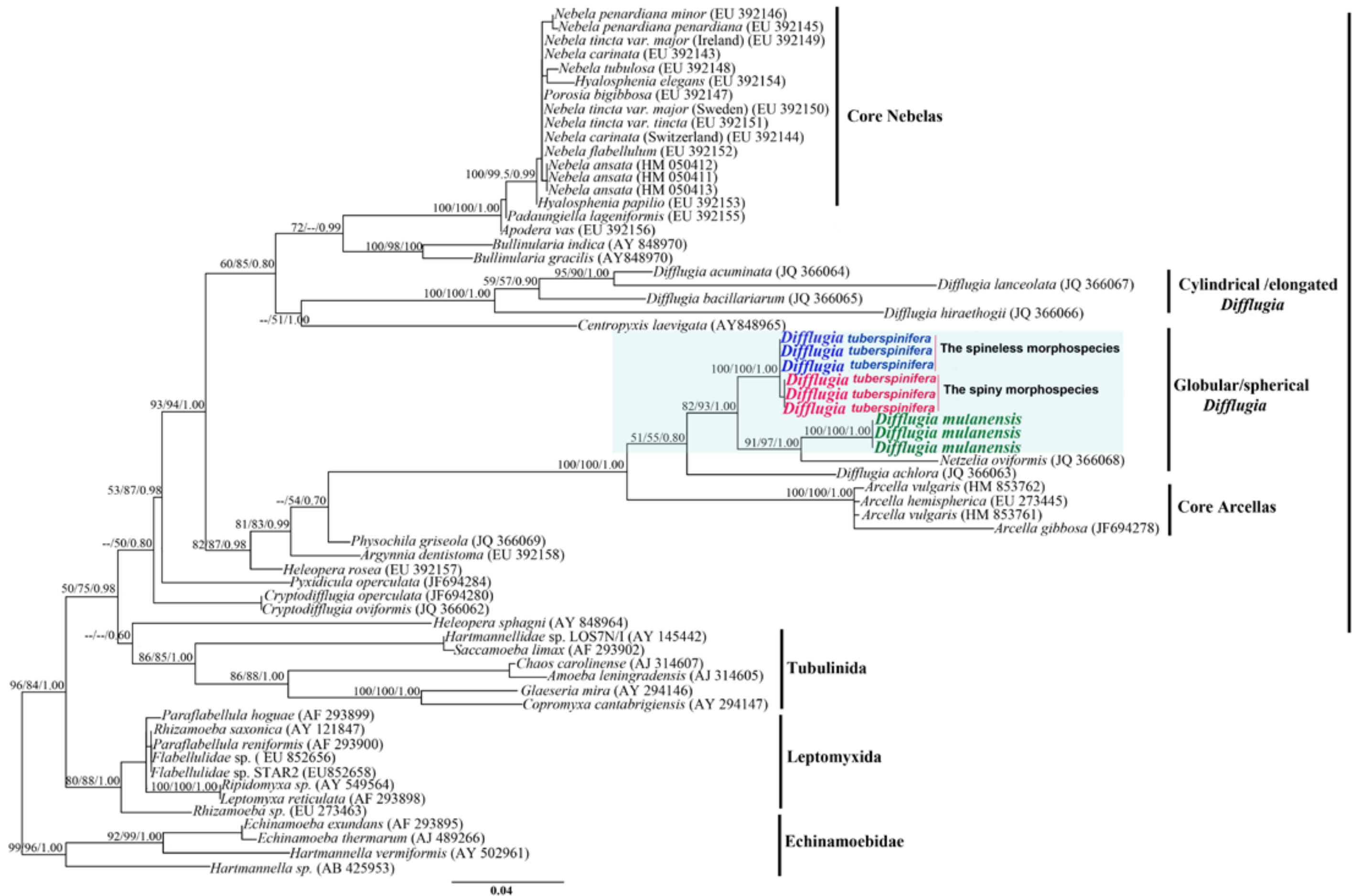

Figure 3: Molecular phylogenetic tree based on SSU rRNA gene sequences illustrating the phylogenetic position of Asian *Difflugia* spp. (*Difflugia tuberspinifera* the spiny and the spineless morphospecies and *D. mulanensis*). The tree is rooted with Echinamoebidae. The tree was derived by Bayesian 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% sequence divergence.

ITS gene analysis of the two morphospecies of *D. tuberspinifera*

The sequenced fragments of ITS1+ ITS2+ 5.8S were 812 bp long in *D. tuberspinifera* the spiny morph, 708 bp in *D. tuberspinifera* spineless morph and 591 bp in *D. labiosa*. We found group 1 intron in the ITS gene sequence of the spiny morphospecies of *D. tuberspinifera* (position 118 to 552) (i.e 434 bp) (Figures 4 and 5). We also observed intron intraspecific sequence variability among the different eight sequences obtained from the spiny morphs of *D. tuberspinifera* (Figure 4). In contrast, no introns were found in the spineless morph of *D. tuberspinifera* or in *D. labiosa*. The sequences of the ITS exon regions of the spiny and spineless morphs of *D. tuberspinifera* showed high inter-morphotype divergence varied from 4% to 10.2%, while the intra-species divergence ranged between 0.3% to 1.9% in the spineless morph of *D. tuberspinifera* and from 0.3% to 5% in the spiny morph of *D. tuberspinifera*.

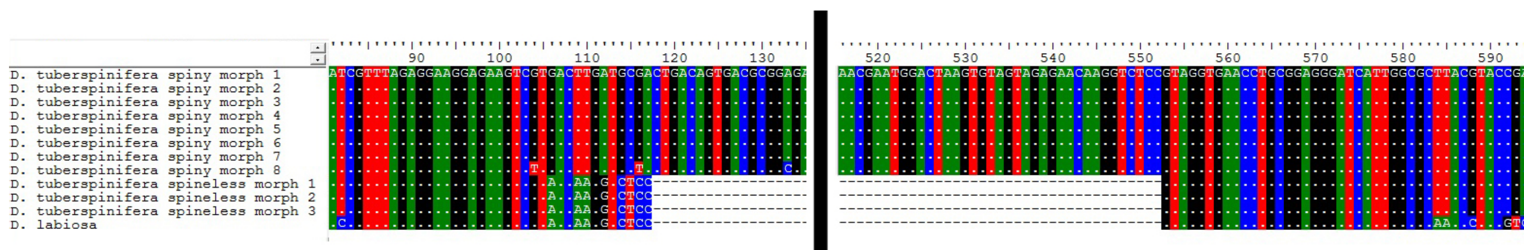

Figure 4: Sequence alignments of the ITS gene obtained from *D. tuberspinifera* the spiny and the spineless morphospecies and *D. labiosa*. The alignments show the introns position, length, and polymorphism in the spiny morph of *D. tuberspinifera*, and the relative nucleotides sequence variation in the exon region. The black bar hides an internal section of the intron sequence alignment.

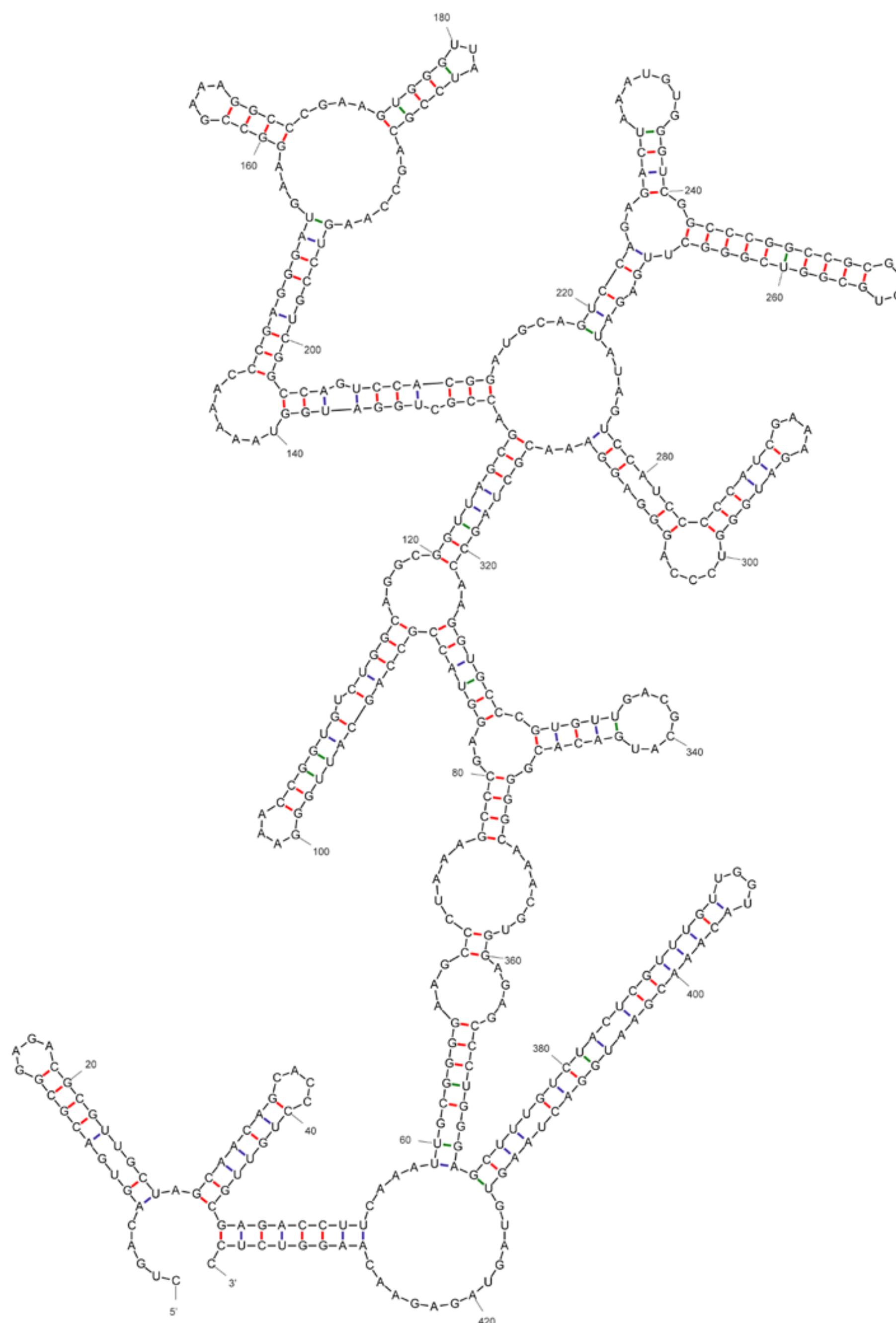

Figure 5: Predicted secondary structure of group I intron in the ITS gene sequence of *D. tuberspinifera* the spiny morphospecies. The 3`and 5` point the exon-intron junctions. The model generated with the online program MFOLD (Zuker, 2003).

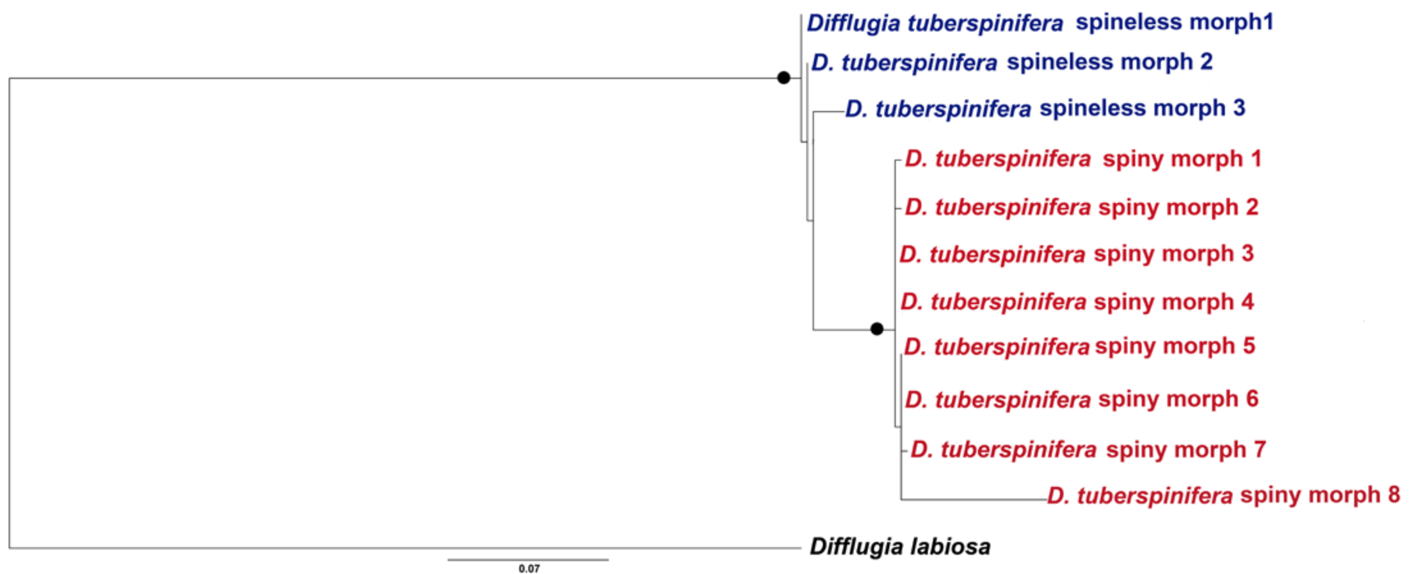

Figure 6: Molecular phylogenetic tree based on ITS gene sequences of the spiny and spineless morphs of *D. tuberspinifera*. The tree rooted by *D. labiosa*. The tree was derived by Bayesian inference using MrBayes, and an identical topology was obtained by maximum likelihood analysis. The scale bar indicates 0.07% sequence divergence.

Discussion

Both *D. tuberspinifera* morphs were included within a clade that also contained *Netzelia oviformis* and other globose taxa such as *Difflugia achlora* and the newly barcoded species *D. mulanensis* as well as *Lesquereusia modesta* as determined with other genes than SSU (Gomaa et al., 2012; Lahr et al., 2013); there is, however, evidence that species with a more elongated shell exist in that group, such as *Difflugia bryophila* (Lahr et al., 2013). It is still difficult to ascertain if globose shape

can be used as a deep phylogenetic criterion, and *D. bryophila* would be an exception, or if general shape can change frequently. Another character that might be stable in time (probably because it is related to a key function, cell division and the ontogenesis of a daughter cell), our results suggest that the presence or absence of apertural collar or necklace (a small raised rim of organic layer surrounding the shell aperture) might be reliable criterion for phylogenetic relationships for some arcellinid genera (*Arcella*, *Difflugia* and *Netzelia*). For example, four species with a collar or necklace (*Netzelia oviformis D. achlora D. mulanensis* and both morphotypes of *D. tuberspinifera*) are closely related to each other. *Difflugia tuberspinifera* has a circular aperture with a distinct collar, *D. mulanensis* has a widely projecting apertural collar, *Netzelia oviformis* and *D. achlora* both have thick organic rim or necklace around the aperture (Ogden, 1980; Ogden and Meisterfeld, 1989; Yang et al., 2004; Yang et al., 2005). Furthermore, both *Arcella vulgaris* and *A. hemisphaerica* 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). On the contrary, none of the four pyriformed-shaped *Difflugia* species have any distinct collar or necklace.

The presence of spines, however, at least in the case of *D. tuberspinifera* spiny morph, can be considered as a fast varying character. It is genetically determined, and not the result of phenotypical plasticity as both SSU and ITS sequences demonstrate; moreover, an intron was inserted only in the spiny morph, which seemed to have evolved from a spineless ancestor (Figure 2). Our results show

clearly that spiny and spineless *D.tuberspinifera* are very closely related, as they differ by only one position in the v4 region, the most variable part of the SSU rRNA. ITS sequences although more distantly related, were perfectly alignable, suggesting genetic proximity between the two forms. The inclusion of a class I intron (Figure 5,6) in the ribosomal operon of *D. tuberspinifera* is common among Arcellinida. The appearance and disappearance of these elements has been documented to occur within a single morphospecies in Arcellinida (Lara et al., 2008).

Dating separation times of the two forms is not yet possible, because of the reduced fossil record of Arcellinida, and also because these are only ITS sequences published for this particular group. However, if compared with other Amoebozoa (i.e. *Vannella* ssp.), a distance of 7.5% in average is consistant with the threshold obtained for separating what has been considered as different species (Nassonova et al., 2010). Such a rapid evolution might have been the result of an evolutionary pressure that favoured spiny forms. It has been demonstrated, for other planctonic organisms such as *Daphnia*, that spines help escaping predation. Interestingly, the development of spines in these cladocerans happened as a product of phenotypic plasticity in presence of predators (Luning, 1992). A similar functioning has been documented in *Keratella* rotifers (Marinone and Zagarese, 1998); in protists, similar effects have been documented on a variety of ciliates (Wicklow, 1997). However, in the case of *D. tuberspinifera*, this trait is fixed genetically. As it is costly to produce, it can be suggested that spined appeared as the result of a particularly strong selective pressure.

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6

Discussion

1. Phylogeny and evolution of Arcellinida

1.1. New Arcellinida phylogeny based on SSU rRNA gene sequence

In this thesis we provided the first molecular phylogenetic data on genus *Difflugia* (the largest arcellinid genus) as well as other arcellinid taxa based on SSU rRNA gene sequences (chapter 2 and 5). Although Arcellinida is a well-known group of eukaryotes since the 19th century, their phylogeny was hitherto only studied from the morphological point of view (Golemansky, 1986; Meisterfeld, 2002; Ogden and Hedley, 1980; Penard, 1902). According to traditional taxonomy, shell composition is a good taxonomic feature and species that share the same type of building materials are phylogenetically related to each other (Meisterfeld, 2002; Ogden, 1983). However, our phylogenetic data analyses showed that the shell composition is not a reliable criterion for reconstructing phylogeny and understanding the phylogenetic relationships among arcellinid taxa. Our results suggested that the general shell shape is a much more relevant criterion for understanding the phylogenetic relationships among the groups. Indeed, organisms with similar shell shape group together, for instance the pyrifom-flattened shapes for Hyalospheniidae (Kosakyan et al., 2012), cylindrical for the *Difflugia acuminata* group, rounded for the *Arcella/Netzelia/Difflugia achlora* group. In addition, we suggested that the presence or absence of apertural collar and necklace might also be a reliable criteria for phylogenetic relationships for at least some arcellinid genera such as *Arcella*, *Difflugia* and *Netzelia* (see chapter 1 and 5).

1.2. Problems and limitations in the current Arcellinida phylogenetic trees

The phylogenetic tree of the Arcellinida still suffers from undersampling of 1) taxa and few genes sequenced for individual taxa, 2) highly divergent sequences that can create long branch attraction artifacts such as *Spumochlamys* spp. (Kudryavtsev et al., 2009), and 3) taxa with unstable (uncertain) position in the current phylogenetic trees such as genus *Cryptodifflugia*. Genus *Cryptodiflugia* belongs to order Phryganellina, a group that differs from Arcellinida by the presence of conical, pointed pseudopods (Meisterfeld, 2002). Increasing taxa sampling from related genera such as *Wailesella* and *Phryganella* will probably place them in an independent lineage within lobose amoebae. In addition, the sequences of *Heleopera sphagni* combine both problems; highly divergent and with uncertain position in Arcellinida. It is however currently unclear if the existing sequence of *Heleopera sphagni* (Nikolaev et al., 2005) is correct and thus suggests a fast evolving taxon, or if it is a contamination from another (symbiont or prey) amoeboid organism associated to *H. sphagni*.

1.3. What are the appropriate genetic molecular markers for Arcellinida phylogeny?

The SSU rRNA gene is one of the most common makers used in inferring phylogenetic relationships at higher taxonomical level among Amoebozoa (Nassonova et al., 2010). This marker successfully separated among different species of arcellinid testate amoebae (Gomaa et al., 2012; Lara et al., 2008).

However, the SSU rRNA is a relatively conservative marker with a low evolutionary rate (low rate of genetic substitution) (Hillis and Dixon, 1991). Thus it might underestimate the genetic diversity among closely related taxa such as in Hyalospheniidae, or for instance such as in the case of recently differentiated species like the endemic *Difflugia tuberspinifera* (spiny and spineless morphotypes) which have highly similar SSU rRNA gene sequences with only two nucleotides polymorphism (see chapter 5).

The relatively low variability observed within SSU rRNA gene calls for searching for additional genetic markers. Lahr et al. (2011) assessed the usefulness of the actin gene for *Arcella* spp. phylogeny. Their results showed that actins have a high number of paralogs (40-50) with divergence reaching up to 29%. In addition, analysis of actin gene on *Spumochlamys* spp. by Kudryavtsev et al. (2009) showed that the Arcellinida is not a monopyletic clade, in contrast to the obtained tree by SSU rRNA gene sequences in the same study that supported it is monophyly. This suggested that the multiple paralogs of actin gene can produce biased phylogenetic analysis. Therefore actin is probably not an ideal marker for Arcellinid phylogeny.

Several studies have demonstrated the effectiveness of COI as a barcoding gene in different animal groups (Hebert et al., 2003; Hebert et al., 2004), amoebozoans (Nassonova et al., 2010) and recently family Hyalospheniidae (Arcellinida) (Kosakyan et al., 2012). In addition to that, we have shown that COI is a good marker to study species-rich groups (i.e. those containing many closely related species or cryptic diversity) such as in *Nebela collaris* s.l. and *Hyalosphenia papilio*

species complex (see chapter 4 and appendix). In both studies COI proved to be an ideal barcode marker to differentiate closely related arcellinid morphospecies.

1.4. Evolution of Arcellinida

Reconstructing comprehensive phylogeny is of crucial importance for understanding the early events in Arcellinida evolution. The non-monophyly of genus *Difflugia* and splitting the arcellinid phylogenetic tree into two different groups, the pyriform to cylindrical taxa in one group and the rounded to spherical taxa in a second group (see chapter 1 and 5) suggested that genus *Difflugia* probably evolved from at least two independent ancestors. This interpretation is supported by the fact that globose-shaped *Difflugia* species are most common and abundant in lakes, while pyriform-shaped *Difflugia* species are more abundant in wet *Sphagnum* or aquatic mosses and that the largest species are found in aquatic habitats (Grospietsch, 1954). Similarly, Alves et al. (2012) reported that the planktonic testate amoebae communities in Brazilian lake are mostly characterized by the globose and hemi-spherical shell shapes and small sizes. However, some spherical and hemispherical species are also found is relatively dry habitats (e.g. *Phryganella acropodia* in forest soils).

This ecological transition from aquatic to terrestrial habitat can explain the evolutionary transformation in the shell and pseudostome morphology from circular shell in cross section with rounded and terminal pseudostome (e.g *Physochila* and *Arcella,* spherical *Difflugia* spp.) to more or less rounded shell in cross section terminal and flat pseudostome (*Nebela* spp.) to dorso-ventrally compressed shell

with sub-terminal (*Centropyxis laevigata*) or even invaginated pseudostome (*Bullinularia* spp.).

Recently, thousands of well-preserved arcellinid fossils have been discovered from a sedimentary outcrop of the Dakota Formation in southeastern Nebraska, dating back to the Late Albian (Cretaceous) deposits (145 to 65 Mya). Most of the recovered species were assigned to the extant species within genera *Difflugia*, *Lagenodifflugia*, *Pontigulasia*, *Lesquereusia* and *Cucurbitella*. A high intraspecific morphological variability was observed among *Difflugia* species suggesting that Arcellinida species in general and *Difflugia* in particular in the geologic past were as variable as modern species.

1.5. The role of adaptive phenotypic plasticity in Arcellinida evolution and adaption to the new environments

The phenotypic variations among populations of testate amoebae are well documented in the literature (Bobrov and Mazei, 2004; Charman, 1999; Lahr et al., 2008; Wanner, 1994, 1999). Several studies suggested that the agglutinated members of arcellinids such as genera *Difflugia, Trigonopyxis,* and *Centropyxis* are oversplit at the species and subspecies level due to minor variation in test composition, size, presence or absence of pores or the number and shape of aperture lobes, etc. (Hoogenraad and De Groot, 1948; Medioli et al., 1987; Ogden and Meisterfeld, 1989). It was shown that a single morphospecies of Arcellinida or testate amoeba in general has the capacity to produce different phenotypes in response to environmental variations or as intraspecific variation within the same

species population (Bobrov et al., 2004; Chardez, 1974; Wanner and Meisterfeld, 1994). In some taxa these phenotypes can represent extreme forms (polymorphism). For example, Medioli and colleagues at (1987) found that clonal laboratory culture of single morphotype of *D. tricuspis* yielded 98 different forms that were found in nature previously and described as different species and subspecies. Thus, according to these authors, these phenotypes represent the "minimum" variability that can be expected in that species (Medioli et al., 1987). Todorov and Golemansky (2007) observed polymorphism in shell size and shape of *D. urceolata* populations. Accordingly, many of the described species cannot be considered as valid taxa.

Currently there are no standard schemes or clear rules by which we can determine the range and the degree of phenotypic plasticity within a given taxon. We usually depend on taxonomical literature and the few available monographs for comparisons (Chardez, 1974; Jung, 1942a, b; Mazei and Tsyganov, 2006; Meisterfeld, 2002; Ogden, 1979, 1983; Ogden and Hedley, 1980).

Recently, several evolutionary studies suggested that "adaptive" phenotypic plasticity which occurs in response to the environment alterations or changes might facilitate the rate and the outcome of adaptive radiation especially when these phenotypic variations become heritable in ecological time scale (Ghalambor et al., 2007; Logares et al., 2008; Wund, 2012). It might promote diversification among different populations of the same species that live in alternative environments.

In chapter 4 we have found that the variability in the number of pores at the shell edge of *Hyalosphenia papilio* does not have taxonomical value and taxa with different pores number (from 2 to 8) branch among different clades in our phylogenetic tree based on COI gene sequences. However, our results together with

previously obtained data by Booth and Meyers (2010) suggested that the variation in the number of pores is adaptive phenotypic plasticity related to the moisture of habitats (species in the wetter part of *Sphagnum* have more pores in comparison to the ones that lives in drier *Sphagnum*). Thus *H. papilio* would be an example in which the environmental changes produce different adaptive phenotypes. However, more investigations including genetic and experiment ecological data is required in this area to understand the evolution of this adaptive plasticity and its contribution in evolutionary innovation and adaptive radiation. Other findings by Kosakyan et al. (accepted) in the *Nebela collaris s.l* complex showed that small variations in shell morphology actually fall within the natural range of plasticity of a given species and does not correlate to genetic and/or ecological changes. Thus we revised the taxonomy of the group based on COI gene sequences (see appendix).

1.6. Cryptic species in Arcellinida

Cryptic diversity within closely-related taxa is of considerable scientific interest due to its potential contribution to evolution and particularly for rapid evolutionary responses (speciation events) which sometimes occur when organisms colonize new environments (Ghalambor et al., 2007). Cryptic genetic diversity was reported in arcellinids such as in *Nebela* group *sensu lato* (Kosakyan et al., 2012). In chapter 4 we have found obvious cryptic diversity (intraspecific genetic diversity) in *H. papilio* morphospecies that were collected from different geographical regions. Our data suggested that cryptic diversity in *H. papilio* reflects possible allopatric speciation events as sequences originate from different geographic locations.

2. Mixotrophic association as a driving force for evolution

Arcellinida, Euglyphida and Amphitremida include species that are largely depend on their photosynthetic *Chlorella* algae for survival, although that they still retain their phagotrophic ability. These species are very useful models to study in details the host-symbiont evolutionary relationships, specificity and diversity of photosynthetic algae. In chapter 4 we unveiled on a novel endosymbiotic *Chlorella* lineage that was adapted for photobiont life in four mixotrophic testate amoebae species (*Hyalosphenia papilio*, *Heleopera sphagni*, *Placocista spinosa*, and *Archerella flavum*). The acquired phototrophy is an important and successful ecological and evolutionary process where the permanent algal enslavement can lead eventually for a complete metabolic dependence and adaptation for intracellular life that sometimes is followed by gene transfer from algae to the host cell nucleus such as in chlorarachniophytes and cryptophytes (Archibald, 2009). Obtaining more genetic data from symbiotic algae will be necessary to investigate the fine scale diversity of symbionts across biogeographic regions.

3. Unveiling of novel testate amoeba clades and their implications

As we presented in chapter 3, the testate amoebae and other related taxa are more diverse in the eukaryotic tree than we previously thought. Amphitremida is a novel clade within Labyrinthulomycete (Stramenopiles). It includes the mixotrophic species of family Amphitremidae, *Archerella flavum* and *Amphitrema wrightianum* that were previously considered as *incertae sedis* taxa among Cercozoa (Adl et al., 2005 ; Meisterfeld, 2002). Their phylogenetic relationships with Labyrinthulomycete is not only supported by the SSU rRNA gene sequences but also morphologically,

since both Amphitremida and Labyrinthulomycete share the presence of two pseudostome at the opposite ends of the shell as a synapomorphic character.

In addition, our phylogenetic analysis suggested that primitive labyrinthulomycetes were phagotrophic and osmotrophy evolved later within the clade. Interestingly, we found that several environmental SSU rRNA gene sequences obtained from a wide range of environments fell within the Amphitremida clade. This suggests that the Amphitremida probably encompasses a very diverse group of eukaryotes inhabiting a wide range of environments, from peat bogs to anoxic zones in the deep sea and activated sludge. Their metabolisms vary from aerobic to anaerobic/micro-aerophilic, they can be phagotrophic or mixotrophic and have a planktonic or a benthic lifestyle.

We also suggested to accurately interpreting the environmental DNA surveys of eukaryotic diversity. It is extremely important to isolate and maintain identifiable and/or cultivable previously uncharacterized protists to conduct morphological observations alongside of the molecular studies. Thus we can understand their evolutionary relationships with other eukaryotes, their life history and fill the remaining gaps in the tree of eukaryotes.

4. Future prospects

The application of molecular methods to the systematics of Arcellinida particularly the SSU rRNA gene sequences started to give a more and more coherent picture about their phylogeny and evolution. In parallel, the application of more variable genetic markers such as COI to Arcellinida identification allowed us to

compare the fine scale genetic divergence within the same population and among closely related species, thus answering fundamental questions concerning cryptic and pseudocryptic diversity of these organisms.

Understanding Arcellinida evolution in terms of taxonomic diversity and ecology is important to understand the different evolutionary hypotheses in eukaryotic evolution as well. Also by improving the arcellinid phylogeny and including other taxa that most likely belong to the Arcellinida, such as *Trigonopyxis*, *Cyclopyxis*, *Phryganella*, *Plagiopyxis*, *Lesquereusia*, etc, will help improve the taxonomy which will increase the utility of using this group in bioindication studies and palaeoecological reconstructions and will make estimates of their diversity and biogeography more meaningful.

We also suggest that conducting experimental controlled studies that expose testate amoebae to different degrees of moisture, food source, temperature, and extraneous materials will provide an insight about the effect of ecological changes on the shell shape and composition. In a second step, the molecular based approaches will assess the genetic diversity and mutations that might be induced in response to these ecological changes in different population of the same species.

The exploration of mixotrophy (acquired phototrophy) in testate amoeba species broadened our knowledge on both host and algal symbiont phylogenetic relationships. Yet, application of more variable markers to the phylogeny of the symbiotic algae plus investigating more mixotrophic organisms (protist, metazoan) in peat bogs will expand our knowledge on this type of association. On a future step we can evaluate the degree of metabolic association between the host and the symbiont through targeting different elements such as Carbon (C) or Nitrogen (N) using the

stable isotope probing. Nevertheless, further analyses of plastid encoded and plastid targeted proteins will clearly demonstrate if these algal symbionts left a detectable “foot print” of plastid genes in the nuclear genome of their host (i.e. *Archerella flavum, Amphitrema wrightianum, Hyalosphenia papilio*, *Heleopera sphagni* and *Placocista spinosa*) via the endosymbiontic gene transfer (EGT) or horizontal gene transfer (HGT) or not. Recently, several studies uncovered putative plastid or algal-derived genes in the nuclear genome of dozen of taxa including several that are currently lacking of plastids : Oomycetes, *Cryptosporidium parvum*, *Plasmodium falciparum*, *Theileria parva*, *Toxoplasma gondii*, *Perkinsus marinus* (oyster parasite), *Tetrahymena thermophila* and *Paramecium tetraurelia* (Eisen et al., 2006; Huang et al., 2004; Slamovits and Keeling, 2008).

Although, the cell division and life cycle of several testate amoebae groups such as (Amphitremida, and most genera of Arcellinida and Euglyphida) still remains unknown, mainly due to unsuccessful attempts to maintain these organisms in laboratory cultures. FISH (fluorescence in situ hybridization) techniques can be used in laboratory mesocosm or controlled filed studies to give us a glimpse into their enigmatic life cycle. The obtained information together with other physiological and ecological data such as their food preferences, pH, temperature and the degree of moisture, could provide solid information on their role in the ecosystem in different stages of their life. Monitoring these organisms using the FISH in controlled filed studies might also shed the light on their growth rates and their seasonal changes as well as other behavioral traits.

Finally, the application of ultra-structure studies through the transmission electron microscope advanced techniques will reveal detailed information on cellular structure of testate amoebae including the shape and numbers nucleus, chromatin structure, number of nucleoli, the structure and spatial organization of their mitochondria, Golgi complex and endoplasmic reticulum. Comparing and analyzing the obtained data together with molecular approaches may clarify the phylogenetic and evolutionary relationships among various testate amoebae species and genera.

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B

Other project in which I have been involved during my PhD

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which should be used for any reference to this work

Using DNA-barcoding for sorting out protist species complexes: A case study of the *Nebela tincta–collaris–bohemica* group (Amoebozoa; Arcellinida, Hyalospheniidae)

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Abstract

Species identification by means of morphology is often problematic in protists. *Nebela tincta–collaris–bohemica* (Arcellinida) is a species complex of small to medium-sized (ca.100 μm) testate amoebae common in peat bogs and forest soils. The taxonomic validity of characters used to define species within this group is debated and causes confusion in studies of biogeography, and applications in palaeoecology.

We examined the relationship between morphological and genetic diversity within this species complex by combined analyses of light microscopy imaging and Cytochrome Oxidase Subunit 1(COI) sequences obtained from the same individual amoeba cells. Our goals were (1) to clarify the taxonomy and the phylogenetic relationships within this group, and (2) to evaluate if individual genotypes corresponded to specific morphotypes and the extent of phenotypic plasticity.

We show here that small variations in test morphology that have been often overlooked by traditional taxonomy correspond to distinct haplotypes. We therefore revise the taxonomy of the group. We redefine *Nebela tincta* (Leidy) Kosakyan et Lara and *N. collaris* (Ehrenberg 1848) Kosakyan et Gomaa, change *N. tincta* var. *rotunda* Penard to *N. rotunda* (Penard 1890), describe three new species: *N. guttata* n. sp. Kosakyan et Lara, *N. pechorensis* n. sp. Kosakyan et Mitchell, and *N. aliciae* n. sp. Mitchell et Lara.

Keywords: Barcoding; COI; *Nebela tincta–collaris–bohemica*; Species complex; Taxonomy

Introduction

Estimating global biodiversity has long been a subject of debate and the main uncertainty lies in the diversity of microorganisms, including bacteria, archaea, unicellular protists and micro-metazoa. A recent analysis suggests that the total species diversity is about 8.7 million species and is dominated by multicellular organisms, mostly animals (Mora et al. 2011). This estimate is in clear conflict with other analyses suggesting much higher diversity and a dominance of microorganisms (Cotterill 1995; Finlay et al. 2004; Foissner 1997, 1998, 1999). There are several causes for this discrepancy, among which: (1) the recognition or not of local distributions among free-living microbes (i.e. the endemism vs. cosmopolitanism debate), (2) the definition of what constitutes a species for micro organisms; it is not known if and

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how far they go through genome recombination (but see Lahr et al. 2011) and the percentage divergence in given genes required to identify separate species (Kosakyan et al. 2012; Mayr 1964; Nassonova et al. 2010). The huge gap in knowledge and research effort that exists between microscopic and macroscopic diversity calls for good model taxa that can be used to address such questions. We focus here on Arcellinid testate amoebae, a group of free-living, mostly heterotrophic protists.

Arcellinid testate amoebae are a good model for studies on the biodiversity, biogeography and evolution of free-living protists because of their ubiquity, diversity, abundance and taxonomically diagnostic test (Alves et al. 2010, 2012; Foissner 2006; Heger et al. 2011b; Smith et al. 2008). Unfortunately, poor taxonomy is one of the curses of the study of free-living protists, including arcellinid testate amoebae, leading, for instance, to endless debates about the existence of biogeographical patterns in the distribution of free-living protists (Foissner 2008; Heger et al. 2009; Mitchell and Meisterfeld 2005), and possibly undermining their use in palaeoecological studies (Payne et al. 2011). DNA-based studies often show that traditional taxonomy underestimates diversity of both macroscopic and microscopic organisms (Harper et al. 2009; Hebert et al. 2004a,b; Heger et al. 2011a), but detailed combined morphological and molecular studies of protist groups remain rare.

Among Arcellinid testate amoebae, the *Nebela tincta–bohemica–collaris* species complex (hereafter referred to as the *N. collaris sensu lato*) is often cited as a problematic group combining at first sight very similar species (Heal 1963) and indeed these taxa are generally lumped together by palaeoecologists (Charman et al. 2000). Numerous species and infra-specific taxa (i.e. subspecies and morphs) have been listed within this group, including: *Nebela acolla* Cash 1909, *N. bohemica* Taránek 1882, *N. collaris* (Ehrenberg, 1848) Leidy 1879, *N. collaris* var. *maxima* Lepsi 1957, *N. flabellulum* Leidy 1874, *N. parvula* Cash 1909, *N. minor* Penard 1902, *N. tincta* (Leidy, 1879) Awerintzew 1906, *N. tincta* f. *galeata* Jung 1936, *N tincta* f. *stenostoma* Jung 1936, *N. tincta* var. *major* Deflandre 1936, *N. tincta* var. *rotunda* Penard 1890, *N. sphagnophila* (Steinecke) van Oye 1933, etc. Morphological identification of these species is often problematic, partly because their original descriptions are often not precise and the main characters used to define the forms such as size, shape and the composition of the test often overlap between descriptions. The criterion of presence or absence of lateral pores on the test is often used to discriminate species, e.g. between *N. tincta* and *N. parvula* (Cash and Hopkinson 1909; Lüftenegger et al. 1988). However pores can be hard to see or completely masked, depending on the composition of the test. The validity of this criterion is therefore source of debate and confusion (Cash and Hopkinson 1909; Deflandre 1936; Jung 1942; Leidy 1879; Taranek 1882). This uncertainly in turn leads to confusion in the study of biogeography and ecology of the organisms (Heal 1961).

We therefore investigated the species delineations and the phylogenetic relationships within *Nebela collaris s.l.* based on a portion of the mitochondrial cytochrome oxydase gene subunit 1 (COI) sequences. This marker is commonly used for DNA barcoding in animals (Hebert et al. 2003a,b) and has been shown to be well suited for delimiting species of many microbial eukaryotes, including ciliates, dinoflagellates, vannellid naked amoebae, euglyphid and arcellinid testate amoebae (Barth et al. 2006; Chantangsi et al. 2007; Heger et al. 2010; Kosakyan et al. 2012; Lin et al. 2009; Nassonova et al. 2010).

In this study, we barcoded members of the *Nebela collaris s.l.* using COI as a genetic marker in order to: (1) improve current taxonomy of the members of this widespread group by comparing morphometric measurements and genetic data, and (2) evaluate the part of the morphological variation that can be due to phenotypic plasticity, and also possible genuine cryptic diversity.

Material and Methods

Sampling and species isolation

Cells were obtained from *Sphagnum*, or other mosses and forest from two geographical sites (Table 1). They were extracted by sieving and back sieving using appropriate mesh size and isolated individually with a narrow diameter pipette under the inverted microscope. Cells were rinsed with tap water. We characterized the morphology of each cell by light microscopy (Figs 1–6, 8). From each clade, we selected some cells from the same sample to be documented by electron microscopy (Fig. 7), and kept as a voucher specimen which are deposited at the Natural History Museum of Neuchâtel, Switzerland.

Scanning electron microscopy

Testate amoeba tests were mounted on stubs and then kept during one week in a desiccator. The tests were coated with gold in vacuum coating unit and then observed either with a JEOL JSM-5510 microscope (Tokyo, Japan) at 10 kV or with a Philips XL30 FEG microscope (Amsterdam, The Netherlands) at 3 kV.

DNA amplification

Single cells were used without DNA extraction for DNA amplification. The mitochondrial COI sequences were obtained by polymerase chain reaction (PCR) using the general primer LCO (Folmer et al. 1994) and a specific primer TINCOX (CCATTCKATAHCCHGGAAATTTC); designed to amplify *Nebela collaris s.l.* species. DNA was amplified in a total volume of 25 μl with an amplification profile consisting of a 5 min initial denaturation step in a 40 cycles program

Table 1. List of sequenced cells and sampling locations.

Cells	Sampling location	Country	Co-ordinates	Sequence lenght (bp)	GenBank number
LC-55	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	392	JX682586
LC-58	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	300	JX682596
LC-62	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	354	JX682597
LC-64	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	486	JX682601
LC-69	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	486	JX682602
LC-71	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	485	JX682595
LC-74	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	498	JX682591
LC-75	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	499	JX682600
LC-86	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	499	JX682594
LC-89	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	417	JX682592
LC-103	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	437	JX682587
LC-117	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	478	JX682589
LC-118	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	485	JX682588
LC-126	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	333	JX682598
LC-135	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	498	JX682593
LC-137	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	482	JX682590
PE-144	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	378	JX682577
PE-145	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	379	JX682578
PE-147	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	379	JX682579
PE-148	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682580
PE-149	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682581
PE-150	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682582
PE-151	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682583
PE-155	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682599
PE-156	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682584
PE-159	*Sphagnum* mosses, Pechora	Russia	62°05,449′N 58°19,050′E	383	JX682585
Nebela aliciae n. sp. CR	Mosses, Volcán Poás	Costa Rica	10°11′N 84°13′W	631	JN849023
N. flabellulum CA	Mosses, Lynn Peak, British Columbia	Canada	49°22′N 123°01′W	665	JN849026
N. tubulosa BG-1	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N 23°17′E	623	JN849020
N. tubulosa BG-2	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N 23°17′E	623	JN849021
N. tubulosa BG-3	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N 23°17′E	618	JN849061
Certesella martiali AR	*Sphagnum* mosses, near Ushuaia, Patagonia	Argentina	54°47′S 68°17′W	586	JN849064

of 15 s at 95 °C, 15 s at 43 °C, and 1 min and 30 s at 72°C with the final extension at 72 °C for 10 min.

The PCR products were purified using the High Pure PCR Purification Kit (Roche, Basel, Switzerland) or the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then directly sequenced. Sequencing was carried out using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed either with

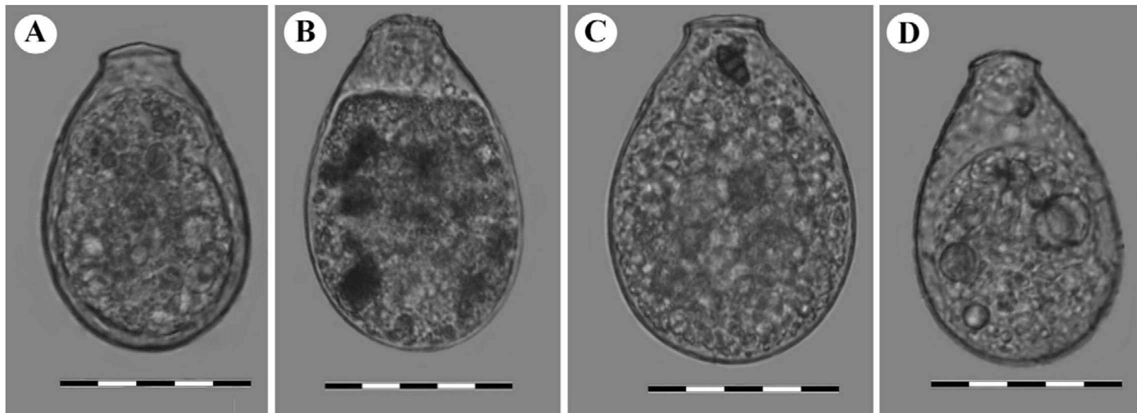

Fig. 1. Light micrographs of clade A cells (*Nebela guttata*): **A.** LC-126 from Le Cachot population, Switzerland, **B.** PE-159 from Pechora population, Russia, **C.** LC-118 from Le Cachot population, Switzerland, and **D.** LC-103 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

an ABI-3130xl or a 3730S 48-capillary DNA sequencer (Applied Biosystems). COI sequences were deposited in GenBank and the accession numbers are given in Table 1.

Phylogenetic analyses

The data set used for phylogenetic analyses (333–665 bp) comprised 32 COI sequences. The sequences were aligned

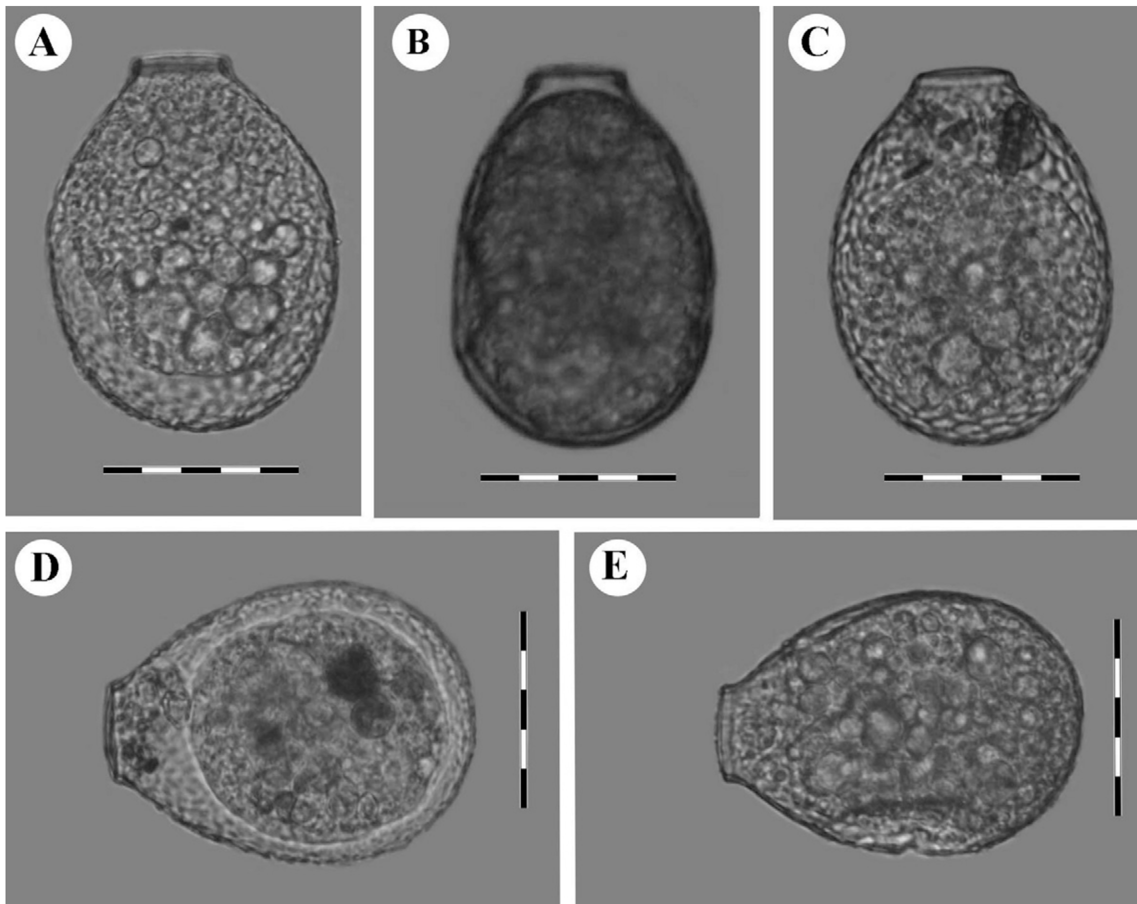

Fig. 2. Light micrographs of clade B cells (*Nebela tincta*): **A.** LC-86, **B.** LC-137, **C.** LC-117, **D.** LC-89, **E.** LC-62 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

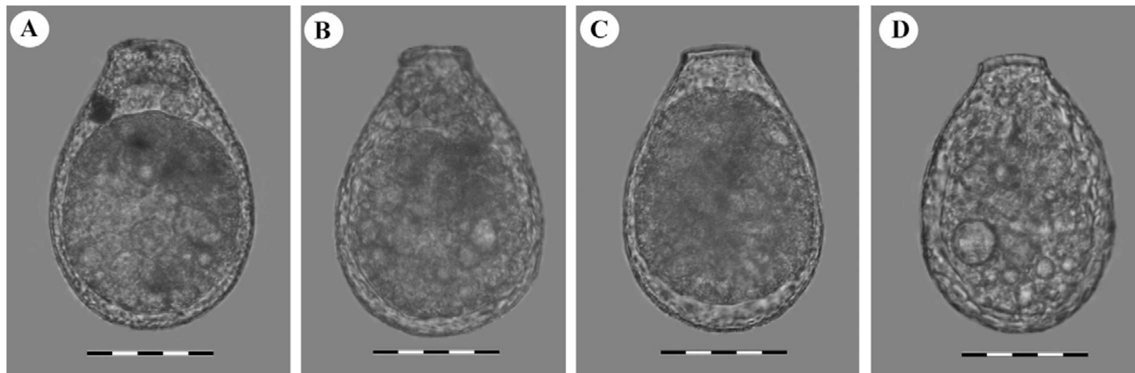

Fig. 3. Light micrographs of clade C cells (*Nebela collaris*): **A.** LC-75, **B.** LC-69, **C.** LC-64, **D.** LC-55 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

manually using BioEdit software (Hall 1999). The alignment is available from the authors upon request. Trees were reconstructed using alternatively a maximum likelihood and a Bayesian approach. The maximum likelihood tree was built using the RAxML v7.2.8 algorithm (Stamatakis et al. 2008) as proposed on the Black Box portal (http://phylobench.vital-it.ch/raxml-bb/) using the GTR+Γ+I model. Model parameters were estimated in RAxML over the duration of the tree search. We used sequences from *Certesella martiali* (GenBank number JN849064) and from *Nebela tubulosa* (JN849020, JN849021, JN849061) to root all tree, based on the fact that these species appear relatively closely related to the *N. collaris s.l.* group in the COI gene-based phylogeny of Hyalospheniidae (Kosakyan et al. 2012). Bayesian Markov Chain Monte Carlo analyses were performed using MrBayes v3.1 (Ronquist et al. 2005) with a general time reversible model of sequence evolution with four gamma-distributed rate variation across sites and a proportion of invariable sites. Bayesian MCMC analyses were carried out with two simultaneous chains, and 1,000,000 generations were performed. The generations were added until the standard deviation of split frequencies fell below 0.01, according to the manual of MrBayes 3.1 (2005). For every 1000th 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 Fig Tree (program distributed as part of the BEAST package http://tree.bio.ed.ac.uk/software/figtree/). The divergences between sequences were calculated using the package ape in R version 2.10 (R Development Core Team 2010). Missing data were not considered in the calculation (Supplementary Table S1).

Results

A total of 32 COI sequences were obtained from 24 single cells plus 6 sequences (one *Nebela aliciae* which was reported as *N. tincta* var. *galeata*, one *N. flabellum*, three *N. tubulosa* and one *Certesella martiali*)) from a previous study (Kosakyan et al. 2012). Three *Nebela tubulosa* and one *Certesella martiali* sequences were used as an outgroup. Single cells investigated in this present study were documented by light microscopy (Figs 1–6). From each population (a population is defined here as “several individuals of a given morphospecies collected from a given moss sample”) a representative cell was documented by electron microscopy and kept as a voucher species (Fig. 7).

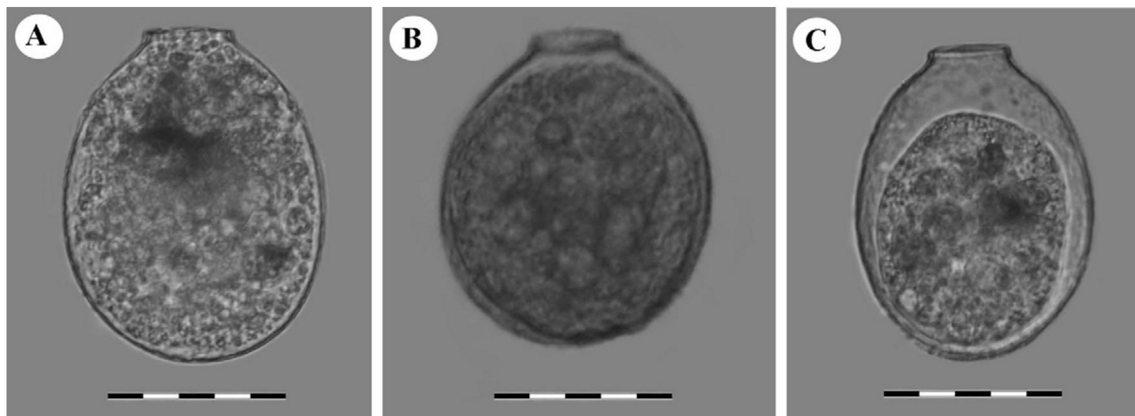

Fig. 4. Light micrographs of clade D cells (*Nebela rotunda*): **A.** LC-58, **B.** LC-71, **C.** LC-74 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

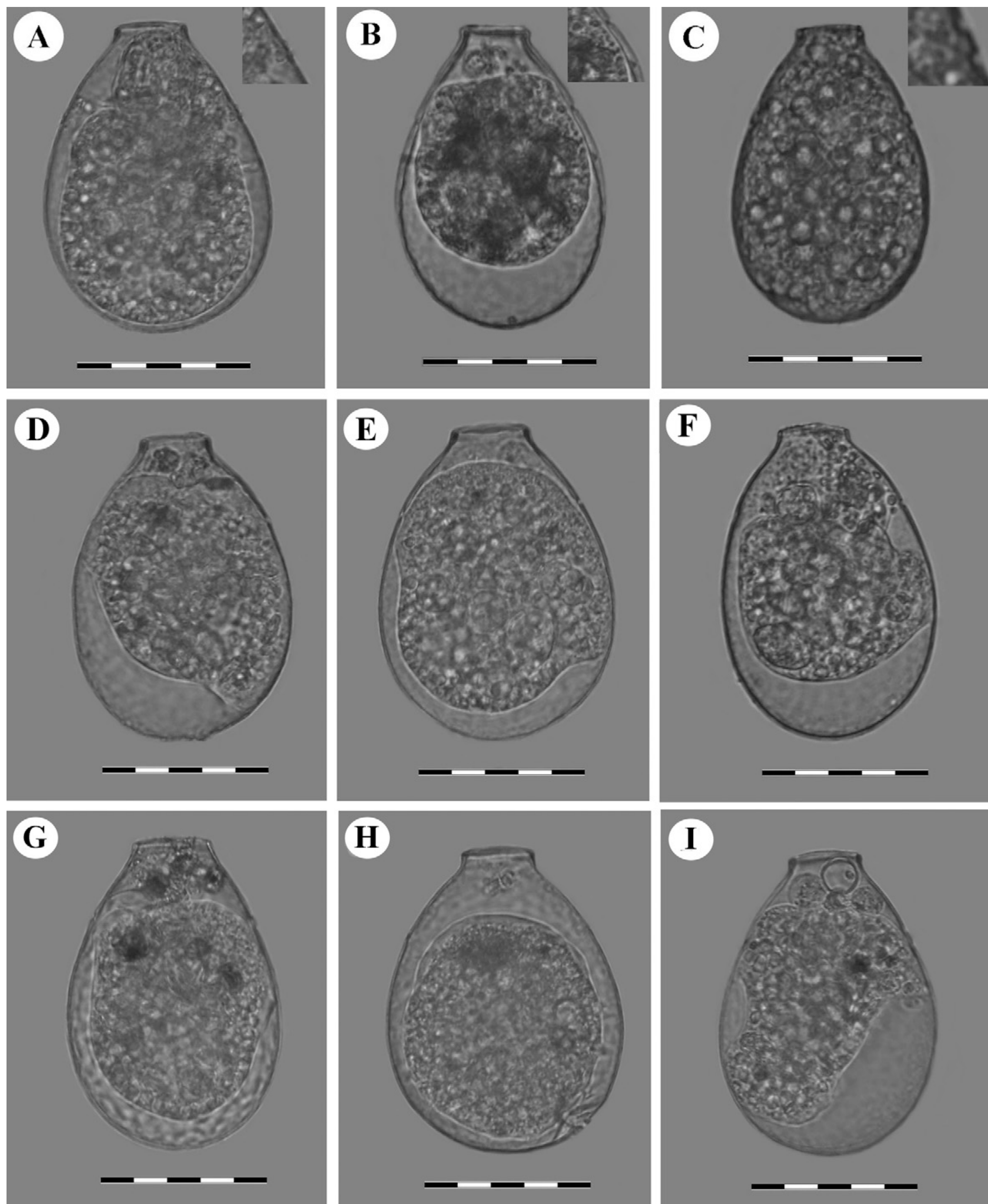

Fig. 5. Light micrographs of clade E cells (*Nebela pechorensis*): **A.** PE-149 from Le Pechora population, Russia, with its detailed picture of lateral pores, **B.** PE-151 from Pechora population, Russia, with its detailed picture of lateral pores, **C.** LC-135 from Le Cachot population, Switzerland, with its detailed pictures of lateral pores, **D.** PE-150 from Pechora population, Russia, **E.** PE-148 from Pechora population, Russia, **F.** PE-156 from Pechora population, Russia, **G.** LC-147 from Pechora population, Russia, **H.** PE-144 from Pechora population, Russia, **I.** PE-145 from from Pechora population, Russia. Scale bars represent 50 μm.

The COI fragment lengths of the newly sequenced cells ranged from 300 bp to 499 bp (Table 1). Our phylogenetic reconstructions showed that taxonomical positions of species within *Nebela collaris s.l.* must be reconsidered (Fig. 9). Topologies of both the strict consensus ML and Bayesian trees were identical. The tree revealed the existence of five main clades (A–E) plus a sequence that could not assign to any group, PE-155. Further phylogenetic analyses together with detailed morphological observations (see Methods) confirmed the existence of five clear morphotypes within the *N. collaris s.l.* complex (see Taxonomic actions) that corresponded well with the clades obtained by genetic

Fig. 6. Light micrographs of the remaining forms: **A**. *Nebela aliciae* n. sp. from Costa Rica, **B.** PE-155 from Pechora population, Russia, **C.** *N. flabellulum* from Canada. Scale bars represent 50 μm for A and B, and 60 μm for C.

means. The cell from which sequence PE-155 derived was clearly distinct from groups A–E. Information on the morphology of the cells is summarized in Table 2.

Clade A is supported respectively with 100% bootstrap (B) and 1.00 posterior probabilities (PP) values (Figs 1, 9). It includes 3 cells (LC-126, LC-118 and LC-103) from Le Cachot (Switzerland) and one cell (PE-159) from Pechora (Russia) populations (Table 1). Cells of this clade are tear- or drop-shaped, with a protruding neck (7.4 ± 0.3 μm high) and with a slightly curved and narrow aperture measuring

Table 2. Morphological characteristics of the studied cells.

Clades	Cells	Aperture (μm)	Length/breadth (μm)	*L*/*B* ratio	Test shape
A	LC-126	20, curved	83/53	1.5	Tear-shaped
	LC-159	20, curved	89/59	1.5	Tear-shaped
	LC-118	21, curved	89/63	1.4	Tear-shaped
	LC-103	20, curved	80/53	1.5	Tear-shaped
B	LC-86	25, linear	94/71	1.3	Round-elliptic
	LC-137	24, linear	90/62	1.4	Round-elliptic
	LC-117	25, linear	95/70.5	1.3	Round-elliptic
	LC-89	26, linear	93/71	1.3	Round-elliptic
	LC-62	25, linear	93/67	1.4	Round-elliptic
C	LC-75	32, slightly curved	112/81	1.4	Wide pear-shaped
	LC-69	29, linear	112/80	1.4	Wide pear-shaped
	LC-64	30, linear	112/77.5	1.4	Wide pear-shaped
	LC-55	28, curved	109/74	1.4	Wide pear-shaped
D	LC-58	26, linear	94/74	1.2	Round-shaped
	LC-71	25, linear	87.5/73.5	1.2	Round-shaped
	LC-74	24, linear	88/67	1.3	Round-shaped
E	LC-149	20, linear	84.6/53.8	1.4	Tear-shaped
	PE-151	19, linear	86/57	1.5	Tear-shaped
	PE-135	19, linear	88.5/63.5	1.5	Tear-shaped
	PE-150	21, linear	90/69	1.4	Tear-shaped
	PE-148	23, linear	92/62	1.3	Tear-shaped
	PE-147	23, linear	90/64	1.5	Tear-shaped
	PE-156	21, slightly curved	90/64	1.5	Tear-shaped
	PE-144	20, linear	92/69	1.3	Tear-shaped
	PE-145	21, slightly curved	92/62	1.4	Tear-shaped

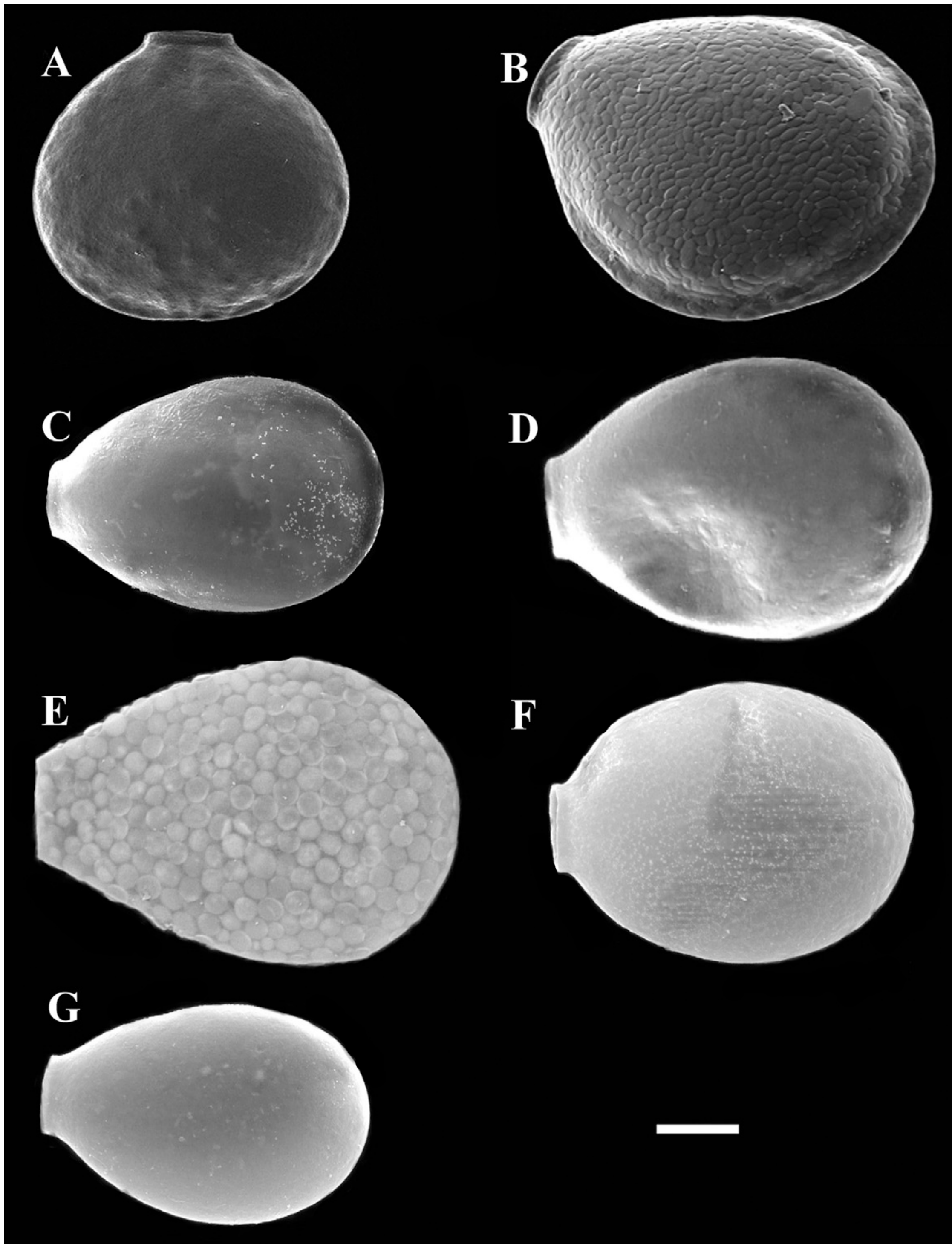

Fig. 7. Scanning electron micrographs of cells from each species-population: **A.** *Nebela flabellulum* from Lynn Peak, Canada, **B.** *N. aliciae* n. sp. from Volcán Poás, Costa Rica, **C.** *Nebela guttata* n. sp. from Le Cachot, Switzerland, **D.** *N. tincta* from Le Cachot, Switzerland, **E.** *N. collaris* from Le Cachot, Switzerland, **F.** *N. rotunda* comb. nov. from Le Cachot, Switzerland, **G.** *N. pechorensis* n. sp., from Pechora, Russia. Scale bars represent 20 μm.
Pictures A and B from Kosakyan et al. 2012.

20–22 µm. Cells of this clade have intermediate size as compared to other members of other clades: 80–89 µm in length (L) and 53–65 µm in breadth (B). The length to breadth ratio (L/B) ranges from 1.4 to 1.5 (Table 2).

Clade B receives moderate support with 70% B and 0.98 PP values. It comprises five cells collected from the same Swiss population (Le Cachot) (LC-86, LC-137, LC-117, LC-89 and LC-62) that are characterized by a round to elongated elliptic shape. The tests from this clade are larger and rounder than clade A: $L = 90–95$ µm, $B = 62–71$ µm, and L/B ratio = 1.3–1.4. Cells from this clade have relatively short neck (4.6 ± 0.2 µm high) and wide linear aperture of 24–26 µm wide, covered with a thick organic lip (Figs 2, 9 and Table 2).

Clade C receives maximum support (100% B and 1.00 PP). All four cells from this clade were collected in Le Cachot (Switzerland) (LC-75, LC-69, LC-64 and LC-55) and share exactly the same morphological characters: wide pyriform shape, relatively large $L = 109–112$ µm, $B = 74–81$ µm, and L/B ratio = 1.4 (Table 2). The neck is almost absent or very short (2.7 ± 0.9 µm high), aperture is wide 28–32 µm, linear, slightly curved or curved, and covered with thick organic lip (Figs 3, 9).

Clade D, also strongly supported (94% B and 0.99 PP), is composed of three cells from Le Cachot population (LC-58, LC.71 and LC-74). Cells from this clade have a typical very rounded shape, a short neck (4.2 ± 0.4 µm high) and a wide linear aperture, which gives an impression of a somewhat square shape (Figs 4, 9). Cells are intermediate sized: $L = 87.5–94$ µm, $B = 67–74$ µm, $L/B = 1.2–1.3$, and the aperture is 24–26 µm wide (Table 2).

Clade E with 89% B and 0.95 PP support comprises 8 cells from Pechora (PE-149, PE-151, PE-150, PE-148, PE-156, PE-147, PE-144 and PE-145) and one cell (LC-135) from Le Cachot populations. The tests are tear-shaped, small to medium size $L = 84.6–92$ µm, $B = 54–69$, $L/B = 1.3–1.5$, with a slightly protruding neck (6.2 ± 0.8 µm high) and narrow linear aperture 19–23 µm (Fig. 5).

Nebela flabellulum, and the sequence PE-155 from Pechora population have uncertain positions in the tree, and one species *N. aliciae* sp. nov. branches robustly with clade A and B.

Discussion

DNA-based studies often show that traditional taxonomy underestimates diversity of both macroscopic and microscopic organisms (Harper et al. 2009; Hebert et al. 2004a,b). Cytochrome Oxidase Subunit 1 (COI) was shown to be a good barcoding gene and successfully separated all studied morphospecies within the family Hyalospheniidae (Arcellinida) (Kosakyan et al. 2012). In this study we used COI together with morphological analyses to assess the phylogenetic relationships within the *Nebela collaris s.l.* species complex and related taxa, and to revise the rank of each species and infraspecific taxa within this group.

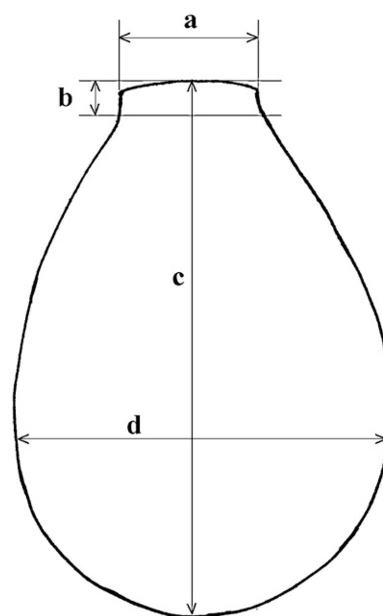

Fig. 8. Schematic sketch and position of the measured axes of the test: **a.** diameter of aperture, **b.** length of the neck, **c.** length of the test, **d.** breadth of the test.

Taxonomic relevance of the characters used to discriminate species within the *N. collaris s.l.* complex

The taxonomic position of species within *N. collaris s.l.* has long been a subject of confusion (Heal 1963; Hoogenraad and de Groot 1937; Lüftenegger et al. 1988). The main characters that defined the species within this complex were the shape and the size of the test, the shape (curved or linear) and size of the aperture, the length of the neck, and the composition of the test: the size and shape of platelets, the presence or absence of an organic layer on the test, and the presence or absence of the lateral pores. However in most cases these characters were used in a confusing and often contradictory way by different authors. For instance, the length of *Nebela tincta* varies between 71–83 µm (Leidy 1879), 85–90 µm and up to 110 µm and more (Cash and Hopkinson 1909), 76–94 µm (Ogden and Hedley 1980), 85–106 µm (Gnekow 1981), 80–110 µm (Lüftenegger et al. 1988). Heal (1963) measured, 1060 individuals and restricted *N. tincta* to the range of 78–97 µm. Larger forms were referred to as *N. tincta* var. *major*, *N. tincta* f. *stenostoma*, *N. bohemica*, or *N. collaris*.

Another variable character is the shape and size of the aperture. Heal (1963) separated large sized taxa (i.e. >100 µm) into two groups: *N. collaris sensu stricto*, with curved apertural lips, and *N. collaris sensu lato* with straight aperture (such as *N. bohemica*). Deflandre (1936) observed tests with

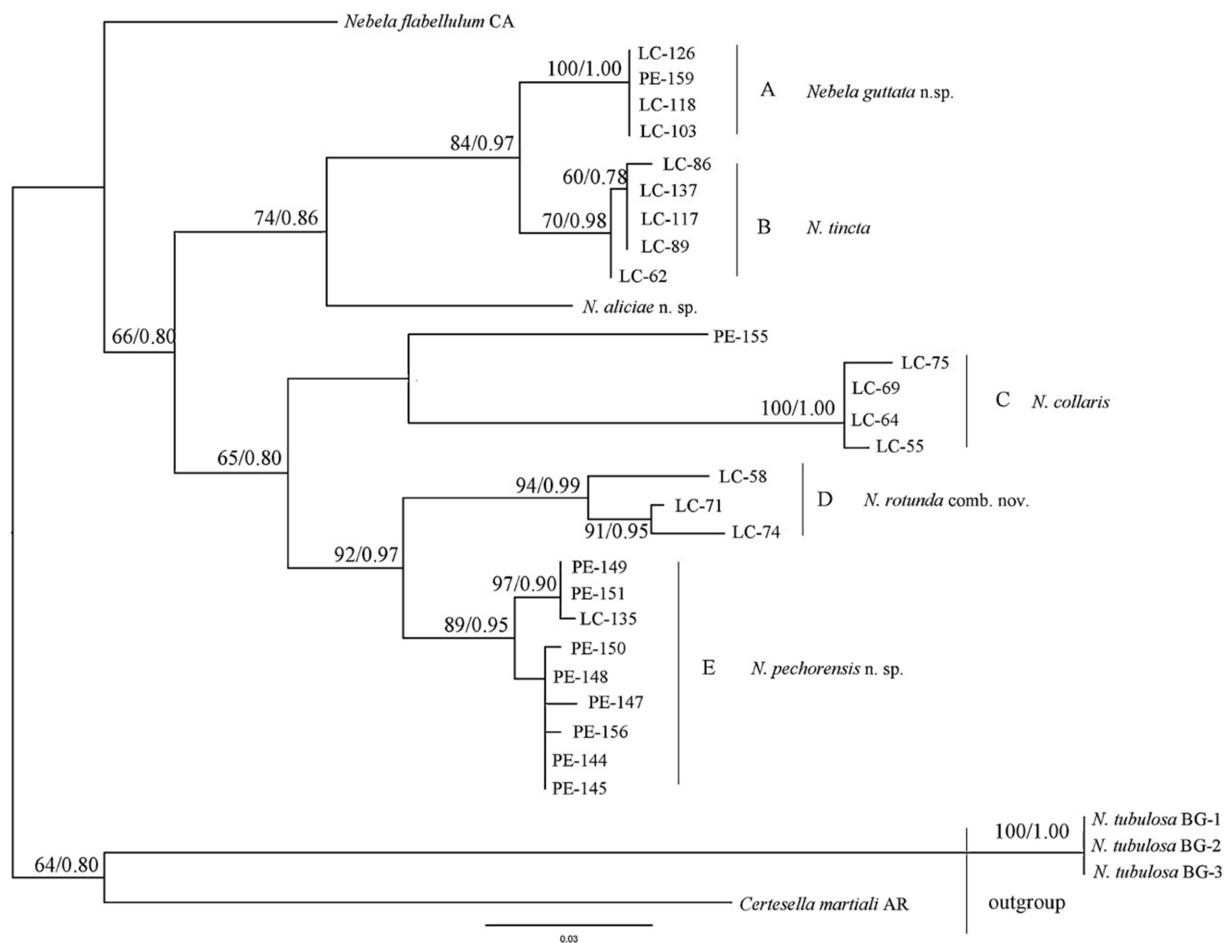

Fig. 9. Maximum likelihood bootstrap consensus tree of 31 *Nebela collaris s.l.* testate amoeba COI sequences based on 300–665 nucleotide positions. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated with Bayesian analyses. Only values above 50/0.50 are shown. The tree was rooted with outgroup *Certesella martiali* and *Nebela tubulosa*.

the features of *N. collaris sensu stricto*, which can usually be distinguished from its varieties, but still the mouth width and general shape do vary (Deflandre 1936, plates XIV and XV).

Another controversial character is the composition of the test. Given the observation that *Nebela* species use prey material to build their tests, Van Oye (1933) tried to separate species that would have different food regimes on the base of the composition of their tests. He discriminated a new species *N. sphagnophila* from *N. collaris* by its angular, irregular plates, which are never round or oval. Ogden and Hedley (1980) illustrated a specimen of *N. tincta* composed of oval or circular test plates, with a thin layer of organic cement overlay. Heal (1963) observed on Penard's slide (20.12.8.501) *N. tincta* individuals with membranous tests and with tests covered with platelets. Some authors documented that *N. collaris* species complex feeds on a wide range of prey and that test composition may change depending on the food source in single clones (Gilbert et al. 2003; MacKinlay 1936). Our molecular data confirm that the type and arrangement of the plates and overall composition of the test cannot be used as a taxonomic character for discrimination of species within *N. collaris s.l.*

The presence or absence of lateral pores is probably the most controversial taxonomic character for *N. collaris s.l.* Many species were described based on this character. Cash and Hopkinson (1909) described *N. parvula* as differing from *N. tincta* only by the absence of lateral pores. *N. minor* described by Penard (1902) differing from *N. tincta* mainly by the absence of lateral pors. Large forms of *N. tincta* such as *N. tincta* var. *major* or *N. tincta* f. *stenostoma* differ from *N. collaris* mainly by the presence of two lateral pores. Deflandre (1936) and Jung (1942) illustrate *N. collaris* without lateral pores. However, Mazei and Tsyganov (2000) and Ogden and Hedley (1980) illustrate it with pores. Hoogenraad and de Groot (1952) describe *N. collaris* var. *galeata* with pores, and Klitzke (1913) describes *N. collaris* var. *bohemica* with pores. In addition to this controversy, Heal (1963) notes that pores are impossible to distinguish when the test is completely covered with platelets. We observed pores in all our specimens, with a number that varies from 1 to 4 per cell,

within single well-supported clades; sequences were identical or differed by only a few base pairs (Fig. 6). Thus, based on our molecular data this criterion has no taxonomical validity for discrimination of the species within *N. collaris s.l.* and should therefore no longer be considered. Number of pores is probably a result of phenotypic plasticity, as it has been suggested for the hyalospheniid *Hyalosphenia papilio*. In this species a higher number of pores was observed in wetter habitats (Booth and Meyers 2010).

Thus based on our results, the morphological characters of proven taxonomic validity were: the size and shape of the test, the presence or absence of lateral ridge, the length of the neck, and the shape and size of the aperture. Characters of no proven taxonomic validity were: the composition of the test, the size and shape of platelets, the presence or absence of an organic layer on the test, and the presence or absence of lateral pores.

Phylogenetic analyses of *Nebela collaris s.l.*

We obtained molecular data for a wide range of morphotypes from the *Nebela collaris s.l.* group. Our phylogenetic analyses separate the studied morphospecies into 5 groups (A–E), which are robustly supported by molecular analyses (B, PP), and also morphologically easy to discriminate. According to Heal's (1963) definition, all the species within *N. collaris s.l.* with test length ranging from 75 to 95 µm belong to the species *N. tincta*. However, our phylogenetic data revealed four distinct clades (A, B, D, E) within this size range (75–95 µm).

Closely related clades can be morphologically quite distinct. For instance, individuals from clade A differ strongly from clade B by their smaller size (length <90 vs. 90–95 µm), general drop-like versus elliptic and rounded shape, and narrow curved vs. straight aperture. However, they branch together in the tree with a strong support ($B = 84\%$, $PP = 0.97$) and share about 96% genetic similarity on the gene considered. In our opinion the combination of morphological and molecular differences clearly indicate that the two clades correspond to two different species. Whether representatives of these clades can be considered separated specific entities is however open to debate, since there is no commonly accepted threshold to separate amoebozoan species, as we do not know how far the biological species concept (Mayr 1964) applies to microbial eukaryotes. In animals, a divergence of 4% is considered as sufficient to separate species in a barcoding approach (Hebert et al. 2004a; Witt et al. 2006). Here, a 4% threshold separates efficiently the different morphotypes, and can be used by analogy with animals, especially if we consider Arcellinida in general (and certainly the *Nebela* group) as mostly sexual (Lahr et al. 2011), and therefore following the same modalities of speciation as metazoans. A similar gap was observed in vannellid naked amoebae, another group of amoebozoa (Nassonova et al. 2010).

Representatives of clade D correspond perfectly to the descriptions of *N. tincta* var. *rotunda*. Genetic distances (up to 10%) between clade D and A, B strongly suggests that *N. tincta* var. *rotunda* is indeed independent species and not a mere variety of *N. tincta* (see Taxonomic actions).

In contrast, relatively far-related forms can harbor relatively similar morphologies. Clade E also falls within the length interval 75–95 µm. The cells in this clade very much resemble the cells of clade A in their general shape and size. The only notable difference is the straight aperture. Based on morphology it is therefore surprising that these clades do not branch together but that they rather from two robustly supported entities (clade A 100% B and 1.00 PP; clade E 89% B and 0.95 PP); sequences similarity is only 88%.

The validity of certain characters can vary among taxa. Aperture shape (i.e. straight or curved) has been shown to be a valid criterion for species discrimination in this study, and was used notably to distinguish *N. collaris* from other large forms of the species complex (*N. tincta* f. *stenostoma*, *N. tincta* var. *major*, *N. bohemica* and *N. sphagnophila*). Although our molecular data clearly separates all larger sized ($L = 109–112$ µm) specimens from all smaller forms into the well-supported (100% B and 1.00 PP) Clade C, their aperture varies from straight to strongly curved; different morphologies form a continuum of shapes, and individuals share an important degree of genetic identity (see Fig. 4). Our results suggest that all large-sized (>100 µm length) *N. collaris s.l.* species and infra-specific taxa such as *N. tincta* f. *stenostoma*, *N. tincta* var. *major*, *N. bohemica* and *N. collaris*, *N. sphagnophila* correspond to one single species: *N. collaris* (see Taxonomic actions).

Some morphospecies such as *Nebela flabellulum*, *N. aliciae* n. sp. and PE-155 branched as different entities than the five main described groups. *N. flabellulum* is a morphologically well-defined species that differs from other *N. collaris s.l.* by being wider than long. *N. aliciae* n. sp. resembles *N. tincta* f. *galeata* (Hoogenraad and de Groot 1952; Jung 1936). The 7% genetic divergence between *N. tincta* and *N. aliciae* n. sp., brings further support to considering it as an independent taxon (see Taxonomic actions). PE-155 probably constitutes another species given its particular morphology (see Fig. 6B); further investigation will be necessary to describe it as a new taxonomic entity.

Our molecular and morphological analyses show that the main characters that define the species within *N. collaris s.l.* are the size and the shape of the cells, and probably the size and shape of aperture (as in case of clade A and E). This generally agrees with our previous observations (Kosakyan et al. 2012; Lara et al. 2008).

However, we should not ignore intra-species morphological variability, which may or may not be driven by environmental conditions, including food sources, etc. (Wanner 1991). Such phenotypic plasticity which can lead to morphological difference that do not correspond to molecular differences is a source of confusion for morphology-based taxonomy and studies such as ecology and palaeoecology that are typically based only on morphological characters. Detailed studies combining morphological and molecular

data are needed first to clarify the taxonomy of different groups of testate amoebae (and protists in general). The following step is then to re-assess the ecology of the re-defined taxa and apply this revised data to ecological and palaeoecological studies.

Taxonomic actions

1. *Nebela tincta*

Nebela tincta was first described as *Hyalosphenia tincta* by Leidy (1879) as follows: "*Shell compressed pyriform, variable in the relation of breadth to length; in transverse section compressed oval; composed of pale yellow transparent, structureless, chitinoid membrane; mouth transversely oval. Sarcode colorless; pseudopods digitate, usually two, three, or more. Size – Smallest specimen, 0.076 mm long, 0.056 mm broad, 0.028 mm thick, with the mouth 0.02 mm by 0.008 mm; second specimen broader than long, 0.06 mm long, 0.08 mm broad with the mouth as in the former; third specimen, 0.08 mm long and broad, 0.026 mm thick, and mouth same as in the former; largest specimen, 0.092 mm long, 0.064 broad, and mouth as in the others*". Leidy distinguished it from *H. cuneata* by its much more pyriform shape, pale tinted test, and habitat (*Sphagnum* mosses vs. ponds). The specimens he observed were laterally compressed, oval, with a short neck composed of a pale yellow or straw-colored transparent chitinoid membrane, without trace of definite structure. Leidy also noted the presence of lateral pores "below the middle" and sometimes also "above the middle" (i.e. at about 1/3 and 2/3, of the distance from the pseudostome to the fundus). Later on Awertinzew (1906) noticed that the test of many of the specimens corresponding to this description bore platelets and the test was not simply a homogenous organic membrane. He therefore transferred this taxon to genus *Nebela* and reported a broader range of test length than in the original description: 70–120 μm. Heal (1963) considered *N. tincta* as a well-defined species with a length ranging from 75 to 95 μm. Two other species, *N. parvula* and *N. minor* with similar length were described by Cash and Hopkinson (1909) and by Penard (1902) respectively as distinct species based on the absence of lateral pores. Our molecular data suggests that the presence of pores is not a valid taxonomical criterion and therefore that these tree species need to be synonymized. The name *Nebela tincta* Awertinzew 1906 takes precedence according to the principle of priority (article 23 of the international code of zoological nomenclature).

Diagnosis of *Nebela tincta* (Leidy) sensuKosakyan et Lara

Taxonomic summary:
Arcellinida Kent 1880
Hyalospheniidae (Schulze) Kosakyan et Lara
Syn.: *Nebela tincta* (Leidy 1879) Awerintzew 1906
Nebela bursella Vejdovsky 1882
Hyalosphenia tincta Leidy 1879
Nebela minor Penard 1902
Nebela parvula Cash 1909

The test is ovoid or elongated elliptic, slightly yellowish or brownish, laterally compressed with small lateral pores (number of pores can vary), which sometimes can be difficult to observe. The test is generally composed mainly of oval or circular test plates but can also be composed mainly or only of organic cement. Length: 90–95 μm, breadth: 62–71 μm, *L/B* ratio: 1.3–1.4. The neck is very short with a linear aperture 24–26 μm wide and surrounded by an organic collar.

2. *Nebela collaris*

The name *N. collaris* has been used to cover a wide variety of forms. It was first described as *Difflugia* by Ehrenberg (1848), as were many other members of genus *Nebela*. Later on, when Leidy (1879) separated *Nebela* from *Difflugia* based on the structure of the test, he considered all pyriform species with test length around 150 μm and breadth 72 μm, as *Nebela collaris*, which then became the type species of genus *Nebela*. Heal (1963) noted that, within this group, individuals with curved apertural lips can be separated from the rest. He referred to these morphotypes as *N. collaris sensu stricto*. However it remained problematic to separate among several large taxa ranging from 95 to 155 μm in length, such as *N. tincta* f. *stenostoma*, *N. tincta* var. *major*, *N. bohemica*, *N. sphagnophila* and *N. collaris*; these taxa were generally referred to *N. collaris sensu lato*. The main discriminating character between all these taxa is (1) the size, which often overlaps, (2) the presence or absence of lateral pores and (3) the shape of the platelets (as in case of *N. sphagnophila*).

We observed pyriform species with the length 109–112 μm length and 74–81 μm breadth, with variable aperture: linear, slightly curved or strongly curved, which together form a separate clade with high 99 B and 100 PP values. We suggest synonymizing the above-mentioned species with *Nebela collaris*. Further extensive sampling focusing especially on this group would be needed to ascertain if *Nebela collaris* is a homogenous species or not.

Diagnosis of *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa

Taxonomic summary:
Arcellinida Kent 1880
Hyalospheniidae (Schulze) Kosakyan et Lara
Nebela collaris sensu Kosakyan et Gomaa
Syn.: *Nebela collaris* (Ehrenberg 1848) Leidy 1879
Difflugia collaris Ehrenberg 1848
Diffluga cancellata Ehrenberg 1848
Difflugia reticulata Ehrenberg 1848
Difflugia carpio Ehrenberg 1854
Difflugia laxa Ehrenberg 1871
Difflugia cellulifera Ehrenberg 1874
Nebela numata Leidy 1874
Nebela bohemica Taranek 1882
Nebela sphagnophila (Steinecke) Van Oye 1933
Nebela tincta var. *major* Deflandre 1936
Nebela tincta f. *stenostoma* Jung 1936

The test is large, pyriform, slightly yellowish or brownish, laterally compressed with small lateral pores (number of pores can vary), which can be difficult to observe. The test can

be composed mainly oval or circular test plates or can be composed only of organic cement. Length: mainly 109–112 μm, extremes reported: 95–115, breadth 74–81 μm, *L*/*B* ratio 1.4. The neck is very short or almost absent, with aperture, which can be linear, slightly or strongly curved, 28–32 μm wide, surrounded by an organic collar.

3. *Nebela tincta* var. *rotunda* Penard 1890 changed to *Nebela rotunda* Penard 1890

Note: A formal change is not required in this case according to the International Code of Zoological Nomenclature: (1) According to article 45.6.3, as the name was published before 1961 using the abbreviation var., it is deemed to be subspecific rather than infra-subspecific and therefore falls under rulings for species-group nominal taxa (Chapter 10). (2) According to article 46.1, names established at either species ranks (species or subspecies) are simultaneously established at the other rank, with same author and same type. Authority thus is unchanged.

4. Description of new species: *Nebela guttata* n. sp. Kosakyan et Lara

Taxonomic summary:
Arcellinida Kent 1880
Hyalospheniidae (Schulze) Kosakyan et Lara
Nebela guttata Kosakyan et Lara

Description: The test is colorless or slightly brownish, tear- or drop-shaped, with a protruding narrow neck (7.4 ± 0.3 μm high), laterally slightly compressed, with small lateral pores (number of pores can vary) (Figs 1A–D, 7C). Test composed of small particles (likely obtained from preys, e.g. euglyphid testate amoebae), which often can be covered with thin layer of organic cement. The aperture is oval, curved (Figs A–D, 7C). Dimensions (based on 5 individuals): length: 80–89 μm, breadth: 53–65 μm, width of aperture: 20–22 μm.

Hapantotype: The tests were collected from *Sphagnum* mosses in a peatland in Le Cachot, Vallée de la Brévine, Switzerland (47.5°N 6.4°E), except one PE-159, which was collected from Pechora, Russia (62°05,449′N 58°19,050′E). One SEM stub with several specimens is deposited at the Natural History Museum of Neuchâtel (Ref Nr. SEM-A-2, UniNe-EM-2). COI sequences were deposited in GenBank with accession numbers JX682598, JX682585, JX682588, and JX682587.

Etymology: The name of this species is derived from the Latin word "gutta" which means drop or tear.

Note: *Nebela guttata* resembles *N. tincta*, from which it differs by its narrow protruding curved aperture and slender drop shape of the test. Our molecular data clearly separates these two species (sequence divergence up to 4%).

5. Description of new species: *Nebela pechorensis* n. sp. Kosakyan et Mitchell

Taxonomic summary:
Arcellinida Kent 1880
Hyalospheniidae (Schulze) Kosakyan et Lara
Nebela pechorensis Kosakyan et Mitchell

Description: The test is colorless or slightly brownish, tear or drop shaped, with a protruding narrow neck (6.2 ± 0.9 μm high), slightly compressed laterally, with small lateral pores (number of pores can vary) (Figs 5A–I, 7G). The test is composed of small particles likely obtained from preys (i.e. euglyphid testate amoebae), which are often covered with a thin layer of organic cement. The aperture is oval, linear (Figs 5A–I, 7G). Dimensions (based on 10 individuals): length: 84–92 μm, breadth: 54–69 μm, width of aperture: 19–23 μm.

Hapantotype: The tests were collected from *Sphagnum* mosses in a peatland in Pechora, Russia (62°05,449N; 58°19,050E), and only one was collected from Le Cachot, Switzerland. Dry moss samples containing this species are deposited in the sample collection of the laboratory of Soil Biology, University of Neuchâtel, Switzerland (codes: EM-1614). One SEM stub with several specimens is deposited at the Natural History Museum of Neuchâtel (Ref. Nr.: SEM-A-3, UniNe-EM-3). COI sequences were deposited in GenBank with accession numbers JX682581, JX682583, JX682593, JX682582, JX682580, JX682579, JX682584, JX682577, JX682578.

Etymology: The name of this species is derived from the name of Pechora River, and the general region where moss samples containing this species were collected.

Note: *Nebela pechorensis* by shape very much resembles *N. guttata*, from which it differs only by the linear aperture. Despite the similar morphology, these two species are clearly genetically different (sequence divergence up to 12%). These two species can therefore be considered as examples of pseudocryptic species.

6. Description of new species: *Nebela aliciae* n. sp. Mitchell et Lara

Taxonomic summary:
Arcellinida Kent 1880
Hyalospheniidae (Schulze) Kosakyan et Lara
Nebela aliciae Mitchell et Lara

Description: The test is wide pyriform, with a lateral keel about 5 μm wide, laterally compressed, with a small lateral pore on each side (Figs 6A, 7B). The test is composed of small oval particles likely obtained from preys (i.e. euglyphid testate amoebae). The aperture is oval, linear (Figs 6A, 7B). Dimensions (based on 7 individuals): length: 104–115 μm, breadth: 76–93 μm, width of aperture: 24–27 μm. (Note: This description is based on *Nebela tincta* var. *galeata* data published in Kosakyan et al. 2012).

Hapantotype: The tests were collected from mosses Volcán Poás, Costa Rica (10.11°N 84.13°W). Dry moss samples containing this species are deposited in the sample collection of the laboratory of Soil Biology, University of Neuchâtel, Switzerland (code: EM-1451). One SEM stub with several

specimens is deposited at the Natural History Museum of Neuchâtel (Ref. Nr.: UniNe-EM-4). The COI sequence was deposited in GenBank with accession number JN849023.

Etymology: The name of this species refers to the name Alice for two reasons. The first is by reference to Lewis Carroll's book "Alice in Wonderland" and the fact that the world of testate amoebae is indeed one of strange and wonderful creatures. The second is in reference to E. Mitchell's first daughter, Alice, who occasionally enjoys looking at testate amoebae through his portable microscope.

Note: *Nebela aliciae* resembles *Nebela tincta* f. *galeata* mentioned by Jung 1936, from which it differs by the smaller size (length: 104–115 µm for *N. aliciae* vs. 117–143 µm for *Nebela tincta* f. *galeata*). Our molecular and morphological data suggests that *N. aliciae* is indeed an independent species from other studied taxa. Molecular data on *Nebela tincta* f. *galeata* is however lacking.

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Identification key of *N. collaris s.l.*

1. → Test wider than long. 2
   ← Test longer than wide. 3
2. → L. 72–111 µm, B. 90–133 µm, strongly flattened, with short neck, with linear or slightly curved aperture 19–34 µm. Mostly in moist *Sphagnum* mosses in raised bogs and heathlands. Fig. 7A________________***Nebela flabellulum* Leidy 1874**
   ← Smaller species, L. 60 µm, B. 70 µm, without neck. In wet *Sphagnum* ____***N. acolla* Cash 1909**
3. → Larger species, test longer than 100 µm 4
   ← Smaller species, test shorter than 100 µm 5
4. → Species with lateral ridge, wide ovoid, L. 104–115 µm, B. 76–93, with wide linear aperture 24–27 µm. In mosses. Fig. 7B ________________***N. aliciae* n. sp.**
   ← Species without lateral ridge, L > 100 µm, L/B = 1.4, pyriform, with very short (2.7 ± 0.9 µm high or almost absent) neck, with aperture slightly or strongly curved, or sometimes linear, 28–32 µm. In moist and wet *Sphagnum* and other mosses in peatlands, forests and acidic humic ponds. Fig. 3_____***N. collaris*** (Ehrenberg 1848) Leidy 1879
5. → Species with protruding neck and narrow aperture 19–23 µm, up twisted. 6
   ← Species with short neck (4.6 ± 0.2 high), with linear wide aperture 24–26 µm 7
6. → Test drop- or tear-shaped, L. 80–89 µm, B. 53–65 µm, L/B = 1.4–1.5, curved aperture. In *Sphagnum* mosses. Fig. 1______________________***N. guttata* n. sp.**
   ← Aperture not curved, L. 84–90(92) µm, B. 54–64(69) µm, L/B = 1.3–1.5. In *Sphagnum* mosses. Fig. 5 ________________________***N. pechorensis* n. sp.**
7. → Test ovoid or elongated elliptic, L. 90–95, B. 62–71, L/B = 1.3–1.4, aperture linear. In *Sphagnum* mosses. Fig. 2___*N. tincta* (Leidy 1879) Awerintzew 1906
   ← Test rounded, L. 87.5–94 µm, B. 67–74 µm, L/B = 1.2–1.3, aperture linear. Lives *Sphagnum* mosses. Fig. 4________________***N. rotunda comb. nov.***

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejop.2012.08.006.

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C

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