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Anush Kosakyan

Phylogeny, Systematics and Ecology of Free Living Protists

Case study: family Hyalospheniidae

Members of the Jury:

Prof. Edward Mitchell (thesis director): University of Neuchâtel, Switzerland

Dr. Enrique Lara: University of Neuchâtel, Switzerland

Prof. Jan Pawlowski: University of Geneva, Switzerland

Prof. Frederick Spiegel: University of Arkansas, USA

Prof. Daniel Lahr: University of Sao Paulo, Brazil

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UNIVERSITÉ DE NEUCHÂTEL

Faculté des sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel - Suisse
Tél: + 41 (0)32 718 2100
E-mail: secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Anush KOSAKYAN

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sur le rapport des membres du jury composé comme suit:

- Prof. Edward Mitchell, Université de Neuchâtel, directeur de thèse
- Dr Enrique Lara, Université de Neuchâtel
- Prof. Jan Pawlowski, Université de Genève
- Prof. Frederick W. Spiegel, University of Arkansas, USA
- Prof. Daniel J.G. Lahr, Universidade de Sâo Paulo, Brésil

Neuchâtel, le 5 juin 2014

Le Doyen, Prof. P. Kropf

— — —

No one knows the diversity in the world, not even to the nearest order of magnitude. We don't know for sure how many species there are, where they can be found or how fast they're disappearing. It's like having astronomy without knowing where the stars are.

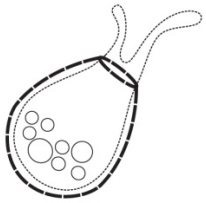
Edward O. Wilson

BUT

— — —

Each species on our planet plays a role in the healthy functioning of natural ecosystems, on which humans depend.

William H. Schlesinger



A process which led from the amoeba to man appeared to the philosophers to be obviously a progress--though whether the amoeba would agree with this opinion is not known.

Bertrand Russell

CONTENTS

APPENDIX II.

Abstract

Despite the fact that free-living protists compose the major part of Earth's eukaryotic biodiversity and play numerous essential roles in ecosystems, knowledge on their true diversity, evolution and ecology remain highly limited.

In this thesis I choose testate amoebae as a model group to address several key questions on the diversity, evolution and ecology of free-living protists. Family Hyalospheniidae is one of the most species-rich and conspicuous families of testate amoebae combining species with a broad range of test shapes and ultra-structures. Some species are easily identifiable but several species complexes are composed of an unknown number of cryptic and pseudocryptic forms. Thus this is an excellent model group to address open questions on the concept of the species in free-living protists, to assess the validity of characters used to define "true species" and to reveal the true diversity and ecology of these organisms. The studies presented in this thesis are based on the combination of morphological (light and scanning-electron microscopy) and molecular approaches (mitochondrial cytochrome oxidase subunit 1 - COI - sequence data).

We used COI to assess the phylogenetic relationships and taxonomy of the family Hyalospheniidae in order to assess the validity of morphological characters within this group (Chapter 1). The COI data successfully separated all studied morphospecies and revealed the existence of several cryptic species. The phylogenetic analysis shows that genus *Nebela* was paraphyletic and could be split into genus *Nebela s.str.* and a newly defined genus, *Padaungiella*. Family Hyalospheniidae Schulze was redefined: Genus *Quadrulella*, one of the few arcellinid genera building its shell from self-secreted siliceous elements, the mixotrophic *Hyalosphenia papilio*, and six other genera of true Nebelids (*Apodera*, *Alocodera*, *Certesella*, *Nebela*, *Porosia*, *Padaungiella*) were included in this family, while genera *Argynnia* and *Physochila* did not. Thus we redefined the family as Hyalospheniidae Kosakyan et Lara, which now includes *Hyalosphenia*, *Quadrulella* (previously in the Lesquereusiidae) and all true Nebelids. We defined the general morphology of the shell and the presence of an organic rim around the aperture as synapomorphies for Hyalospheniidae.

Our next task was to analyse in more depth the complex group of species in the Hyalospheniids.

We examined the relationship between the morphological and genetic diversity within two species complexes, *Nebela collaris s.l.* and *Quadrulella symmetrica s.l.* We combined analyses of light microscopy imaging and COI sequence data obtained from the same individual single cell (Chapters 2 and 3). We showed that small variations in test morphology easily overlooked by traditional taxonomy corresponded to separate, sometimes quite divergent, genotypes. The position of each taxa within species complex was revised, and overall the taxonomy of these two species complexes was redefined.

We developed a method to estimate the qualitative and quantitative community structure of *Nebela collaris* species complex from environmental samples, and validated this approach through microscopic observations (Chapter 4). We assessed the relative biomass and density of species using cloning-sequencing of the mitochondrial cytochrome oxydase (COI) gene amplified from environmental DNA and from artificial communities. Comparisons with direct microscopy counts showed that the COI clone library data were correlated to community counts corrected for biovolume, which allowed making inferences about individual taxon abundance and biomass in a community. We then used this approach to define the ecological niches of closely related /cryptic species in the different microhabitats that compose a peatland complex (Appendix I, contribution as a second author). Our sequence analysis revealed four of the seven barcoded *Nebela collaris s.l.* species, plus two new genotypes of yet unknown morphology. Species ranged from generalists found in most habitats (e.g. *N. collaris*) to specialists, encountered only but pervasively in particular habitats (e.g. *N. rotunda* in forested bogs). Experimental approaches would be needed to assess whether the observed niches correspond to the ecological optimum of the different species or if some at least are pushed towards less favourable habitats by competition. Our study suggests that speciation should have occurred sympatrically by specialization towards divergent niches instead of through geographical isolation.

My direct contribution to the taxonomy of this group was the introduction of one new genus *Padaungiella* Lara et Todorov, and four novel species of *Nebela*: *N. aliciae* Mitchell et Lara, *N. guttata* Kosakyan et Lara, *N. meisterfeldi* Heger et Mitchell, *N. pechorensis* Kosakyan et Mitchell. Additionally at least 3 potentially new *Quadrulella species* will be described (work in progress).

Finally we compiled all known taxonomic, molecular and ecological data on hyalospheniid testate amoeba in a monograph entitled “Family Hyalospheniidae” (Chapter 5).

Based on a careful revision of historical data combined with recent molecular data, this work aims at establishing a clear state of the art of current knowledge on the diversity of this family, providing improved species descriptions of hyalospheniid testate amoebae and hopefully to familiarize a broader audience with these beautiful protists.

Overall my thesis illustrates how traditional taxonomy often underestimates the true diversity of microorganisms, and calls for a renewed research effort on the taxonomy of free-living protists. My work contributes of understanding of a tiny piece of microbial diversity and shows how a combination of morphological and molecular approached can help improving our knowledge on the evolution, systematics and ecology of these organisms.

Keywords: cryptic species, ecology, environmental DNA, Hyalospheniidae, morphology, mtCOI gene, phylogeny, protists, species complexes, species diversity, systematics, taxonomy, testate amoebae

Organisation of the thesis

This thesis is based on four papers (Chapters 1-4) and one monograph (Chapter 5). Chapters 1-5 are preceded by a general introduction and followed by a general discussion and conclusions summarizing the main results of the thesis. I have also included two papers in which I have been involved as co-author during my PhD (Appendix I and II). I have discussed the data concerning Appendix I in my Abstract, Introduction and Discussion and Conclusion parts, since it has direct contribution to the main subject of my thesis and is a logical continuation of Chapter 4.

Chapters 1, 2 and the Appendix II correspond to published papers while chapters 3-5 and the Appendix I are either submitted or still work in progress.

Introduction

Free-living protists in the current biosphere, their diversity and systematic

Free-living protists are a heterogenous group of mostly unicellular eukaryotic organisms and compose the major part of global eukaryotic biodiversity, in terms of lineages, structural and molecular divergence (Medinger et al., 2010; Boenigk et al., 2012; Pawlowski et al., 2012). Indeed, the phylogenetic tree of life comprises mostly protist linages (Figure I-1), even if the plants, fungi and animals visibly dominate terrestrial and aquatic ecosystems. Free-living protists are extremely abundant and present in all environments on Earth with the exception of hyperthermophilic systems (Zettler et al., 2002; Lopez-Garcia et al., 2007; Mora et al., 2011). Protists play numerous roles of major importance in ecological processes at the global scale, and the study of the diversity and functional roles of these organisms is a dynamic field of research (Adl and Gupta, 2006). On the applied side, protists are increasingly used and studied for their potential in the pharmaceutical and biotechnological industries and in environmental monitoring (Patterson et al., 1996; Foissner, 1997a, 1999b; Charman, 2001; Nguyen-Viet et al., 2007, 2008; Mitchell et al., 2008). Taxonomy and historical phylogenetic relationships are fundamental aspects in biology and are central to further understanding in ecology, physiology, biochemistry, and molecular and evolutionary biology.

However, the current knowledge about the diversity, biology, taxonomy and systematics of these organisms are still very limited, because 1) many geographic regions and habitats have been insufficiently sampled, 2) the difficulties to maintaining most species in culture are serious obstacles that prevented the exploration of protistan diversity, 3) many forms, especially the smallest ones, lack diagnostic characters that can be used to build a morphology-based taxonomy, 4) funding agencies often do not perceive the intrinsic interest of many soil and freshwater protists because these organisms are generally poorly known even by biologists and often perceived as not very relevant to process of direct interest for humans (unlike bacteria and, to some extent, fungi), while that phytoplankton and pathogenic protists receive much more attention. Nevertheless, the rate of discovery of new species from

environmental samples is increasing rapidly. Indeed, most soil, freshwater, or marine samples collected contain a multitude of undescribed species (Foissner, 1999a, 2005; Šlapeta et al., 2005).

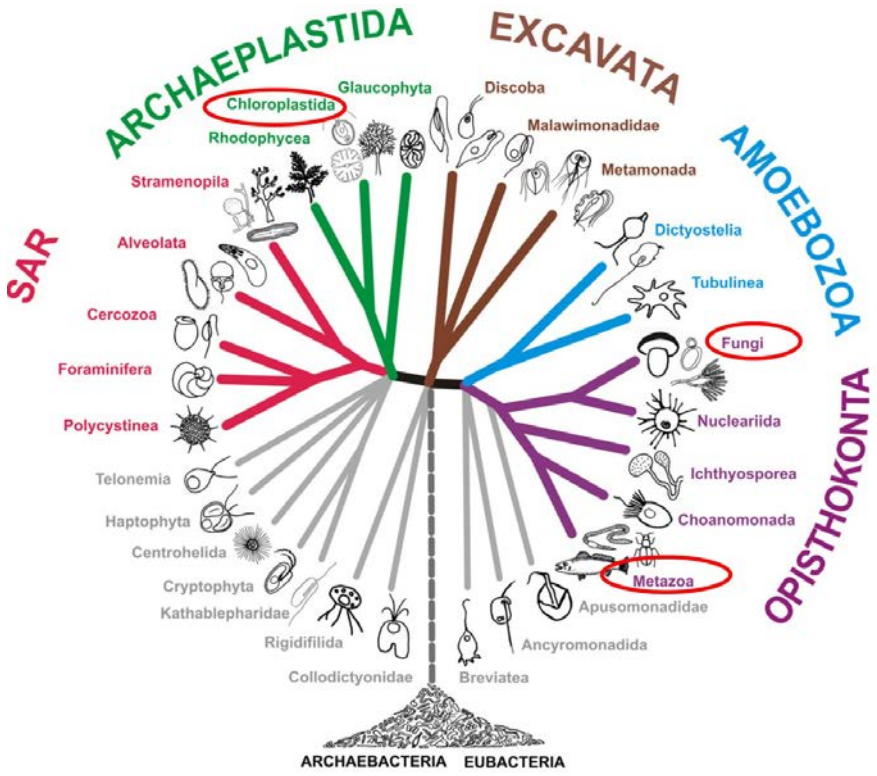


Figure I-1. The phylogenetic tree of life (after Adl et al. 2013) showing the diversity of protist lineages compared with plants, fungi and animals (highlighted by red circles).

"Limited taxonomy" is a central concern in almost all groups of protists. Unfortunately, poor taxonomy is one of the main reasons preventing the correct application of these organisms in related fields of research and also leads to confusion about debates on global biodiversity and biogeography of free-living microorganisms (Finlay and Fenchel, 2004; Mitchell and Meisterfeld, 2005; Foissner, 2006; Heger et al., 2009).

Fortunately, nowadays, molecular methods allow to studying the phylogeny and taxonomy of protists, and help resolve long-standing debates such as their diversity and biogeography. The introduction of molecular markers and the development and refinement of phylogenetic software for their analysis, has led to two main developments:

1) DNA barcoding, which applies DNA sequences as taxon-specific molecular markers, as a tool to identify species and strains (Hebert et al., 2003a, b;

Pawlowski et al., 2012). Necessary requirements for reliable DNA barcode-based species identification are broad sampling of species and well-defined morphological species descriptions (Meyer and Paulay, 2005).

2) Reconstruction of the eukaryotic phylogenetic tree, based first on ribosomal genes and, later, on multi-gene- and genomic approaches (Cavalier-Smith, 1989; Sogin and Silberman, 1998; Baldauf, 2003; Adl et al., 2005; Keeling et al., 2005; Burki et al., 2008; Adl et al., 2013). Remaining open questions require the analysis of poorly sampled groups and especially lineages corresponding to key nodes in the general phylogeny (e.g. deep-branching clades).

Since the beginning of the twenty first century, the first studies based on the retrieval of environmental sequences of the gene coding for the small subunit of the ribosome (18S rRNA), first using the classical cloning and sequencing approach and then followed by the massive sequencing approach (454, illumina) (Cavalier-Smith, 1989; Sogin and Silberman, 1998; Harper et al., 2005; Nikolaev et al., 2005; Sogin et al., 2006; Bik et al., 2012; Egge et al., 2013) revealed an unexpectedly huge diversity of eukaryotes in various environments (López-García P. et al., 2001; Moon-van der Staay et al., 2001; Stoeck et al., 2003; Šlapeta et al., 2005). Many of these environmental sequences appeared not to have any affinity with any cultured strain (Epstein and Lopez-Garcia, 2008; Berney et al., 2004). These new clades and improved phylogenetic numerical tools have regularly brought scientists to rethink the evolutionary relationships among groups of eukaryotes as attested by the changing number and names of eukaryotic super-groups. With the development of molecular phylogenetic studies the position of different protist groups was reconsidered within the phylogenetic tree of eukaryotes (Cavalier-Smith, 1989; Cavalier-Smith, 2009; Sogin and Silberman, 1998; Simpson and Roger, 2002; Roger and Simpson, 2009; Okamoto et al., 2009; Adl et al., 2005, 2013). The position of many genera or individual taxa was redefined, for instance some naked amoebae from Amoebozoa were transferred to Excavates, (Page and Blanton, 1985; Simpson, 2003), or the filose amoebae genus *Nuclearia* (Nucleariids) was transferred from Rhizaria to Opisthokonta (Page, 1991; Zettler et al., 2001) (Steenkamp et al., 2006). Unexpected new diversity within several morphospecies was discovered (Heger et al., 2011a) and entirely new forms were found in environments overlooked in the past (Kudryavtsev and

Pawlowski, 2013). Today the deep relationships among supergroups and the position of the eukaryotic root remain to be confirmed and the position of numerous incertae sedis groups clarified.

The estimation of global eukaryotic diversity is also not yet resolved. Classically, it has been assumed that multicellular forms, especially insects, dominated diversity with a total of about 8.7 million species on Earth (Mora et al., 2011). Others claim that diversity is much higher with the dominance of microorganisms (Cotterill, 1995; Finlay et al., 2004; Foissner, 1997b, 1998, 1999a) recently; an estimation of over 5 million species of Fungi challenged the "macroscopic domination" of diversity (Blackwell, 2011). Estimation of total number of species depends on which species concept is considered. Biological species, in the sense of Mayr (as members of populations that actually or potentially interbreed in nature) are difficult to apply to micro-organisms because sexuality has never been documented for most groups; however it also has not been studied in most groups and existing evidence suggests that it is more pervasive than generally believed (Lahr et al., 2011). Neither morpho-species, which are known to include a large genetic diversity, nor a "genetic species" concept that lacks validation as discrimination thresholds probably vary from clade to clade, are satisfactory. In addition, under-sampling is always a serious issue with micro-organisms, as their detection is not immediate.

Testate amoebae as a model group for evolutionary studies: from classical taxonomy to molecular phylogeny

Testate amoebae are a polyphyletic assemblage of at least three major, unrelated taxonomic groups of mostly heterotrophic unicellular eukaryotes (Fig. I-2-4). Testate amoebae are traditionally separated into two main groups based on pseudopod morphology (Meisterfeld, 2002a,b; Adl et al., 2013). The phylogenetic position of Arcellinida (with lobose pseudopodia) and Euglyphida (with filose pseudopodia) among Amoebozoa and Cercozoa (SAR), respectively, has been established based on ribosomal DNA sequences (Bhattacharya et al., 1995; Cavalier-Smith and Chao, 1997; Wylezich et al., 2002; Nikolaev et al., 2005).

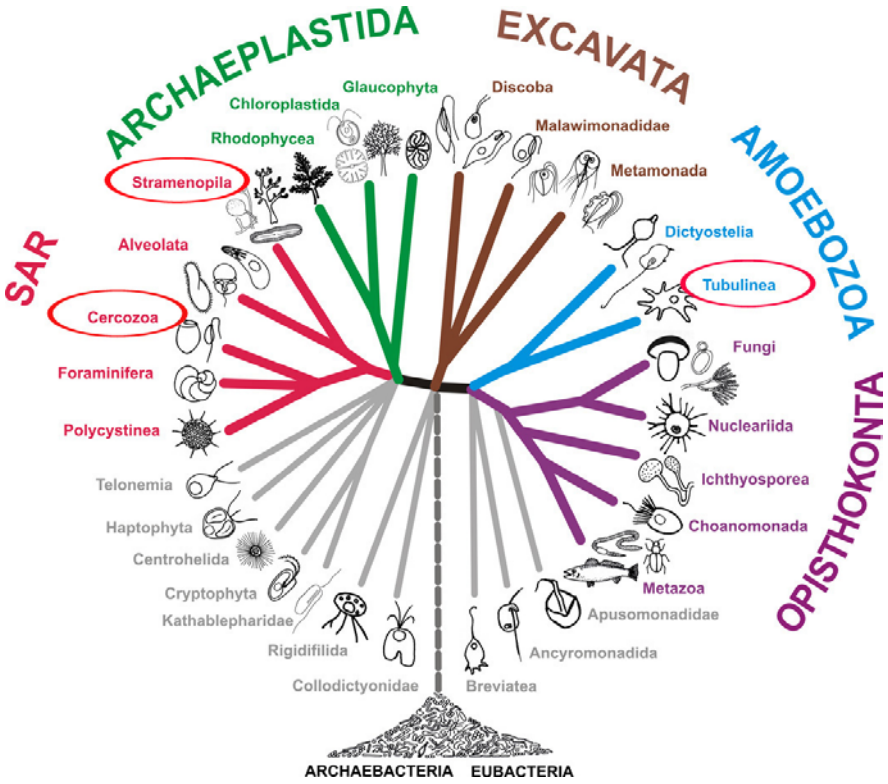


Figure I-2. The phylogenetic position (highlighted by red circles) of Arcellinid, Euglyphid and Stramenopilid testate among Amoebozoa and SAR supergroups in the tree of life. After Adl et al. 2013.

Testate amoebae possessing anastomosing networks of reticulopodia, such as for instance *Amphitrema* spp., were classified in the phylum Granuloreticulosea by Bovee (1985) and later placed in the Rhizaria (Meisterfeld, 2002a). However, Gomaa et al, (2013) based on SSU rRNA gene sequences data showed that this group belonged to the Labyrinthulomycete, a group of Stramenopiles (Fig. I-4).

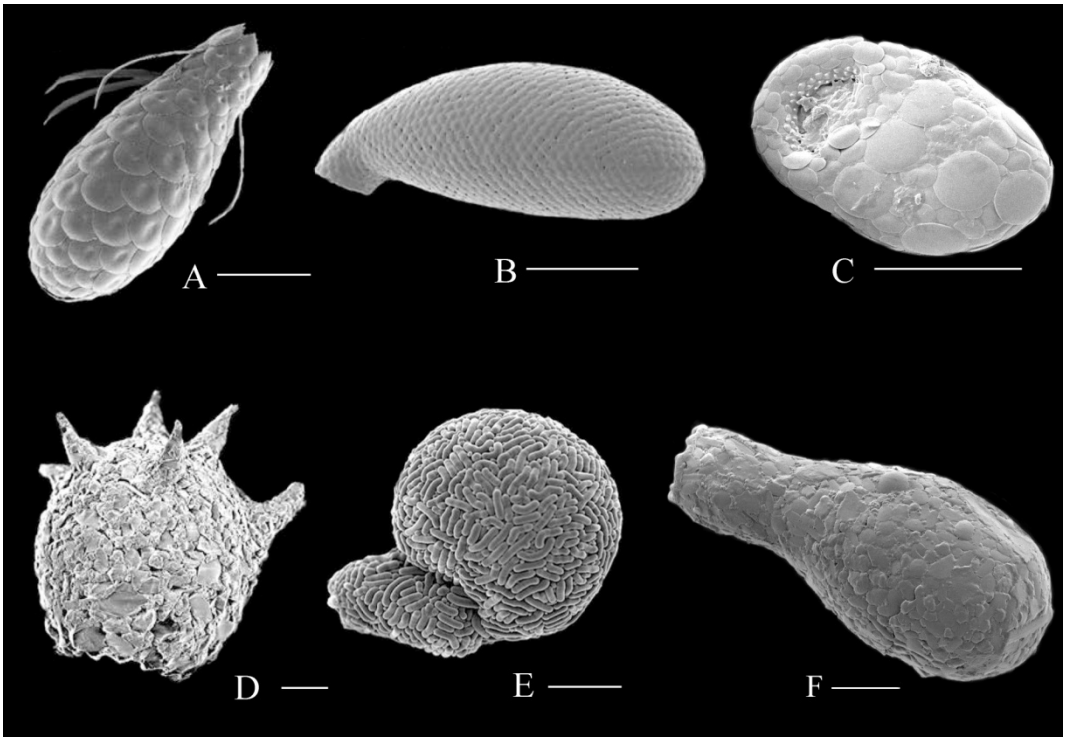


Figure I-3. Scanning electron micrographs of some Euglyphid (A-C) and Arcellinid (D-F) testate amoebae. **A**-*Euglypha brachiata* , **B**-*Cyphoderia ampulla*, **C**-*Trinema complanatum*, **D**-*Difflugia corona*, **E**-*Lesquereusia epistomium*, **F**-*Padaungiella nebeloides*. Scale bars indicate 20 µm. Images by T. Heger (A,B,D), E. Lara (C), M. Todorov (E), and A. Kosakyan (F).

Current knowledge of phylogenetic relationships among members of these three groups is uneven: while the tree of Euglyphida comprises most families and its internal deep relationships are well resolved, information on Arcellinida phylogeny is still very incomplete; most genera have still not been investigated with molecular biology tools. In order to establish a more robust phylogeny based on molecular and morphological characters the sequence data needs to be expanded to cover all families and genera.

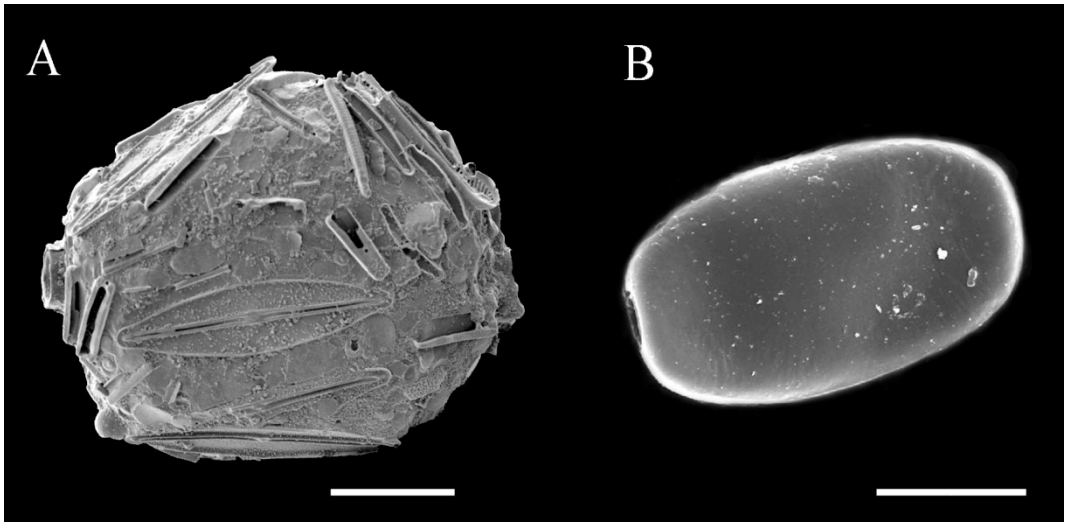


Figure I-4. Scanning electron micrographs of Stramenopilid testate amoebae. **A**- *Amphitrema wrightianum*, **B**- *Archerella flavum*. Scale bars indicate 20 µm. Images by Edward Mitchell.

Among the different groups of protists, the testate amoebae are a good model for taxonomy and evolutionary studies because of their diversity, ubiquity, the presence of a shell which is taxonomically diagnostic, and their long (but discontinuous and still very poorly studied) fossil record (Schönborn et al., 1999; Porter and Knoll, 2000; Schmidt et al., 2006, 2010).

Testate amoebae are fascinating for many reasons. Research on these organisms has increased substantially in the last decade largely due to their increasing use as a bioindicators for paleoecological studies (Mitchell et al., 2008). The shell or test that amoebae produce is preserved in peat and sediments and past conditions can be inferred from the presence of certain bioindicator taxa (Charman, 2001). Also, it has been shown that testate amoebae play important roles in the cycling of elements in terrestrial ecosystems (Aoki et al., 2007; Schröter et al., 2003). They are also increasingly used in ecotoxicology, forensic sciences biomonitoring and in many other applied aspects (Nguyen-Viet et al., 2007; Szelecz et al., 2014).

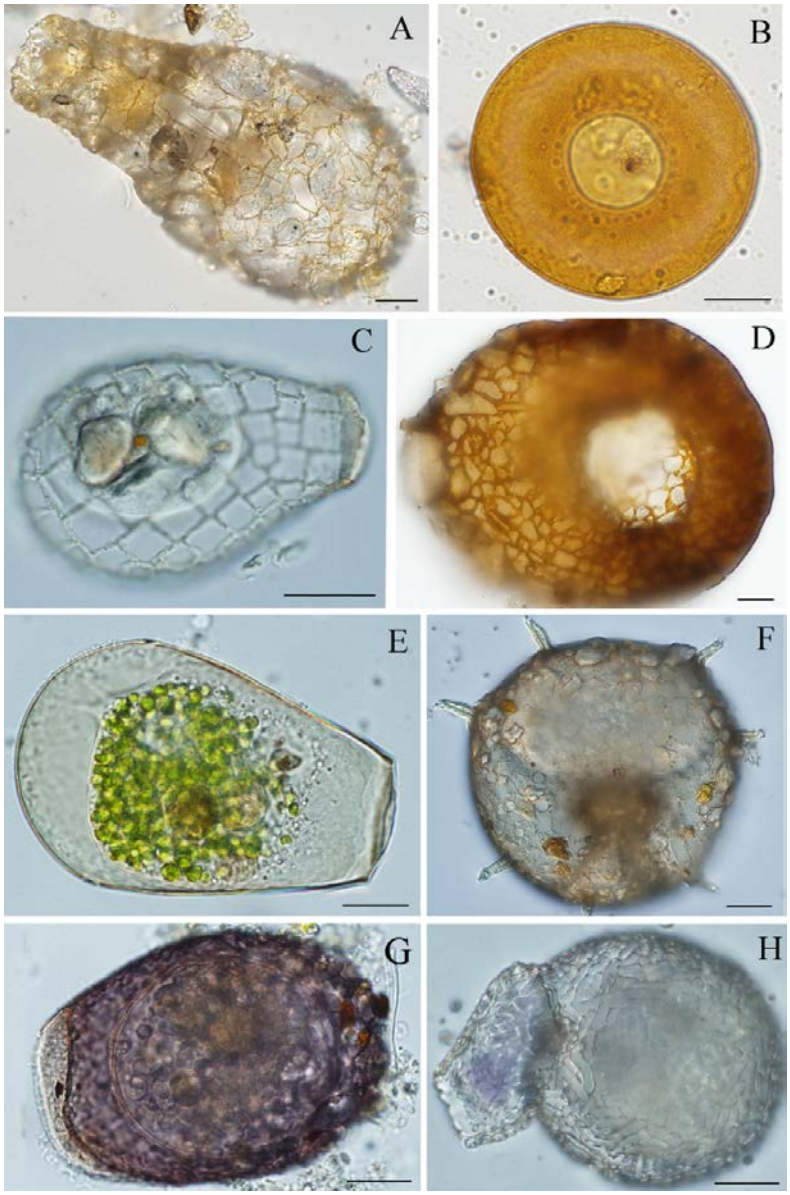


Figure I-5. Light micrographs of some testate amoebae illustrating the range of morphological variability of the test shape and composition. A- *Difflugia* sp., B- *Arcella discoides*, C- *Quadrulella symmetrica*, D- *Centropyxis* sp., E- *Hyalosphenia papilio*, F- *Centropyxis* sp., G. *Heleopera rosea*, H- *Lesquereusia epistomium*. Scale bars indicate 20 µm. Images by A. Kosakyan.

These organisms cover a relatively broad range of sizes: the smallest (*Paulinella*, *Cryptodifflugia*) measure less than 10 µm long while the largest (*Lagenodifflugia*, *Lesquereusia*, *Cyclopyxis*) can reach as much as 450 µm (Meisterfeld, 2002a). Their tests display a wide array of shapes (see Fig. I-5), and generally include a proteinaceous matrix in which mineral elements are embedded, either self-secreted (*Euglypha*, *Quadrulella*, *Lesquereusia*), taken from the surrounding environment (*Centropyxis*, *Difflugia*) or from prey organisms (*Nebela*, *Padaungiella*, *Apodera*). They are common in all freshwater (sediments and plankton), and terrestrial (including soil, litter and mosses) habitats (Nguyen-Viet et al., 2008), some species having also colonized the supra-littoral marine environment (Golemansky, 1991, 1992).

Studies on testate amoebae started in the beginning of the 19th century, when the first species were described; many of these taxa still remain valid to this date (Leclerc, 1816; Ehrenberg, 1838). By the end of the 19th and beginning of the 20th century a considerable amount of material was published on the morphology and systematics of testate amoebae from different parts of the world (Wallich, 1864; Leidy, 1879; Penard, 1890, 1902; Cash and Hopkinson, 1905, 1909, Awerintzew, 1906; Wailes, 1912; Cash and Wailes, 1915; Cash et al., 1905-1921). This accumulated data allowed to building a detailed systematic description of some genera based on morphology (Deflandre, 1928, 1929, 1936), and to developing a global taxonomy of testate amoebae (Saedeleer, 1934; Hoogenraad and de Groot, 1940; Jung, 1942; Deflandre, 1953). In the middle of the 20th century, several monographs were published, covering all aspects of the biology and ecology of testate amoebae, and more simple identification guides (Bartoš, 1954; Grospietsch, 1958; Harnisch, 1958; Schönborn, 1966; Chardez, 1967b). By the 1990s, many taxonomic studies of different genera were available: *Arcella* (Deflandre, 1928; Décloître, 1976), *Centropyxis* (Deflandre, 1929; Décloître, 1978, 1979), *Cyclopyxis* (Deflandre, 1929; Décloître, 1976, 1977b), *Plagiopyxis* (Thomas, 1958), *Nebela* (Deflandre, 1936; Gauthier-Lièvre, 1953; Jung, 1942; Décloître, 1977a), *Hyalosphenia* (Grospietsch, 1965), *Difflugia* (Štěpánek, 1952) (Gauthier-Lièvre and Thomas, 1958; Chardez, 1961; Chardez, 1967a; Ogden, 1979, 1980 a,b, 1983, 1984; Ogden and Hedley, 1980; Ogden and Meisterfeld, 1991; Ogden and Fairman, 1979; Ogden and Zivkovic, 1983), *Lesquereusia* (Thomas and Gauthier-Lièvre, 1959), *Cucurbitella* (Gauthier-Lièvre and Thomas, 1960) *Quadrulella* (Chardez, 1967c), *Paraquadrula* (Décloître, 1961),

Cryptodifflugia (Grospietsch, 1964; Schönborn, 1965), *Trinema* (Chardez, 1960), *Euglypha* (Décloître, 1962), *Cyphoderia* (Chardez, 1991).

Especially useful for the scientific community were the Illustrated Atlas of Freshwater Testate Amoebae (Ogden and Hedley, 1980), and several identification guides (Ellison and Ogden, 1987; Corbet, 1973; Charman et al., 2000) and a recompilation of earlier literature including references from the former Soviet Union, but published in Russian (Mazei and Tsyganov, 2006).

Despite the fact that many common testate amoebae species can usually be identified with confidence, there is a clear need for taxonomic revision and a synthesis of the existing data. There are no recent updated complete monograph on testate amoebae or even species lists and many of the approximately 2000 described taxa are probably never securely identified by most ecologists for lack of appropriate identification criteria, the difficulty in accessing the original descriptions, or simply because no up to date synthesis exists in which the identification characteristics of all species are clearly described (Mitchell et al., 2008). Over the last decades several studies have attempted to clarify the taxonomy of a limited number of taxa (Coûteaux, 1979; Schönborn et al., 1983; Lüftenegger et al., 1988; Foissner and Korganova, 1995, 2000). However, much remains to be done to make the identification of testate amoebae more straightforward for ecologists and paleoecologists, and through this, the comparison among studies more reliable. The amount of work involved in such a task is huge and will require a significant investment.

It is noteworthy to mention that many species have been described based on the extremes of the continuum of morphotypes of the same variable species (Bobrov and Mazei, 2004; Lahr et al., 2008; Heger et al., 2009). For instance, it was shown that abiotic and biotic environmental factors such as food source, temperature, and organic pollutants such as insecticides could affect the shell morphology (Chardez, 1989; Schönborn, 1992; Wanner, 1999) (Wanner and Meisterfeld, 1994; Wanner et al., 1994), and so there could be possibly a high degree of morphological variability both among and within populations (Bobrov and Mazei, 2004). Such variability is apparently at least partly genetically determined and allows the species to adapt to the spatial or temporal heterogeneity of their environment (Bobrov and Mazei, 2004). On the other hand, tiny variations in the scaling pattern of the species that

secrete their own tests can be diagnostic for species discrimination, as for instance with genus *Cyphoderia* (Todorov et al., 2009); these slight morphological differences can correspond to significant genetic distances between strains (Wylezich et al., 2002; Heger et al., 2011a; Chatelain et al., 2013). Such evidence for both phenotypic plasticity and (pseudo)cryptic diversity represent challenges for testate amoeba species identification. However, in spite of these difficulties and uncertainties, testate amoebae are still considered as excellent bioindicators for past and present ecosystems (Mitchell et al., 2008). It can be predicted nevertheless that they could become an even better tool for ecologists if the taxonomy was improved (Birks, 2003; Heiri and Lotter, 2001; Nahmani et al., 2006).

Introduction to family Hyalospheniidae (Arcellinida, Amoebozoa)

In this thesis, my focus is on one of the largest and most conspicuous families of Arcellinid testate amoebae: family Hyalospheniidae. Hyalospheniids are especially abundant and diversified in *Sphagnum* peatlands, but are also found in mosses, soils and freshwater habitats (Meisterfeld, 2002b). This family is composed of vase or flask-shaped species and it was suggested that the ca. 750 Mya old vase-shaped microfossils could belong to this family (Porter and Knoll, 2000; Porter et al., 2003).

The family is valuable in many fields of research, as it contains many common species, which are very abundant, relatively easy to collect and manipulate.

Most hyalospheniid morphospecies have very constant characters, which make them immediately recognizable, such as *Nebela militaris*, *Nebela ansata*, *Hyalosphenia elegans*. However, the family also contains many problematic taxa, such as species complexes (*Nebela collaris s.l.*, *Quadrulella symmetrica s.l.*), or easily overlooked species (*Quadrulella longicollis*, *Hyalosphenia mraconiae*, *Nebela acolla*). Substantial variability in shell construction exists within the same family. The shell is either entirely self-secreted (e.g. *Hyalosphenia,* probably *Alocodera*) composed of an organic matrix, or with addition of self-secreted siliceous plates (e.g. *Quadrulella*) or recycled shell plates of small euglyphids or other similar material such as diatom frustules incorporated in the test (e.g. *Apodera*, *Nebela*, *Padaungiella*) (Fig I-6).

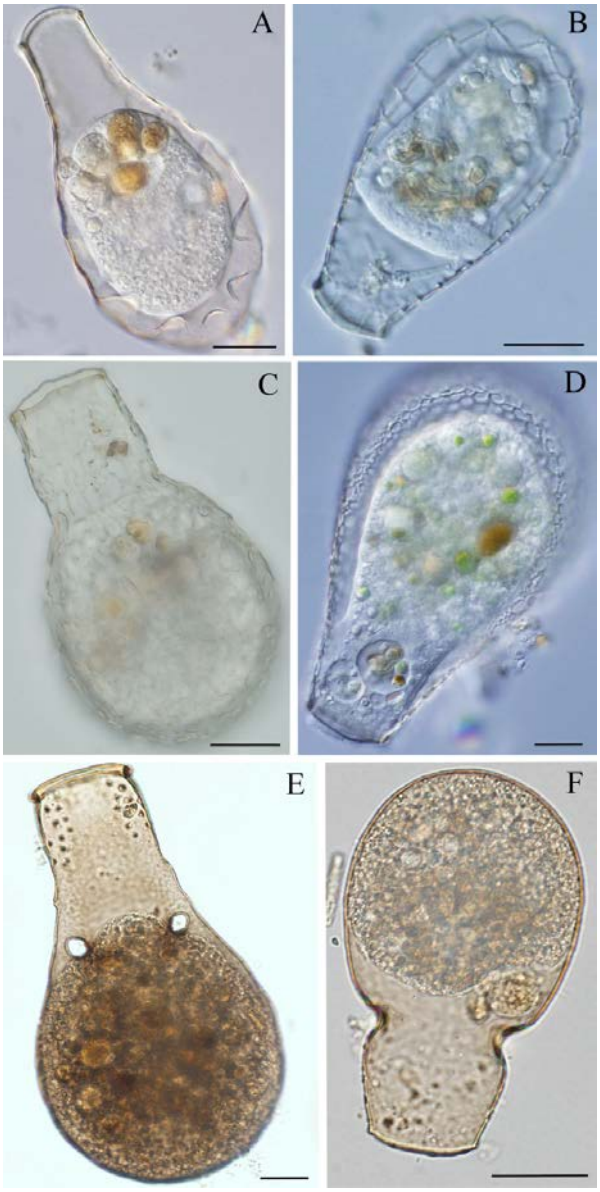


Figure I-6. Light micrograph of representatives of family Hyalospheniidae, demonstrating different composition of the test. **A.** *Hyalosphenia elegans* secreted organic amorphous test. **B.** *Quadrulella symmetrica* test composed of self-secreted siliceous plates. **C.** *Padaungiella lageniformis* test composed of predated plates. **D.** *Nebela galeata* test composed of predated plates. **E.** *Certesella certesi* test composed of plates covered by thick organic layer. **F.** *Alocodera cockayni* organic amorphous test. Scale bars=20 µm. Images by A. Kosakyan (A, B, C, D) and Edward Mitchell (E, F).

Note that in the absence of euglyphid preys, members of the *Nebela tincta* species complex were found with an entirely organic test (MacKinlay, 1936), although it is also possible that the plates were hidden under thick organic layer and the detailed ultrastructure of the test was invisible.

Open questions in Hyalospheniid taxonomy

Attempts to classifying Hyalospheniidae within testate amoebae, as well as internal relationships have lead to a wealth of different classifications, depending on the relative taxonomic value that was given to the observed traits.

Leidy (1874) was probably the first to notice the common characters between the cells having a vase shaped test composed of "*discoid plates and minute rods, apparently siliceous and intrinsic to the structure of the animal*", namely small particles caught within an organic matrix, that he interpreted as originated by the amoeba ("intrinsic"). He grouped species with those characters within genus *Nebela* and restricted them from the already known genus *Difflugia* Leclerc, 1815 - "*those rhizopods with lobose pseudopods, which ordinarily possess a covering or test composed of extraneous bodies, such as particles of quartzose sand, and diatom cases*" (Leidy, 1874), obviously understanding that these large particles were taken from the environment.

Schultze (1877) defined families Arcellidae, Difflugidae, Hyalospheniidiae, and Quadrulidae. He placed the genera with an organic homogenous test such as *Hyalosphenia* Stein, 1859 into family Hyalospheniidae, the genus *Nebela* Leidy, 1874 into Difflugidae and genera with quadratic plates, such as *Quadrula* Schultze, 1875 into Quadrulidae (Schultze, 1877) .

Based on the presence of siliceous plates, Taranek defined family Nebelidae in 1882 that included genera *Nebela*, *Lesquereusia* Schlumberger, 1845, *Corythion* Taranek, 1881 and *Quadrula* (=*Quadrulella*). Later on, genus *Corythion* was excluded and the following genera were added to Nebelidae: *Amphizonella* Greef, *Cochlipodium* Hertw. et Lesser, *Hyalosphenia* Stein 1857, *Leptoclamys* G.S. West, *Zonomyxa* Nusslin (Taranek, 1882).

In 1942 Jung redefined family Nebelidae and organized it into 13 genera: *Alocodera*, *Apodera*, *Argynnia*, *Deflandria*, *Nebela*, *Leidyella*, *Penardiella*, *Physochila*, *Porosia*, *Pterygia*, *Quadrulella*, *Schaudinnia* and *Umbonaria* (Jung, 1942). Unfortunately Jung's classification lacked type designations, and therefore all genera containing more than one species had to be invalidated (after ICZN article 13.3). As a consequence, only monospecific genera such as *Alocodera*, *Physochila* and *Porosia* could be recognized, all other remaining species being assigned to the genus *Nebela*. Loeblich and Tappan (1961) validated *Apodera* and *Certesella*, and Vucetich (1974) *Argynnia.*

The last review of family using only morphological data was done by Meisterfeld (2002b), who replaced *Nebela* and closely related taxa into two families: taxa with rigid, chitinoid, organic and non-areolar test (namely *Hyalosphenia* and *Leptochlamys* West, 1901) were grouped in the Hyalospheniidae and genera with tests composed of plates of small euglyphids or diatom fragments (*Apodera* Loeblich and Tappan, 1961, *Argynnia* Vucetich, 1974, *Certesella* Loeblich and Tappan, 1961, *Nebela*, *Physochila* Jung, 1942, *Porosia* Jung, 1942, *Schoenbornia* Decloitre, 1964) were grouped in the Nebelidae. Meisterfeld did not include genus *Quadrullela* Cockerell, 1909 into family Nebelidae, since he considered Ogden's (1979) classification, who replaced the genus *Quadrullela* Cockerell, 1909 into the Lesquereusiidae Jung, 1942 together with all Arcellinid taxa that included endogenous (self-secreted) siliceous elements (rod-like, nail-shaped or rectangular) in their tests (although some mineral particles may be added, like in the case of *Netzelia* Ogden, 1979).

The first studies on hyalospheniid testate amoebae based on molecular data were done by Nikolaev et al. (2005) and Lara et al. (2008). They covered only a very limited number of species, and were based on a relatively invariable gene, SSU rRNA. According Lara et al. (2008) the Nebelidae sensu Meisterfeld (2002a) was paraphyletic as *Argynnia dentistoma* Penard 1890 appeared only distantly related to members of genus *Nebela* Leidy, 1874. In addition, members of genera *Apodera* Jung 1942, *Hyalosphenia* Stein, 1859, *Nebela* and *Porosia* Jung, 1942, were intermingled in a robust clade informally called "core Nebelas". Unfortunately this study could not show the clear phylogenetic relationship between members of the "core Nebelas" and between closely related taxa, partly because of under-sampling and partly because these close-related species could hardly be discriminated on the

basis of the SSU rRNA gene. Recentlyanother study based on SSU gene harboring several common Hyalospheniids (*Hyalosphenia elegans*, *H. papilio*, *Nebela carinata*, *N. flabellulum*, *N. militaris*, *N. tincta*, *N. tubulosa*, and *Quadrulella symmetrica*) was conducted by Oliverio et al. (2014). This study suggested non monphyly of the genera *Hyalosphenia* and *Nebela*, non monophyly of morphospecies *H. papilio* and *H. elegans*. However some of this studied *H. elegens* cells are resembling more to *H. insecta* than *H. elegans* (see fig. 2 in Oliverio at al. 2014), and besides different gene needs to understand the position of *H. elegans* on the hyalopsheniid phylogenetic tree and its relationships with *H. papilio* and *H. insecta.*

Thus, a new and comprehensive study was needed, gathering at least the most common hyalospheniid taxa using more variable gene.

Hyalospheniids in ecological, biogeographical and biomonitorig research

Hyalospheniid testate amoebae are considered as reliable bioindicators for ecological and environmental monitoring studies, in particular as proxies for hydrological change, and therefore for paleoclimate reconstruction in peatlands (Charman and Warner, 1997; Mitchell et al., 2000; Booth, 2008). They quickly respond to environmental changes such atmospheric pollutions (Payne et al., 2012). The fact that their shells are very well preserved over long periods of time (several thousand years) in peat, added to their sensitivity to environmental changes gives them a prominent role in palaeoenvironmental reconstructions in bogs and fens (Charman, 2001; Mitchell et al., 2008).

Hyalospheniids played also a very important role in the debate about cosmopolitanism vs. endemism in micro-organisms. In a few words, protists were considered as cosmopolitan because of their high dispersal capabilities, and their supposedly large population sizes and high reproduction rates (Finlay, 2002; Fenchel and Finlay, 2004). However, this view based on the famous tenet “everything is everywhere, but, the environment selects” (Beijerinck, 1913) was strongly criticized, mainly based on individual examples of large and conspicuous species (“flagship species”) that obviously were missing in some parts of the world, in spite

of more than 200 years of testate amoebae research (Yang et al., 2004, 2005; Foissner, 2006; Smith et al., 2008).

Hyalospheniid testate amoebae had a central position in this debate because they demonstrated some of the most convincing examples of non-cosmopolitan protists, for instance: *Apodera vas*, *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), or *Nebela ansata* which was found only along the Eastern coast of North America (Nova Scotia, Québec and New Jersey) (Heger et al., 2011b). Further evidence for more or less local endemism have been reported for several other taxa including *Certesella australis* Vucetich (Tierra del Fuego), *Hyalosphenia angulata* Decloitre (Brussels), *H. jirovici* Štepanek (Czech Republic) and *Hyalosphenia gigantea* de Graaf (Netherlands).

Current limitations to use of hyalospheniids in ecological studies (similar species, cryptic/pseudocryptic species)

As mentioned already, many groups of testate amoebae can contain problematic taxa (similar species, cryptic/pseudocryptic taxa) with respect to their limited taxonomy, identification and discrimination of the species. The position of many individual species within several group-complexes is still confusing. Several questions remains such as: Do these taxa represent independent evolutionary lineages? If so, can they be delimited securely? Do these closely-related species differ in their ecological preferences and/or functional roles?

Hyalospheniidae is a good example of testate amoebae that have good potential for use as bioindicators or palaeo-bioindicators, but the presence of similar taxa or cryptic/pseudocryptic species potentially undermines their use in applied research. A good example is the *Nebela tincta-bohemica-collaris* (*N. collaris s.l.*) complex group comprises very similar species that are often misidentified or simply lumped together by palaeoecologists (Heal, 1963; Charman et al., 2000; Booth and Meyers, 2010). One consequence of this taxonomic uncertainty is that the distribution patterns of some species along ecological gradients seem unlikely, such as the multimodal distribution of "*Nebela tincta*" along the moisture gradient (Valiranta et al., 2012) (Fig. I-7).

Such (pseudo)cryptic diversity was also documented among Hyalospheniid testate amoebae (Heger et al., 2013; Oliverio et al., 2014). But it is not known to what extent these species share the same ecology.

Confusion can also exist at coarser taxonomic resolution, as illustrated by Mitchell and Meisterfeld 2005 who discussed the supposed morphological similarity between taxonomically distant species such as *Apodera vas* and *Lagenodifflugia vas*. In this case the possible taxonomic confusion blured the debate on their cosmopolitanism versus local endemism theory.

The present state of testate amoeba taxonomy and the above-mentioned findings shows a critical need for a re-evaluation of previously described taxa, the description of new species with the long-term goal of revising the taxonomy of testate amoebae based on the combination of molecular and morphological data (Mitchell et al., 2008). Focusing on the species composition, morphology, molecular taxonomy, and ecology of problematic groups of species is therefore a research priority.

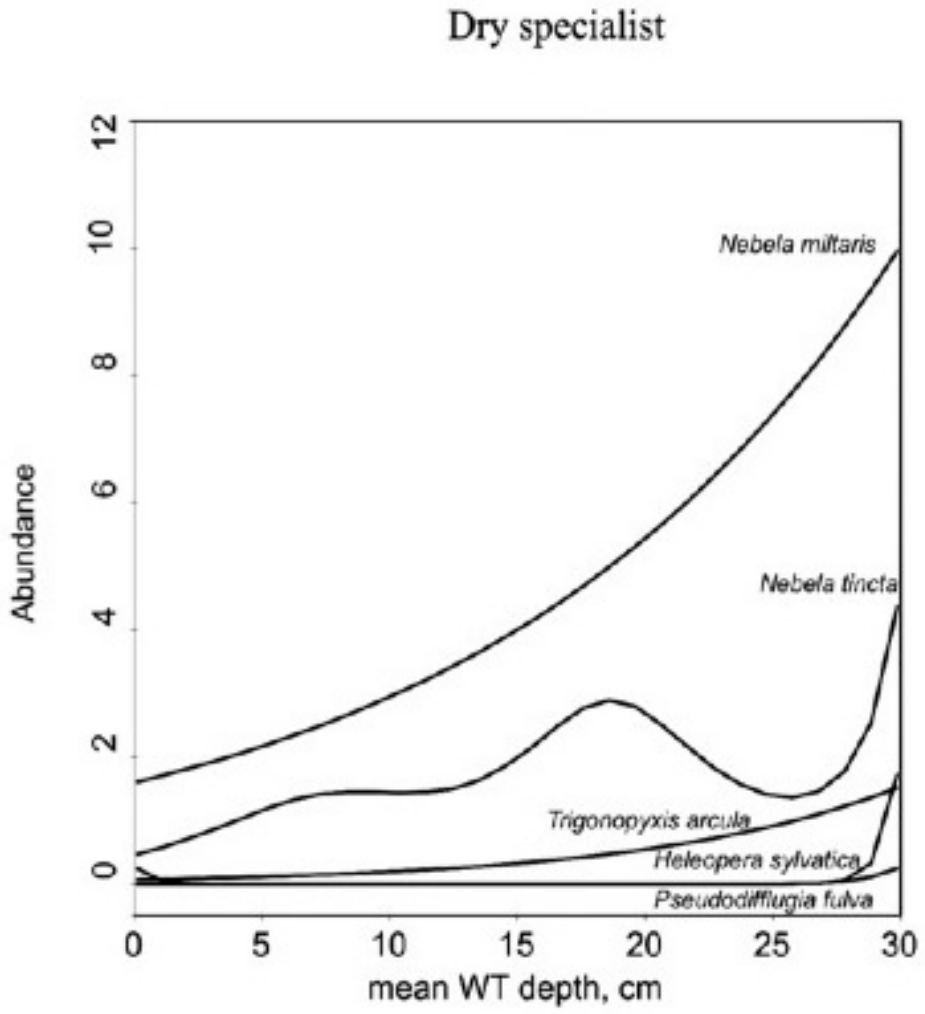


Figure I-7. Figure modified from Valiranta et al. 2012, showing the relationship between *Nebela tincta* and the current mean water table depth (WT) in Kotolanrahka bog (Finland).

Environmental DNA surveys: A fast tool to understand the diversity and ecological interactions of species; advantages and limitations

The development of the environmental DNA cloning/sequencing approach has made it possible to uncover the diversity of microorganisms and to overcome the limitation of culture-based methods. This approach, applied to the prokaryotic world in the early nineties (Giovannoni et al., 1990), opened the way to the discovery of an unknown environmental diversity in eukaryotes about ten years later (López-García et al., 2001). Amongst the many advantages of this approach are its impartiality (DNA-based identification does not rely on the observer's "jeez"), which is very convenient especially when species identification is challenging and time-consuming as it is the case with similar or cryptic taxa. However, it was shown that culture independent methods can be biased, very often revealing an erroneous picture of the environmental community due to the creation of artefacts such as chimerical sequences (Berney et al., 2004). In addition, organism quantification is often biased, because of specific genetic characteristics of the different species (e.g. different gene copy numbers between species), especially between far-related groups or biases in PCR amplification (Weber and Pawlowski, 2013).

Clearly, evaluating these biases using a good candidate barcoding marker is a prerequisite to using the eDNA approach for ecological studies. Choosing the optimal marker is critical in environmental studies. It should allow separating closely related/cryptic taxa while allowing reasonable quantification of the relative abundance of each taxon. If such a marker can be found, it would allow to understanding the ecological preferences or realized niches of individual taxa and their functional roles in microbial food webs.

Why a comprehensive taxonomic revision of family Hyalospheniidae?

Family Hyalospheniidae (Arcellinida, Amoebozoa) includes some of the most remarkable, common and well-studied species of testate amoebae. As discussed above, despite their wide application, current knowledge about their diversity, biology, ecology, taxonomy and systematics remain very limited. For many groups the existing data are partly

unreliable, incoherent, published in different languages, and often in local journals, making it difficult for most researchers to access the information. It is therefore not surprising that many inconsistencies exist and indeed we found that ca. 50% of names were *nomen nudum*, synonymous or doubtful.

The last review of the family was published in 2002 by Ralf Mesiterfeld but covered only part of the diversity (i.e. all genera but not all species). The last comprehensive reviews of the main genera (*Nebela s.l.* and *Hyalosphenia*) date from the early to mid 20th century (e.g. Deflandre, 1936; Grospietsch, 1965). In spite of recent progresses in the knowledge of their molecular diversity, current taxonomy is still based only on morphological data. A revision of the position of each species using modern morphological and molecular tools is clearly necessary. Although, high throughput sequencing open new horizons for biodiversity studies, sound interpretation of these results still depends on basic species taxonomy and ecology. That is why a sound taxonomy is the first prerequisite and become central to further understanding in related area (e.g. ecology, physiology, biochemistry, and molecular and evolutionary biology) of these organisms.

Thesis Objectives

My thesis focuses on the molecular phylogeny, taxonomy, systematic and ecology of testate amoebae with special focus on the family Hyalospheniidae (order Arcellinida). Hyalospheniids are perhaps the most convenient group of Arcellinida to develop a large taxonomic study on because (1) many species are easy to find in large numbers in the some environments (2) shell morphology seems more regular than in some other groups of Arcellinida (3) many genera and species have been described (4) some morphospecies were suspected to contain more than one "true species" (e.g. *Nebela collaris*). Following up on the acquired knowledge on the molecular diversity of this group we developed and tested an environmental DNA approach for exploring the diversity and ecology of these organisms, in order to make them more valuable for environmental monitoring and ecological research.

The six main topics addressed in this thesis are separated into six chapters:

In **Chapter 1** we studied the phylogeny and taxonomy of genus *Nebela* and closely related taxa using mitochondrial Cytochrome Oxidase Subunit 1 (COI) as a barcoding marker. We redefined the family Hyalospheniidae Kosakyan et Lara and the taxonomic position of several taxa. We described several new taxa, a new genus *Padaungiella* Lara et Todorov, and a new species *Nebela meisterfeldi* Heger et Mitchell. We also explored the existence cryptic speciation and species complexes.

In **Chapter 2** we examined the diversity within the *Nebela collaris s.l.*- species complex. We used single cell DNA barcoding and detailed morphological examination approach to assess the taxonomic value of the main morphological characters of the shell. The taxonomy of this group was redefined, additionally three novel species were described: *Nebela guttata* Kosakyan et Lara, *N. pechorensis* Kosakyan et Mitchell, and *N. aliciae* Mitchell et Lara.

In **Chapter 3** using a similar approach to the one used in Chapter 2 we examined another species complex of hyalospheniid testate amoeba: the *Quadrulella symmetrica* group. Variations of test morphology were studied in order to understand which characters are fixed genetically, and which ones are the result of phenotypic plasticity. This work is still in preparation, but preliminary results clearly show that *Quadrulella symetrica* is not a single species and that it will have to be split into at list five independent taxa.

Chapter 4 is a methodological study. Our aim was to test an environmental DNA sequencing approach with species from the *Nebela collaris s.l.* complex - a group of closely related and morphologically very similar taxa, using the barcoding marker COI used in Chapter 2. We compared the clone library data with direct microscopic counts of the communities in order to assess if the sequence data provided an accurate estimation of the real species community and what could be the causes for possible biases between the two approaches.

Finally the **Chapter 5** presents a monograph in preparation devoted to family Hyalospheniidae (Amoebozoa, Arcellinida). Our goal is to provide an up to date treatment of the taxonomy of this family combining all known data about taxonomy, phylogeny, and systematic of these organisms.

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CHAPTER 1

COI barcoding of Nebelid testate amoebae (Amoebozoa: Arcellinida): extensive cryptic diversity and redefinition of the family Hyalospheniidae Schultze

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COI Barcoding of Nebelid Testate Amoebae (Amoebozoa: Arcellinida): Extensive Cryptic Diversity and Redefinition of the Hyalospheniidae Schultze

Anush Kosakyan[a,1], Thierry J. Heger[a,b,c,1], Brian S. Leander[b], Milcho Todorov[e], Edward A.D. Mitchell[a,b,c,d], and Enrique Lara[a]

[a]Laboratory of Soil Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland
[b]Department of Zoology, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada
[c]WSL, Swiss Federal Institute for Forest, Snow and Landscape Research, Ecosystem Boundaries Research Unit, Wetlands Research Group, Station 2, CH-1015 Lausanne, Switzerland
[d]Ecole Polytechnique Fédérale de Lausanne (EPFL), Laboratory of Ecological Systems, Station 2, CH-1015 Lausanne, Switzerland
[e]Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Science, 1113 Sofia, Bulgaria

We used Cytochrome Oxidase Subunit 1 (COI) to assess the phylogenetic relationships and taxonomy of *Nebela* sensu stricto and similar taxa (*Nebela* group, Arcellinida) in order to clarify the taxonomic validity of morphological characters. The COI data not only successfully separated all studied morphospecies but also revealed the existence of several potential cryptic species. The taxonomic implications of the results are: (1) Genus *Nebela* is paraphyletic and will need to be split into at least two monophyletic assemblages when taxon sampling is further expanded. (2) Genus *Quadrulella*, one of the few arcellinid genera building its shell from self-secreted siliceous elements, and the mixotrophic *Hyalosphenia papilio* branch within the *Nebela* group in agreement with the general morphology of their shell and the presence of an organic rim around the aperture (synapomorphy for Hyalospheniidae). We thus synonymise Hyalospheniidae and Nebelidae. Hyalospheniidae takes precedence and now includes *Hyalosphenia*, *Quadrulella* (previously in the Lesquereusiidae) and all Nebelidae with the exception of *Argynnia* and *Physochila*. *Leptochlamys* is Arcellinida *incertae sedis*. We describe a new genus *Padaungiella* Lara et Todorov and a new species *Nebela meisterfeldi* n. sp. Heger et Mitchell and revise the taxonomic position (and rank) of several taxa. These results show that the traditional morphology-based taxonomy underestimates the diversity within the *Nebela* group, and that phylogenetic relationships are best inferred from shell shape rather than from the material used to build the shell.

Key words: Arcellinida; DNA barcoding; COI; cryptic species; Hyalospheniidae; Testate amoeba.

[1]Corresponding authors have contributed equally to this work:
e-mail anush.kosakyan@unine.ch (A. Kosakyan), theger@interchange.ubc.ca (T.J. Heger).

Introduction

Free-living protists make up a large part of the Earth's biodiversity (Medinger et al. 2010) and are of major ecological importance at the global scale (Adl and Gupta 2006). The vast majority of this diversity is, however, not yet described (Piganeau et al. 2011) and existing descriptions are often imprecise (Adl et al. 2008; Caron 2009). Yet reliable taxonomy is an essential prerequisite for understanding the ecology, biogeography, and evolution of any group of organisms. Unfortunately, poor taxonomy is one of the curses of the study of free-living protists, leading, for instance, to endless debates about the existence of biogeographical patterns in the distribution of free-living protists (Finlay et al. 2004; Foissner 2008; Heger et al. 2009; Mitchell and Meisterfeld 2005).

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). Due to a lower taxonomic effort and the lack of easily recognized morphological features, the expectation is that the amount of cryptic diversity (i.e. genetic diversity that is not reflected in observable morphological features) in microscopic organisms is very high.

Arcellinid amoebae are a good model for taxonomy and evolutionary studies of free-living protists because of their diversity, abundance and taxonomically diagnostic shell. The distinct ecological requirements of testate amoebae species, including both the arcellinids (Amoebozoa: Arcellinida) and the euglyphids (Rhizaria: Cercozoa: Euglyphida), and the preservation of their shells in peat and sediments make them good bioindicators for palaeoecological studies and environmental monitoring (Charman 2001). In addition, testate amoebae were shown to play important roles in the cycling of carbon, nitrogen and silica in terrestrial ecosystems (Aoki et al. 2007; Schröter et al. 2003; Wilkinson 2008). However, as for most protists, data on total biodiversity, geographic distribution, morphology, phylogeny and ecology of this group of organisms are still very incomplete and controversial.

Our focus here is on a group of arcellinid testate amoebae including the "core Nebelas" sensu Lara et al. (2008) and most closely related taxa (i.e. the clade containing *Apodera vas* Certes, 1889 and *Nebela lageniformis* Penard, 1890), hereafter referred to as the "*Nebela* group". This group contains some of the most remarkable and common species of testate amoebae, including both easily identifiable species, and problematic species-complexes. Members of this group are especially abundant in mosses and forest litter, and more rarely in other biotopes such as freshwater pools, etc. (Meisterfeld 2002; Todorov 2002). The classification of this group is based on characters of the test such as composition (proteinaceous or agglutinated), shape of the aperture (circular, oval or curved) and shape of the shell (mostly flask-shaped but more or less elongated and in some cases with appendages, a keel, horns etc.). The classification of the *Nebela* group has changed considerably over time (Fig. 1), mainly depending on which morphological trait has been considered as phylogenetically most relevant at the different taxonomical levels.

Molecular tools now make it possible to reassess the validity of this taxonomy. However until now only one study has examined the phylogeny of the *Nebela* group based on molecular methods (SSU rRNA), but with very partial coverage of the described morpho-species (Lara et al. 2008). This study showed that the Nebelidae sensu Meisterfeld (2002) was paraphyletic as *Argynnia dentistoma* Penard,1890 appeared only distantly related to members of genus *Nebela* Leidy, 1874. In addition, members of genera *Apodera* Loeblich and Tappan, 1961, *Hyalosphenia* Stein, 1859, *Nebela* and *Porosia* Jung, 1942, were intermingled in a robust clade informally called "core Nebelas". However, the species delineations and the phylogenetic relationship between members of the "core Nebelas" remained unclear, partly because of under-sampling and partly because these close-related species could hardly be discriminated on the basis of the less variable SSU rRNA gene. We therefore investigated the species delineations and the phylogenetic relationships within genus *Nebela* and related taxa based on 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 ciliates, dinoflagellates, vannellid amoebae or euglyphid testate amoebae (Barth et al. 2006; Chantangsi et al. 2007; Heger et al. 2010; Lin et al. 2009; Nassonova et al. 2010). Our data confirm the usefulness of COI sequences for taxonomic studies of certain Arcellinida species. In addition our combined molecular and morphological results lead us to propose several nomenclatural changes.

Results

A total of 59 sequences were obtained from 24 morphospecies, most of which were also

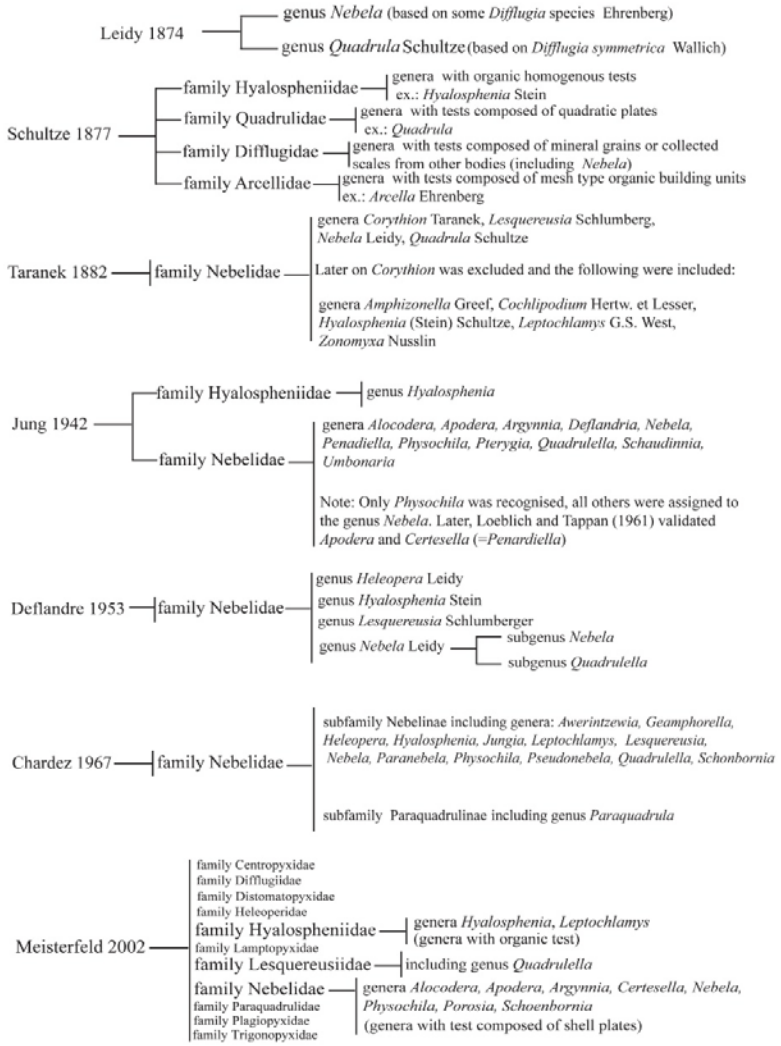

Figure 1. Summarised history of classification of genus *Nebela* and related taxa.

characterized by scanning electron and light microscopy. The fragment lengths ranged from 308 bp to 668 bp. Only 6 sequences were shorter than 560 bp (Table 1). COI separated efficiently the different morphospecies, including closely related ones and our molecular data suggest the presence of cryptic species within several morphospecies.

The results of our phylogenetic reconstructions are shown in Figure 2. Topologies of both the strict consensus ML and Bayesian trees were similar. The tree revealed the existence of five main clades

Table 1. List of sequenced taxa and sampling locations.

Taxa	Sampling location	Country	Co-ordinates		Number of cells used per extraction	Sequence length (bp)	Gen Bank number
Alocodera cockayni AR	*Sphagnum magellanicum* mosses, Lapataya National Park, Tierra del Fuego	Argentina	54°51′S	68°34′W	> 1	637	JN849043
A. cockayni CL-1	Alerce Costero, Cordillera Pelada, Valdivia	Chile	40°11′S	73°28′W	1	617	JN849069
A. cockayni CL-2	Alerce Costero, Cordillera Pelada, Valdivia	Chile	40°11′S	73°28′W	1	308	JN849068
Certesella martiali AR	*Sphagnum* mosses, near Ushuaia, Tierra del Fuego	Argentina	54°47′S	68°17′W	1	586	JN849064
Hyalosphenia papilio CA-1	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45′N	126°28′W	1	664	JN849016
H. papilio CA-2	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45′N	126°28′W	> 1	664	JN849017
H. papilio CA-3	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45′N	126°28′W	1	664	JN849011
H. papilio CA-4	Aquatic mosses, near Unnecessry Mountain, British Columbia	Canada	49°25′N	123°12′W	> 1	664	JN849012
H. papilio CA-5	Aquatic mosses, near Unnecessry Mountain, British Columbia	Canada	49°25′N	123°12′W	1	664	JN849013
H. papilio CA-6	Aquatic mosses, near Unnecessry Mountain, British Columbia	Canada	49°25′N	123°12′W	> 1	664	JN849014
H. papilio CA-7	Aquatic mosses, near Unnecessry Mountain, British Columbia	Canada	49°25′N	123°12′W	> 1	629	JN849015
H. papilio PL-1	Bory Tucholskie, Poland	Poland	53°36′N	18°00′E	1	620	JN849019
H. papilio PL-2	Bory Tucholskie, Poland	Poland	53°36′N	18°00′E	1	620	JN849018
Nebela ansata CA-1	*Sphagnum* mosses, Peggy's Cove, Nova Scotia	Canada	44° 29′N	63° 53′W	1	624	JN849055
N. ansata CA-2	*Sphagnum* mosses, Peggy's Cove, Nova Scotia	Canada	44° 29′N	63° 53′W	> 1	629	JN849054
N. bohemica BG	*Sphagnum* mosses, Vitosha	Bulgaria	42°36′N	23°17′E	> 1	636	JN849042
N. carinata CA-1	Mosses, Grouse Mountain, British Columbia	Canada	49°23′N	123°04′W	1	668	JN849038
N. carinata CA-2	Mosses, Grouse Mountain, British Columbia	Canada	49°23′N	123°04′W	> 1	638	JN849036
N. carinata CA-3	Mosses, Grouse Mountain, British Columbia	Canada	49°23′N	123°04′W	> 1	637	JN849037
N. carinata CA-4	*Sphagnum* mosses, Peggy's Cove, Nova Scotia	Canada	44° 29′N	63° 53′W	> 1	667	JN849039

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N. carinata CA-5	*Sphagnum* mosses, Peggy's Cove, Nova Scotia	Canada	44° 29'N	63° 53'W	> 1	667	JN849040
N. carinata CA-6	Aquatic mosses, Cape Breton, Nova Scotia	Canada	46°50'N	60°24'W	> 1	618	JN849041
N. carinata CH-1	*Sphagnum* mosses, Praz-Rodet, Vaud	Switzerland	46°33'N	06°10'E	> 1	668	JN849034
N. carinata CH-2	*Sphagnum* mosses, Praz-Rodet, Vaud	Switzerland	46°33'N	06°10'E	> 1	640	JN849035
N. carinata SE	Sphagnum mosses, bog pool, Ryggmossen	Sweden	60°00'N	17°15'E	> 1	668	JN849033
N. flabellulum CA	Mosses, Lynn Peak, British Columbia	Canada	49°22'N	123°01'W	> 1	665	JN849026
N. galeata CA-1	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45'N	126°28'W	1	631	JN849059
N. galeata CA-2	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45'N	126°28'W	1	607	JN849058
N. galeata CA-3	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45'N	126°28'W	1	624	JN849060
N. hippocrepis CA-1	Aquatic mosses, Cape Breton, Nova Scotia	Canada	46°50'N	60°24'W	> 1	630	JN849056
N. hippocrepis CA-2	Aquatic mosses, Cape Breton, Nova Scotia	Canada	46°50'N	60°24'W	1	629	JN849057
N. marginata CA-1	Aquatic mosses, near Unnecessary Mountain, British Columbia	Canada	49°25'N	123°12'W	1	668	JN849029
N. marginata CA-2	Aquatic mosses, near Unnecessry Mountain, British Columbia	Canada	49°25'N	123°12'W	> 1	668	JN849027
N. marginata CA-3	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45'N	126°28'W	> 1	668	JN849028
N. marginata CA-4	Aquatic mosses, near Unnecessary Mountain, British Columbia	Canada	49°25'N	123°12'W	> 1	631	JN849032
N. marginata CA-5	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45'N	126°28'W	1	668	JN849030
N. marginata CH	*Sphagnum* mosses, Poor fen on the west side of Lake Piora, Ticino	Switzerland	46°32'N	08°42'W	> 1	615	JN849031
N. meisterfeldi CA-1	*Sphagnum* mosses, Strathcona Park, Vancouver Island, British Columbia	Canada	49°42'N	125°18'W	> 1	668	JN849053
N. meisterfeldi CA-2	Mosses, Grouse Mountain, British Columbia	Canada	49°23'N	123°04'E	1	615	JN849052

Table 1 (*Continued*)

Taxa	Sampling location	Country	Co-ordinates		Number of cells used per extraction	Sequence length (bp)	Gen Bank number
N. penardiana BG	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	360	JN849062
N. speciosa BG-1	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	509	JN849045
N. speciosa BG-2	*Sphagnum mosses*, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	640	JN849044
N. tincta CA	Aquatic mosses, near Unnecessry Mountain, British Columbia	Canada	49°25′N	123°12′W	> 1	665	JN849025
N. tincta var. *galeata* CR	Mosses, Volcan Poás	Costa Rica	10°11′N	84°13′W	> 1	631	JN849023
N. tincta var. *major* CA	*Sphagnum* mosses, Pacific Rim, British Columbia	Canada	48°38′N	124°46′W	1	665	JN849067
N. cf. *tincta* CA	*Sphagnum* mosses, Burns bog, British Columbia	Canada	49°08′N	122°55′W	> 1	614	JN849024
N. tubulosa BG-1	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	623	JN849020
N. tubulosa BG-2	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	623	JN849021
N. tubulosa BG-3	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	618	JN849061
N. cf. *tubulosa* CA	*Sphagnum* mosses, Cape Breton, Nova Scotia	Canada	46°48′N	60°49′W	> 1	631	JN849022
Padaungiella lageniformis BG	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	568	JN849065
P. nebeloides FR	Floating *Sphagnum* mire, Lac de Bellefontaine, Jura	France	46°34′N	6°05′W	1	605	JN849063
P. wailesi CH	Forest litter, Bois du Jorat, Vaud	Switzerland	46°30′N	6°40′W	1	485	JN849066
Quadrulella symmetrica BG-1	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	1	634	JN849047
Q. symmetrica BG-2	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	625	JN849049
Q. symmetrica BG-3	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	340	JN849048
Q. symmetrica CH	*Sphagnum* mosses, les Nicolets, Vaud	Switzerland	46°21′N	07°07′W	> 1	633	JN849046
Q. symmetrica CA	*Sphagnum* mosses, Echo Bay, British Columbia	Canada	50°45′N	126°28′W	> 1	607	JN849051
Q. longicollis BG	*Sphagnum* mosses, Vitosha Mountain	Bulgaria	42°36′N	23°17′E	> 1	640	JN849050

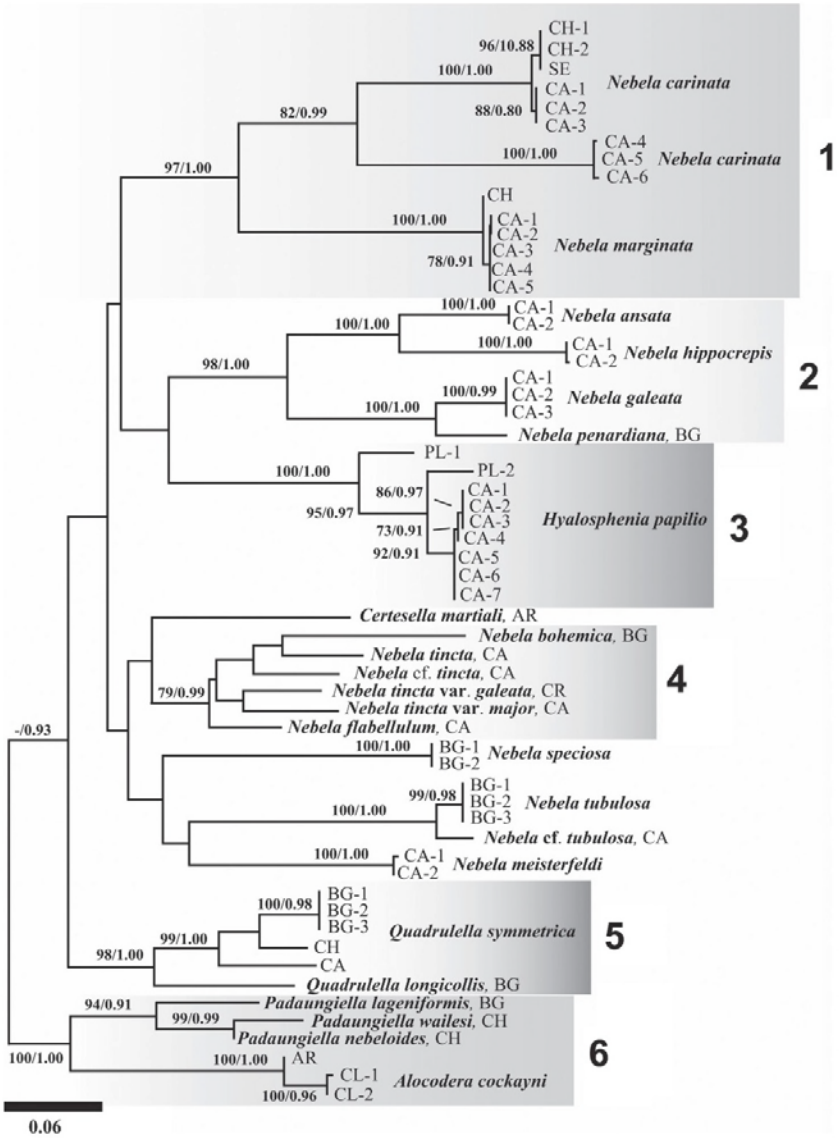

Figure 2. Maximum likelihood bootstrap consensus tree of 59 *Nebela* s.str. and related taxa testate amoebae COI sequences based on 677 nucleotide positions. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated with Bayesian analyses. Only values above 50/0.50 are shown. The tree was rooted with clade 6 (i.e. the group of *Padaungiella (Nebela) lageniformis*).

with several subclades. Further phylogenetic analyses (see Methods) confirmed the existence of these five clades and revealed that *Nebela lageniformis, N. nebeloides* (Gauthier-Lièvre et Thomas, 1958) Todorov et al. 2010, *N. wailesii* Deflandre, 1936 (which we transfer to new genus *Padaungiella*, see section Taxonomic Actions) and *Alocodera cockayni* Penard, 1910 sequences (outgroup used in the tree of the Figure 2) formed an additional independent clade (clade 6).

Clade 1 comprised *N. carinata* Archer, 1876 and *N. marginata* Penard, 1902, two species presenting a keel around the aboral end of the test, and was supported with respectively 97 bootstrap (B) and 1.00 posterior probabilities (PP) values. Whereas all *N. marginata* sequences were closely related and presented limited genetic variation, *N. carinata* sequences clustered into two groups which are clearly split and genetically relatively distant from each other. Clade 2 (node support: B=98; PP=1.00) includes the following species: *Nebela ansata* Leidy, 1879, *N. hippocrepis* Leidy, 1879, *N. galeata* Penard, 1890 and *N. penardiana* Deflandre, 1936 (Fig. 3). *N. ansata* and *N. hippocrepis* are morphologically very distinct species and branch together with maximal B and PP values (100/1.00 for both cases). *N. penardiana* appears to be a sister taxon and closely branching with *N. galeata* with a 1.00 PP value. Clade 3 is robustly supported and represents a group of sequences derived from *Hyalosphenia papilio* Leidy, 1875. There were high genetic distances (up to 8%, Supplementary Table S1) within this morphospecies, suggesting more a species complex than a single species. Clade 4 comprises species of the *Nebela tincta major-bohemica-collaris* species complex (Gilbert et al. 2003; Heal 1963) such as *N. bohemica* Taranek, 1882, *N. tincta* (Leidy, 1879) Awerintzew, 1906, and *N. flabelullum* Leidy, 1874 (Fig. 4). Although the respective position of species within this clade remained unresolved, the whole clade receives relatively high support with 79 B and 0.99 PP values. Clade 5 is represented by isolates of *Quadrulella symmetrica* Wallich 1863 and its variety *longicollis* (Fig. 5). The clade was well supported (98 B and 1.00 PP). As for *H. papilio*, the genetic distances between isolates were relatively high, suggesting the existence of a complex of species. Clade 6, chosen as the outgroup of our tree based on previous results (Lara et al. 2008), comprises species that are characterized by a well-developed neck in the shell: *Alocodera cockaynii, Nebela lageniformis*, *N. nebeloides* and *N. wailesi* (Fig. 6). Finally, some species had an uncertain position in the tree: *Certesella martiali* Certes 1889, the newly found species *N. meisterfeldi* n. sp. (see below), *Nebela speciosa* Deflandre, 1936 and *N. tubulosa* Penard, 1890 (Fig. 7).

Discussion

The use of molecular markers offers a way to reassess the validity of taxonomic systems based on morphology and provides new criteria for species discrimination. Molecular taxonomy has revealed the presence of a large cryptic or pseudocryptic diversity (Hebert et al. 2004b; Heger et al. 2011a; Kolisko et al. 2010) while molecular phylogeny and phylogenomics have led to major revisions in the classification of most groups of organisms (Baldauf 2003; Burki et al. 2008). In this study we have tested and demonstrated for the first time the usefulness of COI as a DNA barcoding marker for the arcellinid testate amoeba. Our results provide evidence for (1) the discrimination of morphospecies and the assessment of cryptic diversity within the Arcellinida and (2) the phylogenetic relationships within the group of "core Nebelas" and related taxa.

Phylogeny of the "core Nebelas" and Related Taxa, Notes on their Ecology

In 1874 Leidy created the genus *Nebela*, for testate amoebae with a test "*composed of discoid plates and minute rods, apparently siliceous and intrinsic to the structure of the animal*". Leidy restricted the genus *Difflugia* Leclerc, 1815 to "*those rhizopods with lobose pseudopods, which ordinarily possess a covering or test composed of extraneous bodies, such as particles of quartzose sand, and diatom cases*" (Leidy 1874). Schultze (1877) first defined families Arcellidae, Difflugidae, Hyalospheniidiae, and Quadrulidae. He replaced the genera with organic homogenous test such as *Hyalosphenia* Stein, 1859 into family Hyalospheniidae, the genus *Nebela* Leidy, 1874 into Difflugidae and genera with quadratic plates, such as *Quadrula* Schultze, 1875 into Quadrulidae. Basing on the presence of siliceous plates Taranek first defined family Nebelidae in 1882 by unifying the genus *Nebela*, *Lesquereusia* Schlumberger, 1845, *Corythion* Taranek, 1881 and *Quadrula* (Quadrulella) (Taranek 1882). In 1942 Jung redefined family Nebelidae and organised it into 11 genera: *Alocodera*, *Apodera*, *Argynnia*, *Deflandria*, *Nebela*, *Physochila*, *Pterygia*, *Penardiella*, *Quadrulella*, *Schaudinnia* and *Umbonaria* (Jung 1942). Unfortunately Jung's classification lacked

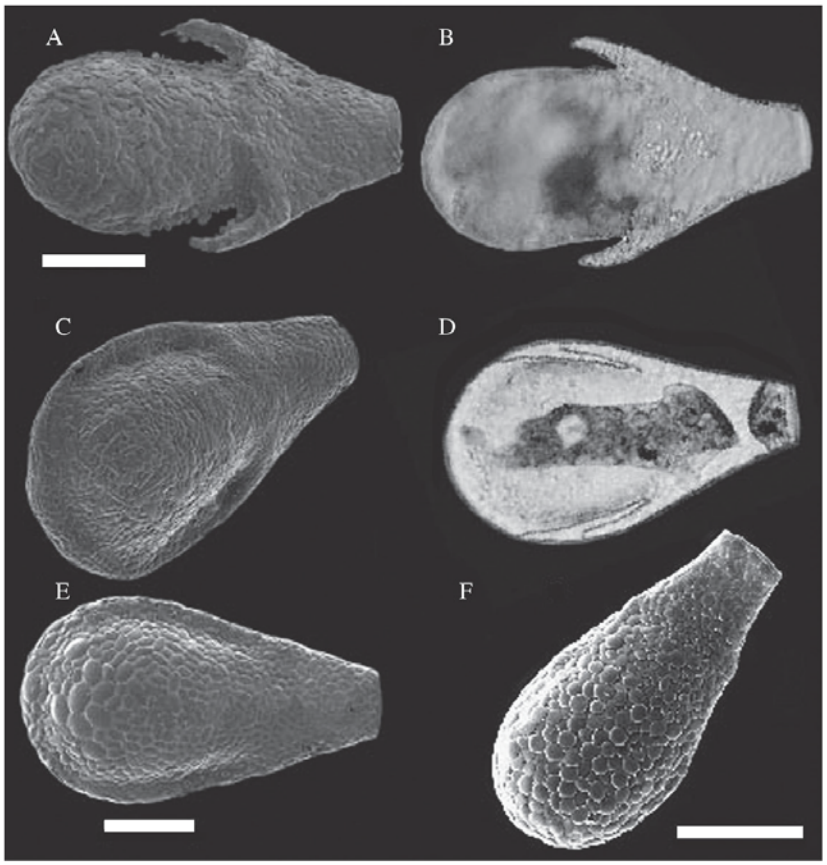

Figure 3. Scanning electron and light micrographs of group 2 morphospecies: **A**. and **B**. *Nebela ansata* from Peggy's Cove, Canada (picture **A** from Heger et al. 2011b). **C**. and **D**. *N. hippocrepis* from Cape Breton, Canada. **E**. *N. galeata* from Cape Breton, Canada. **F**. *N. penardiana* from Vitosha, Bulgaria. Scale bars represent 50 μm.

type designations. All genera containing more than one species and lacking type designation are in discordance with international code of zoological nomenclature article 13.3, and hence do not exist (i.e. they are technically considered "unavailable") for all purposes of the code. Meisterfeld (2002) re-organised *Nebela* and closely related taxa into two families: taxa with rigid, chitinoid, organic and non-areolar test (namely *Hyalosphenia* and *Leptochlamys* West, 1901) were grouped in the Hyalospheniidae and genera with tests composed of plates of small euglyphids or diatom fragments (*Apodera* Loeblich and Tappan, 1961, *Argynnia* Vucetich, 1974, *Certesella* Loeblich and Tappan, 1961, *Nebela*, *Physochila* Jung, 1942, *Porosia* Jung, 1942, *Schoenbornia* Decloitre, 1964) were grouped in the Nebelidae. Ogden (1979) placed the genus *Quadrullela* Cockerell, 1909 into the Lesquereusiidae Jung, 1942 with other taxa building shells from endogenous (self-secreted) siliceous elements (rod-like, nail-shaped or rectangular) to which mineral particles may be added (in the case of *Netzelia* Ogden, 1979).

We obtained molecular data for most common species belonging to the "*Nebela*" group. Our phylogenetic analyses demonstrated that *Nebela*

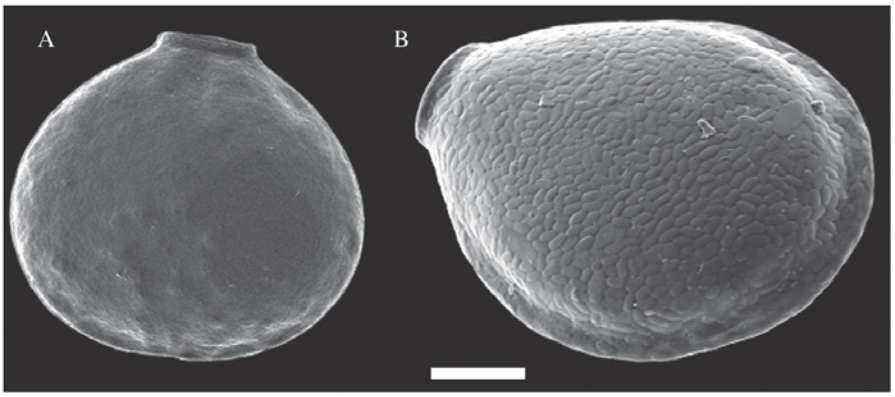

Figure 4. Scanning electron micrographs of *Nebela* morphospecies: **A**. *Nebela flabellulum* from Lynn Peak, Canada. **B**. *N. tincta* ssp. *galeata* from Volcán Poás, Costa Rica. Scale bar represent 20 μm.

sensu stricto is paraphyletic and includes several species-complexes and cryptic species. Generations of taxonomists have classified the different members of this clade primarily on the basis of test composition, such as the secreted organic test in *Hyalosphenia papilio*, the secreted quadrangular plates in *Quadrulella symmetrica*, and the siliceous shell plates recycled from other testate amoebae in *Nebela tincta* (Deflandre 1953, Jung 1992; Meisterfeld 2002; Schultze 1877; Taranek 1882). In agreement with previous molecular phylogenetic studies (Lara et al. 2008; Nikolaev et al. 2005), our results invalidate this approach and show that these genera together constitute a distinct clade; therefore, test composition should not be considered as the primary criterion for distinguishing major taxa within the "*Nebela*" group. It is still possible, however, that phylogenetic relationships among arcellinid taxa could be inferred from the general shape of the test (e.g. compressed bottle-shape in the case of the "*Nebela*" group).

An interesting case is *Argynnia dentistoma*, previously classified as *Nebela* and characterised by a typical compressed vase-shaped test but lacking a neck and with a rougher shell surface and especially apertural rim. *Argynnia* was shown to branch at the base of the "core Nebelas" (Lara et al. 2008). The rougher and less elaborate shell of *Argynnia* thus could be interpreted as representing an intermediate form between the "*Nebela*" group and agglutinating taxa such as *Difflugia*. Also the results of SEM studies on the shell ultrastructure in *A. dentistoma* show that with its structured organic cement network this species is more similar to the species of the genus *Difflugia* than to those of the genus *Nebela* (which have usually an unstructured sheet-like organic cement with a single pores) (Ogden and Hedley 1980; Todorov et al. unpublished data). Further work is required especially on apparently more distant genera such as *Difflugia* and *Centropyxis* Stein, 1857 as well as potentially closely related taxa such as *Microquadrula* Golemansky, 1968 and *Leptochlamys* to test these hypotheses. As more distant taxa are included however less variable genes will need to be sequenced, starting with the SSUrRNA gene as used by Nikolaev et al. (2005), Lara et al. (2008), or Kudryavtsev et al. (2009).

The general tree of the "core Nebelas" consists of a series of strongly supported clades, but the relationships among these clades remain undetermined. As a general rule, members of each clade possess some common morphological features, but many are also characterized by common ecological preferences.

Clade 1, constituted by *Nebela carinata and N. marginata* corresponds to the (unavailable for lack of type species designation) genus *Pterygia* described by Jung (1942) based on the presence of a lateral keel on the side of the test. In *N. carinata* the keel is wide and conspicuous, whereas in the slightly smaller *N. marginata*, the keel is narrower and starts at about the middle of the length of the test. Both species are large (above 120 μm) and are restricted to wet microsites in *Sphagnum*-dominated ecosystems (Booth 2008; Charman and Warner 1997).

Clade 2 includes *N. ansata*, *N. hippocrepis*, *N. galeata* and *N. penardiana*. These species have

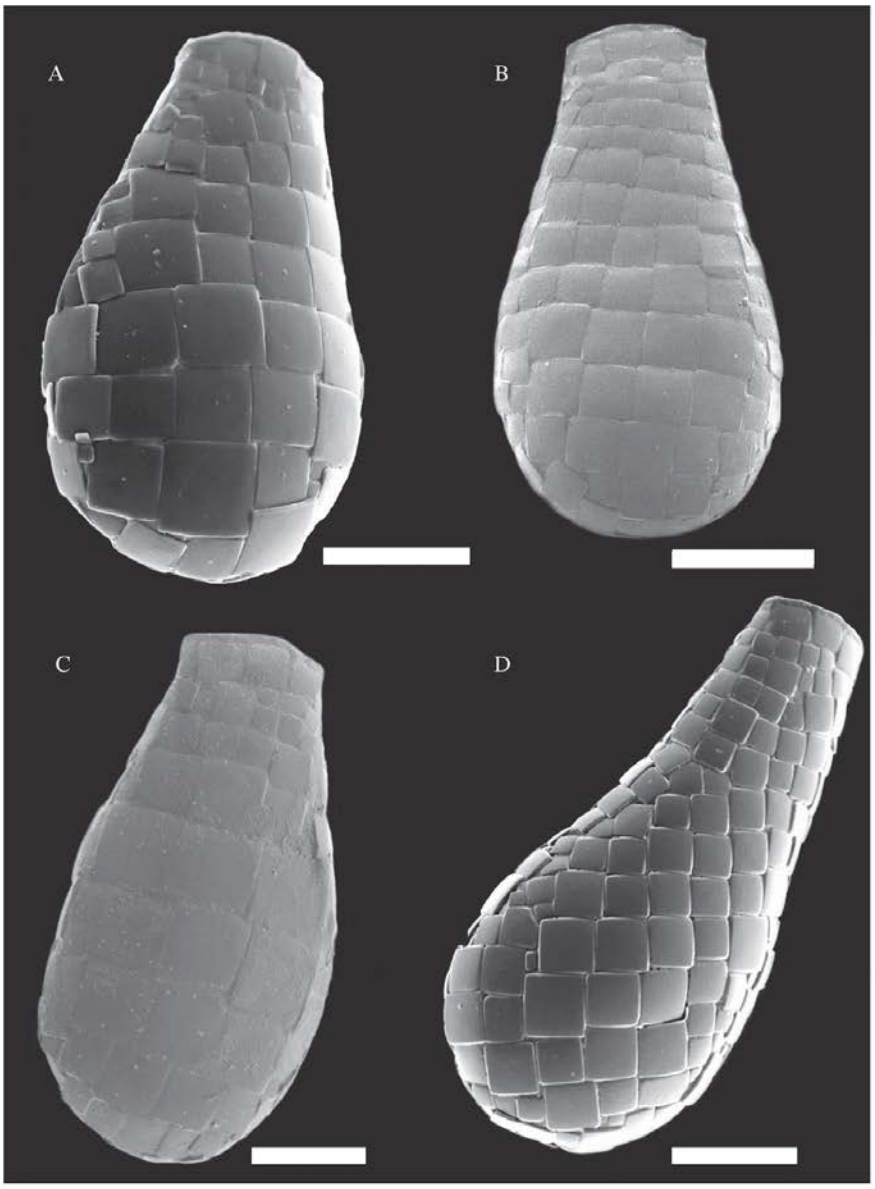

Figure 5. Scanning electron micrographs illustrating morphological variations within the *Quadrulella symmetrica* (*sensu lato*) morphospecies: **A**. *Q. symmetrica* from Bulgaria. **B**. *Q. symmetrica* from Canada. **C**. *Q. symmetrica* from Switzerland. **D**. *Q. longicollis* from Bulgaria. Scale bars represent 20 μm.

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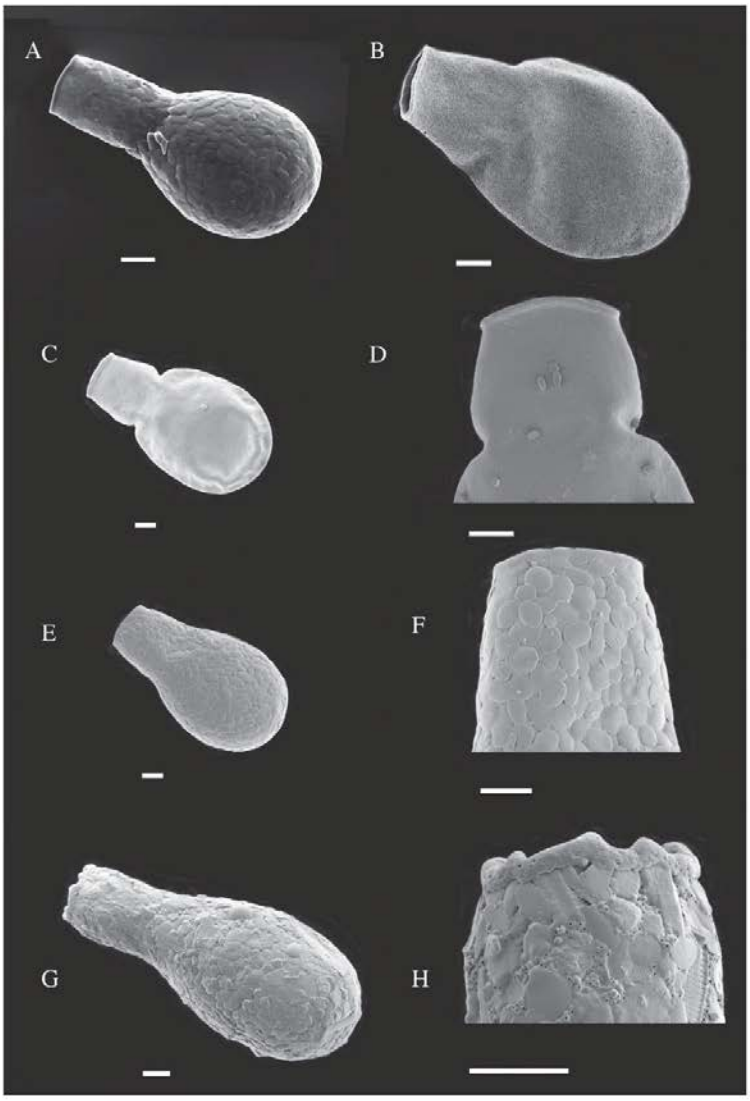

Figure 6. Scanning electron micrographs of outgroup species. **A**. *Padaungiella lageniformis* from Bulgaria. **B**. *Alocodera cockayni* from Argentina. **C-D**. *A. cockayni* from Chile and close view of its aperture respectively. **E-F**. *P. wailesi* from Switzerland and close view of its aperture. **G-H**. *P. nebeloides* from France and close view of its aperture. Scale bars represent 20 μm.

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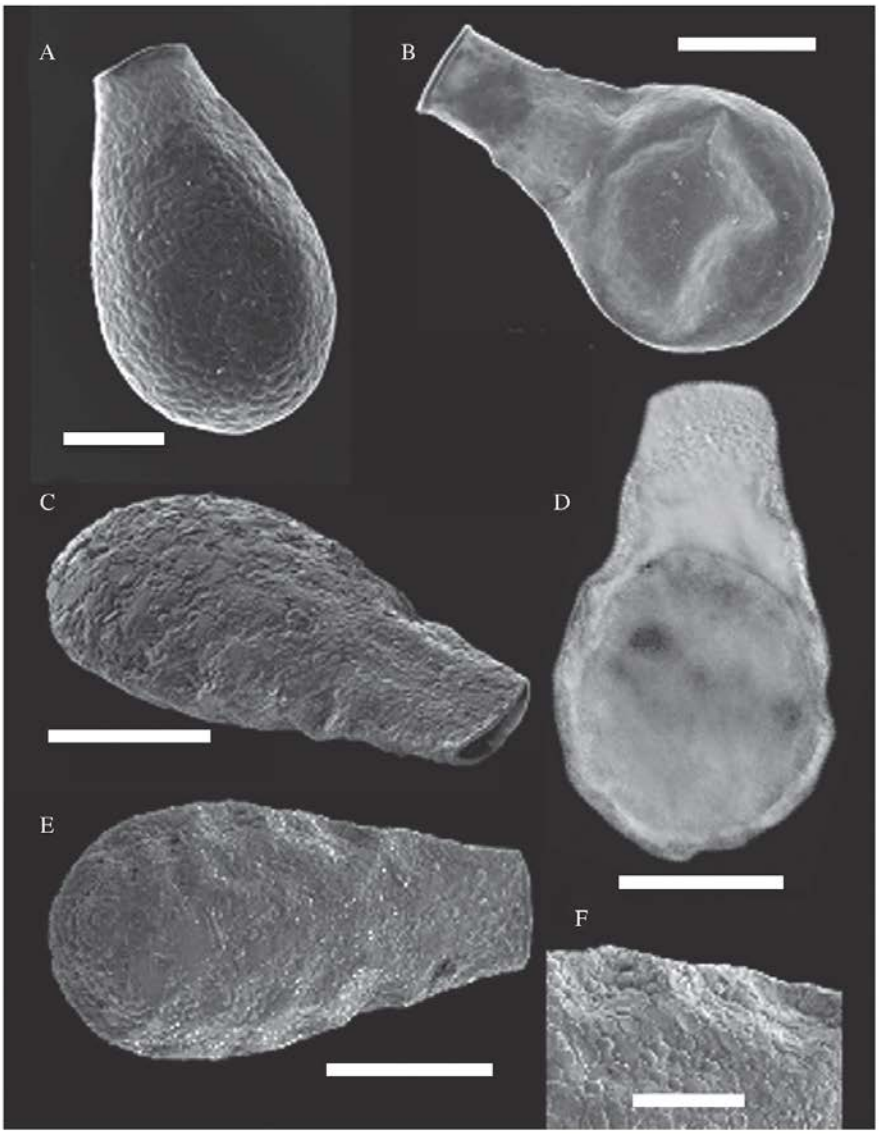

Figure 7. Scanning electron and light micrographs of species having uncertain phylogenetic position: **A**. *Nebela tubulosa* from Bulgaria. **B**. *Certesella martiali* from Tierra del Fuego Province, Argentina. **C**. *Nebela meisterfeldi* from Strathcona Park, Canada. **D**. *N. meisterfeldi* from Grouse Mountain, Canada. **E**. *N. meisterfeldi* from Strathcona Park, Canada. **F**. Detailed picture of the margin of *N. meisterfeldi* test from Strathcona Park, Canada. Scale bars represent 50 μm excepted for the detailed pictures of *N. meisterfeldi* (F): 20 μm.

an elongated test (Fig. 3). They are characteristic for nutrient-poor to nutrient-rich fens (Heger et al. 2011b; Lara et al. 2008; Leidy 1879; Todorov 2010). *N. ansata* and *N. hippocrepis*, two species that branch together in our tree with good support, share some morphological features (almost the same size and comparable shape). However, *N. ansata* is a distinctive species characterised by two lateral hollow horns (Heger et al. 2011b; Leidy 1879). Although *N. hippocrepis*, *N. galeata* and the members of clade 1 (*N. carinata* and *N. marginata*) are characterized by the presence of a keel, these species do not branch together; the shape of the latter being clearly different in members of clade 1 (thin) vs clade 2 (hollow, wide, bump-like), it is likely that the ontogenesis of the two structures differ.

The taxa that constitute clade 3 (*Hyalosphenia papilio*, sensu lato) are common and characteristic for *Sphagnum*-dominated ombrotrophic bogs and poor fens (Booth and Meyers 2010). It is possible that the establishment of mixotrophy through acquisition of zoochlorellae by *H. papilio* ancestors exerted an evolutionary pressure that prompted quick morphological changes. The sequencing of non-photosynthetic *Hyalosphenia*, such as *H. subflava* Cash, 1909, *H. cuneata* Stein, 1857, might bring clues to understand the evolution of this group. Based on the SSUr RNA gene *H. elegans* was shown to branch within the "core Nebelas" but not close to *H. papilio*. We were however unfortunately unsuccessful in obtaining a COI sequence for *H. elegans*. More work is therefore necessary before a possible revision of the taxonomic status of this species can be proposed. The remaining clades appear also related by their respective ecological preferences: clade 4 represents the "*Nebela tincta major-bohemica-collaris*" complex (Gilbert et al. 2003; Heal 1963), a group of small to medium-sized species (Fig. 4) showing a tendency to colonize relatively drier habitats such as forest humus and *Sphagnum* hummocks (Mitchell et al. 1999, 2004). Species belonging to clade 5 and 6 (i.e. respectively *Quadrulella* sp. and the group of *Padaungiella lageniformis* – former name *Nebela lageniformis* Figures 5 and 6), in turn, are respectively linked to minerotrophic habitats such as fens and forest soils (Bamforth 2010; Deflandre 1936), although *Alocodera cockayni* is found in oligotrophic peatlands (Charman 1997; Zapata and Fernández 2009).

Cryptic Speciation within "core Nebelas"

Cryptic speciation is thought to be common among protists. For instance, this was observed in euglyphid testate amoebae, kinetoplastids, foraminiferans and diatoms (Beszteri et al. 2005; Heger et al. 2010; Koch and Ekelund 2005; Kucera and Darling 2002). Our results show that cryptic diversity is also common at least within the "*Nebela*" group and likely the Arcellinida in general.

Our molecular data separated what we considered to be *N. carinata* into two clear-cut, robustly supported groups. The important genetic distance between the two groups (14% divergence in nucleotide sequences) suggests that these two forms should be considered as separate species. This would be in agreement with genetic distances of 7.3%-21.6% among species of the amoebozoan naked amoeba *Vannella* (Nassonova et al. 2010). Further investigation will clarify whether there exist slight morphological differences between these two forms (pseudocryptic diversity) or if no external morphological features can discriminate them (true cryptic diversity) and if the two clades differ with respect to ecology.

Another case of cryptic or pseudo-cryptic speciation is to be found within *Hyalosphenia papilio*, which appears here much more as a species complex than as a single taxonomic unit based on observed genetic distances. Indeed, it is divided into several subclades (Fig. 2) and genetic distances between isolates vary up to 7% in nucleotide sequences. Booth and Meyers (2010) reported morphological (and ecological) variation within *H. papilio* - shells collected in wetter habitats tended to bear more pores. Booth and Meyers (2010) interpreted this as phenotypic plasticity. This situation is further complicated with the possible occurrence of *H. ovalis* Wailes, 1912, a supposed sister species (but lacking zoochlorellae) whose identity remains dubious (Booth and Meyers 2010). A detailed investigation with careful morphological documentation of the obtained isolates will be needed in the future to investigate the limits between the different taxa that compose this clade, and to clarify to which extent variability is the product of phenotypic plasticity or is genetically fixed.

Another clear case of a species complex is the *Nebela tincta major-bohemica-collaris* group (Fig. 4), a group of very similar-looking morphospecies (Deflandre 1936; Heal 1964) which are often not distinguished from each other (Charman et al. 2000; Gilbert et al. 2003; Heal 1963; Warner 1987). *N. flabellulum* with its unusually wide test falls within this group, as in SSU rRNA gene-based analyses (Lara et al. 2008). It appears clearly from Figure 2 that the taxonomic status of the varieties of *N. tincta* would deserve specific status, as suggested in a previous work (Lara et al. 2008).

Our results show that genus *Quadrulella* Cockerell, 1909 branched within the "*Nebela*" group. Partial SSU sequences also indicate that *Q. symmetrica* belong to the "core Nebelas" (T. Heger unpublished data). The studied specimens corresponded morphologically in all cases to the species *Q. symmetrica*, including a variety described as *longicollis*. Here also, genetic distances (up to 11%) suggest rather a species complex than intraspecific diversity. SEM micrographs revealed some differences of the shell shape, as well as differences in the size and disposition of the secreted plates between different isolates (Fig. 5), and calls for a detailed study of this specific group. *Quadrulella* is one of the few arcellinid genera building its shell from self-secreted siliceous particles (Meisterfeld 2002). Differences in the size and shape of self-secreted scales (idiosomes) have proved to be taxonomical characters that could be used for species discrimination in the euglyphid testate amoebae of genus *Cyphoderia* (Heger et al. 2010). The position of *Q. longicollis* (former name: *Q. symmetrica* var *longicollis*) in our tree, its genetic distance from *Q. symmetrica* species as well as described morphological differences (Deflandre 1936), confirm that it is indeed an independent taxon.

Clade 6 contains species with bottle-shaped test and an elongated tubular neck (*Nebela lageniformis*, *N. nebeloides*, *N. wailesi* and *Alocodera cockayni*, Fig. 6). *N. nebeloides* was initially described as *Difflugia nebeloides* by Gauthier-Lièvre and Thomas (1958) but was recently transferred to genus *Nebela* by Todorov et al. (2010). In an earlier study based on SSU rRNA gene sequences (Lara et al. 2008), *Apodera vas* branched with *N. lageniformis*. We therefore transfer the *Nebela* species of this group, which constitute a sub-clade, to new genus *Padaungiella* (see the section Taxonomic Actions).

Taxonomic Actions

1. ***Quadrulella symmetrica* var. *longicollis*** (Taranek 1882) **to *Quadrulella longicollis*** (Taranek 1882)

   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 infrasubspecific 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.

2. **Description of a new species: *Nebela meisterfeldi* n. sp. Heger et Mitchell**

   Taxonomic summary
   Arcellinida Kent 1880
   Nebelidae Taranek 1882
   Nebela meisterfeldi n. sp. Heger et Mitchell

   Description: The shell is acrostome, elongated pyriform, laterally slightly compressed, with wavy lateral margin, brownish in colour (Fig. 7 D and F). Shell composed of small particles likely obtained from preys (i.e. euglyphid testate amoebae). The aperture is oval, surrounded by a very thin collar of organic cement (Fig. 7C and D). Dimensions (based on 6 individuals): length 147-160 μm, breadth 69-85 μm, diameter of aperture 37-42 μm.

   Hapantotype: The shells were collected from *Sphagnum* mosses in a peatland in Strathcona Park, Vancouver Island (49°42′N; 125°18′W) and from aquatic mosses at the border of a small stream in Grouse Mountain (49°23′N; 123°04′E), British Columbia, Canada. Dry moss samples containing this species are deposited in the sample collection of the laboratory of Soil Biology, University of Neuchâtel, Switzerland (codes: EM-286, 299). One SEM stub with several specimens is deposited at the Natural History Museum of Neuchâtel (Ref Nr. SEM-90, UniNe-EM-1). COI sequences were deposited in Genbank with accession numbers JN849052 and JN849053.

   Etymology: This species was named in honor of Dr. Ralf Meisterfeld, one of the most distinguished researchers in testate amoebae systematics and ecology in recognition for his contribution to this field.

   Note: *Nebela meisterfeldi* resembles *N. gracilis*, *N. gracilis* var. *stomata* Wailes, 1912 and *N. penardiana* by the shape of its shell. It is distinguished from the above mentioned species by the presence of wavy lateral margins. Our molecular data did not reveal a close affinity with *N. penardiana*.

3. **Genus *Padaungiella* Lara et Todorov**

   In 1942 Jung described genus *Schaudinnia* as follows: "Von *Nebela* s. str. durch den deutlich abgesetzten Hals, der den Schalen eine flaschenartige Gestalt verleiht, von den übrigen Gattungen durch das Fehlen von Merkmalen zu unterscheiden, die den anderen Nebelien Genera das Gepräge geben". English

translation: "Differs from *Nebela* s. str. by a distinct elongated neck that gives the shell a bottle shape, and from other Nebelid genera by the lack of distinctive features (that characterises each of them)". He included in this genus *Nebela lageniformis*, *N. tubulata* and *N. wailesi*. Unfortunately as Jung's classification lacked type designations, the name *Schaudinnia* is unavailable, and these species remained in genus *Nebela*. Our molecular data shows that genus *Nebela* is paraphyletic. We therefore transfer *Nebela lageniformis* and its closely related species (*Nebela nebeloides* and *N. wailesi*) to a new genus *Padaungiella* Lara et Todorov. Here, we propose *Padaungiella lageniformis* (Penard, 1890) Lara as the new type species for the genus. Consequently, the following names are changed:

Nebela lageniformis Penard, 1890 to *Padaungiella lageniformis* comb. nov. (Penard, 1890) Lara et Todorov

Nebela wailesi Deflandre, 1936 to *Padaungiella wailesi* comb. nov. (Deflandre, 1936) Lara et Todorov

Nebela wetekampi Jung, 1942 to *Padaungiella wetekampi* comb. nov. (Jung, 1942) Lara et Todorov

Nebela tubulata Brown, 1911 to *Padaungiella tubulata* comb. nov. (Brown, 1911) Lara et Todorov

Nebela nebeloides (Gauthier-Lièvre et Thomas, 1958) Todorov et al. to *Padaungiella nebeloides* comb. nov. (Gauthier-Lièvre et Thomas, 1958) Lara et Todorov

Syn.: *Difflugia nebeloides* Gauthier-Lièvre et Thomas, 1958

Nebela nebeloides (Gauthier-Lièvre et Thomas, 1958) Todorov, Golemansky et Meisterfeld, 2010

Etymology : The name of this genus is derived from the name of a tibeto-burmese ethnic minority of Burma, called "Padaung". The women of this tribe traditionally wear very long, coiled neck rings, which are constituted of a single brass coil placed around the neck. The length of the coil (which is gradually increased) and the added weight presses the clavicle and the rib cage, resulting in the appearance of a very long neck.

4. **Families Hyalospheniidae and Nebelidae**

Hyalospheniidae and Nebelidae were described respectively by Schultze in 1877 and Taranek in 1882 and revised on several occasions (Fig. 1). Following the latest revision of the two families (Meisterfeld 2002) the Nebelidae included genera with tests composed of collected or predated round or oval siliceous plates, fragments of diatoms or mineral grains: *Alocodera*, *Apodera*, *Argynnia*, *Certesella*, *Geamphorella*, *Jungia*, *Nebela*, *Physochila*, *Pseudonebela*, *Porosia*, and *Schoenbornia* and the Hyalospheniidae included genera with chitinoid, clear, completely organic, non-areolar test: *Hyalosphenia* and *Leptochlamys*. Given that genus *Hyalosphenia* clearly branches within the "core Nebelas" clade and that the distinguishing character of Hyalospheniidae (shell transparent and entirely secreted) can also be observed in some Nebelidae (*Alocodera, N. tincta*) the two families need to be synonymised. The name Hyalospheniidae Schultze 1877 takes precedence according to the principle of priority (article 23 of the international code of zoological nomenclature).

Diagnosis of the Hyalospheniidae Schulze, 1877 emend. Kosakyan et Lara

The test is rigid, colorless or yellowish-brown, flask-vase shaped, oval or pyriform, dorso-ventrally compressed. The shell is either entirely self-secreted (e.g. *Hyalosphenia*) composed of an organic matrix, or with addition of self-secreted siliceous plates (*Quadrulella*) or recycled shell plates of small euglyphids or other similar material such as diatom frustules incorporated in the test. The pseudostome is terminal and is bordered by a thin organic collar.

Physochila and *Argynnia* do not form a monophyletic clade with the Hyalospheniidae based on molecular phylogenetic data (Lara et al. 2008; Gomaa et al., unpublished data) and also differ from other Nebelidae by their morphology, hence are excluded from the Hyalospheniidae and are *incertae sedis*. Similarly, *Leptochlamys* differs from all Hyalospheniidae by a unique combination of characters: shell circular in cross-section, round pseudostome, and unique hyaline pseudopod (Cash and Hopkinson 1909) and is now deemed *incertae sedis*. As a consequence, the Lesquereusiidae now includes *Lesquereusia, Netzelia, Microquadrula* and *Pomoriella*.

Methods

Sampling and species isolation: Testate amoebae were obtained from *Sphagnum*, other mosses and forest litter collected from different 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 dissecting microscope. Cells were rinsed with demineralized water. We characterized the morphology of these distinct "populations" (=individuals of a given morphospecies isolated from one sample) by scanning electron or light microscopy when enough cells were available. To make sure that the ultrastructure of the cells did not differ within populations, several SEM micrographs from different individuals were taken and subsequently compared.

Scanning electron microscopy: Testate amoeba shells were mounted on stubs and then kept during one week in a desiccator. The shells 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: A guanidine thiocyanate protocol (Chomczynski and Sacchi 1987) was used to extract DNA (1 to 10 cells were isolated per DNA preparation) or single cell were used without DNA extraction. The mitochondrial COI sequences were obtained by polymerase chain reaction (PCR) in two steps: for first using the LCO1490 and HCO2198 "Universal" COI primers designed for diverse metazoan invertebrates (Folmer et al. 1994). A second PCR was performed on the products, using again the general primer HCO and a specific primer Arcelcox1F (CAA AAT CAT AAA GAT ATT GGD AC); designed to amplify most "core Nebelas" or / the general primer LCO with Apocox R (CCW GGA TGD CCT TCA ATA CTA CT), specific for the group 6, situated on position 366 of the *Padaungiella lageniformis* COI sequence. The PCR cycling profile was the same for the first and second PCRs (except for group 6 species for which we used specific pair of primers LCO and Apocox). DNA was amplified in a total volume of 25 μl with an amplification profile consisting 3 min initial denaturation step in a 40 cycles program of 15 s 95 °C, 15 s 40 °C, and 1 min at 72 °C with the final extension at 72 °C for 8 min. For species of clade 6 the following program was used: 5 min initial denaturation step in a 40 cycles program of 15 s 95 °C, 15 s 55 °C and 1 min 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 analysed either with an ABI-3130xl or a 3730S 48-capillary DNA sequencer (Applied Biosystems). COI sequences are deposited in GenBank with the accession numbers given in Table 1.

Phylogenetic analyses: The data set used for phylogenetic analyses (668 bp) comprised 59 COI sequences. The sequences were aligned 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 the group of *Padaungiella lageniformis* (i.e. clade 6) to root all trees, based on the fact that these species appear as sister clade of the "core Nebelas" in the SSU rRNA gene-based phylogeny (Lara et al. 2008). We performed similar phylogenetic analyses using *Vannella* spp. as outgroup (GQ354136; GQ354148; GQ354154; GQ354165; GQ354171; GQ354177; GQ354184; GQ354191). This tree revealed six Arcellinida groups (data not shown) which correspond to the six clades of the Figure 2. 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 standard deviation of split frequencies fell below 0.01 according to the manual of MrBayes 3.1 (2005). 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 (program distributed as part of the BEAST package). The sequence divergence between sequences were calculated using the program R version 2.9.1 (R Development Core Team 2009). Missing data was not counted during the calculation relative % of sites that differ between each pair of sequences (Supplementary Table S1).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.10.003.

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CHAPTER 2

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

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

Anush Kosakyan[a,*], Fatma Gomaa[a,b], Edward A.D. Mitchell[a], Thierry J. Heger[c], Enrique Lara[a]

[a]*Laboratory of Soil Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland*
[b]*Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt*
[c]*Biodiversity Research Centre, University of British Columbia, Vancouver, BC V6T 1Z4, Canada*

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

*Corresponding author. Tel.: +41 767742980.
E-mail address: anush.kosakyan@unine.ch (A. Kosakyan).

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

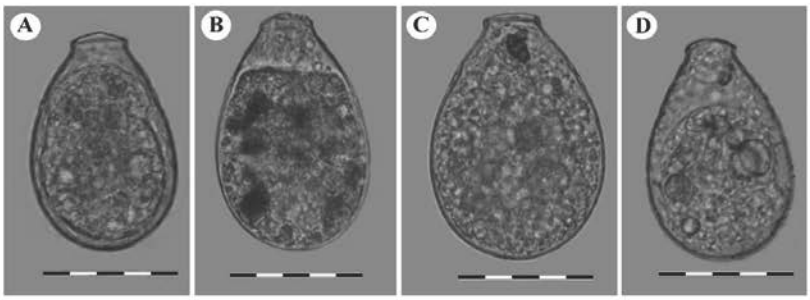

Fig. 1. Light micrographs of clade A cells (*Nebela guttata*): **A.** LC-126 from Le Cachot population, Switzerland, **B.** PE-159 from Pechora population, Russia, **C.** LC-118 from Le Cachot population, Switzerland, and **D.** LC-103 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

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

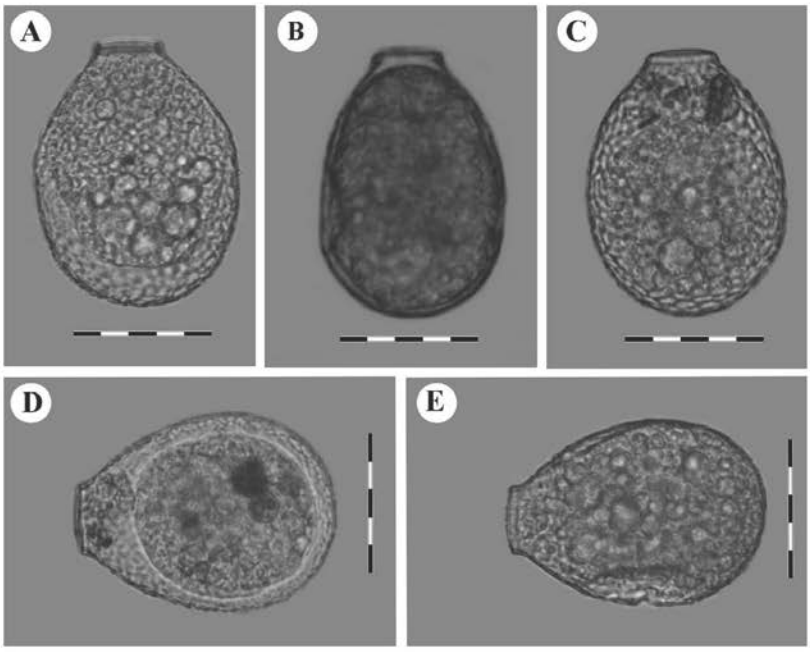

Fig. 2. Light micrographs of clade B cells (*Nebela tincta*): **A.** LC-86, **B.** LC-137, **C.** LC-117, **D.** LC-89, **E.** LC-62 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

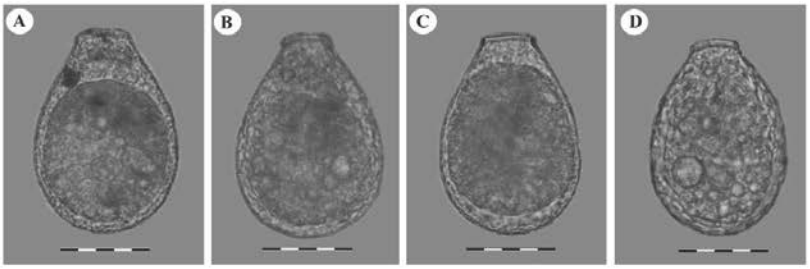

Fig. 3. Light micrographs of clade C cells (*Nebela collaris*): **A.** LC-75, **B.** LC-69, **C.** LC-64, **D.** LC-55 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

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).

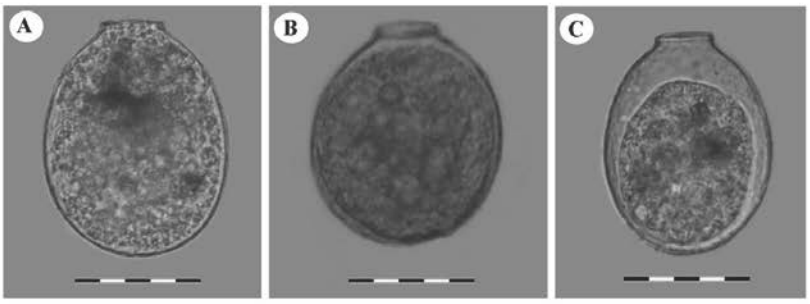

Fig. 4. Light micrographs of clade D cells (*Nebela rotunda*): **A.** LC-58, **B.** LC-71, **C.** LC-74 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

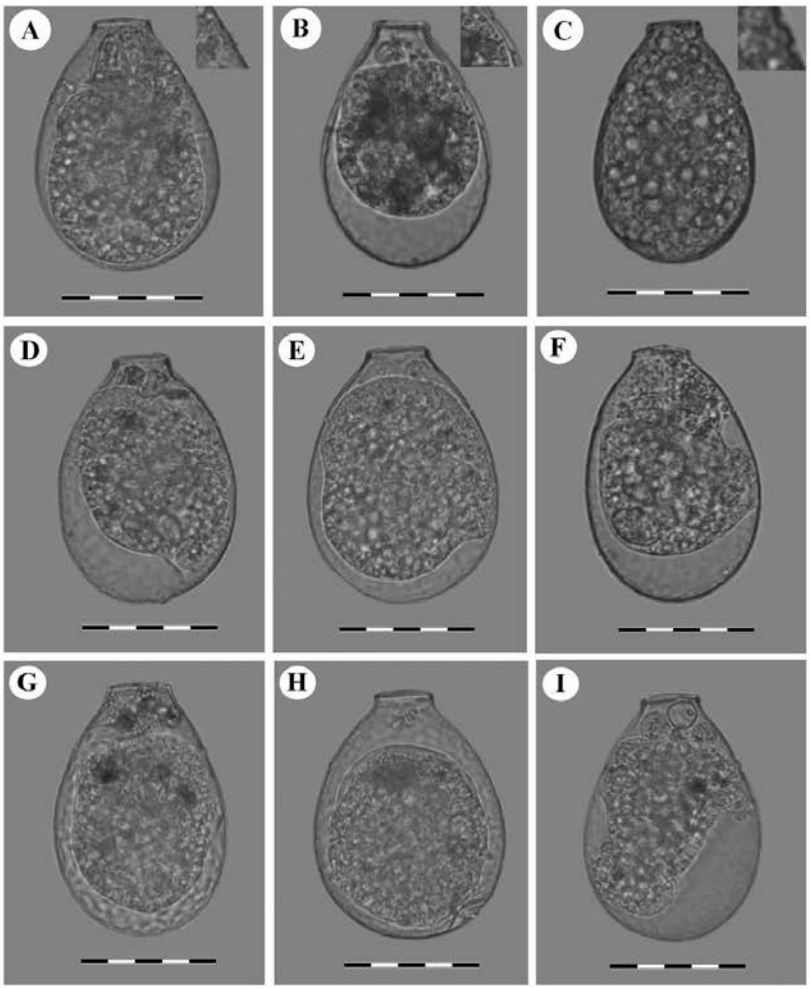

Fig. 5. Light micrographs of clade E cells (*Nebela pechorensis*): **A.** PE-149 from Le Pechora population, Russia, with its detailed picture of lateral pores, **B.** PE-151 from Pechora population, Russia, with its detailed picture of lateral pores, **C.** LC-135 from Le Cachot population, Switzerland, 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

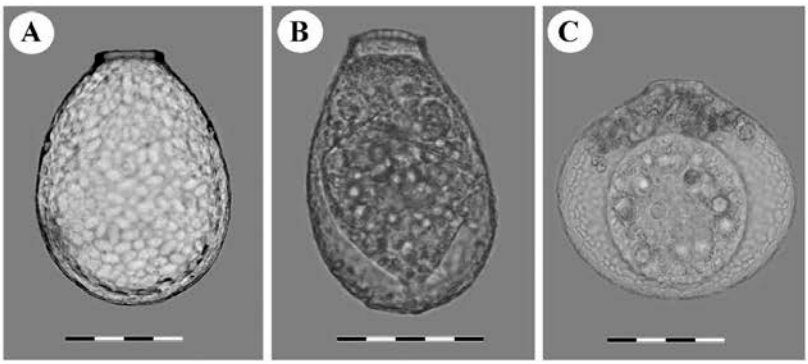

Fig. 6. Light micrographs of the remaining forms: **A**. *Nebela aliciae* n. sp. from Costa Rica, **B.** PE-155 from Pechora population, Russia, **C.** *N. flabellulum* from Canada. Scale bars represent 50 μm for A and B, and 60 μm for C.

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

8

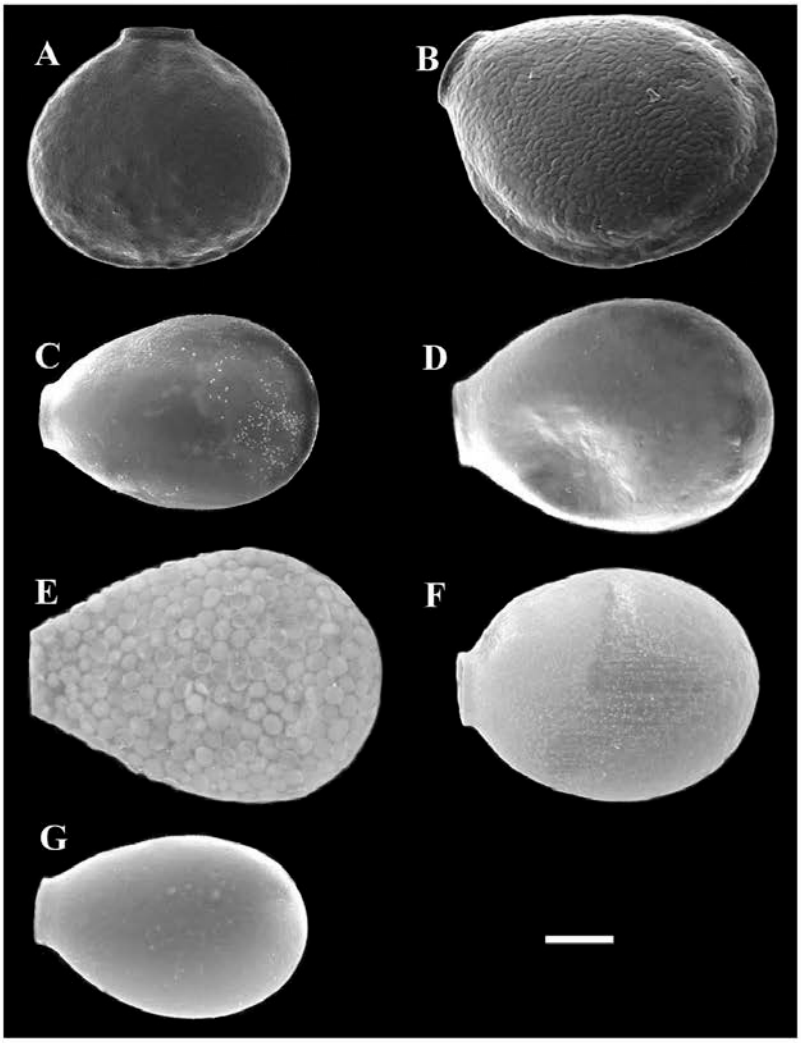

Fig. 7. Scanning electron micrographs of cells from each species-population: **A.** *Nebela flabellulum* from Lynn Peak, Canada, **B.** *N. aliciae* n. sp. from Volcán Poás, Costa Rica, **C.** *Nebela guttata* n. sp. from Le Cachot, Switzerland, **D.** *N. tincta* from Le Cachot, Switzerland, **E.** *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.

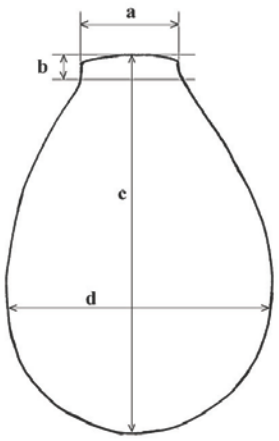

Fig. 8. Schematic sketch and position of the measured axes of the test: **a.** diameter of aperture, **b.** length of the neck, **c.** length of the test, **d.** breadth of the test.

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

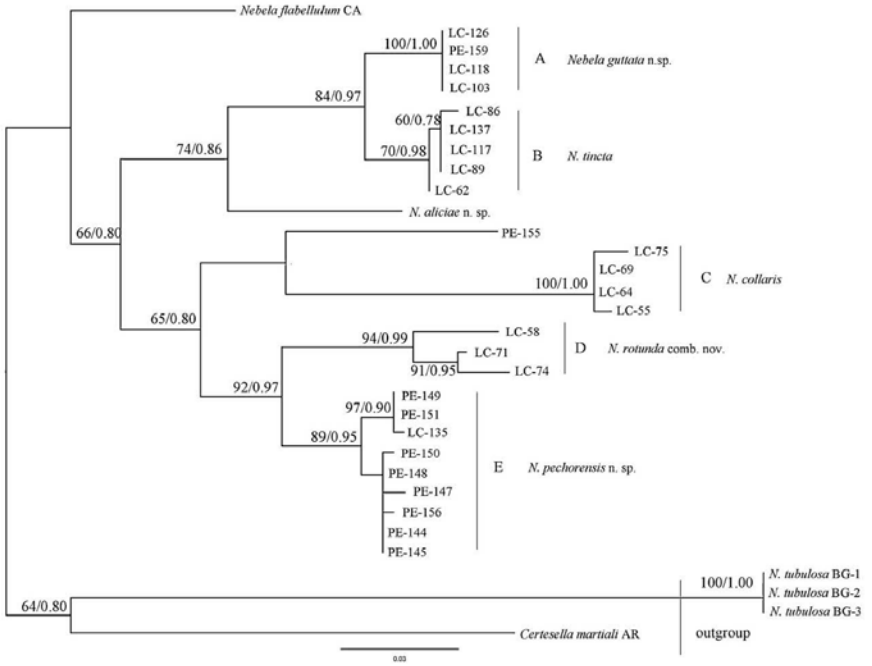

Fig. 9. Maximum likelihood bootstrap consensus tree of 31 *Nebela collaris s.l.* testate amoeba COI sequences based on 300–665 nucleotide positions. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated 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 rel****ation 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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CHAPTER 3

Exploring the true diversity of protist groups using DNA-barcoding: A case study of genus *Quadrulella* (Amoebozoa, Arcellinida, Hyalospheniidae)

Paper in preparation, it will be submitted to Journal of Eukaryotic Microbiology

Exploring the true diversity of protist groups using DNA-barcoding: A case study of genus *Quadrulella* (Amoebozoa, Arcellinida, Hyalospheniidae)

Anush Kosakyan, Edward A. D. Mitchell and Enrique Lara

Laboratory of soil Biology, University of Neuchatel, Rue Emile-Argand 1, 2000 Neuchatel, Switzerland.

Abstract

The species diversity of most protistan groups is very uncertain. The taxonomic value of morphological characters that were used for species descriptions are in many cases unclear, leading to confusion and undermining the use of protists as models in ecology, biogeography and palaeoecology. Here we focus on genus *Quadrulella* (Arcellinida, Hyalospheniidae). These testate amoebae produce very characteristic tests (shells) from self-secreted quadrangular siliceous plates. It consists of mostly rare species occurring mainly in wet mosses. The most common, taxon in central Europe is *Quadrulella symmetrica*, which was hitherto believed to be a single species.

We combined analyses of test ultrastructure and Cytochrome Oxidase Subunit 1 (COI) sequence data to assess if morphological difference corresponded to genetic data, or if they rather reflected possible phenotypic plasticity.

Morphological and molecular data showed that *Quadrulella symmetrica* is a species complex that can be split into at least five genetically and morphologically distinct species. The main morphological characters that define the taxa within this species complex are the overall test size and the arrangement and size of plates.

Note: This paper is in preparation. The next step will be to add more samples and characterise the ecological characteristics of sampling sites to assess if closely related species differ in their ecological preferences.

Introduction

Estimation of global biodiversity has long been a key question in biology. While describing species is in most cases relatively straightforward for macroscopic organisms, which have a multitude of useful morphological characters the situation is more complicated to microorganisms including microbial eukaryotes, since the characters used to define the species in micro-organisms are less obvious to find (i.e. morphology is most often simpler than for macro-organisms) and/or difficult to observe (i.e. requiring electron microscopy). As a result, the true level of microbial diversity is underestimated by traditional taxonomy. It is therefore not surprising that DNA-sequence based studies have revealed a high diversity in many microbial eukaryotic groups (Epstein and Lopez-Garcia, 2008; López-García P. et al., 2001; Heger et al., 2011; Kosakyan et al., 2013). The introduction of molecular methods has made it easier and faster to estimate microbial eukaryotic diversity. This in turn has made it possible to address new fundamental questions about their evolution and to envision new applications in a number of research fields (e.g. ecology, environmental monitoring, health monitoring, etc.). The accumulation of new data also revealed that some errors in morphology-based identification as well as a high number of new species and thus clearly showed how incomplete is our current knowledge about micro-eukaryotic diversity, and how urgent is the need to conduct careful revisions of the taxonomic position of many protistan taxa.

Among microbial eukaryotes, hyalospheniid testate amoebae (Hyalospheniidae, Arcellinida, Amoebozoa) are considered as important bioindicators and are commonly used in environmental monitoring and palaeoecology (Charman, 2001; Mitchell et al., 2008). Recent molecular studies have showed that the classical taxonomy underestimated the true diversity within this group. About 200 Hyalospheniidae taxa have been described to date (Meisterfeld, 2002) but detailed studies conducted of some of these taxa have all revealed the existence of cryptic diversity (i.e. genetically distinct taxa but morphologically indistinct even using electron microscopy) (Kosakyan et al., 2012, 2013; Heger et al., 2013; Oliverio et al., 2014). In some cases this diversity does not seem to be correlated to any morphological differences and the taxa can be considered as cryptic species (Heger et al., 2013; Oliverio et al., 2014). But in other cases this diversity corresponds to pseudo-cryptic diversity as careful examination of the ultra structure, notably with scanning electron microscopy (SEM), has

allowed to identify subtle morphological variations (e.g. small differences in the general shape of the shell) possibly visible only using electron microscopy among genetically distinct organisms (Kosakyan et al., 2013).

Genus *Quadrulella* is composed of medium sized (ca. 100 µm) testate amoebae that are found in peatlands, wet forest mosses and soils. They build their shell from self-secreted square siliceous elements. The genus is currently composed of 22 species and infraspecific taxa, most of which were described from material collected in the tropics and not observed since. All European specimens have been to date assigned to the species *Q. symmetrica*, or its subspecies and varieties. In a previous phylogenetic study of family Hyalospheniidae using COI sequence data (Kosakyan et al., 2012) and which included six *Quadrulella symmetrica* sequences, we found large differences between DNA sequences that corresponded to morphological differences and clearly showed that more than one species exist within *Q. symmetrica s.l.* For example, *Q. symmetrica* var. *longicollis* is clearly an independent taxon based on the test morphology and 11% nucleotide divergence from the rest of the studied *Q. symmetrica* cells. Besides differences in general test shape, SEM micrographs revealed some differences in the size and disposition of the secreted plates among the studied *Q. symmetrica* isolates (Kosakyan et al., 2012, Fig 5). These results strongly suggested that a more detailed study would reveal more diversity within this species complex.

Our aims were 1) to characterize the morphological and molecular diversity within *Q. symmetrica s*.l., 2) to assess the degree of the morphological variability within genetic lineages as an indication for possible phenotypic plasticity, and 3) to estimate is these variations are related to environmental conditions.

Material and Methods

Sampling, species isolation and documentation

Sphagnum mosses were collected in Le Cachot bog and the Botanical Garden of Neuchâtel (originally collected in Le Cachot bog, Jura Mountains), Switzerland (Table 1). Amoebae 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. We selected some cells from each sample which are going to be documented by electron microscopy (SEM) and kept as a voucher specimen deposited at the Natural History Museum of Neuchâtel, Switzerland. Each cell was measured in order to obtain the following values: length of the test, maximum breadth of the test, and breadth of aperture. In order to obtain a value for average dimensions of the scales, we measured 10 plates taken at random in the middle part of the shell, and measured the minimum and maximum dimension.

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.

Table 1. List of sequenced cells and sampling locations.

Cells	Sampling location	Country	Co-ordinates	Sequence length (bp)	Gen Bank number
Q. sym.-52	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	256	
Q. sym.-48	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	227	
Q. longicollis BG	*Sphagnum mosses, Vitosha Mountain*	Bulgaria	42°36'N 23°17'E	640	JN849050
Q. sym.-54	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	375	
Q. sym.-53	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	375	
Q. symmetrica CH	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	633	JN849046
Q. symmetrica BG	*Sphagnum mosses*, Vitosha Mountain	Bulgaria	42°36'N 23°17'E	634	JN849047
Q. symmetrica BG	*Sphagnum mosses*, Vitosha Mountain	Bulgaria	42°36'N 23°17'E	625	JN849049
Q. symmetrica BG	*Sphagnum mosses*, Vitosha Mountain	Bulgaria	42°36'N 23°17'E	340	JN849048
Q. sym.-63	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	364	
Q. sym.-75	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	361	
Q. sym.-83	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	361	
Q. sym.-81	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	363	
Q. sym.-73	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	572	
Q. sym.-82	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	567	
Q. sym.-51	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47.5°N 6.4°E	264	
Q. symmetrica CA	*Sphagnum mosses*, Echo Bay, British Columbia	Canada	50°45'N 126°28'E	607	
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

Principal component analyses (PCA)- were carried out using RDA methods of the Vegan package (Terbraak, 1986; Legendre and Legendre, 2012).

DNA amplification- Single cells were transferred into PCR tubes and the mitochondrial COI gene amplified by polymerase chain reaction (PCR) using general primer LCO (Folmer et al. 1994) and specific primer QR1 (5' TAA CAT HGT WAT TCC AGC AGC 3') designed to amplify *Quadrulella* species. DNA was amplified with an amplification profile consisting of a 5 min initial denaturation step in a 40 cycles program 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 PCR products 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). COI sequences were deposited in GenBank under numbers xxxx-yyyyy (nb. will be done after manuscript acceptance).

Phylogenetic analyses

The data set used for phylogenetic analyses (227–640 bp) comprised 11 COI *Quadrulella* sequences together with six sequences from Genbank from our previews study (Kosakyan et al., 2012). The sequences were aligned 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/). Model parameters were estimated in RAxML over the duration of the tree search. We used sequences from *Nebela tubulosa* (GenBank number JN849020, JN849021, JN849061) to root all trees, based on the fact that these species appear relatively closely related to the *Quadrulella.* 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 500000 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 (Ronquist et al., 2005). 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 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 BioEdit. Missing data were not considered in the calculation.

Results and discussion

Morphological data

Illustrations of *Quadrulella* individuals studied for molecular phylogeny (light microscopy) and individuals from the same population (SEM) shown in Figures 2-5. The results of the test measurement are presented in Table 2 and Figure1.

Table 2. Summary of morphological characteristics of *Quadrulella* individuals isolated for molecular analysis.

Clades	Cells	Lenght (µm)	Breadth (µm)	L/B ratio	Aperture (µm)	Plate characters, min -max size (µm)
A	Q. sym.-52	69	36	1.9	18	small, 3-7.5
	Q. sym.-48	71.5	40.5	1.8	18.5	small, 4-7.5
	Q. longicollis BG	96	45	2.1	17	small, 4-8(9)
	Q. sym.-54	66	37	1.8	18	small, 5-7.5
	Q. sym.-53	67	37	1.8	18.5	small, 3-7
B	Q. symmetrica CH	87	44.5	1.9	23	big, 4-14 (15)
	Q. symmetrica BG	75	42	1.8	20	big, 3-12.5
	Q. symmetrica BG	75	42	1.8	20	big, 3-11
	Q. symmetrica BG	75	42	1.8	20	big, 3-11
	Q. sym.-63	80	44.7	1.8	23	big, 7-10.5
	Q. sym.-75	81	45	1.8	23	big, 5-11
	Q. sym.-83	71.7	43	1.7	21	big, 4-12
	Q. sym.-81	84	45.5	1.8	22	big, 5-11
	Q. sym.-73	77.7	46	1.7	21	big, 5-9.5
	Q. sym.-82	76.6	46	1.7	21	big, 5-9
	Q. sym.-51	79	46	1.7	21.5	big, 4.5-9.7
	Q. symmetrica CA	71.5	40	1.8	20	big, 4.5-9

*-Entered data is based on only one SEM picture from the same population from where sequences were obtained.

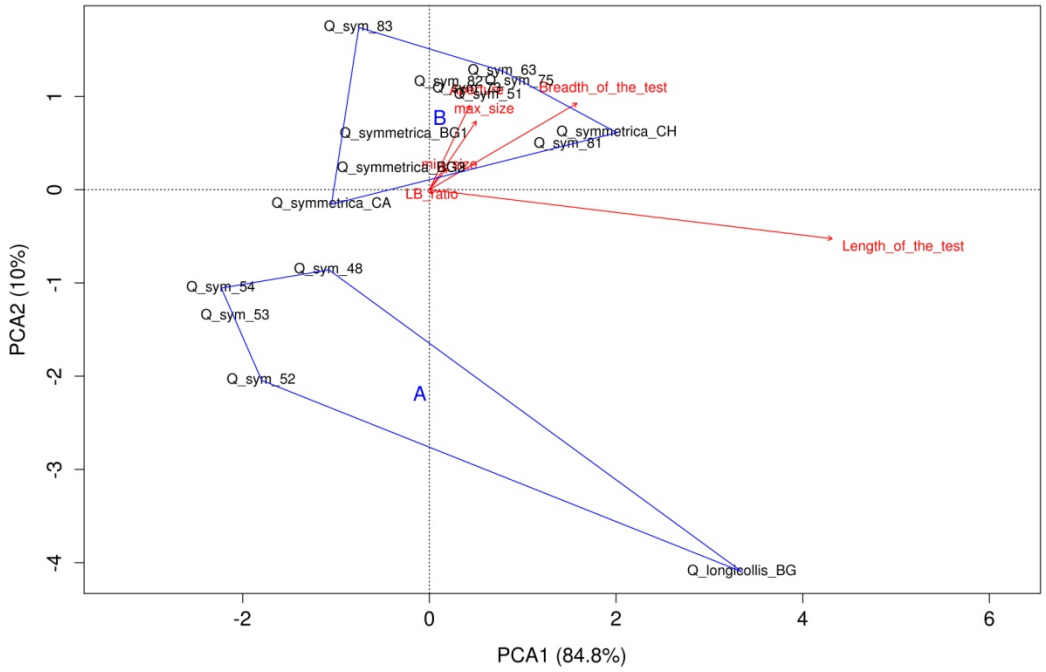

Figure 1. PCA analyses on studied *Quadrulella* cells morphology according to the following descriptors: length (L) of the test, breadth (B) of the test, L/B ratio, size of the plates (min and max values).

Molecular data

A total of 20 COI sequences were obtained from 11 single cells plus 9 sequences (five *Quadrulella symmetrica*, one *Q. longicollis* and three *Nebela tubulosa*) from a previous study (Kosakyan et al., 2012). Three *N. tubulosa* sequences were used as outgroup.

Our phylogenetic reconstructions showed that *Q. symmetrica* is composed of two main clades (A and B) each of which included several groups (Fig. 2). Topologies of both the strict consensus ML and Bayesian trees were identical. Sequence divergence between clades A and B ranges from 11 % to 13%. Information on the morphology of the cells constituting each clade is summarized in Table 2.

Clade A is moderately supported respectively with 54% bootstrap (B) and 0.90 posterior probabilities (PP) values. It comprised five cells from the current study (Q. sym.-52, Q. sym.-48, Q. sym.- 54, Q. sym-53) and *Q. longicollis*_BG from a previous study (Kosakyan et al., 2012). Except for *Q. longicollis*, all the cells in this clade are relatively small (L = 66.5-

72, B = 36-40.5 µm, aperture width 18-18.5 µm). The shell plates are also relatively small (3-7.5 µm). *Q. longicollis* (L=96, B=45µm, aperture=17µm) is larger than the other representatives of clade A, but the shell plates are also small (4-8(9) µm). Sequence divergence among members of clade A is 2-3%.

Clade B is supported with 75% B and 0.97 PP values. It comprised seven cells from the current study (Q. sym.-63, Q. sym.-75, Q. sym.-83, Q. sym.-81, Q. sym.-73, Q. sym.-82, Q. sym-51) and five *Q. symmetrica* sequences from a previous (Kosakyan et al., 2012) study (*Q. symmetrica*_CH, three *Q. symmetrica*_BG, and *Q. symmetrica*_CA). All the cells in this clade are much larger than those of clade A (L = 75-87, B = 42-46 µm, aperture = 20-23 µm). They also have relatively large plates 3-14(15) µm, although the ranges overlap. Clade B is further divided into 4 groups (Fig. 2): (1) *Q. symmetrica* _CH, (2) *Q. symmetrica* _BG group, (3) group of *Q. symmetrica* cells from current study, and (4) *Q. symmetrica*_CA. Sequence divergence among these groups is 4-10 %.

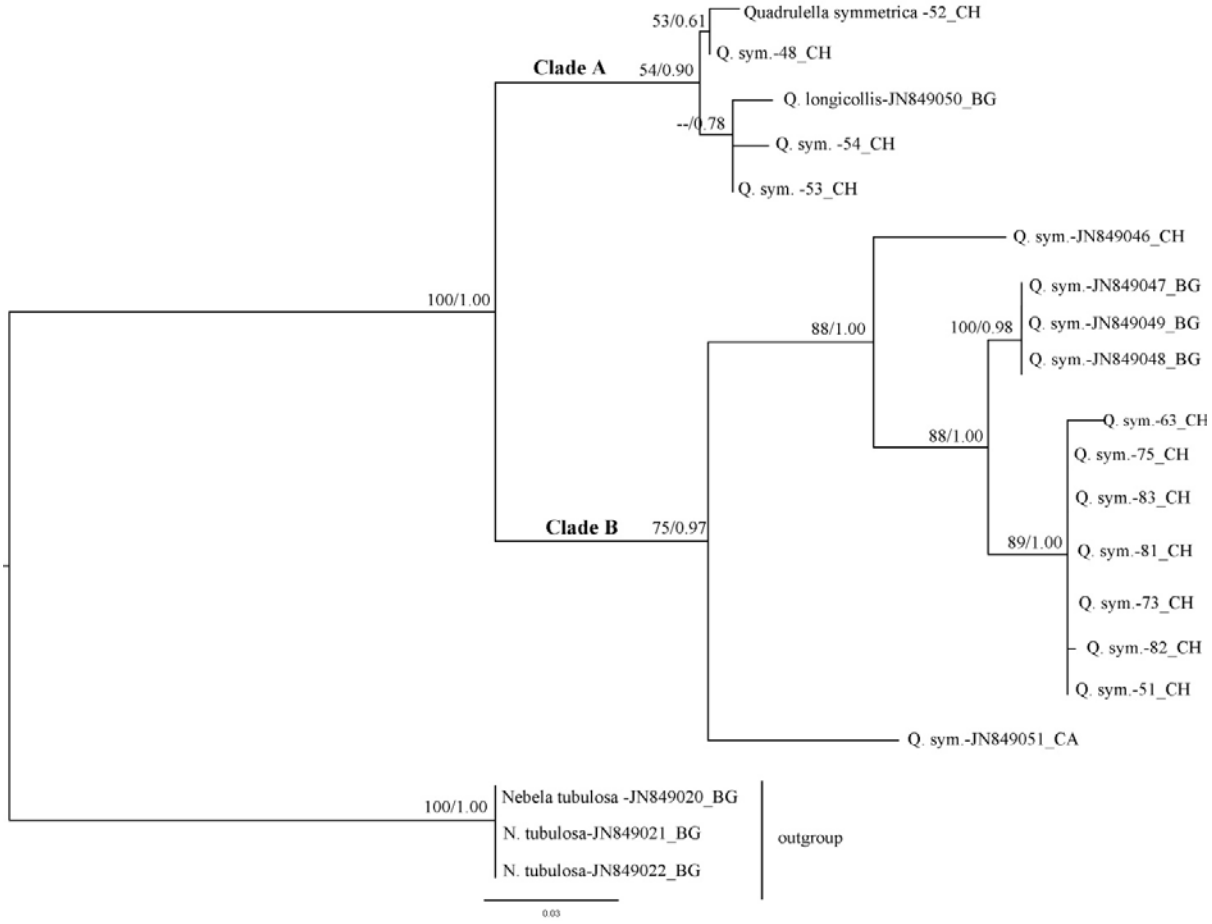

Figure 2. Maximum likelihood bootstrap consensus tree of 17 *Quadrulella symmetrica* s.l. COI sequences based on 640 nucleotide alignment. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated with Bayesian analyses. Only values above 50/0.50 are shown. Three was rooted with three *Nebela tubulosa* sequences as outgroup.

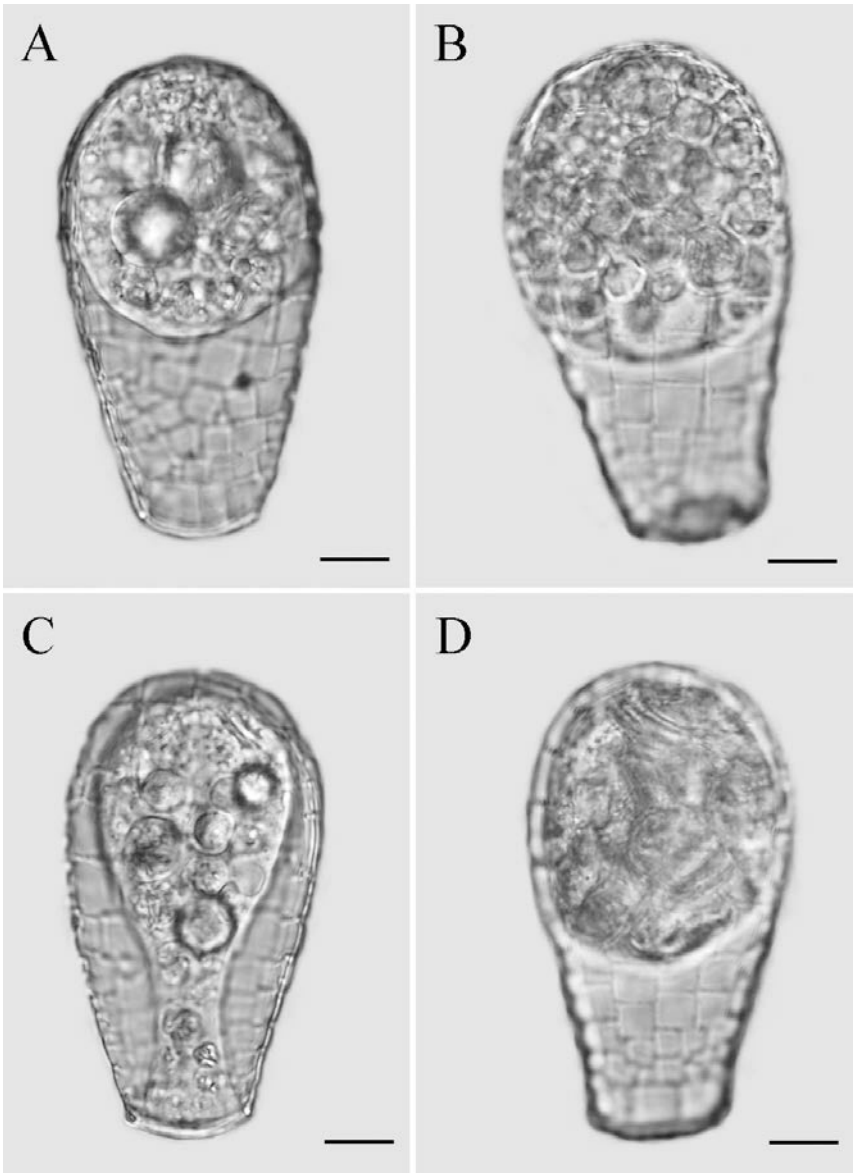

Figure 3. Light micrographs of representatives of *Quadrulella symmetrica* individuals from from Clade A: **A.** Q.sym.-52 cell, **B.** Q. sym.-48 cell, **C.** Q.sym.-54 cell, **D.** Q. sym.-53 cell from Le Cachot bog, Switzerland. Scale bars =10 µm.

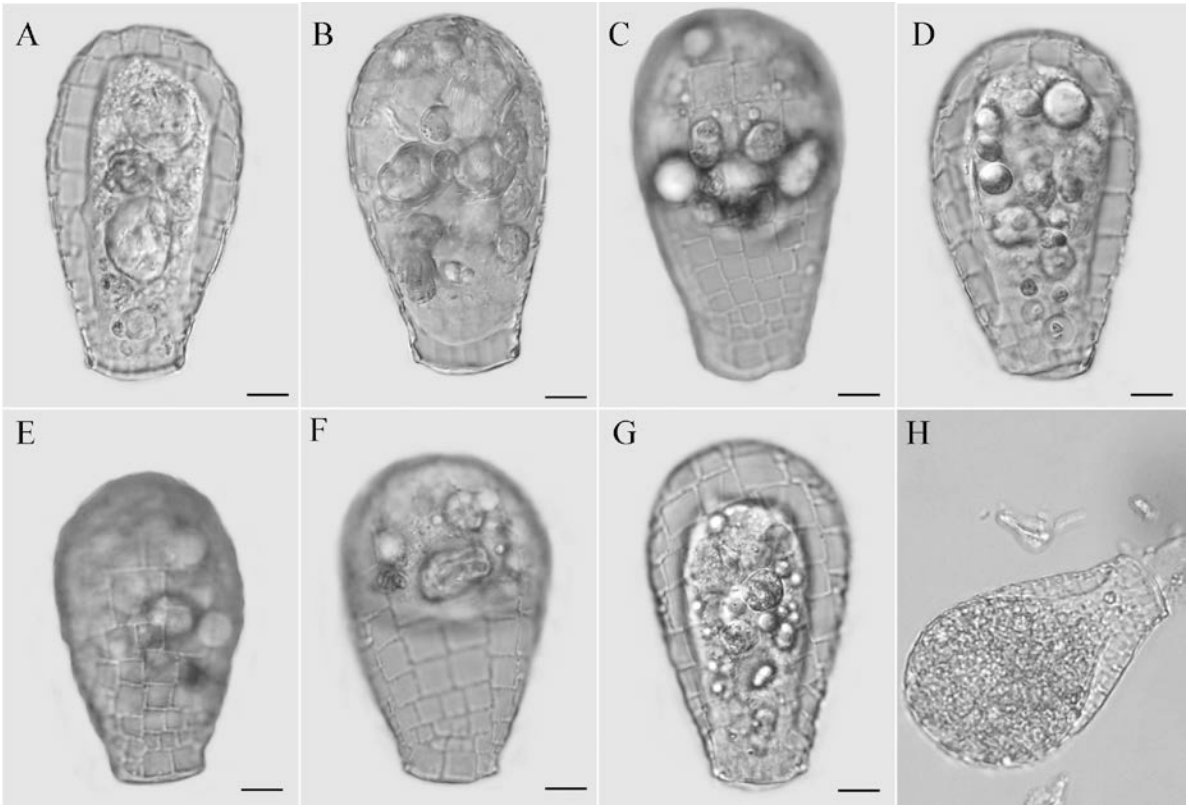

Figure 4. Light micrographs of *Quadrulella symmetrica* individuals from Clade B: **A.** Q.sym.-63 cell, **B.** Q. sym.-75 cell, **C.** Q.sym.-81 cell, **D.** Q. sym.-73 cell, **E.** Q.sym.-83 cell, **F.** Q.sym-82 cell, **G.** Q. sym.-51cell from Le Cachot bog, Switzerland, **H.** light micrograph from Bulgarian *Q. symmetrica* population, Vitosha mountains , Bulgaria. Scale bars =10 µm.

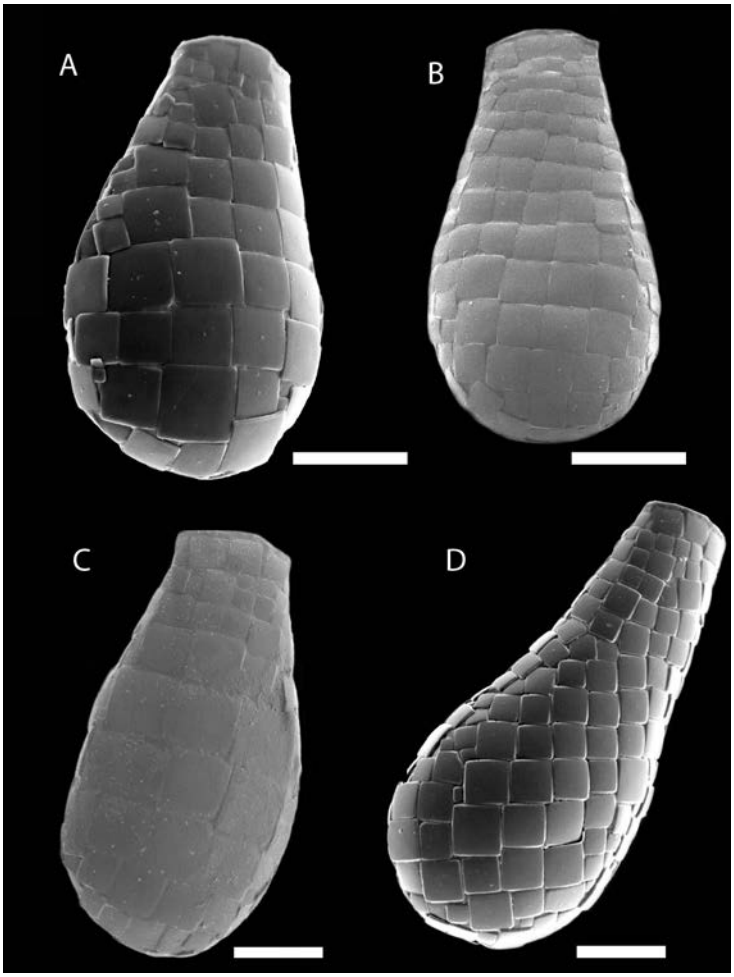

Figure 5. Scanning electron micrographs illustrating morphological variations within the *Quadrulella symmetrica* (*sensu lato*) morphospecies. **A.** *Q. symmetrica*_BG from Bulgaria, **B.** *Q. symmetrica*_CA from Canada, **C.** *Q. symmetrica*_CH from Switzerland, **D.** *Q. longicollis* from Bulgaria. Scale bars =20 µm. The image is taken from Kosakyan et al. 2012.

Note: an expanded figure will be prepared for a final version of this study including new material.

DNA-based studies have repeatedly demonstrated that traditional taxonomy underestimates diversity of both macroscopic and microscopic organisms (Harper et al., 2009; Hebert et al., 2003a; Hebert et al., 2003b). Cytochrome Oxidase Subunit 1 (COI) was initially chosen as a barcoding gene for animals proved also to be a good marker for barcoding Hyalospheniidae (Arcellinida) (Kosakyan et al., 2012, 2013) and several other groups of protists (e.g. Euglyphida, naked lobose amoeabe) (Heger et al., 2011) (Nassonova et al., 2010). In this study we used COI sequence data to assess the phylogenetic relationships within the *Quadrulella symmetrica s.l.* species complex in order to understand what is the range of morphological and molecular variability within this group, to what extent the two are correlated, and to what extent morphological characters can vary under different environmental conditions (phenotypic plasticity), and based on this, to (re)define taxa within this group.

Qudrulella symmetrica was believed to be a single species until the recent study by Kosakyan et al. (2012) revealed unexpected morphological and genetic variability among the five studied populations. The current study confirms that *Q. symmetrica* is indeed a species complex. Our phylogenetic reconstruction separated the studied populations into two clades A and B (Fig. 2). While clade B receives a good support at its node, clade A is less robust. However, the size of the plates is identical in all its representatives in each clade (small test, aperture and plates in clade A; and large tests, aperture, and plates in clade B), corroborating its monophyly (Fig 3, 4). Sequence divergence between these two clades (13%) is much higher than the 4% threshold generally accepted to separate two animal species. Thus, representatives of clade A and B should thus clearly be considered as independent taxa.

Things are less clear within clade B. Members of this clade share the same morphological characters (large test and plate sizes) and are clearly distinct from those in clade A. However sequence divergence among representatives of clade B (4-10%) also equals or exceeds the 4% limit. If we consider clade B as being constituted of four entities, 1) *Q. symmetrica*_CH, 2) the group of *Q. symmetrica*_BG, 3) a group of cells studied in the current study and 4) *Q. symmetrica*_CA, then we can observe that group 2 and 3 are closely related and have 96% sequence similarity; and *Q. symmetrica*_CH and *Q. symmetrica*_CA have only 90-92% similarity between each other and with the other representatives of the clade. A carefully look at morphological data shows that these groups can generally be

separated based on test and plate sizes. However there is also some overlap in morphology and therefore in some cases it is impossible to identify the species based on these criteria. For instance (1) *Q. symmetrica*_CH is the largest in the clade, morphologically very well separated from the rest (L = 87 µm, = 44.5 µm, aperture = 23µm), and the plates are the largest of all observed cells (4-15µm). (2) from the *Q. symmetrica*_BG only one SEM figure was available for morphological observations (L = 75 µm, B = 42 µm, aperture = 20 µm, plates 3-12.5 µm). By the size of the test it is overlapping with group 3, may be sharing slightly bigger plates. The representatives of group 3 have tests L = 71.7-80.7 µm, B =43-46 µm, aperture 21-23 µm, and plates 4-12 µm. (4) *Q. symmetrica* is slightly smaller, but also overlapping with the size of the test and plates with the once in group (2) and (3): L =71.5 µm, B =40 µm, aperture 20 µm, plates 4.5-9 µm. Considering the 4-10 % COI sequence divergence among these groups, we can clearly say that we are dealing with four different species, however additional sampling is needed to understand whether these taxa are cryptic, pseudocryptic or morphologically well-defined species.

Another interesting case is *Q. longicollis*, which was described as a variety of *Q. symmetrica* described by Taranek (1882), and recently was suggested as an independent taxon based on different shape of the test (with elongated neck distinct from typical *Q. symmetrica*, see Fig. 5D) and up to 11 % sequence divergence from other studied *Q. symmetrica* morphospecies (Kosakyan et al., 2012). In the current study, it may be surprising to see it branching in clade A with small sized morphotypes. As mentioned above representatives of clade A have relatively small tests (L = 66-71.5 µm, B = µm, aperture = 18-18.5 µm, plates 3-7.5 µm). *Q. longicollis* has a larger test L = 96 µm, B = 45 µm but has a small aperture (17 µm) and small plates (4-8µm - one 9 µm), and thus falling into morphological limitations of clade A.

From these preliminary results we can conclude that *Q. symmetrica* it is not a single species and will need to be split into at least five independent taxa. The main taxonomic characteristic that define the two main clades is the size of the siliceous plates. The elongated neck and thus larger overall size of the test of *Quadrulella longicollis* either results from phenotypic plasticity or is genetically determined. Further sampling combining ecological data is needed to clarify this.

This project is on-going, and our next step will be the sampling diverse habitats, carefully documenting environmental characteristics (e.g. humidity, hydrochemistry) and possibly biotic aspects (e.g. microbial community composition, vegetation) to determine the relative potential influence of genetic vs. environmental factors on shell morphology in *Quadrulella*.

Acknowledgments

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CHAPTER 4

Environmental DNA COI barcoding for quantitative analysis of protists communities at fine taxonomic level: a test using the *Nebela collaris* species complex (Amoebozoa; Arcellinida; Hyalospheniidae)

Paper to be submitted to Microbial Ecology

Environmental DNA COI barcoding for quantitative analysis of protists communities at fine taxonomic level: a test using the *Nebela collaris* species complex (Amoebozoa; Arcellinida; Hyalospheniidae)

Anush Kosakyan, Matthieu Mulot, Edward A.D. Mitchell and Enrique Lara

Laboratory of Soil Biology, University of Neuchatel, Rue Emile-Argand 11, CH-2000, Switzerland.

Abstract

Environmental DNA (eDNA) surveys are increasingly used for screening eukaryotic diversity. However it is unclear how quantitative this approach is and thus to what extent results from eDNA studies can be used for ecological studies requiring quantitative data. Mitochondrial cytochrome oxydase (COI) is used for species-level taxonomic studies of testate amoebae and should allow assessing the community composition from environmental samples bypassing the biases due to morphological identification. .

We tested this using a COI clone library approach and focusing on the *Nebela collaris s.l.* complex - a group of common, closely-related taxa in *Sphagnum* peatlands.

Comparisons with direct microscopy counts showed that the COI clone library diversity data matched the morphologically identified taxa, and that community composition estimates using the two approaches were similar. However, this correlation was further improved when microscopy counts were corrected for biovolume.

Higher correlation with biovolume-corrected community data suggests that COI clone library data matches the ratio of mitochondria and that within closely-related taxa such as the *Nebela collaris s.l.* complex the density of mitochondria per unit biovolume is approximately constant.

Further developments of this metabarcoding approach including quantifying the mitochondrial density among closely-related taxa, experiments on other taxonomic groups and using high throughput sequencing should make if possible to quantitatively estimate

community composition of different groups of micro-eukaryotes, which would be invaluable for studies of microbial food webs.

Keywords: environmental DNA; metabarcoding; protist diversity; community ecology; testate amoebae; *Sphagnum* peatlands

Running title: environmental DNA metabarcoding for community analysis of soil protists

Introduction

Environmental DNA (eDNA) surveys are revealing a huge unknown diversity of microbial eukaryotes both globally and within individual samples[1-3]. This high diversity in turn suggests the existence of extremely complex, but mostly undocumented ecological interactions. To understand the ecological roles played by species quantitative estimates of their abundance and biomass are required. For soil protozoan groups such as ciliates and testate amoebae, this is currently done using highly time-consuming direct counting of known taxa [6-9] rather than eDNA approaches.

Assessment of environmental micro-eukaryotic diversity is almost exclusively done by sequencing partial or entire ribosomal genes [3]. Besides biases in DNA extraction, PCR and possibly cloning, copy numbers of ribosomal genes in eukaryotes are known to vary over more than four ranges of magnitude, from one in the picoplanctonic *Nannochloropsis salina* to 12000 in the large dinoflagellate *Akashiwo sanguinea* [10]. Such biases, already known and documented in prokaryotes [11], can be expected to be even higher in protists given their larger, more complex and more variable genomes. Thus, in many cases the community structure or biomass evaluation inferred from eDNA studies not always reliable [11-14]. Selecting the optimal barcoding marker is not trivial either; ribosomal genes (SSU and LSU rRNA) are most useful for coarse taxon discrimination, while ITS has been shown to present intra-genomic variation in some groups and is therefore not suited as a barcoding gene for all eukaryotic groups [3].

The analysis of protist communities by light microscopy is time-consuming. Precise identification of taxa is often hindered by poor taxonomy, and the existence of morphologically similar, but genetically distinct species, which may have different ecological niches. As their identification is highly dependent on the observer if possible at all results are difficult to compare among studies. This problem, often referred to as cryptic diversity, is recurrent in eukaryotic micro-organisms [5, 15-18].

An alternative to existing approaches would be to apply molecular methods but to focus on small groups that are genetically and morphologically well-characterised. In such a context, the use of a variable marker is possible and even required to reach a higher level of taxonomic accuracy. Candidate markers have to be tested and validated for DNA barcoding before being applied to environmental DNA samples. A good candidate marker for such an

approach is the mitochondrial cytochrome oxidase subunit 1 (COI) first used for Amoebozoa in species-level taxonomical studies of vannelids [19], and later in Arcellinida [18, 20, 21]. In the aim to overcoming limitations of both microscopy counting and environmental DNA surveys, we developed and tested an eDNA based method, using a cloning-sequencing approach and COI as metabarcoding marker. The method allows to assess the taxonomic diversity within a selected group of closely related protist taxa and to obtain quantitative estimates of community structure from environmental samples. We used as model the *Nebela collaris s.l.* species complex (Amoebozoa; Arcellinida; Hyalospheniidae), a common group of testate amoebae in northern hemisphere *Sphagnum* peatlands and acidic forest humus. We tested the method by comparing the relative abundance of clones vs. known community composition using artificial communities (i.e. of known composition) and environmental samples. However COI is a mitochondrial marker and if we assume that mitochondrial density remains comparable in close-related taxa with similar lifestyle, mitochondria numbers can be expected to increase proportionally with cell size. Quantitative estimates could be biased if the taxa differ markedly in biovolume, as it is indeed the case even within closely related testate amoeba taxa such as the *Nebela collaris* group. We therefore assessed if the accuracy of COI-based quantitative community structure estimates increased with correction for cell biovolume.

Material and methods

Analyses of natural and artificial communities

Testate amoebae were extracted from *Sphagnum* samples collected from four peatlands in the Jura Mountains of Switzerland and France (Table 1). Testate amoeba cells were extracted and concentrated by sieving (150µm) and back sieving (20µm). This material was used for two complementary sets of experiments schematically shown in Figure 1 and detailed hereafter:

For the first experiment the extracted community of each sample was split into two parts and left to settle in centrifuge tubes. One part was used for bulk eDNA extraction of natural communities: Approximately 0.40 g of microbial community extract was collected from the bottom of the centrifuge tube for environmental DNA extraction. The other was used for direct microscopy observation of communities: A total of 100-150 testate amoebae from *N. collaris s.l.* group were identified from each sample.

For the second experiment, individual species were isolated one at a time with a narrow diameter pipette under an inverted microscope from the same set of samples to build artificial communities. Each cell was documented carefully for species identification, washed several times with distilled water and deposited in an eppendorf tube according to pre-defined community composition (Supplementary Table 2). Two contrasted artificial communities were created (namely Art-A and Art-B). DNA was then extracted from each of these samples.

DNA extraction, amplification and cloning

Power Soil DNA isolation kit (MO BIO) was used to extract the environmental DNA from both environmental and artificial communities following the manufacturer's instructions. Partial COI PCR products were obtained using the wide-spectrum primer LCO [22] in combination with a specific primer TINCOX (CCATTCKATAHCCHGGAAATTTC) designed to amplify only *Nebela collaris s.l.* species [20]. 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 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 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. Cloned inserts were amplified with vector M13F and M13R primers. PCR products 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).

COI sequences are deposited in GenBank with the following accession numbers XX-XX (nb. accession numbers will be added after paper acceptance).

Phylogenetic analyses

The data set used for phylogenetic analyses (300–665 bp) comprised 201 COI new environmental sequences that were analysed together with 31 COI *Nebela collaris s.l.* sequences taken from Kosakyan et al. [20] sequence data. The sequences were aligned manually. 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 [23] 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 the 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 [18]. Bayesian Markov Chain Monte Carlo analyses were performed using MrBayes v3.1 [24] 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 1000 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 [24]. The tree with the best likelihood score was saved every 10 generations, resulting in 100,000 trees. The burn in value was set to 25%. Trees were viewed using FigTree (program distributed as part of the BEAST package http://tree.bio.ed.ac.uk/software/figtree/).

Comparison of morphological and molecular data

We compared community composition as estimated by light microscopy to the proportion of clones recovered. Predicting that the number of mitochondria in a given cell (and, therefore, the number of COI gene copies) will be directly proportional to the biovolume of the amoeba, we compared both (1) raw proportions of clones and (2) proportions of clones as normalised by the ratio between the largest biovolume (namely *N. collaris s.str.*) and the biovolume of the given cell after the following equation:

The biovolume was calculated according to [25] using the following equation:

$$B_x = \frac{2}{3} \times L \times W \times D \quad (1)$$

where:

B_x = biovolume of species x [μm^3]

L = length of the shell [µm]

W = width of the shell [µm]

D = depth of the shell [µm]

In order to calculate **corrected clone number of given species in the community**, we used the following equation:

$$C_{x\_corr} = \frac{B_{x(max)}}{B_x} \times C_x \quad (2)$$

where

C_{x_corr} = corrected clone number of species x in the community

$B_{x(max)}$ = biovolume of the largest species in the community (e.g. *Nebela collaris s.str.*) [µm^3]

B_x = biovolume of species x [µm^3]

C_x = number of clones of species x in the community (from clone library data)

Note: We have used data on corrected relative abundance [%] of species x in the community calculated from the data on corrected clone numbers (C_{x_corr}) to build our biplots.

Statistical analyses

Similarity in communities structure estimated by each method was explored using standard Pearson correlation test, in addition with linear regression of the relative abundances of each species obtained by microscopic count (x), against their relative abundances obtained by either molecular count or molecular count corrected with biovolume (y). The regression formula being $y = a.x + b$, if two communities are identical $a=1$ and $b=0$.

Results

Most of the obtained clones were affiliated to known species of *Nebela collaris s.l.*, as defined by Kosakyan et al. [18], with the exception of two groups of clones which in all likelihood form two independent taxonomic units (*Nebela* unknown *sp*.1 and *N.* unknown *sp*. 2) respectively sister to *N. guttata* and basal to both *N. tincta* and *N. guttata*. Both of these clades are fully supported (100 B and 1.00 PP; Figure 2) and were detected in low numbers only in the environmental surveys.

Microscopy counts were significantly and strongly correlated (using paired Pearson correlation tests) to both raw and biovolume-corrected proportions of clones (r=0.844 and r=0.974, respectively, n=5, $p<0.05$ in both cases) in the two artificial communities (Supplementary Tables 1 and 2, Figure 3). A similar increase was observed in environmental sample 3 (r=0.900 and r=0.962 respectively before and after correction with biovolume, n=7, $p<0.05$), and to a lesser degree in sample 2 (r=0.831 and 0.852, $p<0.05$, n=7) where an unknown species was found (*N. sp*.1). In sample 4 we retrieved only *Nebela collaris* in the clone library in agreement with microscopic observations (Supplementary Table 3, Figure 4). The correlation decreased in sample 1 (r=0.964 and 0.902, respectively before and after correction by biovolume, $p<0.05$ and n=7), where a second unknown species was found (*N. sp*. 2).

Assuming 1) that *N. sp*. 1 & 2 were cryptic species that were morphologically undistinguishable from some other species in the sample, and 2) that they looked exactly like the species that showed the largest deviation to counted numbers proportions, we normalized their biovolume accordingly (i.e. respectively *N. collaris* in S_1 for *N.* sp. 2 and *N. guttata* in S_2 for *N.* sp. 1). The resulting correlation increased for the overall dataset (Figure 5).

Discussion

Community ecology requires reliable identification of species as well as an accurate assessment of the relative abundance of each species. Most communities are constituted of species differing in life history traits such as sizes and reproduction rates. An optimal method to assess community composition should therefore allow the precise identification of all taxa

as well as of the relative biomass of each. The COI-based metabarcoding approach we tested on the *Nebela collaris s.l.* group shows that this method is promising. Other traits can only be included in analyses if enough is known about the organisms.

In this study our aim was to develop a method that allowed a quantitative screening of environmental communities at species level using a molecular approach. This is, to our knowledge, the first time that a metabarcoding approach that is not based on ribosomal genes is applied to a group of heterotrophic protists. The advantage of the COI-based metabarcoding approach is to provide a much finer level of taxonomic resolution than that the SSU rRNA gene usually used in eDNA studies. COI has also been shown to give high taxonomic resolution in many microbial eukaryotic groups [4, 17, 19, 26, 27]. For example, in the Hyalospheniidae, SSU rRNA does not allow to discriminating among closely related taxa [28] while COI does [18] [20]. The COI-based metabarcoding approach allows discrimination among taxa that are generally interpreted as biological species, for Metazoa [29-30] (but see [31-32]) and our recent work on Hyalospheniidae also supports this view [20-21].

Thus the first advantage of this approach is to overcome taxonomic limitation of morphology-based analyses and allow fine-level ecological studies regardless of the current state of taxonomy for individual groups. Indeed this approach can be used in the *Nebela collaris s.l.* group as well as other groups for which a representative amount of COI sequence data is available, even if the total diversity of the group is currently unknown. In this latter case this environmental metabarcoding approach is even the only available option to assess the ecological significance of hidden diversity. The discovery of two new phylotypes of species level shows that the full taxonomic diversity within the *Nebela collaris* group is still not known. This is not surprising given recent results of phylogenetic studies on this group. For example the existence of closely related but distinct species that have geographically limited distributions has recently been demonstrated in hyalospheniids for the *Hyalosphenia papilio* species complex [21]. The application of our approach in other areas of the world will most likely allow the discovery of more unknown taxa. This could be achieved relatively easily using the eDNA-metabarcoding approach, either using cloning-sequencing as done here or using high throughput sequencing.

A second advantage of this method is that it allows the retrieval of reliable quantitative data, and our data suggests that raw clone proportions provide an accurate estimate of the

relative biovolume of each species in a community, even for cryptic or unknown species whose biovolume is unknown. Although this may be seen as a bias if the goal is to estimate proportions of individuals it may actually be an advantage for community ecology studies. Community ecology studies of testate amoebae are indeed increasingly based on biovolume-corrected community data obtained by direct microscopy [33]. The rationale behind this approach is that biovolume-corrected community composition informs better on the true ecological impact of the species. As our results suggest that clone-library analysis directly provides community estimates that closely match a biovolume-corrected community count we thus believe that the clone-library results could be used for ecological analysis without any need for further corrections by biovolume.

The next steps will be to use this approach to determine the full diversity of protist communities in the environment and the degree to which closely related taxa differ in their ecological niche, and functional roles and to assess biotic interactions (e.g. competition). This study represents a first step towards these broader goals, taking as a model a particular group of hyalospheniid testate amoebae, *N. collaris sensu lato.* Obviously, the approach presented in this work can at first only be applied to small groups of reasonably well-known protist species. Species must have been barcoded and their morphologies must have been properly documented. When new (i.e. non-barcoded) species appear within an otherwise well documented group the clone numbers may be used to estimate relative biovolume, assuming that the density of mitochondria per unit volume is approximately constant. This approach can only be applied if members of the studied group share a similar lifestyle (e.g. aerobic) otherwise mitochondrial density might change and ideally the assumption of constant mitochondrial density should be tested. Nevertheless, we believe that this approach can easily be developed for other well-documented groups of testate amoebae, as well as other protists such as ciliates, diatoms, haptophytes etc., thus bringing an invaluable tool for answering many fundamental questions on the ecology of protist communities and revealing their true diversity.

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Figures and Tables

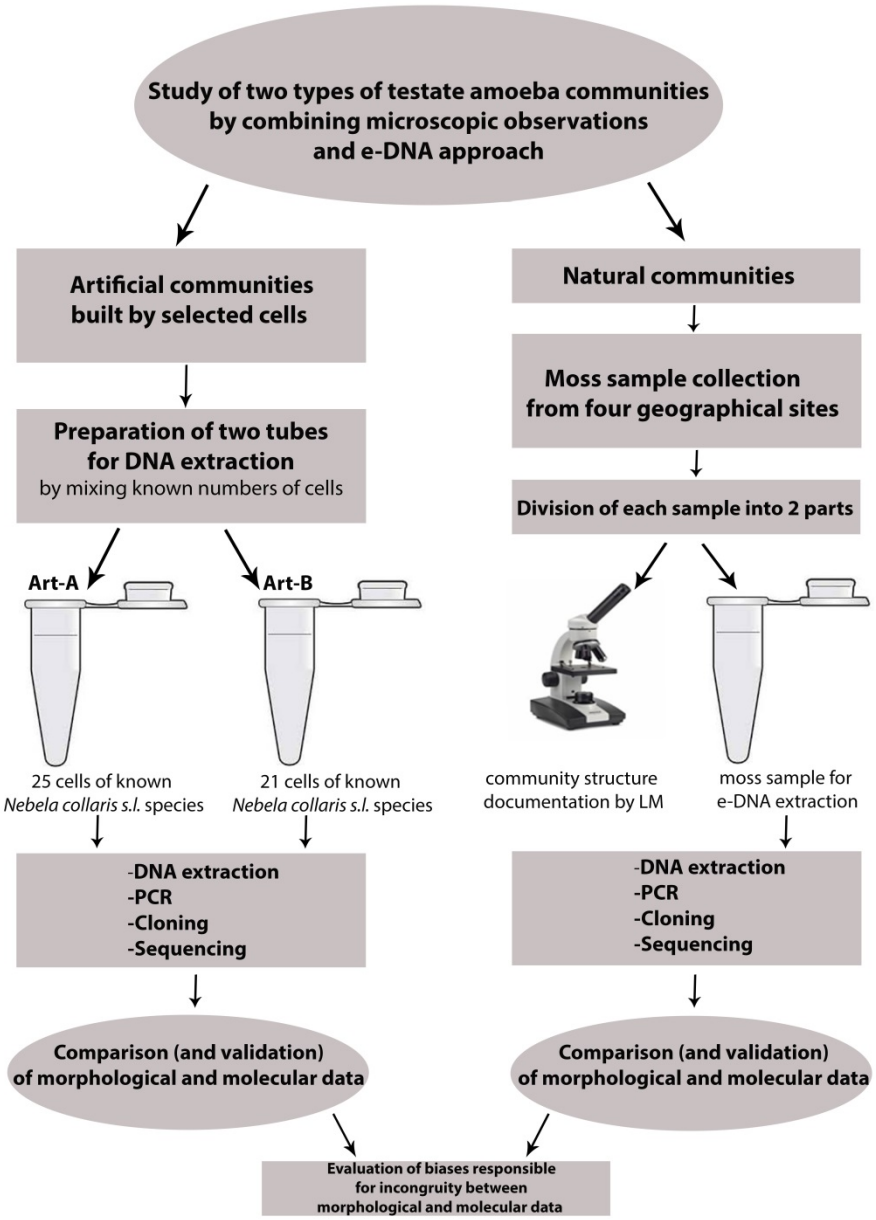

Figure 1. The schematic flow of experiment described in "Material and Methods" part.

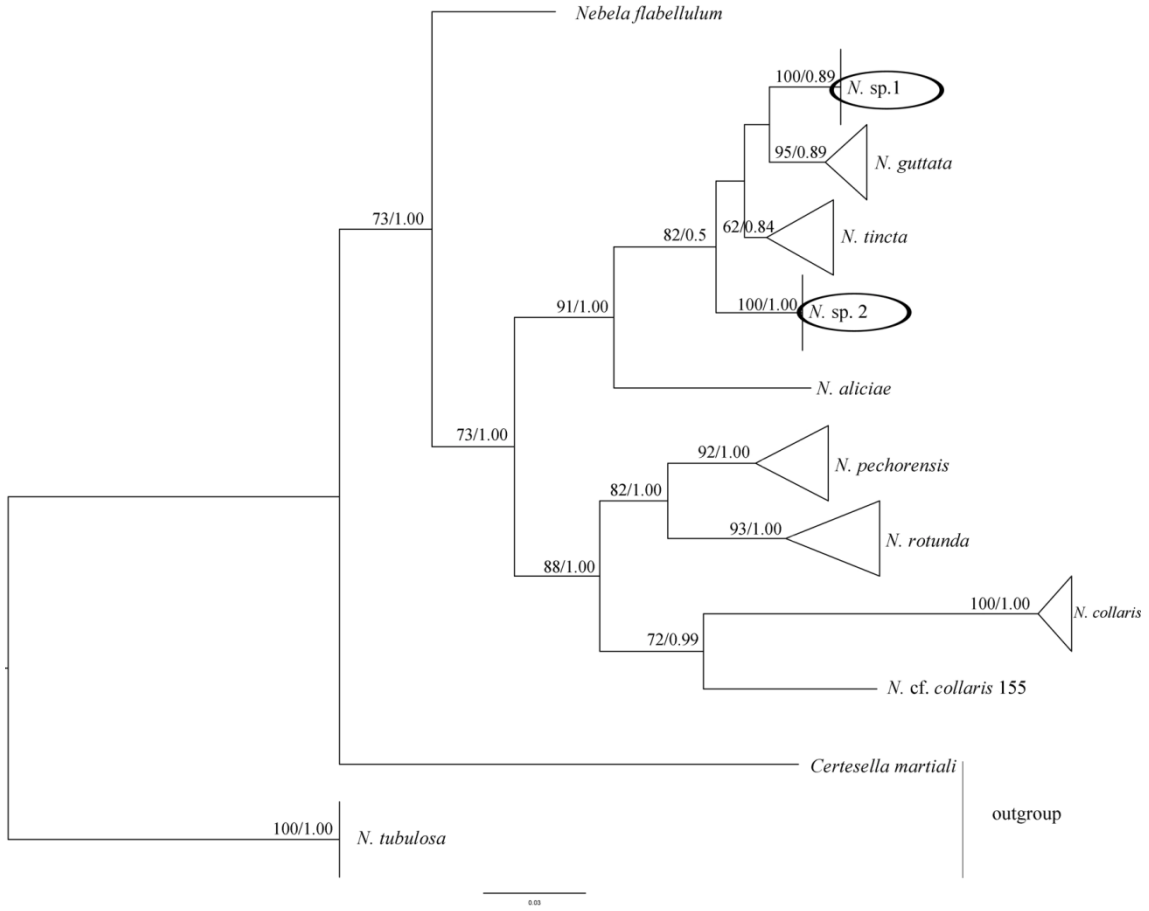

Figure 2. Maximum likelihood bootstrap consensus tree including 31 sequences of *N. collaris s.l.* derived from single cell PCR and adding 201 environmental mtCOI sequences based on a 665-nucleotide alignment. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated with Bayesian analyses. Values under 50/0.50 are not shown.

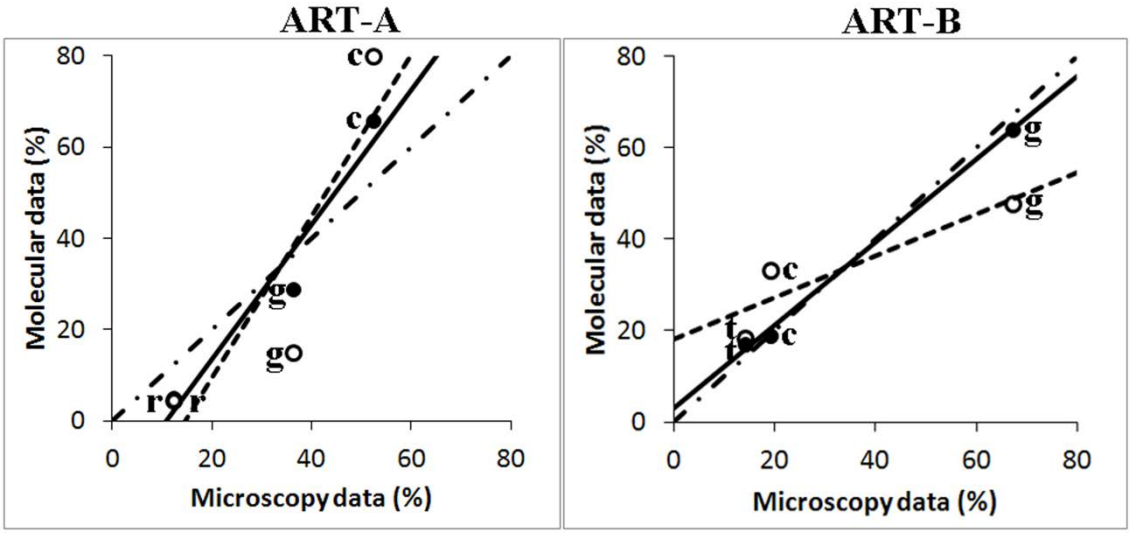

Figure 3. Biplots of relative abundance of *Nebela collaris* s.l. taxa as assessed by direct microscopy observation vs. cloning and sequencing of the COI gene from two artificial communities before and after correction with biovolume. Semi dashed lines indicates the 1:1 slope corresponding to a perfect correlation. Dashed lines and full lines indicate actual correlation before (dashed) and after (full) biovolume correction. Species names are indicated as follows: c: *Nebela collaris*, g: *N. guttata*, r: *N. rotunda*, and t: *N. tincta.* Open and full symbols correspond to values before and after biovolume correction, respectively.

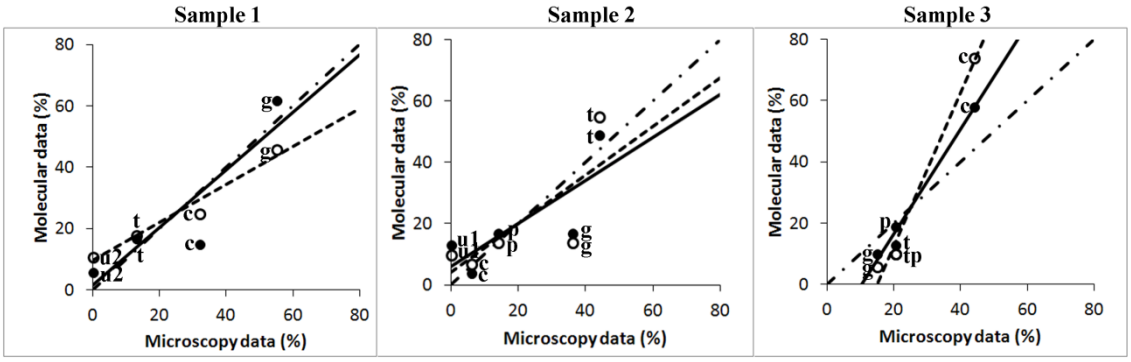

Figure 4. Biplots of relative abundance of *Nebela collaris* s.l. taxa as assessed by direct microscopy observation vs. cloning and sequencing of the COI gene from natural communities before and after correction with biovolume. Semi dashed lines indicates the 1:1 slope corresponding to a perfect correlation. Dashed lines and full lines indicate actual correlation before (dashed) and after (full) biovolume correction. Species names are indicated as follows: c: *Nebela collaris*, g: *N. guttata*, r: *N. rotunda*, and t: *N. tincta*, u1: unknown species 1, u2: unknown species 2. Open and full symbols correspond to values before and after biovolume correction, respectively.

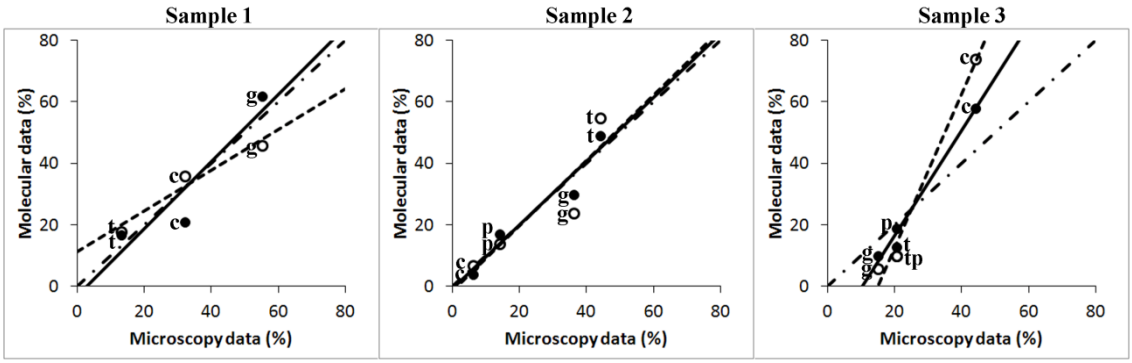

Figure 5. Same data as in Figure 3 but with unknown species 1 and 2 referred to *Nebela guttata* and *N. collaris*, respectively.

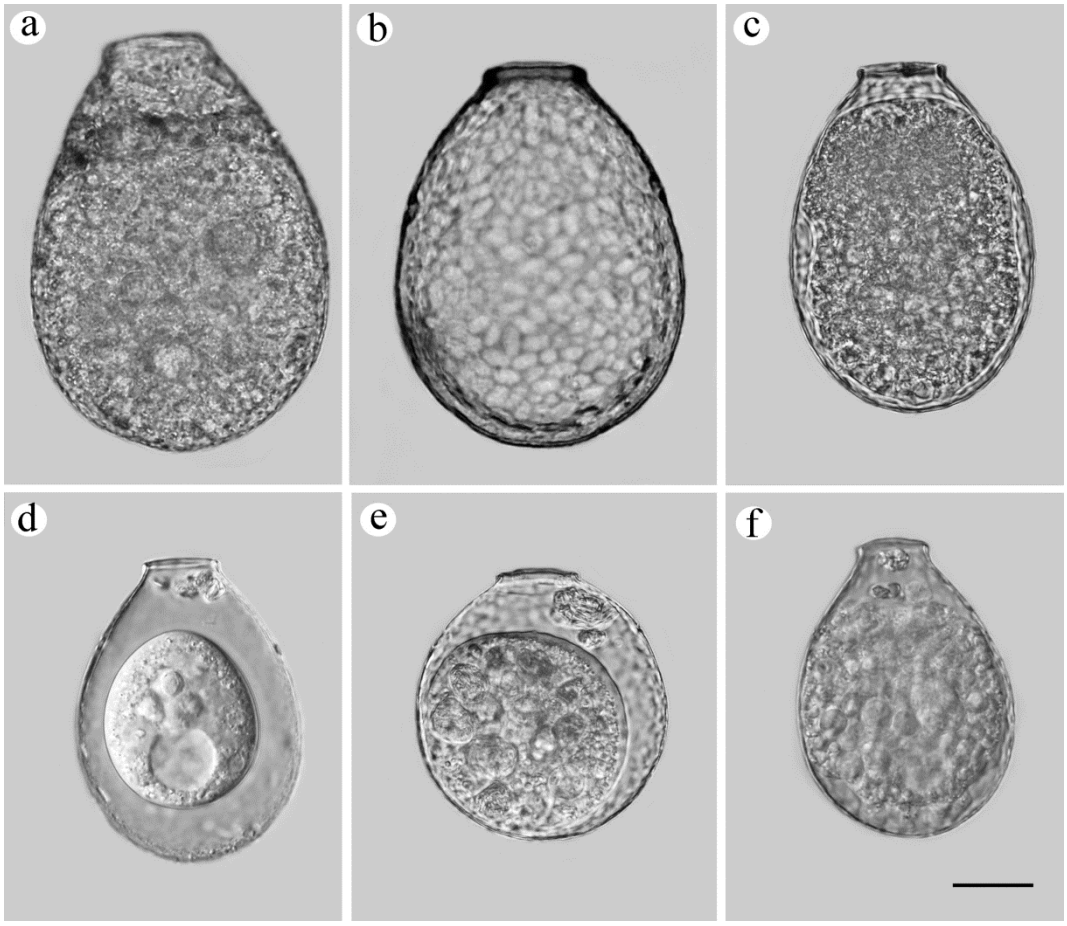

Figure 6. Light microscopy images of the six taxa of the *Nebela collaris s.l.* group included in this study, a: *Nebela collaris*, b: *N. aliciae* (modified from [18]), c: *N. tincta*, d: *N. pechorensis*, e: *N. rotunda*, and f: *N. guttata*.

Table 1. Sampling locations for environmental communities

Sample name	Location	Country	Coordinates	Habitat
Sample 1	Les Pontins	Switzerland	47°01'27.37" N; 6°98'96" E	Poor fen, *Sphagnum* mosses
Sample 2	Le Cachot	Switzerland	47°00'20.93" N; 6°39'52.78" E	Center of peatland with *Eriophorum vaginatum*
Sample 3	Le Russey	France	47°10'128" N; 6°46'263" E	Poor fen, bog margin, *Sphagnum fallax, Eriophorum vaginatum*
Sample 4	Trédudon	France	48°24'44.13" N; 3°50'33.21" W	Hummock in rich fen, *Sphagnum palustre*

Supplementary Table 1. Regression parameters according to the formula y=ax+b of the relationship between counted (microscopy) and COI clone library data.

Artificial communities		**a**	**b**	**p**	**R^2**	**adj R^2**
ART-A	After corr. biovol.	1.18	-3.66	<0,01	0.93	0.91
	Before corr. biovol.	1.29	-5.75	0.053	0.76	0.69
ART-B	After corr. biovol.	0.95	1.04	<0,01	1.00	1.00
	Before corr. biovol.	0.69	6.14	0.321	0.83	0.77
Natural communities		**a**	**b**	**p**	**R^2**	**adj R^2**
Sample 1	After corr. biovol.	0.96	0.55	<0,01	0.87	0.84
	Before corr. biovol.	0.77	3.35	<0,01	0.93	0.92
Sample 2	After corr. biovol.	0.79	3.05	0.015	0.73	0.67
	Before corr. biovol.	0.85	2.12	0.021	0.69	0.63
Sample 3	After corr. biovol.	1.23	-3.24	<0,00	0.92	0.91
	Before corr. biovol.	1.48	-6.85	<0,01	0.80	0.76
Natural communities after refering unknown species 1 &2 to *Nebela guttata* and *N. collaris*						
		a	**b**	**p**	**R^2**	**adj R^2**
Sample 1	After corr. biovol.	1.04	-0.77	<0,01	0.93	0.91
	Before corr. biovol.	0.86	2.72	<0,01	0.95	0.94
Sample 2	After corr. biovol.	1.02	-0.46	<0,01	0.95	0.94
	Before corr. biovol.	1.04	-0.79	0.024	0.86	0.81
Sample 3	After corr. biovol.	1.36	-7.12	<0,01	0.92	0.90
	Before corr. biovol.	1.75	-15.07	0.032	0.83	0.77

Supplementary Table 2. Relative proportions of *Nebela collaris* s.l. taxa in two artificial communities as assessed by clone library without (raw clones) and with (corr. clones) correction for biovolume and in microscopy counts.

Taxon		Artif-A (66 / 25) *	Artif-B (27 / 21)
Nebela collaris	Raw clones	80% (53) °	33.5% (9)
	Corr. clones	66% (53)	19% (9)
	Microscopy	52% (13)	19% (4)
N. guttata	Raw clones	15% (10)	48% (13)
	Corr. clones	29% (23)	64% (29.9)
	Microscopy	36% (9)	67% (14)
N. tincta	Raw clones	0% (0)	18.5% (5)
	Corr. clones	0% (0)	17% (8)
	Microscopy	0% (0)	14% (3)
N. rotunda	Raw clones	5% (3)	0% (0)
	Corr. clones	5% (4,2)	0% (0)
	Microscopy	12% (3)	0% (0)

* For each sample: number of clones sequenced / number of individuals counted by microscopy.

° Data are given as a relative percentage of the total community and absolute numbers (in brackets). Correction is based on *N. collaris* biovolume. The absolute numbers do not change but percentages do.

Supplementary Table 3. Relative proportions of *Nebela collaris* s.l. taxa in four natural communities as assessed by clone library without (raw clones) and with (corr. clones) correction for biovolume and in microscopy counts.

Taxon		Sample1 (28 / 75) *	Sample 2 (29 / 141)	Sample 3 (19 / 122)	Sample 4 (32 / 51)
Nebela collaris	Raw clones	25% (7) °	7% (2)	74% (14)	100% (32)
	Corr. clones	15% (7)	4% (2)	58% (14)	100% (32)
	Microscopy	32% (24)	6% (8)	44% (54)	100% (51)
N. pechorensis	Raw clones	0% (0)	14% (4)	10% (2)	0% (0)
	Corr. clones	0% (0)	17% (9.2)	19% (4.6)	0% (0)
	Microscopy	0% (0)	14% (20)	20.5% (25)	0% (0)
N. guttata	Raw clones	46% (13)	14% (4)	6% (1)	0% (0)
	Corr. clones	62% (30)	17% (9.2)	10% (2.3)	0% (0)
	Microscopy	55% (41)	36% (51)	15% (18)	0% (0)
N. tincta	Raw clones	18% (5)	55% (16)	10% (2)	0% (0)
	Corr. clones	17% (8)	49% (25.6)	13% (3.2)	0% (0)
	Microscopy	13% (10)	44% (62)	20.5% (25)	0% (0)
N. rotunda	Raw clones	0% (0)	0% (0)	0% (0)	0% (0)
	Corr. clones	0% (0)	0% (0)	0% (0)	0% (0)
	Microscopy	0% (0)	0% (0)	0% (0)	0% (0)
Unknown sp.1	Raw clones	0% (0)	10% (3)	0% (0)	0% (0)
	Corr. clones	0% (0)	13% (7)	0% (0)	0% (0)
	Microscopy	0% (0)	0% (0)	0% (0)	0% (0)
Unknown sp.2	Raw clones	11% (3)	0% (0)	0% (0)	0% (0)
	Corr. clones	6% (7)	0% (0)	0% (0)	0% (0)
	Microscopy	0% (0)	0% (0)	0% (0)	0% (0)

* For each sample: number of clones sequenced / number of individuals counted by microscopy.

° Data are given as a relative percentage of the total community and absolute numbers (in brackets). Correction is based on *N. collaris* biovolume. The absolute numbers do not change but percentages do.

Chapter 5

This chapter presents a monograph in preparation, which is devoted to the family Hyalospheniidae. The manuscript will be submitted to **Protozoological Monographs.** The finalizing date is estimated August 2014.

Family Hyalospheniidae sensu Kosakyan et Lara

University of Neuchâtel, Institute of Biology

Switzerland, 2014- XX pp.

Edited by XXX

This book is devoted to taxonomy of Hyalospheniid testate amoebae (Amoebozoa, Arcellinida). In total, 102 species and infraspecific taxa belonging to 8 genera are outlined. The book includes taxonomic keys, improved descriptions of each taxa, ecological, geographical and taxonomical remarks, an annotated list of doubtful species, a list of possible synonymous names, notes to existing molecular data, and detailed plates containing light microscopy and scanning electron microscopy pictures and original drawings for each taxa. The book is designed to be of benefit to protistologists and taxonomists in order to make Hyalospheniid testate amoeba species identification easier and more accurate. It should also be a useful basis for future taxonomic work on these organisms.

A. Kosakyan[1], E. Lara[1], R. Meisterfeld[2] & E.A.D. Mitchell[1]

[1] Institute of Biology, University of Neuchâtel, Emile-Argand 11, 2000 Neuchâtel, Switzerland.

[2] Institute for Biology II (Zoology), Unit for cellular Neurobionics,RWTH Aachen University, Helmertweg 3, 52056, Aachen, Germany

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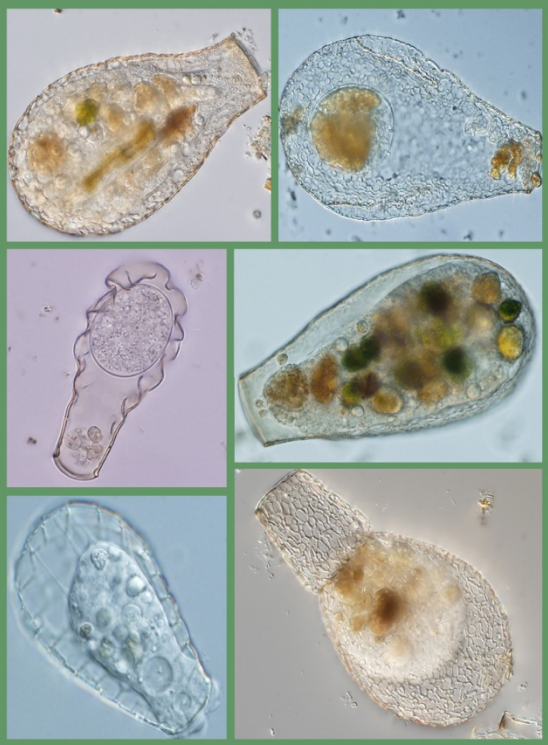
PROTOZOOLOGICAL MONOGRAPHS

Anush KOSAKYAN, Enrique LARA, Ralf MEISTERFELD, and Edward MITCHELL

FAMILY HYALOSHENIIDAE

Edited by xx



SHAKER VERLAG

CONTENTS

Introduction

Estimation of global biodiversity is an old but still unresolved question in biology. The biodiversity of protists has and still is not well understood and often neglected. The recent accumulation of new data based on modern morphological and molecular tools has clearly shown how incomplete is our knowledge about protist diversity, and how urgent is the need of taxonomic revision of many taxa. Species identification is especially challenging for groups for which taxonomy is based only on morphological data. These data are often limited, the original publications difficult to find and/or published in different languages. However, recent studied combining molecular and morphological approaches are now sheding new light on the true diversity of many groups. Hyalospheniid testate amoebae are a typical example of this and we believe they constitute a useful representeative example illustrating the biodiversity of free-living protists.

Family Hyalospheniidae (Arcellinida, Amoebozoa) includes some of the most remarkable, common and well-studied species of testeate amoebae. Despite their wide use in palaoecological studies and environmental monitoring, current knowledge about their diversity, biology, taxonomy and systematics remain very limited. For many groups the existing data is partly unreliable, incoherent and published in different languages, making it difficult for most researchers to access the information. Many names are *nomen nudum*, synonymous or doubtful. There is no recent monograph (based on molecular and morphological data) or illustrated guides comprising at least the most common species of this group. The last review of the family was published over 10 years ago Mesiterfeld (2002) and the last comprehensive reviews of the main genera date from the early to mid 20th century (e.g. Deflandre 1936, Grospietsch 1965).

Sound taxonomy is a prerequisite for the use of groups of organisms in ecological research and a necessity for inter-study comparisons. With this monograph we aim to start a series of books revising the larger groups of testate amoebae. Our goal here is to provide an up to date treatment of the taxonomy of family Hyalospheniidae (Amoebozoa, Arcellinida) combining all known data about the phylogeny, taxonomy and systematic of these organisms. The book is designed to be of benefit to protistologists and taxonomists and will hopefully

stimulate similar efforts in other groups to make testate amoeba species identification easier and more accurate.

The preparation of this book was made possible by the close cooperation and help of several colleagues. Therefore, we would like to express our gratitude to the xxxx **acknowledgments will be here.**

Family Hyalospheniidae: historical survey

The classification of Hyalospheniid testate amoebae has varied over time depending on which morphological trait has been considered as taxonomically most relevant.

Leidy (1874) probably was the first who noticed the common characters between the cells having a vase shaped test composed of "*discoid plates and minute rods, apparently siliceous and intrinsic to the structure of the animal*". He grouped species with these characters within genus *Nebela*, separating them from genus *Difflugia* Leclerc, 1815 - "*those rhizopods with lobose pseudopods, which ordinarily possess a covering or test composed of extraneous bodies, such as particles of quartzose sand, and diatom cases*" (Leidy 1874).

Schultze (1877) defined families Arcellidae, Difflugidae, Hyalospheniidae, and Quadrulidae. He replaced the genera with organic homogenous test such as *Hyalosphenia* Stein, 1859 into family Hyalospheniidae, genus *Nebela* Leidy, 1874 into Difflugidae and genera with quadratic plates, such as *Quadrula* Schultze, 1875 (*Quadrulella* Cockerell, 1909) into Quadrulidae.

Based on the presence of siliceous plates Taranek first defined family Nebelidae in 1882 by unifying genera *Nebela*, *Lesquereusia* Schlumberger, 1845, *Corythion* Taranek, 1881 and *Quadrula* (*Quadrulella*). Later on genus *Corythion* was excluded and the following genera were included: *Amphizonella* Greef, *Cochlipodium* Hertw. et Lesser, *Hyalosphenia* Stain 1857, *Leptoclamys* G.S. West, *Zonomyxa* Nusslin (Taranek 1882).

In 1942 Jung redefined family Nebelidae and organized it into 13 genera: *Alocodera*, *Apodera*, *Argynnia*, *Deflandria*, *Nebela*, *Leidyella*, *Physochila*, *Porosia*, *Pterygia*, *Penardiella*, *Quadrulella*, *Schaudinnia* and *Umbonaria* (Jung 1942). Unfortunately Jung's classification lacked type designations. All genera containing more than one species and lacking type designation are therefore not valid (ICZN article 13.3). From all genera only *Alocodera*, *Physochila* and *Porosia* has been recognized and all other species were remained within genus *Nebela*. Loeblich and Tappan (1961) validated *Apodera* and *Certesella*, and Vucetich (1974) *Argynnia.*

The last review of the family by Meisterfeld (2002) was based only on morphological data. He placed *Nebela* and closely related taxa into two families: taxa with rigid, chitinoid, organic and non-areolar test (namely *Hyalosphenia* and *Leptochlamys* West, 1901) were grouped in the Hyalospheniidae and genera with tests composed of plates of small euglyphids or diatom fragments (*Apodera* Loeblich and Tappan, 1961, *Argynnia* Vucetich, 1974, *Certesella* Loeblich and Tappan, 1961, *Nebela*, *Physochila* Jung, 1942, *Porosia* Jung, 1942, *Schoenbornia* Decloitre, 1964) were grouped in the Nebelidae. Meisterfeld did not include genus *Quadrullela* Cockerell, 1909 into family Nebelidae, but accepted Ogden's (1979) classification, who placed the genus *Quadrullela* Cockerell, 1909 into the Lesquereusiidae Jung, 1942 with other taxa building shells from endogenous (self-secreted) siliceous elements (rod-like, nail-shaped or rectangular) to which mineral particles may be added (in the case of *Netzelia* Ogden, 1979).

The first studies of family Hyalospheniidae using molecular data (based on ribosomal small subunit r SSU sequences) were done by Nikolaev et al. (2005) and Lara et al. (2008), but included a small number of hyalospheniid species. According to these studies the Nebelidae sensu Meisterfeld (2002) was paraphyletic as *Argynnia dentistoma* Penard 1890 appeared only distantly related to members of genus *Nebela* Leidy, 1874. In addition, members of genera *Apodera* (Jung 1942**)** Loeblich & Tappan 1961, *Hyalosphenia* Stein, 1859, *Nebela* Leidy 1874 and *Porosia* Jung, 1942, were intermingled in a robust clade informally called "core Nebelas". Unfortunately this study could not clearly show the phylogenetic relationship among members of the "core Nebelas" and other closely related taxa, partly because of under-sampling and partly because these close-related species could hardly be discriminated based on the SSU rRNA gene. Another study based on SSU gene including several common hyalospheniids (*Hyalosphenia elegans*, *H. papilio*, *Nebela carinata*, *N. flabellulum*, *N. militaris*, *N. tincta*, *N. tubulosa*, and *Quadrulella symmetrica*) was recently published by Oliverio et al. (2014). The results of this study quite puzzlingly suggested that genera *Hyalosphenia* and *Nebela* and the morphospecies *H. papilio* and *H. elegans* were not monphyletic. A few molecular based studies have focused on individual Hyalospheniid testate amoeba genera or species complexes: *Nebela collaris s.l.* group (Kosakyan et al 2013), *Hyalospehia papilio s.l.* group (Heger et al 2014), *Quadrulella symmetrica s.l* group (Kosakyan et al. in prep.). The most comprehensive study including the

most common species from these groups was done by Kosakyan et al. (2012), based on the mitochondrial cythochrom oxydase subunit 1 (COI) gene, which is more variable than the SSU rRNA gene. This study showed that 1) genus *Nebela* is paraphyletic and can be split into two sub-clades: i) pear shaped *Nebela*s (i.e. *N. carinata*, *N. tubulosa*, *N. collaris*, etc.) and ii) newly defined genus *Padaungiella* Lara et Todorov combining the "long necked" *Nebela* species (i.e. *Padaungiella* (*Nebela*) *lageniformis*, *P. wailesi*, *P. tubulata*, etc.). (2) Genus *Quadrulella*, one of the few arcellinid genera building its shell from self-secreted siliceous elements, and the mixotrophic *Hyalosphenia papilio* branch within the *Nebela* group in agreement with i) the general morphology of their shell shape rather than its composition and ii) the presence of an organic rim around the aperture (synapomorphy for Hyalospheniidae). Families Hyalospheniidae and Nebelidae were described respectively by Schultze in 1877 and Taranek in 1882. Acoording to the latest revision of the these two families (Meisterfeld 2002) the Nebelidae included genera with test composed of collected or predated round or oval siliceous plates, fragments of diatoms or mineral grains: *Alocodera*, *Apodera*, *Argynnia*, *Certesella*, *Geamphorella*, *Jungia*, *Nebela*, *Physochila*, *Pseudonebela*, *Porosia*, and *Schoenbornia* (remark: Meisterfeld considered genera *Geamphorella, Jungia, Pseudonebela* as *incertae sedis* and *Schoenbornia* as *sedis mutabilis* even though he include them in the key of the family) and the Hyalospheniidae included genera with chitinoid, clear, completely organic, non-areolar test: *Hyalosphenia* and *Leptochlamys*. Given the fact that genus *Hyalosphenia* clearly branches within the "core Nebelas" clade and that the distinguishing character of Hyalospheniidae (shell transparent and entirely secreted) can also be observed in some Nebelidae (*Alocodera*, *N. tincta*) the two families were synonymised. The name Hyalospheniidae Schultze 1877 took precedence according to the principle of priority (article 23 of the international code of zoological nomenclature, cited. from Kosakyan et al. 2012).

Thus the emended diagnosis of family Hyalospheniidae Schulze, 1877 emend. Kosakyan et Lara is: *"The test is rigid, colorless or yellowish-brown, flask-vase shaped, oval or pyriform, dorso-ventrally compressed. The shell is either entirely self-secreted (e.g. Hyalosphenia) composed of an organic matrix, or with addition of self-secreted siliceous plates (Quadrulella) or recycled shell plates of small euglyphids or other similar material such as diatom frustules incorporated in the test. The pseudostome is terminal and is bordered by a thin organic collar"*. *Physochila* and *Argynnia* do not form a monophyletic

clade with the Hyalospheniidae based on molecular phylogenetic data (Lara et al. 2008; Gomaa et al. 2012), and also differ from other Nebelidae by their morphology, hence are excluded from the Hyalospheniidae and are Arcellinida *incertae sedis*. Similarly, *Leptochlamys* differs from all Hyalospheniidae by a unique combination of characters: shell circular in cross-section, round pseudostome, and unique hyaline pseudopod (Cash and Hopkinson 1909) and is now deemed Arcellinida *incertae sedis*. As a consequence, family Lesquereusiidae now includes *Lesquereusia, Netzelia, Microquadrula* and *Pomoriella* (cited from Kosakyan et al. 2012).

Taxonomic composition of the family

Based on a critical investigation of the existring literature we compiled a total of 222 Hyalospheniid names. Of these, we consider 96 as synonymous, 16 as inquirenda (doubtful), 8 as nomen nudum, and the remaining 102 as valid names. Here is important to note that all the names of the species and infraspecific taxa are used as in the original papers, without any nomenclature rule considerations. In the final verson of the monograph all the names will be presented according to a nomenclature code.

Currently the family is composed of 8 genera and 102 species and infraspecific taxa. Several species (we have called them as *'problematic species'*) within valid names require additional morphological and/or molecular data to fully confirm their validity and position within the group. The list of Hyalospheniid genera, species and infraspecific taxa names that we consider as valid is:

Genus *Alocodera* (1 species)

- *A. cockaynii* Penard, 1910

Genus *Apodera* (4 species and infraspecific taxa)

-*A. crenata* Jung 1942
-*A. vas* Certes 1889
-*A. vas*. f. *reticollaris* Jung 1942
-*A. (Nebela) wellingtonia* Decloitre 1964

Genus *Certesella* (4 species)

-*C. australis* Vucetich 1973
-*C. certesi* Penard, 1911
-*C. martiali* Certes, 1889
-*C. murrayi* Wailes, 1913

Genus *Hyalosphenia* (20 species and infraspecific taxa)

-*H. angulata* Schouteden 1905
-*H. cuneata* Stein, 1857
-*H. elegans* Leidy, 1879
-*H. elegans* var. *cylindricolis* Chardez, 1962
-*H. gigantea* de Graaf, 1952
-*H. humicola* Decloitre 1973
-*H. inconspicua* West, 1903
-*H. jirovichi* Štěpánek 1953
-*H. lucerna* Štěpánek 1967
-*H. minuta* Cash, 1891
-*H. ovalis* Wailes, 1912
-*H. papilio* Leidy, 1879
-*H. papilio* var. *stenostoma* Deflandre 1931
-*H. penardi* Lauterborn, 1908
-*H. platystoma* West, 1903
-*H. punctata* Penard 1891
-*H. shoutedeni* van Oye 1926
-*H. shoutedeni* var.*rotundata* van Oye 1926
-*H. subflava* Cash, 1909
-*H. tamdaoensis* Balik 1995

Genus *Nebela* (38 species and infraspecific taxa)

-*N. acolla* Cash, 1909
-*N. aliciae* Mitchell et Lara 2012
-*N. ansata* Leidy, 1879
-*N. carinata* (Archer, 1867) Leidy, 1879
-*N. carinata* var. *acarinata* Jung 1942
-*N. carinatella* Bayens et Chardez, 1982
-*N. carinulata* Jung 1942
-*N. collaris* (Ehrenberg, 1848) Leidy, 1879
-*N. cylindrica* Bonnet 1979
-*N. d'ydevallei* Van Oye 1953
-*N. fagni* Chardez 1957
-*N. flabellulum* Leidy, 1874
-*N. galeata* Penard, 1902
-*N. galeata* var *orbicularis* Deflandre 1936
-*N. golemansky* Todorov 2010
-*N. gracilis* Penard, 1910
-*N. gracilis* var. *stomata* Penard 1912
-*N. guttata* Kosakyan et Lara 2012

-*N. hippocrepis* Leidy, 1879
-*N. meisterfeldi* Heger et Mitchell 2012
-*N. marginata* Penard, 1902
-*N. maxima* Awerintzew, 1907
-*N. militaris* Penard, 1890
-*N. pechorensis* Kosakyan et Mitchell 2012
-*N. penardiana* Deflandre, 1936
-*N. penardiana* var. *elongate* Gauthier-Lièvre 1957
-*N. penardiana* var. *minor* Gauthier-Lièvre 1957
-*N. penardiana* var. *retorta* Decloitre 1977
-*N. penardiana* var. *suecica* Grospietsch 1954
-*N. pulchra* Bartos 1963?
-*N. rotunda* Penard 1890
-*N. saccifera* Wailes, 1913
-*N. semimarginata* Van Oye, 1949
-*N. spumosa* Awerinzew 1906
-*N. speciosa* Deflandre, 1936
-*N. tincta* (Leidy, 1879) Awerintzew, 1906
-*N. tubulosa* Penard, 1902
-*N. tubulosa* var. *adami* Laminger 1973

Genus *Padaungiella* (13 species and infraspecific taxa)

-*P. cordiformis (*Heinis, 1914) Lara et Todorov 2011
-*P. (Nebela) himalayana* Chattopadhyay & Das 2003
-*P. lageniformis* (Penard 1890) Lara et Todorov 2011
-*P. longicollis* (Penard 1890) Lara et Todorov 2011
-*P. longitubulata* (Gautier-Lièvre, 1953) Lara et Todorov 2011
-*P. nebeloides* (Gautier-Lièvre, 1958) Lara et Todorov 2011
-*P. (Nebela) pulcherrima* Awerintzew 1907
-*P. (Nebela) tubulata* Brown 1911
-*P. (Nebela) tubulata* var. *spatha* Thomas 1960
-*P. (Nebela) varia* Decloitre 1966
-*P. wailesi* (Deflandre 1936) Lara and Todorov 2011
-*P. (Nebela) wailesi* var. *magna* van Oye 1956
-*P. wetekampi* (Jung 1942) Lara and Todorov 2011

Genus *Porosia* (2 species)

-*P. biggibosa* Jung 1942
-*P. (Nebela) japonica* Bobrov et Shimano 2011

Genus *Quadrulella* (20 species and infraspecific taxa)

-*Q. acuminata* van Oye, 1958
-*Q. alata* Gautier-Lièvre, 1957
-*Q. camerounensis* Gautier-Lièvre, 1957
-*Q. constricta* Lopretto & Vucetich 1997
-*Q. cordobensis* Vucetich 1983
-*Q. debonti* Van Oye 1959
-*Q. elegans* Gautier-Lièvre, 1953
-*Q. elongata* van Oye, 1956
-*Q. lageniformis* van Oye, 1949
-*Q. longicollis* Taranek 1882
-*Q. nunciae* Vucetich 1983
-*Q. quadrigera* Deflandre, 1936
-*Q. plicata* Hoogenraad et de Groot, 1940
-*Q. scutellata* Wailes, 1912
-*Q. subcarinata* Gautier-Lièvre, 1957
-*Q. symmetrica* (Wallich, 1863) Schulze, 1875
-*Q. symmetrica* var. *curvata* Wailes 1912
-*Q. symmetrica* var. *kivuensis* Van Oye 1958
-*Q. symmetrica* var. *tubulata* Gautier-Lièvre, 1953
-*Q. tropica* Wailes, 1912

Taxonomic keys

Family Hyalospheniida (Schulze) Kosakyan et Lara

The test is rigid, colorless or yellowish-brown, flask-vase shaped, oval or pyriform, dorso-ventrally compressed. The shell is either entirely self-secreted composed of an organic matrix, or with addition of self-secreted siliceous plates or recycled shell plates of small euglyphids or other similar material such as diatom frustules incorporated in the test. The pseudostome is terminal and is bordered by a thin organic collar.

Key to the genera in the family

1. → Test oval to flask shape, rigid, chitinoid, clear, completely organic, non – areolar…….**3**

1. ← Test compost of different material……...…**2**

2. → Test composed of self secreted quadratic plates…….**genus *Quadrulella*** (p.xx)

2. ← Test composed either of recycled shell plates of small euglyphids or other similar material such as diatom frustules incorporated in the test (plates sometimes covered by very thick organic layer giving the impression of a chitinoid, clear, organic and non structured test) or composed of agglutinated particles as in genus *Diffluga*……**4**

3. → Test oval elongated, pyriform, flask shaped sometimes with an elongated neck or wedge shaped, totally transparent without structur.**genus *Hyalosphenia*** (p. xx)

3. ← Test pyriform, with a lateral indentation at each side, test often organic, totally hyaline, without structure, but sometimes with a (probably self secreted) rough granulated structure (visible by SEM)…… ……**genus *Alocodera*** (p. xx)

4. → Test pyriform or flask shaped, composed of euglyphid idiosomes embedded in an unstructured organic cement, or often covered with a thick layer of organic cement giving the test a smooth appearance. In the broad view two large (2-5µm) and conspicuous pores in depressions connected by internal tubes situated at the base of the neck (ca. 2/3 from the fundus of the test)…… ……**5**

4. ← Test lacking pores in depressions connected by internal tube. Test composed of siliceous plates or diatom frustules, sometimes covered with a thick organic layer giving an organic hyalinous unstructured impression (e.g. in the *Nebela collaris s.l.* group *N. tincta*, *N. guttata*, *N. pechorensis*, *N. flabelululum*, cf. p. xx, xxx, xxx) or composed of agglutinated particles (unique case of *Padaungiella nebeloides*, p.xx)…….**6**

5. → Test flask-shaped, with an elongated neck. Internal side of neck with pointed protuberances visible in LM as lines of points…….**genus *Certesella*** (p. xx)

5. ← Test lacking pointed protuberances in the internal side of the neck ……**genus *Porosia*** (p. xx)

6. → Test flask-shaped, oval-elongated, pyriform, neck either absent, short or if long tapering toward the aperture and never clearly seprated from the base of the test……**genus *Nebela*** (xx)

6. ← Test bottle-shaped. Neck elongated, often with approximately parallel sides and always very distinct …….. …**7**

7. → Neck deeply constricted at the junction with the main body of the test ……**genus Apodera** (p. xx)

7. ← No constriction at the base of the neck…….....**genus *Padaungiella*** (p. xx)

Genus *Alocodera* Jung 1942

Test pyriform, laterally compressed with a well-developed neck separated from the posterior part of the test by two lateral indentations. Two lateral pores are situated in the indentations, but these can be difficult to observe especially in LM. Test almost transparent, yellowish or slightly brownish, with a smooth or tiny granulose surface. Monospecific genus. Type species: *Alocodera cockayni* (Penard) Jung. Description: page xx.

Genus *Apodera* Loeblich & Tappan 1961

Test composed of two clearly distinct parts, a subspherical or ellipsoidal, compressed basal part and a neck. The two parts are separated by a deep constriction. The sides of the neck range are more or less convex in broad view. Type species: *Apodera vas* (Certes) Loeblich & Tappan. Four species and infraspecific taxa are known.

Key to the species

1.→ Neck unilaterally incurved, L=93µm, B=39 µm…….***A. crenata*** (p. xx)
1.← Neck without incurvation…….**2**

2.→ Neck straight cylindrical……**3**
2.← Neck somehow swollen at the base, tappering from its junction with the body towards the aperture, L=130-170µm, B=55-103 µm…….***A. vas*** (p. xx)

3. → Smaller species, test brown to dark brown. Neck rather straight not swollen at the junction separating the main part of the test from the neck. L=118-158µm, B=75-97 µm…….***A. vas* f. *reticollaris*** (p. xx)
3.← Larger species, L=185-220µm, B=125-130 µm……***A. (Nebela) wellingtonia*** (p.xx)

Genus *Certesella* Loeblich & Tappan 1961

Test pyriform elongated or flask shaped. The development of the neck varies among species from slightly to well differentiated. The main characteristics of the genus are the presence of two lateral depressions with large central pores connected by two tubes located at approximately 2/3 of the distance between the fundus of the test and the pseudostome, and the presence of internal teeth on the neck giving punctuated impression. Type species: *Certesella martiali* (Certes) Loeblich & Tappan. Four species are described.

Key to the species

1. → Test without distinct neck, the main body tapering gradually toward the aperture, one sole line of the teeth situated parallel to the rim of the aperture. Lateral keel present. Largest species in the genus. L=199.5-277,5µm, B=119-140µm) ……..……***C. australis*** (p. xx)

1. ← Test with a distinct neck…….**2**

2. → Neck short subcylindrical, sharply differentiated from the rest of test. Internal teeth scattered with no apparent organisation (L=120-136µm, B=95-100µm) ……...…….***C. murrayi*** (p. xx)

2. ← Test gradually tappering from the front pores toward the aperture …….....**3**

3. → Internal teeth separated arranges into two groups separated by a longitudinal ridge. Narrow lateral keel present (L=80-157µm, B=70-90 µm) …….....***C. certesi*** (p. xx)

3. → Internal teeth separate in two groups by a smooth area without longitudinal ridge. Lateral keel absent. L=147-238µm, B=77-130 µm…….....……...***C. martiali*** (p.xx)

Genus *Hyalosphenia* (Stein 1857) Schulze 1877

Test rounded, ovoid or elongated elliptical or flask shaped, laterally compressed, aperture variable from linear to strongly curved, with or without thickened lip. Test hyaline or slightly yellowish, with a smooth organic surface (exception: *Hyalosphenia punctata* which has a punctuated surface). Type species: *Hyalosphenia cuneata* (Stein) Schulze. 20 species are considered as valid, among which many are problematic and need molecular data to confirm their position within the genus.

Key to the species

1. → Test with a distinct neck ……...……**2**
1. ← Test without distinct neck, rounded, ovoid, or elongated elliptical …….**9**

2. →Neck long, test flask- or bottle-shaped……..……..……..….**3**
2. ←Neck very short…….……..……...**6**

3. → Lateral margin of test not smooth……...…….**4**
3. ← Lateral margin of test smooth or slightly wavy……...……**5**

4. → Main part of test with round to oval depressions, giving the outline a inrregular-wavy apprearance, L=(68)85-110(130)µm, B=40-65 µm, aperture curved, with a well-developed 15-20 µm wide organic collar……***H. elegans*** (p. xx)
4. → Round to oval depressions not limited to the main part of the test but extending toward the neck……***H. insecta (***p. xx)

5. → Lateral margin of the test very smooth, neck widening near aperture, L=84µm, B=49 µm …..…….. ***H. jirovici*** (p. xx)
5. ← Lateral margin of the test smooth or slightly wavy, neck cylindrical and not widening near the pseudostome,dimensions as *H. elegans.*
….……..…….**H. elegans var. *cylindricolis*** (p. xx)

6. → Neck very short, test oval-rounded ……..……**7**
6. ← Neck longer, test more elongated ……....**8**

7. → Smaller species, very rounded (L/B=14-17/12-16 µm. Aperture is 6.5-7.7 µm wide, ellipsoid, in the side view curved inside) ……***H*. *inconspicua*** (p. xx)

7. ← Larger species, L=65-70 µm, B=50 µm, pseudostome 23-25 µm wide ……..……...***H. humicola*** (p.xx)

8. →Smaller species, L<45 µm, L =42µm, B=22 µm, pseudostome narrow 5 µm wide, shape described as "resembling the piston of a small automotive bulb" ……***H. lucerna*** (p. xx)
8. ← Larger species, L>45 µm (test wide ovoid L =130-180µm, B = 90-140 µm, including the keel……..***H. ovalis*** (p.xx)

9. → Pseudostome thickened ……**17**
9. ← Pseudostome not thickened ……..**10**

10. → Test very elongated ellipsoid……...……**16**
10. ← Test ovoid ……..**11**

11. → Test very wide ovoid, length almost equalt to the breadth, pseudostome linear, L=40-42 µm, B=28-32 µm, aperture 28-32 µm .....***H. platystoma*** (p. xx)
11. ← Test narrower, lateral sides gradually tapering toward the pseudostome, which is strongly or slightly curved ……..……..……. …..**12**

12. → Test composed of small organic building units, similar to *Arcella.* ……...***H. punctata*** (p. xx)
12. ← Test totally smooth, without any structure……..……...……**13**

13. → Smaller species, L< 90 µm …….……...**15**
13. ← Larger species L> 90 µm ……..…..**14**

14. → Mixotrophic species, always with zoochlorellae. L =90-175µm, B=60-155 µm, aperture 30-40 µm wide, slightly curved or linear ……..,,,,,,,,,,,,,,,….***H. papilio*** (p. xx)
14. → Pseudostome narrower, similar in shape to that of *Nebela militaris* or *N. collaris s.l.*. …….....***H. papilio* var. *stenostoma*** (p .xx)

15. → Tiny lateral keel present, L = 52-76µm, B=44-60 µm…….....***H. cuneata*** (p. xx)
15. ← Lateral keel absent, but in side view the aboral of the test is slightly pointed, L=45µm, B=60 µm…….....***H. angulata*** (p. xx)

16. → L<200 µm…… ……....***H. penardi*** (p. xx)
16. ← L >200 µm …… ……..***H. gigantean*** (p. xx)

17. →Pseudostome clearly thickened, L=40-65µm, B=23-40 µm. ...***H. shoutedeni*** (p. xx)
17.← Pseudostome not clearly thickened ……..……...……**18**

18. → Smaller species L<45 µm…….,……...……**19**
18. ← Larger species L>45 µm ……....……**20**

19. →Test ovoid, elliptical in side view. L=26-43 µm, B=16-27µm, aperture 13 µm wide ……..……..***H. minuta*** (p. xx)
19. ←Test more rounded, L =20µm, B=17 µm …***H. schoutedeni*** **var. *rotundata*** (p. xx)

20. →Test more elongated, L/B ratio = 3 or more, L = 107-115 µm, B = 31-36 µm ……***H. tamdaensis*** (p. xx)
20. ←L/B ratio always < 2, L = 45-87 µm, B = 30-53µm ……..…..***H. subflava*** (p. xx)

The taxonomic keys for genera *Nebela, Padaungiella, Porosia* and *Quadrulella* are in preparation.

Descriptions and Illustrations

Genus *Alocodera* Jung 1942

Type species: *Alocodera cockayni* (Penard) Jung

Genus monospecific. It was proposed by Jung at 1942 with species *Alocodera cockayni* comb. nov. *Alocodera* is only known from the southern hemisphere.

***Alocodera cockayni* (Penard 1910) Jung 1942,** Arch. Protistenk. Bd. 95. H.3: 313-314.

1910 *Hyalosphenia cockayni* Penard, Brit. Ant. Exp.: 238.

1913 *Nebela cockayni* Wailes 1913, Journ. Lin. Soc. Zool. XXXII: 215.

Icon. : Penard 1910 Pl. 22, fig. 5 ; Jung 1942 fig. 49 ; Deflandre 1936 fig. 107, 108; Meisterfeld 2000, fig. 68; Smith et al. 2007, fig. 1c; Kosakyan et al. 2011, fig. 6b-d.

Description: Test pyriform, laterally compressed with a well developed neck which is separated from the posterior part by indentation from two sides, where two lateral pores are situated, but sometimes can be difficult to observe. Test is almost transparent, yellowish or slightly brownish, with smooth surface, however sometimes can have rough and tiny granulose surface (Fig. 1 A, B). We observed tests with length (L- hereafter) 92-100 and breadth (B- hearafter) = 55-60 μm, with 28-30 μm wide linear aperture surrounded by thick organic collar. However, Wailes observed bigger specimens with dimensions L= 120-126, B=74-75 μm, with 25-28 μm wide aperture.

Habitat: Sphagnum mosses.

General distribution: Argentina, Auckland Islands, Australia, Chile, New Zealand.

Note: *Alocodera cockayni* first time was described by Penard at 1910 as *Hyalosphenia cockayni*, when he observed tests with yellowish color or almost transparent, with homogenous structure. Later on Wailes (1913) find the same shaped tests, with little bit larger dimensions in Chile, and observed structure fitting more to the genus *Nebela* such as small xerosomes in organic cement. In 1942 Jung redefined family Nebelidae and presented it with 11 genera, where genus *Alocodera* was presented with *Alocodera (Nebela) cockayni comb. nov.*

By shape of the test *Alocodera cockayni* is similar to *Apodera vas* (Cert.) Loeblich & Tappan and *Padaungiella lageniformis* (Penard) Lara et Todorov, from which differs by indentated from both sides neck. Molecular data (Kosakyan et al. 2012) showed that *A. cockayni* is closely related with *Padaungilla* species (e.g. *P. lageniformis*, *P.wailesi*, *P. nebeloides*).

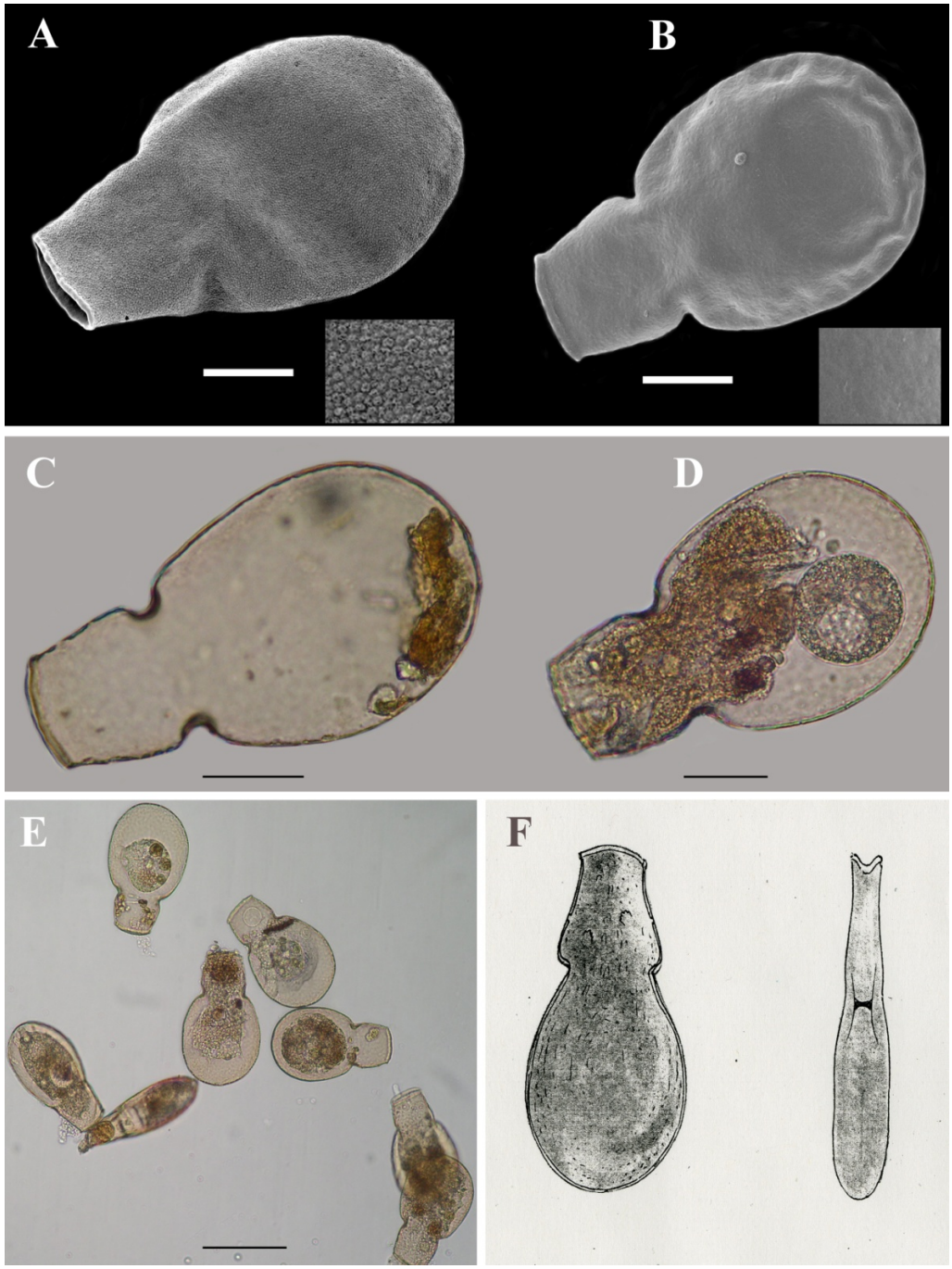


Figure 1. *Alocodera cockayni*. **A**- SEM image of *A. cockayni* from Tierra del Fuego, together with close view of test granular structure. **B**- SEM image of *A. cockayni* from Chile, together with close view of test organic smooth structure. **C**- LM image of *A. cockayni* from Tierra del Fuego. **D**- LM image of *A. cockayni* from Argentina, Patagonia. **E**- some cells of *A. cockayini* Chile. **F** - *A. cockayni* drawing according to original description (Penard 1911). Scale bars =20 µm, except E (40 µm). Images by E. Lara (A,C,D, E) and A. Kosakyan (B).

Genus *Apodera* Loeblich & Tappan 1961

Type species: *Apodera vas* (Certes) Loeblich & Tappan

This genus was proposed by Jung (1942) without type designation. Later on it was validated by Loeblich and Tappan (1961). By the shape of the test it is closely related to *Alocodera* and *Padaungiella*, which all have distinct elongated neck, however in the genus Apodera the constraction between body and neck is very deep giving of well separated from each other impression. Four species and infraspecific taxa are known.

Apodera crenata Jung 1942, Arch. Protistenk. Bd. 95. H.3: 314.

Icon.: Jung 1942, fig.55.

Description: Shell is almost colorless or yellowish, subspherical or ellipsoidal, compressed, with constriction between body and swollen neck which tapers from its junction with the body towards the aperture, neck, with the unilateral incurvation of in front part. Length of the shell is 93 µm, breadth of the shell 39 µm, diameter of aperture 20 µm (Fig. 3D).

Habitat: *Sphagnum* mosses.

General distribution: Chilie

Note: This is problematic species. Jung (1942) described this new species, however after him there is no any other record in the literature. Morphologically, this species is well defined and very much differs from common *Apodera vas*, by its curved test and small size.

Apodera vas (Certes) Loeblich & Tappan 1961; Proc. Biol. Soc. Wash. 74: 205.

1889 *Nebela vas* Certes, Cap Horn t. VI :53
1932 *Nebela goudinii* Gericke 1932, South African J. Sci.: 624-625
1942 *Apodera vas* Jung 1942; Arch. Protistenk., 95:256 nomen nudum.

Icon.: Certes 1889 Pl. I, fig 4-5; Penard 1911 Pl. XXIII, fig 10; Wailes 1913 p127; Deflandre, G. 1936 fig. 109-111; Smith et al. 2007, fig. 1a.

Description: Shell subspherical or ellipsoidal, compressed, constriction between body and swollen neck which tapers from its junction with the body towards the aperture, neck. Aperture oval slightly arched. Test composed of collected or predated euglyphid idiosomes. Length of the test is 130-170 µm, breadth of the shell 55-103 µm, diameter of aperture 26-32 µm (Fig. 2).

Habitat: mosses (often *Sphagnum*), litter and organic soils.

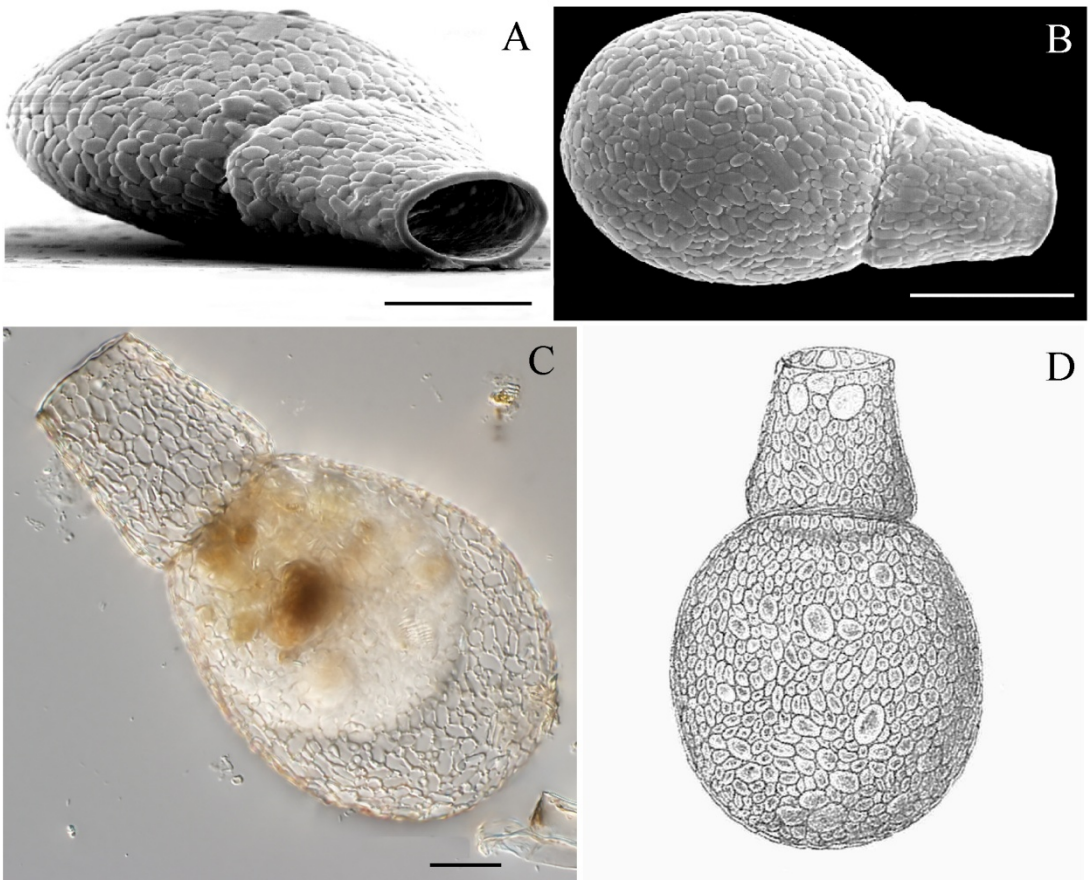


Figure 2. *Apodera vas*. **A** and **B**- SEM imagies of *Apodera vas* from Tanzania (lateral and front view). **C**- LM image of *A. vas* from Tierra del Fuego. **D**-*A. vas* according to original description (after Certes 1889). Scale bars = 50µm in A and B, 20 µm in C. Images by J. Kudenov and E. Mitchell (A, B modified from Mitchell and Meisterfeld 2005) and E. Mitchell (C) .

General distribution: Amsterdam Island, Australia, Brazil, Chile, Columbia, Congo, Costa Rica, Iles Crozet, Falkland Islands, Guatemala, Guadeloupe, Guinea, Hawaii, Ivory Coast, Java, Kerguelen, Macquarie Island, Mexico, Nepal, New Zealand, Papua New Guinea, Philippines, South Africa, South Georgia, Sumatra, Lake Tanganyika , Tanzania, Tasmania, Tierra del Fuego, Venezuela.

Note: *Apodera vas* is frequently cited as evidence of a microorganism with restricted distribution (Mitchell and Meisterfeld 2005; Smith and Wilkinson 2007). It is closely related to *Padaungiella lageniformis* (Penard) Lara et Todorov and *Alocodera cockaynii* Penard, from which differs mainly by deep constriction at the junction with the main part of the test. Lara et al. (2008) have shown in a SSU rRNA sequences based tree that *Apodera vas* is closely related to *Padaungiella lageniformis*.

Jung (1942) described new form of ***Apodera vas* f. *reticollaris***, which differes from the type by the straighter neck and absence of bulb on the junction separating body from the neck. It is darker than type species, almost dark brown. Dimensions of the test are not differing very much from type: length 118-158um, breadth 75-97um, diameter of the aperture 28-38um. Whether this is just phenotypic plasticity or independent species need to be proven by molecular methods. Zapata and Fennandez (2008) already observed polymorphism within *A. vas* species. Based on morphology and morphometry they were able to separate at list 2-3 morphotypes within the species. The polymorphism of *A. vas* need to be proven by molecular methods.

Apodera (Nebela) wellingtonia Decloitre 1964, Expeditions Polaires Francaises (Missions Paul-Emile Victor) 259: 41.

Icon.: Decloitre 1964, fig. 44.

Description: Shell ellipsoidal, compressed, with deep constriction between body and neck like in *Apodera vas*, but neck is much cylindrical without any curvations. Test composed of collected or predated euglyphid idiosomes. Length of the test is 185-220 µm, breadth is 125-130 µm, diameter of aperture 51-55 µm.

Habitat: it was found in puddles created due to melting snow, and near the rotten wood in wet lichens.

General distribution: Australia: Tasmania

Note: It is very rare and problematic species, it was found only once in two different habitats. In the beginning Decloitre thought that it could be an *Apodera vas*, however the differences in the shape of the neck and the size of the test (neck with curvation, tapering from the junction to its aperture, test L=130-170, B=55-103 µm in *A. vas* vs. neck more correct, almost cylindrical, test L==185-220, B=125-130 µm in *A. wellingtonia*) made him to describe the new species. Nevertheless several authors mentioned of finding big sized *Apodera vas* (90-234 µm) without referring it to the possible *A. wellingtonia* (Zapata and Fernandez 2008, Smith and Wilkinson 2007). Molecular tools need to understand if this is independent species or result or possible phenotypic plasticity.

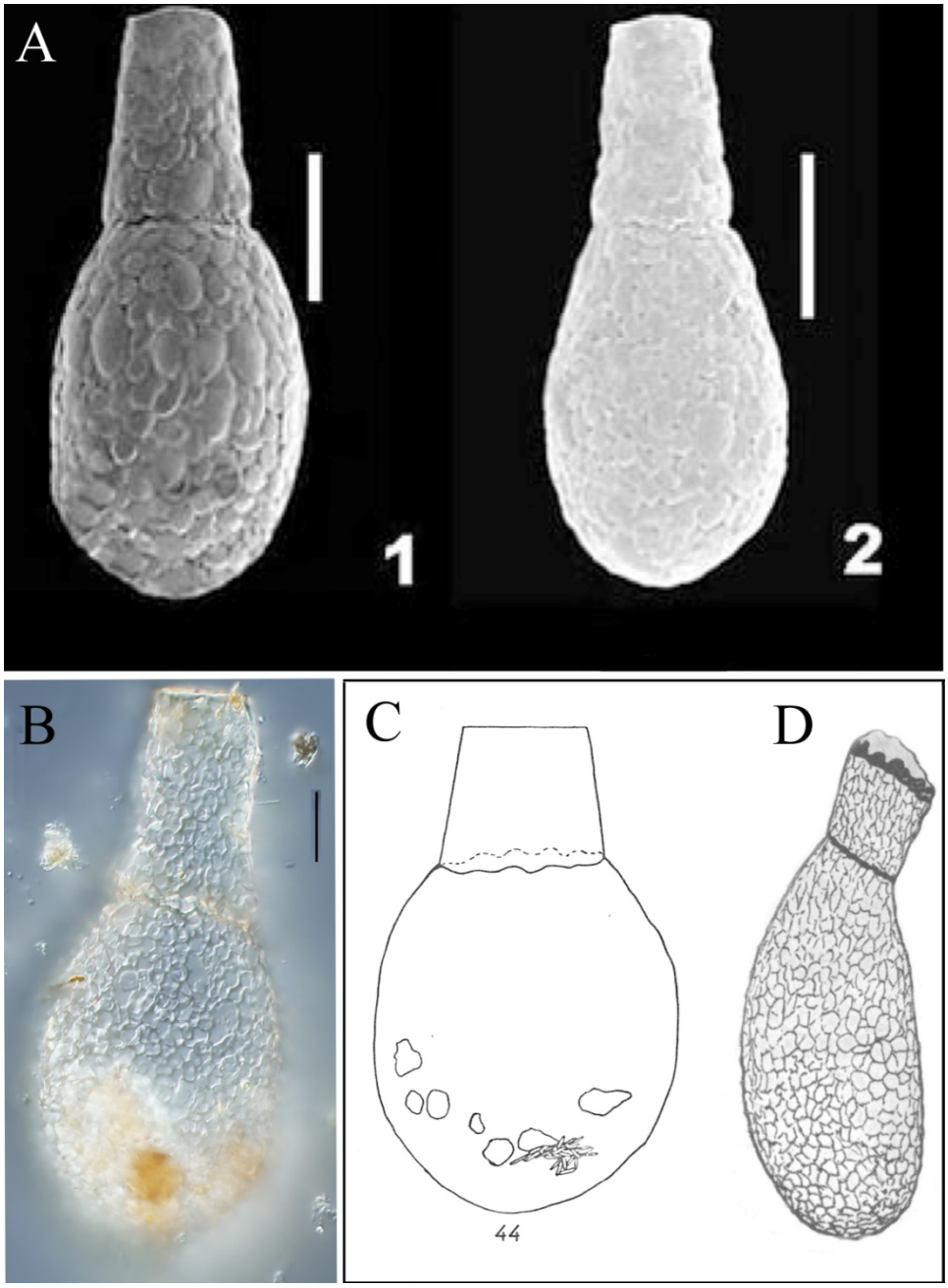


Figure 3. Problematic taxa within genus *Apodera*. A- Tests resembling somehow *Apodera vas* f. *reticollaris* (image modified from Zapata and Fernandez 2008, with L. Fernandez's permission). B- Test from Thierra del Fuego resembling *Apodera vas* f. *reticollaris*. C- *A. wellingtonia* according to original description (after Decloitre 1964). D- *A. crenata* according to original description (after Jung 1942).

Genus *Certesella* Loeblich & Tappan 1961

Type species: *Certesella martiali* (Certes) Loeblich & Tappan

This genus was proposed by Jung (1942) as *Penardiella* without type designation. Later on it was validated by Loeblich and Tappan (1961). Genus is restricted to the southern hemisphere (Meisterfeld 2000). The main characteristics of the genus is the presence of two lateral depressions with large central pores connected to each other with internal tube on the 2/3 of the test where the main body is passing to the neck, and the presence internal teeth situated on the neck. Four species are described:

Certesella australis Vucetich 1973a, Obra del Museo de La Plata IV: 310.

1973b Nebela australis Vucetich, Neotropica 19 :80.

Icon.: Vucetich 1973a, L. III; Vucetich 1973b, fig. 1-2.

Description: Shell is elongated-pyriform, gradually tapering toward the aperture, without neck. Test is laterally compressed with the distinct keel covering all the lateral margin of the test. According to original description two little pores on the lateral margin are present but very difficult to observe (see Fig. 4D). On the 2/3 part of the test there are two lateral depressions are situated with the large central pores connected to each other with internal tube. Test is yellowish-brownish, composed of circular or oval shell plates, covered with thick organic layer, which are sometimes hard to observe. This species is the biggest one in the genus L= 199.5-277.5, B=119-140 μm. Aperture is curved surrounded with thick organic lip, 40-46 μm wide. One sole line of the teeth (around 10) situated in parallel to the board of the aperture, giving punctuated impression (Fig. 4).

Habitat: Sphagnum mosses, wet green mosses.

General distribution: Argentina (Thierra del Fuego).

Note. This is very rare species, probably with restricted distribution. Till know was found only in different localities of Thierra del Fuego.

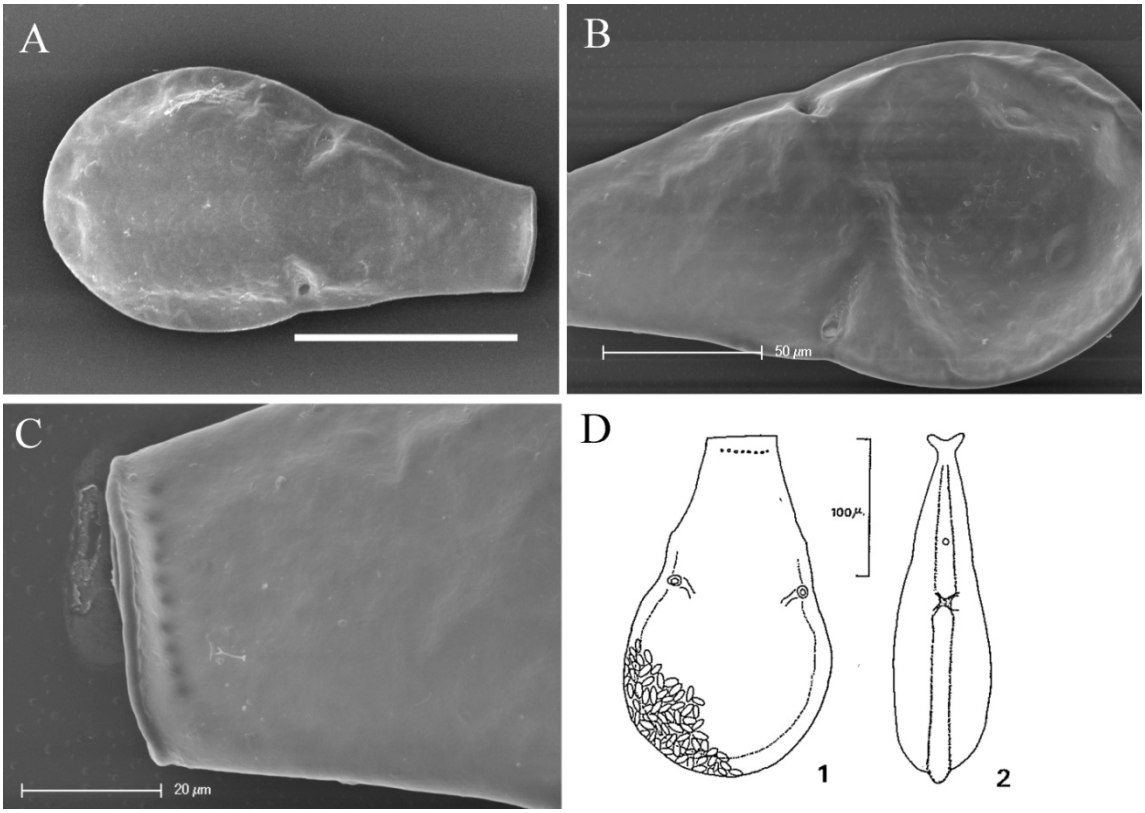


Figure 4. *Certesella australis*. **A**- SEM image of *C. australis* from Thierra del Fuego. **B**- Closer view of lateral depressions with the pores and the lateral keel. **C**- Closer view of the line of the teeth (around 10) situated in parallel to the board of the aperture, giving punctuated impression. **D**- *C. australis* according original description, frontal and lateral views pointing lateral keel, and the pore situated on the lateral margin (after Vucetich 1973b). Scale bars=100 µm (for A, D), 50 µm (for B) and 20 µm (for C). Images by A. Kosakyan and E. Lara.

Certesella certesi (Penard) Loeblich & Tappan 1961, Proc. Biol. Soc. Wash. 74: 213-234.

1889 *Nebela collaris* var. a, b, Certes 1889, Cap Horn VI : 13-14.

1911 *Nebela certesi* Penard, Brit. Ant. Exp. 1907-9, I, Biology I : 241.

1942 *Penardiella certesi*, Arch. Protistenk. 95: 21 (nomen nudum).

Icon.: Certes 1889, Pl. II, figs. 2,3,5 ; Penard 1911, Pl. XXIII, fig. 7; Jung 1942, fig. 56; Vucetich 1973a, Pl. II; Meisterfeld 2000, fig. 70.

Description: Shell is elongated-pyriform, with the distinct elongated neck, that gives a shell a bottle shape. Shell composed of circular or oval shell plates, covered with thick organic layer, which are sometimes hard to observe. Faint lateral keel is present. According to literature tests size length can vary 80-157/70-90 μm. Aperture is curved surrounded with thick organic lip, 30-45 μm. In the base of the neck, on 2/3 part of the test there are two lateral depressions with large central pores connected to each other with internal tube. Little down to these pores, on the neck it is easy to observe two triangular shaped additions, where lateral pores are situated. On the neck often can be observed a longitudinal bar separating two rows of teeth (Fig. 5).

Habitat: *Sphagnum* mosses

General distribution: Australia, America: Argentina (Thierra del Fuego), Cape Horn, Chilie, Colombia; Islands of Pacific Ocean, New Zeland.

Note: The species first was described by Certes (1889) as a variety of *Nebela collaris*. In fact Certes mentioned two variety (a, b), which very similar to each other, except the presence of 2 lateral depression with the big pores in case of var. b. (Fig 5E). Later on Penard (1911) consider these characters (combining var. a and b) quiet distinct from those *N. collaris* and described new species *Nebela certesi*. In 1942 Jung redefined family Nebelidae including *Nebela certesi* into genus Penardiella. Unfortunately Jung's classification lacked type designations and the genus was invalidated (ICZN article 13.3). Later on Loeblich and Tappan (1961) validated it as genus *Certesella*. Discussion concerning *Certesella certesi* and its closely related species *C. martiali* see below.

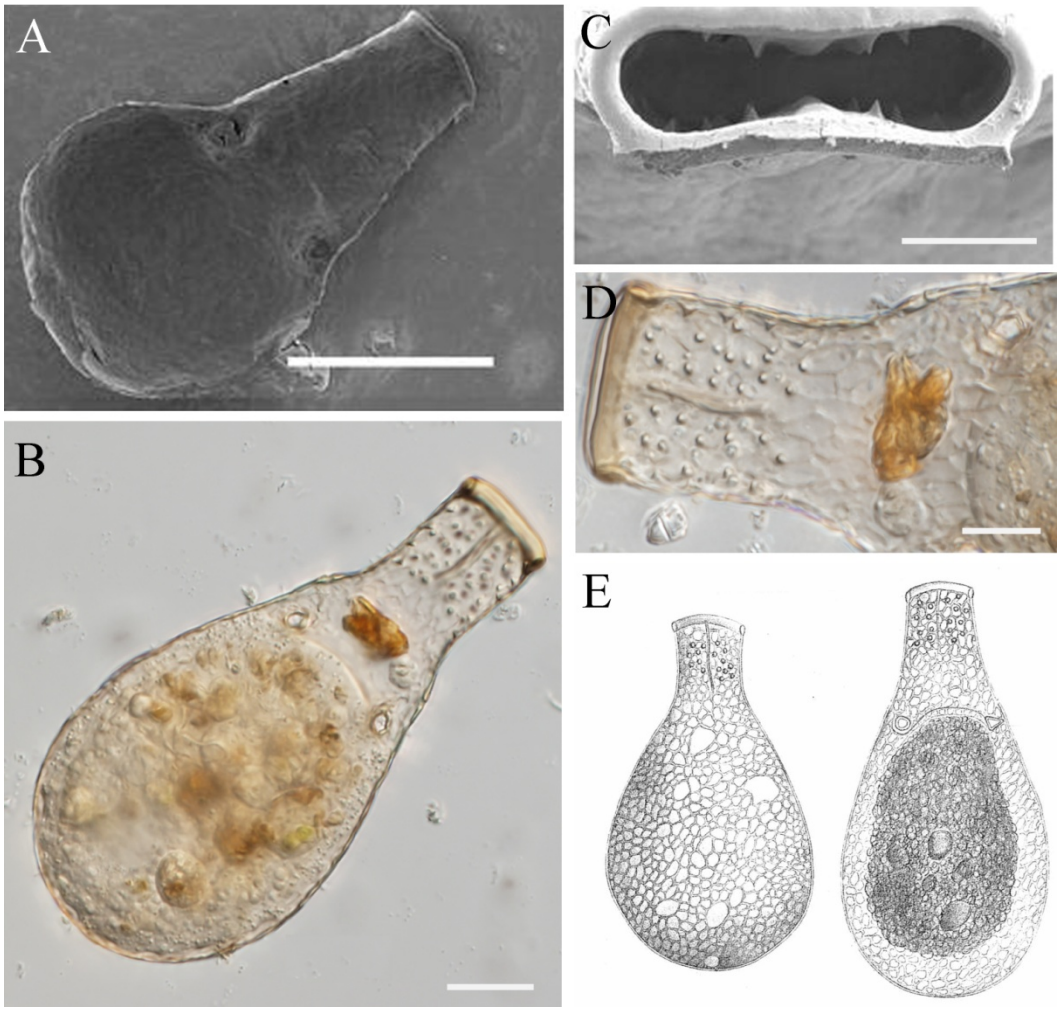


Figure 5. *Certesella certesi*. **A**- SEM image of *C. certesi* from Marion Island. **B**- LM image of *C. certesi* from Tierra del Fuego. **C** –SEM image of internal view of the neck with the teeth and the longitudinal bar. **D**- LM image of external view of the neck with the longitudinal bar and the punctuation. **E**- *C. certesi* according to original description (*N. collaris* var. a and b respectively, after Certes 1889). Scale bars=50 µm (for A), 20 µm (for B) and 10 µm (for C, D). Images by E. Lara (A,C- note: those images are taken from Smith et al. 2008, fig. 1 B,D, where the species considered as *C. murrayi*, however it has clear futures of *C. certesi*- need discussion), E. Mitchell? (B, D).

Certesella martiali (Certes) Loeblich & Tappan 1961, Proc. Biol. Soc. Wash. 74: 213-234.

1889 *Nebela martiali* Certes, Mission Sci. Cap. Horn, T.VI: L14.

1942 *Penardiella martialli* Jung 1942, Arch. Protistenk. Bd. 95. H.3: 381 (nomen nudum), non *Penardiella* Kahl 1930.

Icon. : Certes 1889, Pl. I, fig. 3; Deflandre 1936 figs. 142-144 ; Vucetich 1973a, Pl. I; Kosakyan et al. 2012, fig. 7b.

Description: Shell is elongated-pyriform, with the distinct elongated neck, that gives a shell a bottle shape. Shell composed of circular or oval shell plates, covered with thick organic layer, which are sometimes hard to observe. We observed tests from Argentina with length and breadth 175-178/ 90-92 um. However, according to literature tests size can vary L=155-170, B=85-91 μm, Vucetich (1973) observed tests with L= 147-238, B=77-130 μm. Aperture is curved surrounded with thick organic lip, 38-45 um wide. In the base of the neck, where it is connecting with the main body there are two lateral depressions with large central pores connected to each other with internal tube. Little down to these pores, on the neck it is easy to observe two triangular shaped additions, where lateral pores are situated. On the neck in two parallel rows of many little pores are situated, which are expression of many internal little teeth, separated with kind of space, however there is no distinct longitudinal line as in *C. certesi* (Fig. 6).

Habitat: *Sphagnum* mosses

General distribution: America: Argentina, Cape Horn, Colombia, Macquarie Islands (Pacific Ocean): Australia, Tasmania, New Zeland.

Note: *Certesella martiali* and *C. certesi* are very similar species in appearance, and mainly differ by size: small (80-157 μm) in case of *C. certesi* and big (147-238 μm) in case of *C. martiali*, which has a little risk of overlapping. The similarity of these species and being possible variety or forms are also discussed in Deflandre monograph (Deflandre 1936). He mentioned also that the longitudinal line in the neck situated before aperture in *C. martialy* does not exist while in *C. certesi* is always present. Another differentiating characters mentioned by Vucetich (1973a) is the presence of faint lateral keel in *C. certesi*, and also the size and the shape of pores situated in lateral depressions. The pores are relatively smaller, with irregular margin in *C. certesi*, while the pores are much bigger and have much regular margin in *C. martiali*. But again this can be overlapping character and not convincing enough criteria. Taxonomic position of these two species needs to be clarified using molecular markers.

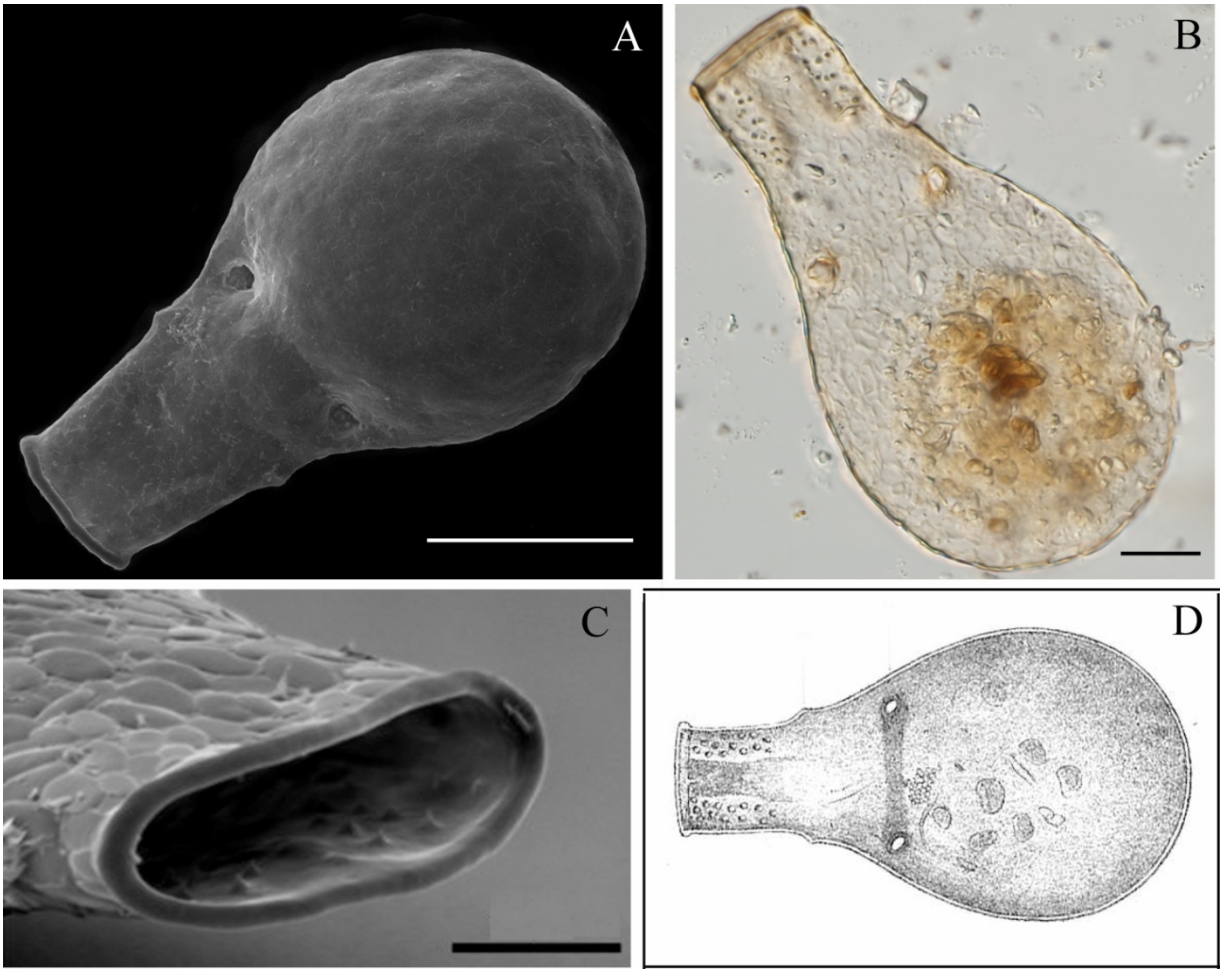


Figure 6. *Certesella martiali*. **A-** SEM image of C. martiali from Argentina. **B**- LM image of *C. martiali* from Chile. **C**- Near view of aperture with theet. **D**- *C. martiali* according to original description (after Certes 1889). Scale bars =50 μm (for A), 20 μm (for B), and 10 μm (for C). Images by A. Kosakyan (A), E. Mitchell? (B), and R. Meisterfel (C).

Certesella murrayi (Wailes) Loeblich & Tappan 1961, Proc. Biol. Soc. Wash. 74: 213-234.

1913 *Nebela murrayi* Wailes 1913, Journ. Lin. Soc. Zool. XXXII: 201-218.

Icon.: Wailes 1913, fig. 18-19; Smith et al. 2007, fig. 1b,d.

Description: Test wide pyriform, laterally compressed with well developed but short subcylindrical neck. According to literature test size vary L==120-136, B=95-100 μm. Aperture is surrounded with thick organic lip, 30-35 μm wide. In the base of the neck, where it is connecting with the main body there are two lateral compressions where the large central pores are situated and connected to each other with internal tube. In a lateral view claviform and keel can be observed. On the neck many little pores are spread, which are expression of many internal little teeth.

Habitat: *Spahgnum* mosses.

General distribution: Brazil, Chili, Marion Island.

Note: This is very rare species, and so far was reported endemic to South America (Vucetich 1978).

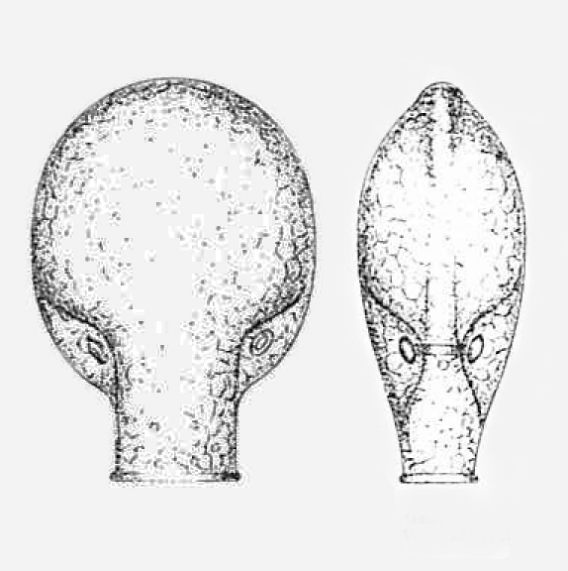


Figure7. *Certesella murrayi* according to original description (after Wailes 1913)- **to be compleated**

Genus *Hyalosphenia* (Stein 1857) Schulze 1877

Type species: *Hyalosphenia cuneata* (Stein 1857) Schulze 1875

The story behind the genus *Hyalosphenia* is rather complicated. Loeblich and Tappan (1964) discussed this in details as follows: *"Seemingly a type species has not been selected previously for this genus, since it has commonly but erroneously been regarded as fixed by monotypy. Stein's original description of Hyalosphenia contains no mention of a specific name. The type reference has been cited both as a publication in the Transactions of the Czechoslovakian Academy for 1857 and as published in the Bericht of the Academy in 1859. Some bibliographies have listed these as two separate publications of differing date but with identical titles and pagination. In January, 1857, Stein orally presented a classification of fresh-water Rizopoda before the Academy in Prague, describing several genera, including Hyalosphenia. No formal paper was published and the transactions of the meetings of this academy were first publish in 1859 (including those of 1857 and other years), in the Bericht. Stein's only published reference to Hyalosphenia was in the transactions of the Academy meeting of 1857, published in 1859. No specific name was given to the form described, hence the genus remained without valid species. About 20 years later Schulze found a species in Germany which he believed to fit the description of Stein's still unnamed and unfigured species of Hyalosphenia from Prague. Schulze and Stein exchanged illustrations of their respective forms, believed by both workers to represent distinct species, and Schulze, 1875 described his as Hyalosphenia lata comparing it in publication to Hyalosphenia cuneata Stein. The latter name must have been included on Stain's unpublished sketches of the unnamed species that he had earlier described, but was first introduced into the literature by Schulze in 1875. Owing to the rarity of the publication containing Stein's description , later workers have referred only to Schulze's publication, and all subsequent texts and treatises have cited H. cuneata Stein, although Stein gave only the description and generic name , without any mention of the specific name cuneata. Hyalosphenia dates from 1859 (date of publication of transactions of the 1857 meeting), but remain without included species until 1875, when the specific name H. cuneata was published, and H. lata was described by Schulze. Either of these nominal species is thus available for selection as type of genus. In the intervening years Tatem (1870) had described Diffluga ligata, which was made the basis for the genus Catharia Leidy, 1874. After Schulze's paper appeared Tatem stated that Hyalospehia lata Schulze was a junior synonym of D. ligata. Catheria Leidy was also a homonym of Catheria Lederer, 1863. Leidy, 1879 stated that he had not seen Stein's publication, but the description quoted by Schulze from Stein did not give sufficient differences to distinguish two species, and added that "-the specific names of caudate, ligata and lata are expressive of characters common to any or all the examples described by Stein, Tatem, Schulze and myself-''. Leidy recognized the species as H. cuneata, but this name was not published until 1875 by Stein in Schulze, which posted Tatem's publication. Hence the*

valid name for the type species in Hyalosphenia ligata (Tatem), and H. cuneata Stein in Schulze and H. lata Schulze, 1875 are both junior synonyms".

For nowdays, *Hyalosphenia cuneata* (Stein) Schulze is considered as type species of the genus *Hyalosphenia*, while *H. lata* Schulze and *H. ligata* (Tatem) Leidy are treated as its synonyms. There are 20 valid species and infraspecific taxa are known for this genus.

***Hyalosphenia angulata* Schouteden 1905**, Ann. Soc. Roy. Soc. Zool. et Malacol. de Belgique 40: 3.

Icon.: Schouteden 1905, fig. 1-2.

Description: Test in broad view oval, and slowly becoming narrow in front; laterally compressed, in profile slim oval, with slightly pointed end. Test is transparent, very smooth, without any structure. Aperture is narrow without any significant collar. Test is relatively small: L= 45-60 µm.

Habitat: freshwater, between algae.

General distribution: It was found only once in algae culture from Botanical garden of Brussels.

Note: This is very rare and problematic species, and as Grospietsch (1965) mentioned that it is even not so clear whether it belongs to genus *Hyalosphenia* or not, since pseudopodia have different form. There is slightly similarity with *Cryptodifflugia compressa* Penard, however latter on is smaller by size (L=13-35 µm). Grospietsch (1965) noted that it is difficult to make any clear decision, since the species was found only once. New findings need to confirm the position of the species within the genus.

***Hyalosphenia cuneata* Stein 1857 (1859)**, Sitzungsb. Böhm. Akad. Wissens 1859: 42.

1870 *Difflugia ligata* Tatem, Month. Micros. Jour. IV: 313.

1874 *Catheria ligata* Leidy, Proc. Ac. Nat. Sc.: 79.

1875 *Hyalosphenia ligata*, Proc. Ac. Nat. Sc.: 415.

1870 *Hyalosphenia lata* Schulze, Archiv mikr. Anat. XI: 335.

Icon.: Stein 1857 fig.´?; Tatem 1870 Pl. lxviii, fig. 1; Schulze 1875 Pl. xviii, figs. 15-18; Leidy 1879 Pl.XX, figs. 1-10; Penard 1902, figs. 1-7; Cash, Wails, Hopkinson 1909 T. 31, figs. 1-4.

Description: Test ovoid, slightly elongated, gradually tapering toward the aperture, with little lateral kill. By shape very much resembling *Hyalosphenia papilio*. Test is very transparent without any structure, no pores were observed. The size of the test according to literature can vary between L=60-76, B=44-60um, however Siemensma (www.arcella.nl) observed smaller tests with L=52-64 (Fig xx). Aperture is linear without any lip or collar.

Habitat: Fresh water, or peat bogs among Sphagnum mosses, an isolated pond in a meadow and the sandy shore a deep lake, in spring in which grows water-crass.

General distribution: Argentina, Austria, Cap Horn, Canada, Costa Rica, Czech Republic, Germany, Hungary, Kongo, Madagascar, Netherlands, Pyrenees, Russia Switzerland, UK, USA.

Note: *Hyalosphenia cuneata* reported as a rare species.

***Hyalosphenia elegans* Leidy 1879,** Rep. US Geol. Surv. Terr. 12: 140.

1874 *Difflugia* (*Catharia*) *elegans* Leidy, Proc. Ac. Nat. Sc.: 156.

1881 *Hyalosphenia turfacea* Taranek 1881, Sitzungsber. Königl. Böhm. Ges. Wiss.: 229.

Icon.: Leidy 1879 Pl. XX, figs. 19-29; Penard 1890 T. 7, figs. 36-39; Penard 1902, figs.1-3; Van Oye 1933, fig. 4; Grospietsch 1965, fig. 16; Charman, Hendon and Woodland, fig. 24a.

Description: Test is pyriform or flask-shaped, laterally compressed, in the broad side elongated with the ellipsoid body and long cylindrical neck; in the profile long elongated tapering toward aperture. There are two little pores in each side, which are very often difficult to observe. Test is almost transparent or slightly yellowish-brownish, totally organic without any structure. Many hemispherical insertions are situated on the body, toward the margin, which are giving wavy impression. Test size vary between L=(68)85-110(130), B=40-65 µm. Aperture is curved, surrounded with the organic collar with 15-20 µm wide.

Habitat: *Sphagnum* mosses, often found in association with *Hyalosphenia papilio*.

General distribution: Africa: Madagascar, Cap-Horn, Seychelles; America: Canada, Chile, Columbia, USA; Asia: China, Japan, Indonesia Siberia (Russia); Europe: Austria, Belgium, Czech Republic, Faroe Islands (Denmark), Finland, France, Hungary, Italy, Germany, Luxemburg, Netherlands, Russia, Sweden, Switzerland, UK.

Note: *Helosphenia elegans* is very common species, very difficult to confuse with other once. Leidy (1879) note that *Difflugia spirigera* described by Erhenberg (1853) can be very similar or probably the same species as *Hyalosphenia elegans*, if when he described “the four internal longitudinal lines” he meant the same as Leidy described as “a series of

hemispherical inflections" on the body of the test. However, the Erhenberg description (in Erhenberg 1853) together with illustration (in Erhenberg 1871, Pl. 3, fig. 4) is not sufficient to understand whether we can treat *Difflugia spirigera* as a synonymous of *Hyalosphenia elegans* or no.

Resent molecular data based on r SSU data (Oliverio et al. 2014) suggested non monophyly of the studied morphospecies of *H. elegans*. However some of this studied cells are resembling more to *H. insecta* than *H. elegans* (see fig. 2 in Oliverio at al. 2014), besides more variable gene such as COI needs to understand the position of *H. elegans* on the Hyalopsheniid phylogenetic tree and its relationships with *H. papilio* and *H. insecta.*

Hyalosphenia elegans var. cylindricollis was described by Chardez (1962, fig. 1-4), which is differing from the type by absence of hemispherical insertions on the body, although the margin of the test has slightly wavy outline. Test is bigger than type species: 130-145 µm in *H. elegans var. cylindricollis* vs. 85-110 µm in *H. elegans*. Molecular data needs in order to understand whether these two taxa are independent or not.

Hyalosphenia gigantea **de Graaf 1952,** Beaufortia 23: 1-4.

Icon.: de Graaf 1952, fig. 1a-c.

Description: Test is quiet big, oval or ellipsoid, in the profile narrow ellipsoid. Lateral pores are not observed. Test is almost transparent or slightly yellowish-brownish, totally organic without any structure. Test size mentioned in the literature is L=204-272, B=65-96 µm. Aperture is oval, with 52-57 µm wide.

Habitat: Freshwater, plankton or benthos.

General distribution: Netherlands

Note: This is very rare species, it was documented only once from Netherlands.

Hyalosphenia humicola **Decloitre 1973,** Extrait des Annales de la S.S.N.A.T.V. (25): 149-156.

Icon.: Decloitre 1973, fig. 2.

Description: Test oval rounded, with very short neck, laterally compressed. Test is very transparent with some foreign elements attached on it. Size L=65-70, B=50 µm, depth of the

test is 10 μm . Aperture is 23-25 μm wide, covered with organic lip, which is shining in some parts giving somehow amorphous impression.

Habitat: Pine forest litter.

General distribution: Europe: France, Sweden?

Note: This is problematic species. It was found only once by Decloitre (1973) in southeastern France, in coniferous forest of Mantrieux (Var) reagion. He mentioned that the test he found did not resemble to any other Hyalosphenia by size and by shape that is why he described it as a new species. It was another finding (one photography) by Chardez (1990) from Sweden but very different from the original description. New findings need to confirm its position within the genus.

***Hyalosphenia inconspicua* West 1903,** J. Linn. Soc. 29: 108-117.

Icon.: West 1903, T.13, fig. 7-11; Cash, Wailes and Hopkinson 1909, T. 31, fig. 5-6; Decloitre 1948, fig. 56.

Description: Test is small, almost rounded, laterally tapering toward the aperture, in the profile wide ellipsoid. Size L=14-17, B=12-16 um. Aperture is 6.5-7.7 μm wide, ellipsoid, in the side view notched.

Habitat: Freshwater, *Sphagnum* mosses.

General distribution: Africa: West Africa; Europe: France, UK.

Note: This is rare and problematic species. Description is very much similar to the one of *Cryptodifflugia compressa* (see Fig. xx for comparison) described by Penard 1902, where the young shells are transparent and hyaline, and older shells are yellowish-brownish . New finding, careful morphological observations, as well as molecular data need to confirm the position of this species within the genus.

***Hyalosphenia insecta* Harnisch 1938,** Zool. Anz. 124: 138-150.

Icon.: Harnisch 1938, fig. 1b; Cash, Wailes and Hopkinson 1909, T. 31, fig. 13-14; Bonnet-Tomas 1955, fig. 3.

Description: Test is pyriform or flask-shaped, very much resembling to *Hyalosphenia elegans* (see description in page xx). The only difference is that hemispherical insertions are situated not only towards the margin but on all the body. Test size vary between L=68-84, B=29-42 μm.

Habitat: *Sphagnum* mosses, wet green mosses, soil.

General distribution: Europe: Belgium, France, Sweden.

Note: This is rare species, reported only from Europe; however one doubtful finding is reported from Sunda Islands (Southeast of Asiatic mainland) by Harnisch (1938).

***Hyalosphenia jirovici* Štěpánek 1953**, Přírodověd. sb. ostrav. kraje 14: 470-505.

Icon.: Štěpánek 1953, fig. 8.

Description: Test elongated bottle shaped, with the long developed neck and rounded body. Test slightly compressed laterally. It is transparent, with L= 84, B=49 µm. Neck is widening near aperture. According to figure by author, aperture is curved, nothing is mentioned about aperture size.

Habitat: aquatic benthos, among decaying plants.

General distribution: Check Republic.

Note: This is problematic species. It was recorded only once, with the short description and one illustration. By the size and the shape shell is very much similar to *Padaungiella wailesii* (L=75-100, B=52-58 µm). The only difference is the composition of the shell: transparent, hyaline in *Hyalosphenia jirovici* and covered with plates in *P. wailesii*. As in other Hyalospheniidae cases (e.g. Nebela tincta) plates are present but very often are very difficult to observe, it may be possible that author missed the details of test composition in the absence of powerful microscope in that times. New findings are need to confirm the position of this species within genus *Hyalosphenia*.

***Hyalosphenia lucerna* Stepanek 1967**, Hydrologia 29: 1-66.

Icon.: Stepanek 1967, fig. 19 (20).

Description: Test shape resembles of the piston of a small automotive bulb. In side view it is flattened as with all representatives of the genus *Hyalosphenia*. Test is light brown transparent, L=42, B=22 µm, Aperture is narrow 5 µm wide.

Habitat: Fresh water.

General distribution: Czech Republic

Note: This is problematic species. It was reported only once from Thaya river. The original description is quite short:

"Länge 42u, Breite 22 u, Öffnung 5 u. In der Gestalt erinnert die Hülle an den Kolben einer kleinen Automobilglühbirne. Bei Seitenansicht ist sie jedoch wie bei allen Repräsentanten der Gattung Hyalosphenia abgeflacht. Die Hülle ist an der Öffnung verengt. Die Öffnung selbst ist spaltenförmig. Farbe der Hülle ist hellbraun, des Plasma vacuolisiert mit einem Stich gelber Hyalosphenia papilio Leidy 1876",

The original drawing is poor (fig. xxx). New findings are needed to confirm the position of this species within the genus.

***Hyalosphenia minuta* Cash 1891**, Trans. Ann. Rept. Manchester Microsc. Soc.: 49-50.

Icon.: Cash 1891, fig. 3-4; Cash, Wailes and Hopkinson 1909, T. 24, fig. 5-11; Wailes 1928, T. 7, fig. 40.

Description: Test, ovoid in front view, in profile narrow elliptical, laterally compressed, with the straight cut aperture slightly rounded at the corners. Author mentioned that "*The slightly convex sides sloping gradually downward to the mouth, which forms a shallow notch. Great care is needed in order to see this owing to the delicate transparency of the shell membrane*". Test is transparent, hyaline, considerably very little with L= 26-43, B=16-27µm, with aperture 13 µm wide.

Habitat: Sphagnum and green wet mosses.

General distribution: Africa: Congo, West Africa; America: Canada, Venezuela; Asia: Japan; Europe: British Islands, Italy, Hungary, Netherlands, Spitsbergen, Sweden.

Note: It is rare species, by shape very similar to *Hyalosphenia subflava*, from which differing mainly by its smaller size. *H. subflava* is always bigger than 50 µm.

Hyaloshpenia ovalis Wailes 1912, Scott. Naturalist: 59-65.

Icon.: Wailes 1912, fig.1; Cash and Hopkinson 1918, T.62, fig.3-4; Jung 1936, fig. 25, Charman et al. 2000, fig. 24c (misapplied).

Description: Test is wide pyriform or wide-ovoid, laterally compressed, where body gradually turning to wide neck like in Hyalosphenia papilio. In the profile the test is elliptic with thin tiny keel, like in *Nebela galeata*. Test is transparent, with 2-12 pores situated on the lateral margin. L=130-180, B=90-140 µm. Aperture linear, possible with tine organic lip.

Habitat: Sphagnum mosses.

General distribution: Europe: Germany, UK?, Netherlands?

Note: *Hyalosphenia ovalis* is very problematic species. The problem is very well discussed in Booth and Mayers (2010): *"Considerable confusion exists regarding the identification of H. papilio and H. ovalis in recent peatland studies. Although early descriptions separated H. ovalis from H. papilio by its larger size, more oval shape, and rounded keel, some recent peatland work has focused more on the pronounced convex tapering of the aperture in H. ovalis (Charman et al. 2000). However, using this criterion, tests identified as H. ovalis are generally smaller than H. papilio, which is inconsistent with early descriptions. In fact, even specimens of H. ovalis in Penard's slides at the British museum would be classified as H. papilio if the convex tapering of the aperture were used as the primary diagnostic feature (Charman et al. 2000). To add to the confusion, individuals of Nebela tincta sometimes lack plates, as is common in some modern samples and most fossil samples, yet these would be identified as H. ovalis using the approach of Charman et al. (2000). N. tincta is generally smaller than H. papilio, and confusion between these taxa may help explain the smaller size of tests identified as H. ovalis in recent studies (Charman et al. 2000)"*.

We are absolutely agreeing that description and the picture of *H. ovalis* given by Charman et al. 2000 is not corresponding to original description of the species.

According to original description, this species is very much similar to *H. papilio*, and differing from it by number of the pores, smaller size and lateral keel. As it was shown by many studies, the number of the pores can't be a taxonomical criterion (Booth and Mayers 2010, Kosakyan et al. 2013, Goma et al. 2014; Mulot et al. in prep.). The size also is not convincing discriminating character, since in some cases it can overlap (L/B= 130-180/90-140 in case of *H. ovalis* vs. L/B=90-175/60-155 in case of *H. papilio*). Thus, the only discriminating character in this case is the presence of lateral keel in *H. ovalis*.

Hyalosphenia papilio Leidy 1879, Rep. Unit. Stat.Geol. Surv. 12: 324.

1874 *Difflugia (Catharia) papilio* Leidy 1874, Proc. Acad. Philad.: 156.

Icon.: Leidy 1879, T. 21; Penard 1902, fig. 1-4; Cash, Wailes and Hopkinson 1909, T. 24, fig. 1-4, Deflandre 1931, fig. 1-2; Ogden and Hedley 1980, Pl. 25; Meisterfeld 2002, fig. 61; Heger et al. 2013, fig 1.

Description: Test is wide pyriform or oblong-ovoid, laterally compressed, with convex fundus, with the lateral sides gradually tapering toward aperture. In the profile the test is elliptic, with the slightly elongated rounded fundus, and with the concave aperture. Test is transparent, often with buff or yellowish tint, with various number of pores (1-10) situated on the lateral margin. L=90-175, B=60-155 μm. Aperture is slightly curved or linear, sometimes with tiny collar, with 30-40 μm wide.

Habitat: Sphagnum mosses

General distribution: Africa: Madagascar, Marion Island, West Africa; America: Argentina, Brazil, Canada, Colombia, USA; Asia: Japan, Russia; Europe: Austria, Belgium, Bulgaria, Czech Republic, Finland, France, Germany, Hungary, Iceland, Netherlands, Romania, Spain, Sweden, Switzerland, UK.

Note: *Hyalosphenia papilio* is one of the most common species in Sphagnum peatbogs. It is mixotrophic species and it was never documented without its host symbiont, which was shown to belong to genus *Chlorella sensu stricto* (Gomaa et al 2014).

Heger et al. (2013) investigate the genetic diversity and phylogeography of *Hyalosphenia papilio* in 42 Sphagnum dominated peatlands in North America, Europe and Asia using mt COI gene sequence data. The sequence data from 301 *H. papilio* single cells revealed 12 different genetic lineages corresponding to evolutionary independent units (i.e. cryptic species). This data also showed a high degree of genetic heterogeneity within different geographical regions.

They evaluated the contributions of climate and dispersal limitations on the distribution patterns of the different genetic lineages. It was shown that the distribution patterns of *H. papilio* genetic lineages in the Northern Hemisphere are more influenced by climatic conditions than by dispersal limitations. Recent molecular data based on rSSU gene also (Oliverio et al. 2014) also showed non monophyly of *H. papilio*.

Hyalosphenia papilio* var. *stenostoma Deflandre 1931, Ann. De Protistologie 3: 81-95.

Icon.: Deflandre 1931, T. 14, fig. 2, 6.

This is problematic taxa. It is differing from the type by the shape of the test and by narrower aperture. Test of *H. papilio* var. *stenostoma* gradually tapering toward the aperture,

resembling to the shape of *Nebela militaris*. L=110-127 μm. It was documented from France in *Sphagnum* mosses.

In the original description it is not mention the actual size of the aperture. Giving the fact that there are many *Nebela collaris* with the same shape and size (see figxxx), and transparent test, it is very easy to be confused. The validity of this infraspecific taxa need to be confirmed by using molecular data.

Hyalosphenia penardi Lauterborn 1908, Z. wiss. Zool. 90: 645-699.

Icon.: Lauterborn 1908, T. 41, fig. 1-2.

Description: Test elliptical, laterally strongly compressed, with rounded fundus, with a strongly curved aperture. Test is transparent, colorless or yellowish-brownish. Pores were not observed. L=180, B=70 μm .

Habitat: Fresh water, among diatoms.

General distribution: Germany.

Note: This species is very rare, was recorded only once in the Upper Rhaine from Germany.

Hyalosphenia platystoma West 1903, J. Linn. Soc. 29: 108-117.

Icon.: West 1903, T. 13, fig. 3-6; Cash, Wailes and Hopkinson 1909, T. 31, fig. 7-8.

Description: Test is wide oval shaped, with almost equal length and breadth, very slightly narrowing right near aperture. In profile elongated elliptical with notched aperture. Test is transparent and hyaline. L=40-42, B=28-32 μm. Aperture linear, with 28-32 um wide.

Habitat: Sphagnum mosses.

General distribution: Europe: Austra, France, UK.

Note: Rare species.

***Hyalosphenia punctata* Penard 1891,** Arch. Sci. Phys. Nat. Genève 26: 134-156.
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Icon.: Penard 1891, T. 2, fig. 1-3; Penard 1899, T. 4, fig. 20-29; Penard 1902, fig. 1-7. Torok 2001, fig. 1-14.

Description: Test wide oval, laterally compressed, tapering toward the aperture. In profile the fundus is widly rounded, tapering toward aperture. Test is transparent, can be colorless, yellowish or brownish. Test composed of small organic building units, similar to Arcella structure. L and B vary from 35-95 and 16-54 µm repectivaly. Aperture is linear, with tiny lip, with 15-32 um wide.

Habitat: It was recorder from different habitats: deep alpine lakes (Penard, 1891, 1902), swamps (Penard, 1902) and running waters (Opravilova, 1974,1980, Török, 2001), sediment of a ditch of a nature reserve.

Note: This is rare and particular species, but very problematic. Török (2001) stated that this species possibly belongs to the genus *Nebela*, because of its shell structure. Still, the structure is quite different from those that genus *Nebela* have. Molecular data needs to confirm the position of this species within genus *Hyalosphenia*.

Hyalosphenia schoutedeni van Oye 1926, Arch. de Zool. Exp. Et Génér. 65: 64-74.

Icon.: Van Oye 1926, Abb. 2, fig. 1-5; Decloitre 1948, fig. 54.

Description: Test is oval shaped, in the profile narrow elliptical. Test is transparent, hyaline. Pores are not observed. L and B vary from 40-65 and23-40 µm respectivaly. Aperture is elliptical, greatly thickened inwards and thus clearly visible.

Habitat: Different substrates? On the bat guano in the cave (Decloitre 1948).

General distribution: Africa: Congo, West Africa.

Note: Very rare species. One variety is known: **var. *rotundata***, which is different from type by more my smaller size and rounded shape L=20, B=17 µm.

Hyalosphenia subflava Cash 1909, Brit. Fr. Rhiz. 2: 87-89.

Icon.: Cash, Wailes and Hopkinson 1909, Pl. XXXI, fig. 9-12; Jung 1936, fig. 8; Bonnet and Thomas 1960, T. 9, fig. 125-126; Chardez 1963, fig. 9; Ogden and Hedley 1980, Pl. 26, a-c; Lüftenegger 1988, fig. 7; Charman, Hendon and Woodland 2000, fig. 24 b; misapplied: Penard 1911, fig. 6 a-c.

Description: Test is ovoid, laterally compressed, with the convex lateral sides, slightly tapering right near the aperture. In the profile it is elliptical with the notched little aperture. Test is colorless or yellow, very smooth. L=45-87, B=30-53. Aperture is curved, 13-14um wide.

Habitat: Mainly *Sphagnum* mosses. Grospietsch (1965) mentioned finding also in soil samples.

General distribution: Africa: Angola, Seychelles; America: Brazil, Canada, Chili, Columbia, Falkland, USA; Australia: New Zealand; Asia: Java; Europe: Czech Republic, France, Germany, Netherlands, Pyrenees, UK.

Note: This is common species. By shape it is very much similar to *Hyalosphenia minuta*, from which it differs by larger size (L=45-87, B=30-53 in *H. subflava* vs. L= 26-43, B=16-27 in *H. minuta*) and by having more substantial looking texture (Cash et al 1909).

Cash et al. (1909) observed cells with several variations in different samples of Britain, and mentioned that these variations could be due to different environments.

Hyalosphenia tamdaoensis Balik 1995, Acta soc. Zool. Bohem. 59: 1-16.

Icon.: Balik 1995, fig. 6.

Description (according to original text): Test bilaterally symmetrical, long, narrow oval, egg-shaped or vase-shaped, on the cross section bean-shaped or kidney-shaped. Dorsal side vaulted, ventral side depressed, depression elliptical. Test organic, with relative robust shell wall, surface smooth. Lateral pores absent. Test is pellucid, translucent, light yellow or yellow-orange. L= 107-115, B=31-36 μm. Aperture is small, elliptical, with thick rim, 9-11 μm wide.

Habitat: Leaf litter in rain forest.

The shell shape signalized that this species is the soil inhabitant with the typical ecological adaptation (thin, arched test, small aperture). The species is probably attached to humic soil horizon (Balik 1995).

General distribution: Vietnam.

Note: The species by general shape somehow similar to *H. subflava, H. schoutedeni* and *H. giganteae* from which differs by size of the test and size of the aperture (Balik 1995).

The plates for genus *Hyalosphenia* are in preparation.

The descriptions and illustrations for the genera *Nebela, Padaungiella, Porosia* and *Quadrulella* are in preparation.

List and notes to inquirenda, nomen nudum and synonymous names

Inquirenda (nomen dubium) names

Here we included the species with doubtful identity. For some of the species in this list the given description is scares, the given figure is very low quality and without further identification is not possible to understand the main futures of the species. Other species in this list have very detail and clear description and drawings, however taxonomic characters of these species overlapping between more than one genus, that is why without molecular data is not possible to understand to which genus they belong. We are listing here **16** inquirenda names for family Hyalospheniidae.

From genus *Hyalosphenia*:

***Hyalosphenia insuetua* Stepanek 1967**, Hydrologia 22: 1-66.

Icon.: Stepanek 1967, fig. 19 (16).

Description: Test is elliptical, narrowing near aperture. Test is transparent collorles with all the feutures of Hyalosphenia. Test is tiny L=42, B=22 µm, aperture is 10 µm um wide.

Habitat: freshwater

General distribution: Czech Republic

Note: This is very rare species, only one finding from Thaya river is recorded. Taking into consideration very brief description: "*Länge 42u, Breite 22 u, Öffnung 10 u. Kleine, elliptische an der öffnung eingeengte Hülle ist farblos durchsichtig. Sie besitzt alle Merkmale von Individum der Art Hyalosphenia . Die öffnung ist eng, spaltenförmig*", and very poor drowning (see figxxx), we suggest to include this species into list of inquirenda taxa, till new findings will confirm the correct position of this species within the genus.

***Hyalosphenia mraconiae* Godeanu 1972**, Rev. Roum. Biol.-Zoologie 17(4): 227-236.

Icon.: Godeanu 1972, fig.7.

Description: Test pyriform, with the rounded body, and with lateral sides tapering toward the aperture, as in *Nebela collaris*. In the 2/3 part of the test, approximately 40 µm far from

aperture 2 clearly seen pores are situated. Test is transparent, hyaline, without any structure, very tiny, with L=60-65, B=42-45 µm. Aperture is 11-13 µm wide, with serrated margin. Cross section aperture is cercular

Habitat: fresh water, among microphytobenthos.

General distribution: Europe: Romania.

Note: The species is very rare, it was found only once from River Mraconia, among microphythobenthos, pH=8.8, T=18^{o}C (Godeanu 1972). Author also noted that although the test has characters similar to different species of genus *Nebela*, however the totally organic structure of the test allowing to assign it to genus *Hyalosphenia*. This is the first *Hyalosphenia* species with serrated aperture. Molecular data needed to confirm the true taxonomic position of this species.

***Hyalosphenia nobilis* Cash 1909,** Brit. Fr. Rhiz. (Printed for the Ray. Soc.), vol. 2: 92.

Icon.: Cash and Hopkinson 1909, Pl. XXV, fig. 1-3.

Description (according to original description): Test is flask-shaped, with the elliptic body and long cylindrical neck like in genus *Padaungiella*. In transverse view (see fig xx) the test is uniformly circular and its whole surface pitted with irregularly-formed depressions. Test is transparent, with the L=200, B=90 µm. Aperture is slightly undulated, with 30 um wide.

Habitat: Aquatic mosses (mainly among the rootlets of *Aulacomnium palustre*).

General distribution: Europe: UK.

Note: This is rare species, was reported only once from Knutsford Moor, Cheshire, UK. Author note that the test was totally colorless and transparent, but with a rugose surface caused by the pits, and devoid of incrustation. Because of very different form of the test from other members of the genus, Cash first thought to replace it within genus *Difflugia*, but the fact of homogenous test made him finally to assign it to the genus *Hyalosphenia*.

Still, the shape of the test and the “rugose surface” as was mentioned in original description call for taxonomic reconsideration of this species using molecular tools. Good example in this case can be *Alocodera cockaynii*, which also has homogenous and slightly rogues surface, but situated quite far from genus *Hyalosphenia* in the phylogenetic tree.

One intraspecific taxa known for this species:

var. compressa Playfair 1918, Proc. Linn. Soc. New South Wales 42: 658.

Icon.: Playfair 1918, Pl. xxxix, fig. 1.

The main differences from the type is that body of the test compressed somewhat, and test is smaller: L= 154-175, B= 70 µm, aperture 27-30 µm wide.

***Hyalosphenia obliqua* Decloitre 1979,** Annales de la Société des Sciences Naturelles et d'Archéologie de Toulon et du Var, 31: 156-159.

Icon.: Decloitre 1979, fig 7.

The description of species is given very short: species is different from other *Hyalosphenia* species by its curved neck, L= 30-40, B=10-12 µm, aperture is 4-6 µm wide It was found in France (2 times) on litter of vine plant. Drawing is very brief and not reliable. We include this species in to the list of inquirenda names till new findings will confirm the position of the species within the genus.

***Hyalosphenia sinuosa* Cash 1909**, Brit. Fr. Rhiz. 2: 91-92. inquirenda
Icon.: Cash, Wailes and Hopkinson 1909, Pl. XXIV, fig. 1,2, and fig. 83-84 in text.

Description (according original test): Test in front view elongated-pyriform, compressed; its crown semi-circular, with the sides tapering, each with a crenulate outline, downwards to the truncated mouth. The entire surface pitted with circular depressions; hyaline, colorless; the mouth , in narrow lateral view, forming a shallow notch. Plasma, seen through the transparent membranous envelope, not filling the cavity, but reaching very nearly to the fundus, in which region the nucleus- visible as a circular pale space-is situated; pseudopodia numerous, bifurcated or simple. L=200-236 µm, verage B in narrow lateral view is 65 µm .

Habitat: Green moses.

General distribution: Europe: UK.

Note: This species is very rare. Due to its particular structure (such as circular pitted test which is giving rogues impression) it differs from other *Hyalosphenia* species. Deflandre (1936) also mentioned that it is difficult to include this species into genus *Hyalosphenia* and it is probably insufficiently studied *Nebela*. Considering the test structure, which is one of the main discriminating characteristic of the genus *Hyalosphenia*, we are including this species into the list of inquirenda taxa, until molecular data will confirm its true position within the genus.

Hyaloshenia schönborni Stepanek 1967, Hydrologia 29: 1-66.

Icon.: Stepanek 1967, fig. 19 (14).

Description: Test has lenticular shape, transparent and colorless. L= 55, B=50 μm. Aperture is column-shaped, small in comparison with the whole test, with 8 um wide.

Habitat: In the river benthos.

General distribution: Czech Republic.

Note: It was found only once in Thaya river benthos, in Vranov. The original description is not detailed enough, without any differenciating character from similar species (" *Länge 55 µ, Breite 50 µ, Höhe 28 µ, Öffnung 28 x 8 µ. Die Hülle hat linsenförmige Gestalt, ist praktisch farblos, ohne kennbare Struktur. öffnung ist spaltenförmig, im Vergleich mit der Hüll klein. Innerhalb der Hülle eines Individuums wurde eine Cyste beobachtet, auch linsenförmiger Form, braungelber Farbe. Die übrigen Merkmale entsprechen den Merkmalen der Gattung Hyalosphenia. Die Benennung der Art ist dem deutschen Rhizopodolgen Dr. V. Schönborn gewidmet worden*"), and the illustration (figxxx) is very poor. We are including this species till new findings will give a possibility to obtain a molecular data.

From genus *Nebela* :

Nebela barbata (need a detailed discussion)

Var. psilonata ????

***Nebela bartosi* Haager & Haagerova 1970**, Acta Univ. Carol. Biol. 1-10.

Icon.: Haager & Haagerova 1970, Pl. 13, fig. 17.

Description (according to original text): Test is bottle-to pear-shaped, with the developed small neck. Two pores are situated on the lateral borders of the neck near aperture . L= 213-215, B=135-137 μm. The surface of the test covered with three types of plate: large disc-shaped platelets around 11-12 to large, small disc-shaped platelets, around 4 um large and elliptical plate, around 8 x 4 at large. Furthermore, some individuals were found which had

also some quadratic plates (like once from genus Quadrulella) attached on the test. Test is bright yellow with slightly rose tint. Aperture is curved, with 56-59 um wide.

Habitat: Sphagnum mosses

General distribution: Czech Republic.

Note: According to author (Haager & Haagerova 1970) this species is very similar to *Nebela tubulosa*, but it differs from later one by its size, the form and nature of shell composition (plates) and by the placement of the pores. However, it was shown that the shell plates and the pores are not taxonomic criteria for Hyalospheniidae, since it can depend on their feeding and environmental conditions (Kosakyan et al. 2012). The size is slightly different, and it is possible to overlap (L=213-215, B=135-137 in *N. bartosi* vs. L=190-215, B=80-125 in *N. tubulosa*). We include this species to the list of inquirenda taxa, till new findings and molecular data will confirm the position of *N. bartosi* as an independent taxon.

Nebela cornuta Voronkoff 1910, Trudy Otdela Ikhtiologii Obshchestva Akklimatizatsii Moskva 7: 217-218.

Icon.: Voronkoff 1910, fig. 1.

This species was found in Sphagnum bogs in Russia. The test by shape is very much similar to the one *Argynnia bipes* (Carter) Murray 1870, however it is much smaller (L=108 µm in case of *Nebela cornuta* and 132-155 µm in case of *Argynnia bipes*), and what is important is that auther is clearly noting that the aperture in *N. cornuta* is very linear, while *Argynnia* do not have linear aperture. In any case, molecular data as well as detail morphological examination need to understand taxonomic position of both described morphospecies.

***Nebela deflandrei* Decloitre 1955 (1977),** Arch. Protistenk. Bd. 119: 325-352.

Icon.: Decloitre 1977, fig. 15.

Description: In the general shape test resembles *Physochila tenella*. Author mention the following discriminating characters from *P. tenella*: it is covered with round scales or with rectangular once with rounded corners., some scales can overlap; the scales reach till bourder of pseudostome; the edges of test are clearly returning to the pseudostome; pores are situated on the middle of the lateral bords of the test, and very difficult to see, test is without color; scales are easily visible at low magnification unlike *P. tenella*.

Habitat: Green mosses

General distribution: Venezuela.

Note: Giving the fact of not totally convincing discriminating characters, original drawing (see fig xxx) and sole finding of this species, we suggest to include it in the list of inquirenda taxa, till new findings will confirm its correct position within the genus.

Nebela penardiana* var. *falcata Wailes 1912

Inquirenda because figure is missing??? (need a discussion)

Nebela strangularia Decloitre 1977, Arch. Protistenk. Bd. 119: 344.

Icon.: Decloitre 1977, fig. 49

Description: The test resembles *Alocodera cockayni* but differs in the shape of the constriction at the base of the neck which is much stronger. Aperture is linear or almost linear with the straight sides of the neck. The test surface is smooth or with only a few plates without order. L=170-180, B=100-105 µm, aperture is 40 µm.

Habitat: it was found in the small pond.

General distribution: Island

Note: There is no doubt that description and the figure are cleary demonstrating that the species is belong to genus *Alocodera* or *Apodera.* But the fact that it was found in Island making the things confusing, since it is well known that *Alocodera* and *Apodera* are genera with districted distribution to Southern hemisphere. One explanation could be that the species was mixed with the similar species *Lagenodifflugia vas* as it was mentioned in Mitchell and Meiterfeld 2005. However in the description Decloitre clearly mention that the surface was smooth or with few disorderd plates '*La membrane parait lisse avec seulment quelques plaques sans ordre'*. We include this species to the list of inquirenda taxa till new findings will be available.

***Nebela tuberculata* (Wallich) Owen and Jones 1976**, Journal of Protistology: 485-487.

1864 Difflugia proteiformis subsp. globularis Wallich 1864, xxxx add ref

1867 Difflugia tuberculata Archer 1867, xxxx add ref

1879 Difflugia lobostoma f. tuberculata Leidy 1879, Rep. US. Geol. Surv. Terr. 12: 113

Difflugia tuberculata var. laevis Penard, xxxx add ref

1942 Cingodifflugia laevis Jung 1942, Arch. Protistenk. 95: 357-90.

Netzelia ????????

Icon.: Jung 1942, Abb. 15a,b; Leidy 1979, Pl. XV, fig 21,22; Owen and Jones 1976, fig. 1-9.

Description (according to Owen and Jones, 1976): Test round to ovoid, 120-130 µm in diameter, with mammilations covering entire test. Test composed of autogenous regular and irregular silicious rods, with longer straight rods between mammilations. Aperture angular, ofter hexagonal with a lip 5-6 um long.

Habitat: Ponds, swamps

General distribution: Africa; America: USA; Europe: Owen and Jones (1976) mentioned that amoeba was said to be cosmopolitan in its distribution.

Note: This species was described under several names, with slightly variable descriptions. Its taxonomic history described in detail in Owen and Jones 1976.

In 1976, Owen and Jones, make a detail observation of this species from Alabama (USA), and because of the mammilated structure of the test and aperture, they suggested that species fitting more into genus Nebela.

This species is quiet interesting, since it is sharing the characters of four genera: *Netzelia*, *Difflugia*, *Pseudonebela* and *Nebela* (see comparing fig. xxx). Molecular data need to confirm the true position of this species.

From genus *Padaungiella*

***Padaungiella americana* Taranek 1882**

We decided to include this species into list of inquirenda taxa, since it is sharing overlapping characters: *P. americana* L=90-130 µm, it is somehow overlapping with *N. lageniformis*, (L=125-130 µm) and with *N. wailesii* (L=75-100 µm).

One infraspecific taxa is known : *Nebela americana* var. *falcata* Wailes 1912

Non valid, nomen nudum or excluded names

In this list we have included the names with not adequate description, the fossil records, or the names with mistaken identification. We are listing **8** such a names for Hyalospheniids.

***Hyalosphenia baueri* Schonborn**, **Dorfelt, Foissner, Krienitz & Schafer 1999** – fossil from amber, that is why we excluded it from list of valid names.

***Hyalosphenia coogeeana* Playfair 1917**- mistaken identification, description is corresponding to the rotifer.

***Hyalosphenia papilio* f. *multiporifera* Jung 1936**- non valid

Note: It is published in Abh. Landesmus. Provinz. West., Mus. Fur Naturkd. 7: 1-87.

Icon.: Jung 1936, fig.7a,b; De Graaf 1956, fig. 30; Chardez 1963, fig. 6; Gomaa et al. 2014, fig. 1.

It is differing from the type only by the presence of more than 2 pores. As it was shown (Boots and Mayer, Gomaa et al. 2013, Mulot et al. in prep.) the number of pores is not taxonomic criterion and thus we suggest invalidating this infraspecific taxon name.

***Hyalosphenia triquetra* Imhof 1895**- nomen nudum (add the REF)

***Hyalosphenia turfaceae* Taranek 1881**- nomen nudum

Note: Description in R. Böhm. Ges. Wiss. Prog. 229-230.

The test by shape is very similar to *Hyalosphenia elegans.* On the body have a short membrabous keel, like in Nebela carinata. It was found in peatbogs, in Sphagnum mosses.

Original test: "*Die schale ist der H. elegans sehr ähnlich, also knieförmig gegen die Pseudopodienöung durch einen breiten verengert. Der hals ist aber in dem ersten Dritttheil knieförmig geobogen und das hintere Ende der hyalinen Schale trägt eine kurze membranöse Carina, wiche der bei Nebela carinata ähnlich ist. In den Torfmooren auf dem Sphagnum von Wittingau, Wittmanov ect*".

We placed this species in to list of numen nudum taxa , because of insufficient decription, such as size of the species and the luck of the drawing.

***Hyalosphenia undans* Couteaux & Munsch 1978** – mistaken identification, description of rotifer ?

***Nebela labiata* Tarnogradskij 1946-** nomen nudum (add Ref)

Nebela ciliata – nomen nudum

Note: the name is mentioned in Fantham Porter 1945, without the name of the author and the test dimensions.

Synonymous names

We are listing here **102** synonymous names for Hyalospheniid teseta amoebae. Based on comprehensive literature revision for some names synonymous names are suggested (indicated in the brackets).

-***Hyalosphenia lata* Schultze 1875-** current name *Hyalosphenia cuneata* Stein, 1857
-***Hyalosphenia ligata* Tatem 1870**-current name *Hyalosphenia cuneata Stein*, 1857
-***Hyalosphenia tincta* Leidy 1879**- current name *Nebela tincta* (Leidy) sensu Kosakyan et Lara 2013
-***Nebela acuminata* Van Oye 1959 -** current name: *Difflugia acuminate* Ehrenberg 1838
-***Nebela ambigua* Leidy -** current name (suggestion): *Nebela collaris* sensu Kosakyan et Gomaa 2013
-***Nebela antarctica* Grospietsch 1971**- current name : *Argynnia* (ref)
-***Nebela batekensis* Gauthier-Lievre 1957-**current name *Physochila batekensis* Gauthier-Lievre 1957
-***Nebela bipes* Carter -** current name: *Argynnia bipes* (Carter) Murray 1870
-***Nebela bicornes* West 1905-** current name : *Argynnia bipes* (Carter) Murray 1870
-***Nebela bursella* Vejdovsky 1882**- current name: *Nebela tincta* (Leidy) sensu Kosakyan et Lara 2012
-***Nebela bursella* var. *rotunda* Penard 1890**- current name (suggestion): *Nebela rotunda* Penard 1980
-***Nebela bohemica* Taránek, 1882**- current name: *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela bohemica* var. *adelia* Decloitre 1977**- current name (suggestion): *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela ertli* Laminger 1973**- current name: *Argynnia* (ref)
-***Nebela caudata* Deflandre 1936** (misspelled also as *Nebela cavdata*) – current name: *Argynnia caudata* Leidy 1879

-***Nebela circulata*** **Bartosh 1963**- current name : *Argynnia* (ref)
-***Nebela collaris*** **var. *retorta* (ref)** - current name: *Argynnia retorta* (ref)
-***Nebela columbiana*** Wailes 1925- current name: *Arginnia*
-***Nebela columbiana*** **var. *ivorensis*** Gauthier-Lievre (ref) -current name: *Arginnia* (ref)
-***Nebela columbiana*** **var. *minor*** Laminger- current name: *Argynnia* (ref)
-***Nebela cordiformis*** (Heinis 1914) Jung 1942-current name: *Padaungiella cordiformis (*Heinis 1914) Lara et Todorov 2011
-***Nebela corniculata*** Jung 1942- current name: *Physochilla* (ref)
-***Nebela cratera*** **Wailes 1912-** current name: *Physochilla* (ref)
-***Nebela crenulata*** **Cash 1891**- current name: *Argyynia dentistoma*?
-***Nebela dentata*** **(Lepsi) Godeanu (year)**- current name: *Cyphoderia dentata* Lepsi 1957
-***Nebela denticulata*** **Chattopadhyay & Das 2003-** current name: *Argynnia*?
-***Nebela dentistoma*** **Penard 1890** – current name: *Argynnia dentistoma*
-***Nebela dentistoma*** **var. *lageniformis*** **Playfair 1918** – current name: *Argynnia dentistoma*
-***Nebela dentistoma*** **var. *major*** **Grospietsch** – current name: *Argynnia dentistoma*
-***Nebela dentistoma*** **var. *oblonga*** **Gauthier-Lièvre** – current name: *Argynnia dentistoma*
-***Nebela digitiformis*** **Vucetich 1973**- *Nebela barbata* var . *psilonata*?
-***Nebela duttoni*** **Gericke 1932-** current name (suggestion): *Nebela tincta* (Leidy) sensu Kosakyan et Lara 2013
Note: The species was found in South Africa, and was proposed as a new species. Gericke (1932) in his original description mentioned that species very similar to *N. collaris*, but differs from it in having the mouth surrounded by a thickened collar. Kosakyan et al. (2013) conducted study on *Nebela collaris s.l.* group using detailed morphological and molecular data. The original description of *N. duttoni* completely correspond to description of *N. tinca* (Leidy) sensu Kosakyan et Lara (also see fig. 2 C,E in Kosakyan et al. 2013). Thus we suggest to synonymies *N. duttoni* Gericke 1932 with N. tinca (Leidy) sensu Kosakyan et Lara.
-***Nebela ertli*** **Laminger 1973**- current name: *Argynnia* (ref)
-***Nebela galeata*** **var. *orbicularis*** **f. *minor*** **Tarnogradskij 1959**- current name (suggestion): *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela gauthier-lievri*** **Stepanek 1963**- current name (suggestion): *Physochilla*
-***Nebela globulosa*** **Štěpánek, 1963**- current name: *Difflugia globulosa*?
-***Nebela gertrudiana*** **Jung 1942**- current name: *Argynnia* (ref)
-***Nebela goudinii*** **Gericke 1932**- current name (suggestion): *Apodera vas* (Certes) Loeblich & Tappan 1961.
Note: The description of this species is totally corresponding to the one of *Apodera vas*. The only difference is the size of the test (L/B=120-138 /62-67µm in *Nebela goudinii* vs. L/B=130-170 /55-103µm in *Apodera vas*), which can easily overlap. That is why we suggest to synonymies *N. goudinii* with *A. vas*.
-***Nebela griseola*** **Penard 1911**- current name: *Physochila* (ref)
-***Nebela hesperia*** **Wailes 1913**- current name: *Argynnia dentistoma* var . *hesperia*

-***Nebela himalayana*** **Chattopadhyay & Das 2003** – current name (suggestion) *Padaungiella himalayana*
-***Nebela intermedia*** (ref) – current name: *Argynnia* (ref)
-***Nebela japonica*** **Bobrov et Shimano 2011** – current name (suggestion) : *Porosia japonica*
-***Nebela kundulungui*** **Oye 1959**- current name: *Argynnia* (ref)
Nebela kivuense **Gauthier-Lievre-Thomas**- current name: *Quadrulella symmetrica* var . *kivuensis* Van Oye 1958
-***Nebela lacustris*** **(ref)** - current name: *Argynnia dentistoma* var. *lacustris* (ref)
-***Nebela lageniformis*** **Penard 1890**-current name *P. lageniformis* (Penard 1890) Lara et Todorov 2011
-***Nebela lageniformis*** **var.** ***cordiformis*** **Heinis 1914**- current name: *Padaungiella cordiformis* Lara et Todorov 2012
-***Nebela lageniformis*** **var.** ***minor*** **Wailes 1912** – current name (suggestion):- -*Padaungiella wailesi* (Deflandre 1936) Lara et Todorov 2012
-***Nebela lobostoma*** **Stepanek**- current name: *Netzelia*?
-***Nebela longicollis*** **Penard 1890**- current name: *Padaungiella longicollis* (Penard 1890) Lara et Todorov 2011
-***Nebela longitubulata*** **Gautier-Lièvre 1953**- current name: *Padaungiella longitubulata* (Gautier-Lièvre, 1953) Lara et Todorov 2011
-***Nebela minor*** **Penard 1902**- current name: *Nebela tincta* (Leidy) Kosakyan et Lara 2013
-***Nebela nebeloides*** **Gautier-Lièvre 1958**- current name: -*Padaungiella nebeloides* (Gautier-Lièvre, 1958) Lara et Todorov 2011
-***Nebela numata*** Leidy 1874- current name: *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela parvula*** **Cash 1909**- current name: *Nebela tincta* (Leidy) Kosakyan et Lara 2013
-***Nebela petricola*** **del Pilar Gracia** – current name: *Heleopera petricola* (typographic error)
-***Nebela playfairi*** **Jung 1942?**- current name: *Argynnia* (ref)
-***Nebela playfairi*** **var.** ***elongata*** Grospietsch- current name: *Argynnia* (ref)
-***Nebela playfairi*** **var.** ***lata*** Grospietsch- current name: *Argynnia* (ref)
-***Nebela podzolica*** **Korganova 1981**- current name: *Schoenbornia humicola* Schönborn 1987
-***Nebela pulcherrima*** **Awerintzew 1907**- current name (suggestion): *Padaungiella pulcherrima*
-***Nebela pusilla*** **Vucetich 1973**- current name: *Argynnia* (ref)
-***Nebela rampii*** **Stepanek 1963**- *Argynnia*?
-***Nebela repanda*** **Jung**- *Argynnia*?
-***Nebela retorta*** **Leidy 1879**-*Argynnia retorta* (Leidy) Stepanek 1953
-***Nebela scotica*** **Brown 1911**- current name: *Argynnia* (ref)
-***Nebela silesiaca*** **Kotulla 1936**-current name: *Physochila* (ref)
-***Nebela similis*** **Vucetich 1973**-current name: *Argynnia* (ref)

-***Nebela sphagnophila*** **Van Oye**- current name: *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela spicata*** **Wailes 1913**- current: *Argynnia*
-***Nebela subsphaerica*** **Van Oye 1956**- current name (suggestion): *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela schwabei*** **Jung 1942**- current name: *Argynnia* (ref)
-***Nebela tenella*** **Jung 1936** – current name: *Physochila* (ref)
-***Nebela teres*** **Jung 1942**- current name: *Argynnia* (ref)
-***Nebela tincta*** **var. *grandis*** Bunescu & Matic 1982 – current name (suggestion): *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela tincta*** **var. *major*** **Deflandre 1936**- current name: *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela tincta*** **var. *rotunda*** **Penard 1890**- current name: *Nebela rotunda* Penard 1890
-***Nebela tincta*** **f. *stenostom*a Jung 1936**- current name: *Nebela collaris* (Ehrenberg 1848) sensu Kosakyan et Gomaa 2013
-***Nebela triangulata*** **(Leidy) Cash 1909**- current name: *Argynnia bipes* (Carter) Murray 1870
-***Nebela triangulate*** **var. *senegalensis*** **Gauthier-Lièvre** - current name: *Argynnia ?*
-***Nebela triangulala*** **Lang 1865**- *Argynnia triangulala* Cash 1909
-***Nebela tropica***- current name: *Quadrulella tropica* Wailis1912
-***Nebela tubulata*** **Brown 1911-** current name (suggestion): *Padaungiella tubulata*
-***Nebela tubulata*** **var. *spatha*** **Thomas 1960**- current name (suggestion): *Padaungiella tubulata* var. *spatha*
-***Nebela tylophora*** **Jung 1942** – current name: *Argynnia* (ref)
-***Nebela varia*** **Decloitre 1966**- current name (suggestion) : *Padaungiella*
-***Nebela vitraea*** **Penard 1899**-current name: *Argynnia* (ref)
-***Nebela vitraea*** **var. *elongata*** **Gauthier-Lièvre** -current name: *Argynnia* (ref)
-***Nebela wailesi*** **Deflandre 1936**- current name: *Padaungiella wailesi* (Deflandre 1936) Lara and Todorov 2011
-***Nebela wailesi*** **var. *magna*** **van Oye 1956**- current name (suggestion): *Padaungiella wailesi* var. *magna*
-***Nebela wellingtonia*** **Decloitre 1964 -**current name (suggestion): *Apodera wellingtonia*
-***Nebela wetekampi*** **Jung 1942-** current name: -*Padaungiella wetekampi* (Jung 1942) Lara and Todorov 2011
-***Quadrulella globosa***-current name: *Paraquadrulla* (ref)
-***Quadrulella irregularis***- current name: *Paraquadrulla* (ref)
Quadrulella symmetrica Wallich **var. *irregularis*** Penard- current name: *Paraquadrulla* (ref)

Some names need discussion (or waiting for original description) prior to include them to any section

-*Nebela wailesi* var. *obliqua* Bunescu et Matic 1982 (missing ref)

-*Nebela militaris* var. *penardiana* (mentioned in del Pilar Gracia, no description)

- *Nebela leidyana* Vejdovsky (missing ref)

-*Nebela fabulosa* Sudzuki 1965 (missing ref)

- *Nebela complanata* Levander 1900 (missing ref)

- *Nebela carinata* var. *brevicarinata* Jung (could it be syn. for *N. margianata*?)

-*Nebela vas* var. *longicollis* Grospietsch (could it be syn. with A. vas f. reticolaris?)

-*Nebela vas* var. *obliqua* Grospietsch (could it be syn. with A. crenata?)

- *Nebela americana* var. *bryophila* Van Oye 1956 (without description?)

-*Nebela lageniformis* var. *elegans* Stepanek 1963 (non Hyalospheniid structure, what it could be?)

- *Nebela patagonica* Vucetich 1975 (missing ref)

Discussion and general conclusions

Testate amoebae diversity is underestimated,

case study: Family Hyalospheniidae

In this thesis we used the family Hyalospheniidae to illustrate a clear example of underestimated diversity in protist groups. The shape and the structure of the test in hyalospheniids allow a much easier identification of morphospecies than in other protist groups. However the main taxonomic characters used to delimit species are still unclear, and thus the true diversity within this group, similarly to other protistan groups remains still uncertain.

Chapters 1, 2 and 3 present the hyalospheniid diversity at the species level study using both morphological and molecular data. We used both LM and SEM to carefully analyse the ultrastructure of each test used in the study. Our molecular data were based on Cytochrome Oxidase Subunit 1 (COI) sequences, which successfully discriminated closely related species within hyalospheniids and appeared to be suitable barcoding marker for this group. Our data illustrate that the hyalospheniid diversity is much higher than previously recognized. Cryptic species (i.e. that have identical morphology, but are genetically divergent)/pseudocryptic species (i.e. that differ only in small ultrastructural details and are genetically different) diversity was revealed in hyalospheniids as also in other groups of protists such as foraminiferans, dinoflagellates and euglyphids (Darling et al., 2004; Ellegaard et al., 2008; Heger et al., 2011).

Therefore, a revision of hyalospheniid diversity in the light of this study had to be made. The position of many species and groups within the family was redefined.

One new genus *Padaungiella* Lara et Todorov, and four novel species *Nebela aliciae* Mitchell et Lara, *Nebela guttata* Kosakyan et Lara, *Nebela meisterfeldi* Heger et Mitchell, *Nebela pechorensis* Kosakyan et Mitchell were described. Additionally at the time of the

writing of this thesis we could describe at least 3 new species of *Quadrulella* (see Chapter 3, work in progress).

Cryptic/ pseudocryptic speciation within Hyalospheniids

COI data allowed the assessment of cryptic/pseudocryptic diversity within family Hyalospheniidae. The examples of possible cryptic/ pseudocryptic speciation are discussed in the Chapter 1 (the cases of *Nebela carinata* and *Hyalosphenia papilio*), in Chaper 2 (the case of *Nebela guttata* and *N. pechorensis*), in the Chapter 3 (case of *Quadrulella symmetrica* clade B), and in Chapter 4 (case of Unknown sp. 1 and Unknown sp. 2 in *N. collaris s.l.* group).

The question of the existence of cryptic/pseudocryptic species implies that these taxa have been lumped in the past, blurring potential biogeogeographical or niche driven patterns, or both. Encountered mitochondrial haplotypes can be either randomly distributed across ecosystems or world regions (and then haplotypes are the product of genetic drift), or can be correlated with geographic distance or physicochemical parameters, suggesting limited dispersal or local adaptation/speciation. A nice example is the study of Heger et al. 2013 revealing a large genetic diversity within *Hyalosphenia papilio* morphospecies (12 distinct genetic linages corresponding to single morphotype). This study also presents the influence of climatic and spatial factors on the genetic structure of *H. papilio* morphospecies, and suggests that the distribution patterns of *H. papilio* genetic lineages in the Northern Hemisphere are more influenced by climatic conditions than by dispersal limitations.

In my thesis, Appendix I clearly demonstrates that similar, cryptic/pseudocryptic species occupy different ecological niches. This suggests that these organisms have differential adaptations to environmental parameters, moisture being the most influential in the case of the members of the *Nebela collaris* complex. Possibly, a stabilizing selective pressure prevented tests from changing shape fast in evolution. Nevertheless, the different

"molecular species" have non-random distribution in the different microhabitats of the bog, suggesting genetically determined adaptations ("ecological species).

Alternatively, it is always interesting to conduct correlation analyses considering the influence of environmental factures such as vegetation, community composition, as well as soil chemical composition on genetic lineages. In fact, as it was mentioned before, this is one of the ongoing tasks of the studies presented in Chapter 3 and Appendix I.

Species complexes within Hyalospheniids

Traditional taxonomy grouped all the problematic species having a very similar look and overlapping morphological characters into so called "species complexes". Fortunately, new molecular techniques and powerful microscopes allow documenting features that would not have been noticed before. When discriminating characters are indeed present, a combination of microscopic and molecular approaches is optimal to identify them and allow visual separation of the organisms. A good example is given in chapter 4 with the similar looking *N. guttata* and *N. pechorensis* that can be differenciated based on the shape of their pseudostome (straight or curved) a detail that would have been systematically overlooked.

In this thesis we studied two species complexes within family Hyalospehiidae: already known *Nebela collaris s.l.* species complex (see Chapter 2) and newly defined *Quadrulella symmetrica s.l.* species complex (see Chapter 3).

We show that the small variations in the test morphology that were overlooked by traditional taxonomy correspond to distinct genotypes. We have defined the main characters discriminating the taxa within these groups: the size and shape of the test, neck and the aperture in *Nebela collaris s.l.* complex; and the size of the tests and siliceous shell plates in the *Q. symmetrica s.l.* complex. The taxonomy of these two groups were redefined based on combined morphological and molecular data and several new species were described (see in the section of discussion "Testate amoebae diversity is underestimated, case study: Family Hyalosphenidae").

Application of accurate biodiversity data in ecological studies: does closely related species have the same ecological preferences?

Accurate estimation of microbial biodiversity is one of the major tasks that occupy biologists since the beginning of the century. Environmental DNA sequencing approach was introduced to estimate microbial diversity without a cultivation step or microscopic observation using suitable group-barcoding genes (Pawlowski et al., 2012). However this approach is considered to be partially informative, because of methodological biases consisting, amongst others, on systematic flaws in the quantitative aspects, because of different gene content of the cells. In Chapter 4 we showed that COI was a suitable barcoding marker to estimate these biases. Our results demonstrated that, when surveying mitochondrial genes within a limited group, a correction of the proportion of clones by the biovolume of the organisms gives more accurate results as compared with microscopic counts.

We used this approach to correlate the presence of morphologically similar species and demonstrate different niche occupation, a task that can only be achieved if relative abundance data are available. The preliminary results summarised in the Chapter 5 shows how we addressed this approach helping fast estimation of species diversity in *Nebela collaris s.l* complex group and to highlight individual ecological preferences of its members.

Combination of molecular and morphological date

Overall, the main goal of my thesis is to address critical questions concerning the diversity of protists using hyalospheniid testate amoebae as a model group. The solution lies at the convergence of two approaches often considered as conflicting, but yet complementary:

1) Traditional taxonomy based on morphological observation- most often, specific diversity has been underestimated in Hyalospheniids. As illustrated in Chapter 2, each morphospecies was actually hiding a series of similar-looking species, impossible to distinguish from each other without an *a priori* molecular study.

2) Molecular approaches- introduction of molecular tools revolutionised our understanding of molecular diversity. However, blind sequencing cannot answer to ecological questions without a proper characterisation of the biological entities present –a task that still requires "traditional naturalist activities" such as species and environment description.

Either way, the progress in this field should be attributed to the accumulation of new data (combing both: 1. morphological studies with expanded sampling and 2. molecular studies based on several markers) as well as the maintenance of one of the most important principles of systematics: ***the totality of traits and combinations of characters is always more important than one trait or one character alone – no matter how important this sole character seems to be (Zmitrovich and Wasser, 2011).***

Why a monograph on Hyalospheniidae ?- An accurate and complete taxonomical survey of the group as grassroots for any further application to any taxonomic group in any research field

After discussing limitations of taxonomy and systematics in hyalospheniid testate amoebae, the need for comprehensive revision of the taxonomy of the family became obvious.

The need of this kind of study is discussed in many papers (Gilbert et al., 2003; Mitchell and Meisterfeld, 2005; Mitchell et al., 2008; Booth and Meyers, 2010), however considering the amount of the work and the huge time investment, it is often being postponed and then never accomplished.

With the monograph "Family Hyalospheniidae" we are aimed to conduct a comprehensive revision of the taxonomy and systematics of the family combing all the possible detailed morphological, molecular and the ecological data on these organisms, including also ancient literature. We believe that this will be significant scientific contribution, since such a work was clearly missing, an evident obstacle for scientists that kept research on.

The monograph is still in preparation: user-friendly taxonomic keys, improved descriptions of each species, as well as helpful notes on their ecology, geographical distribution and taxonomy, the list of possible synonymous names, notes on existing molecular data, the detailed plates containing LM, SEM and original sketches for each taxa will be provided.

The book is designed to be of benefit to protistologists and taxonomists and will hopefully build a database that will allow to make Hyalospheniidae an attractive subject of interest for research in (palaeo)ecology, microbial diversity and evolution but also symbiosis or cell biology investigation.

With this monograph we aim to start a series of books revising the larger groups of testate amoebae, and hope also to stimulate similar efforts in other groups to make testate amoeba species identification easier, more accurate, and improve their utility as model organisms in both fundamental and applied aspects.

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Appendix I

Cryptic testate amoebae species occupy different realised ecological niches in a peatland: the case of the *Nebela collaris* complex.

Paper in preparation, it will be submitted to ISME

Cryptic testate amoebae species occupy different realised ecological niches in a peatland: the case of the *Nebela collaris* complex.

David Singer, Anush Kosakyan, Matthieu Mulot, Edward A.D. Mitchell, Enrique Lara

Laboratory of Soil Biology, University of Neuchâtel, Switzeralnd

Abstract

Protists that share a similar morphology but that are genetically well distinct are a common case; however, the question of their divergent ecology remains of major interest in bioindication studies. Testate amoebae of the *Nebela collaris* complex are frequent inhabitants of peatlands and comprise at least seven different genetic species defined on their COI gene sequences (mitochondrial cytochrome oxidase, subunit I). We studied their species composition in five different micro-habitats (hummock, lawn, forest, fen and peatland border) in a peat bog in Switzerland (Le Cachot, NE). We applied a protocol for specific amplification of COI gene of this group to *Sphagnum* DNA extractions and cloned the PCR products. Sequence analysis revealed four of the seven barcoded species, plus two whose morphology is still unknown. Species ranged from generalists found in most of habitats (like *N. collaris*) to specialists, encountered only but pervasively in particular habitats (like *N. rotunda* in forests). Our study suggests that different genetic species have divergent ecological niches and should be considered differently when used for bioindication purposes.

Key word: Protists, DNA Barcoding, communities, moisture, Ecological niches, cryptic species

Introduction

Free-living protists have a major impact on the global nutriment cycling (Adl and Gupta 2006, Dawson and Hamner 2008). But a large part of the diversity is not yet documented (Piganeau et al 2011) and the existing descriptions are frequently imprecise (Caron and Countway 2009). Cryptic genetic diversity (i.e. organisms that share an identical morphology but are genetically divergent) increase the observed molecular diversity in the environment (Hausmann et al 2006). It is however unclear to which extent these cryptic species play a similar role in the environment, and remains still debated within the scientific community. From one hand, some authors argue that a large part of the molecular diversity is the mere product of genetic drift, and thus does not reflect any change in the ecology (Fenchel 2005). To the contrary, some studies show a correlation between genotypes and ecological niches in planktonic foraminiferans (de Vargas et al 2000). The function of cryptic species of microeukaryotes are still poorly studied and could have a strong influence in the habitat of such organisms. These limitations explain the very few studies about the ecology of protists(Wilkinson et al 2012).

The *Nebela collaris* species complex is a monophyletic group often used in paleoecology studies (Booth 2001). It comprises some species that are morphologically and genetically distinct such as *N. flabellulum* or *N. aliciae*, but most of them are very difficult or impossible to identify morphologically (Kosakyan et al 2013). As a consequence, they were often pooled together in various studies. All these species live apparently in sympatry, and it is still questionable how this diversity arose. The possible existence of an ecological separation of members of this species complex was suggested by (Väliranta et al 2012), who showed a multimodal distribution of *N. collaris sensu lato* (called *N. tincta*) in a moisture gradient (water table depth) in a *Sphagnum* dominated peatland.

Water table depth is considered classically as the variable that affects most strongly diversity in peatlands. Indeed, the distribution of *Sphagnum* species is chiefly influenced by this particular environmental parameter (Bragazza 1997). Vascular plant communities (Strack et al 2006) follow also that trend, and it has been shown that general testate amoeba communities follow also the same pattern (Marcisz et al 2014). We expect therefore that, if

cryptic species of *N.collaris* species complex have to follow a given pattern, it will be most likely correlated to water table depth.

We investigated the diversity of species from the *Nebela collaris s.l.* from five different micro-habitats from the peatland of Le Cachot (Swiss Jura Mountains) using the molecular tools that were developed in Chapter 4 for species discrimination and quantification. We surveyed different microhabitats (fen, lawn, hummock, pine forest and peatland border), and compared species composition with water table depth.

Materials and methods

Sampling and DNA extraction

Testate amoebae were extracted from *Sphagnum* ssp. samples collected from three sampling sites from five different microhabitats (Fen, Lawn, Forest, Hummock and Peatland border) from the peatland of "Le Cachot" (Jura Mountains, Switzerland, Co-ordinate: 47.00°N 6.39°E). Water table depth was measured in each station. Testate amoeba cells were extracted from 20 g of fresh *Sphagnum* and concentrated by sieving (150µm); the resulting fraction was observed by light microscopy to confirm the presence/absence of the cells of the *Nebela collaris* complex and the filtrate are used for global DNA extraction with a MoBio Power Soil® DNA Isolation kit.

DNA amplification and cloning

We amplified a fragment of the mitochondrial COI gene by using the general primer LCO (Folmer 1994) and a specific primer TINCOX (CCATTCKATAHCCHGGAAATTTC) following the same protocol recommended for the amplification of *Nebela collaris* s.l. species (Kosakyan et al 2012). PCR steps consist of a 5 min initial denaturation step in a 40 cycles program 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 amplicons were cloned into pCR2.1 Topo TA cloning vector and transformed into *E.coli* TOP10' One Shots cells (Invitrogen kit) according to the manufacturer's instructions. 50 inserts per PCR product were amplified with M13f, M13R primers.Sequencing was carried out using a BigDye197 Terminator Cycle Sequencing Ready

Reaction Kit (Applied Biosystems) and analyzed with a ABI-3130XL DNA sequencer (Applied Biosystems). COI sequences are deposited in GenBank with the following accession numbers XX-XX (accession numbers will be added after paper acceptance).

Statistical analyses

The data set used for statistical analysis comprised 386 sequences of COI (390bp). Sequences were manually aligned using BioEdit v. 7.1.11 (Hall 1999). A phylogenetic tree was constructed using MEGA 5.0 software (Tamura et al. 2011) with a maximum likehood test and a Jukes-Cantors model (results not shown). This tree was used to infer the phylogenetic position of the clonal sequences obtained; we inferred the proportions of the different cells based on calculations as recommended in Chapter 4. We then performed a PCA and clustering analyses to determine the association between the encountered species and the different environments using the R package "vegan" (Oksanen et al. 2013).

Results

We sequenced a total of 386 clones from 9 clone libraries, resulting in a total of six different haplotypes distributed between all samples. These haplotypes corresponded to four out of the five species commonly encountered in Swiss peatlands as described in (Kosakyan et al. 2013); the only species that has not been found was *N. pechorensis*. We also found two probable cryptic species that were also found in Chapter 4, called there New clade 1 and 2. These mitochondrial haplotypes were not distributed evenly across the samples; a cluster analysis based on Jaccard dissimilarity (Fig 1) shows that different environments cluster logically together. The same analysis showed that the wettest (lawn, fen) and driest (hummock, forest, peatland border) microhabitats clustered together, suggesting an effect due to wetness. Likewise our principal component analysis also grouped microhabitats clearly together. It also revealed that some species (like *N. tincta*) were associated with dryer conditions whereas others (*N. collaris*) were correlated with low water table depth. It seems however that other environmental parameters were also relevant, as *N. rotunda* was consistently associated with samples originating from the Pinus forest (Figure 2).

Bar plots representing communities further illustrated the distribution of the different haplotypes. Here, *N. collaris* is the only species detected in (wet) fens and the most abundant in lawns. Its abundance decreases in the forest and hummocks, and is totally absent in the peatland border. Conversely, *N. tincta* is not detected in the wettest sites, and distribution with *N. collaris* overlaps only in the forest (Fig. 3). Maximum diversity was encountered in forest environments, where *N. rotunda* appears as well. These patterns were even more marked when the biovolume corrective factor was introduced (Fig 3, see Chapter 4).

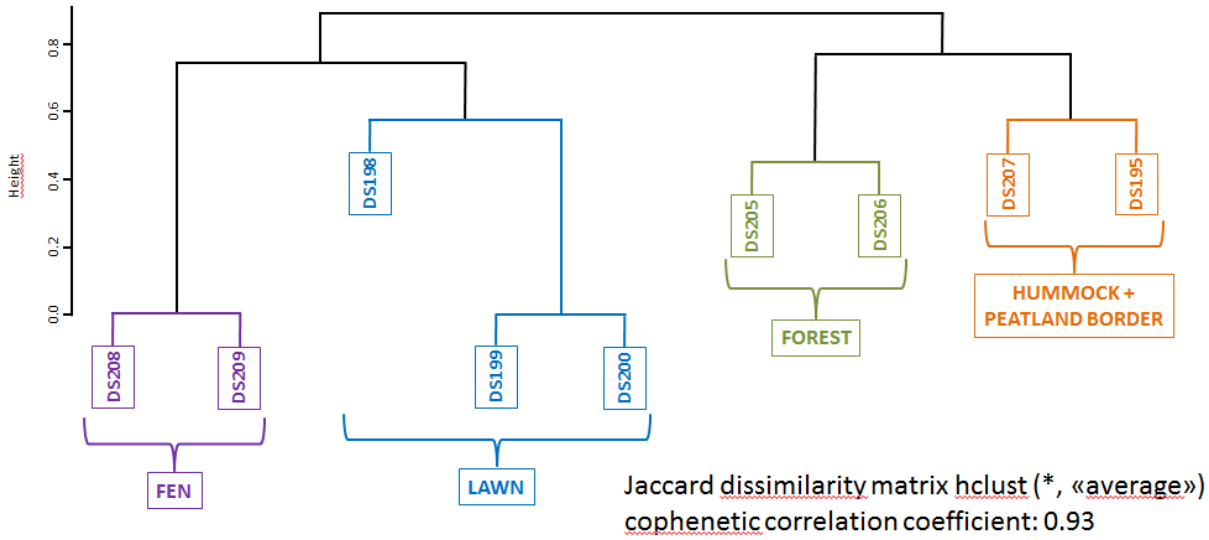

Figure 1. Cluster dendrogram built on Jaccard dissimilarity matrix of the *Nebela* communities.

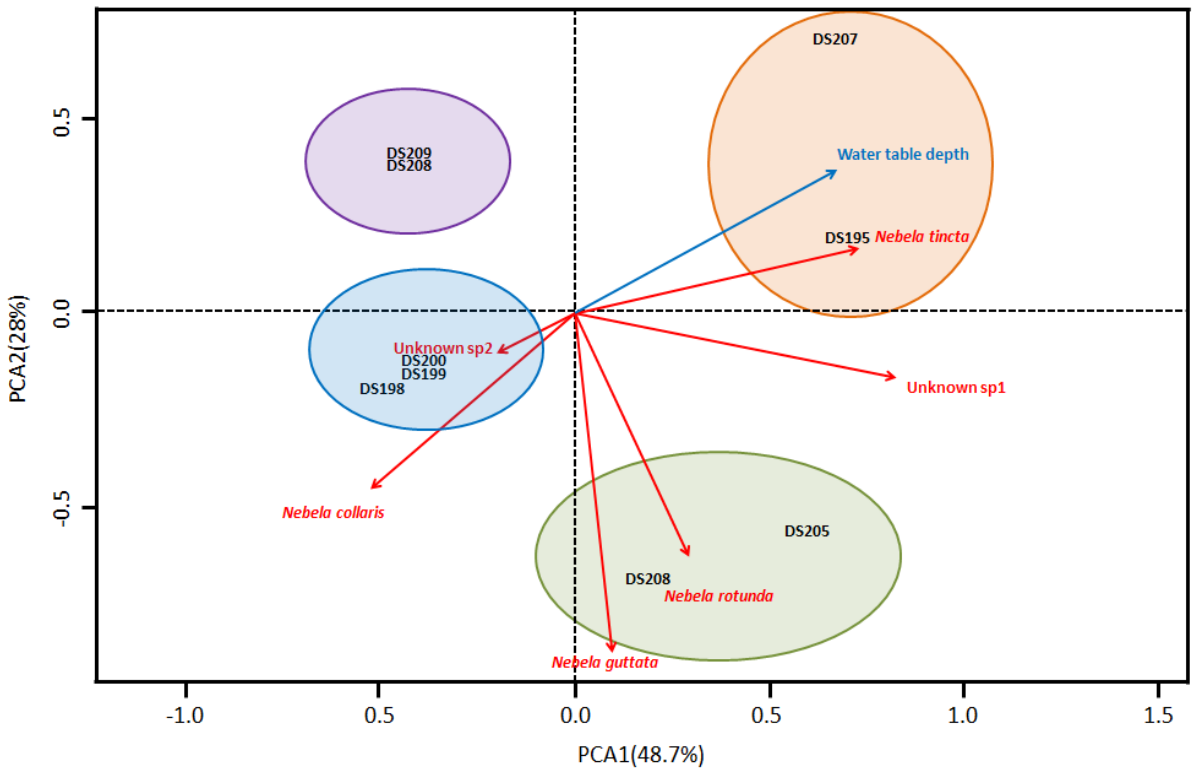

Figure 2. Principal component analysis (PCA) of species and samples with projection of the water table depth. The colours correspond to the following environments: violet-fen, blue-lawn, green-forest, orange- hummock.

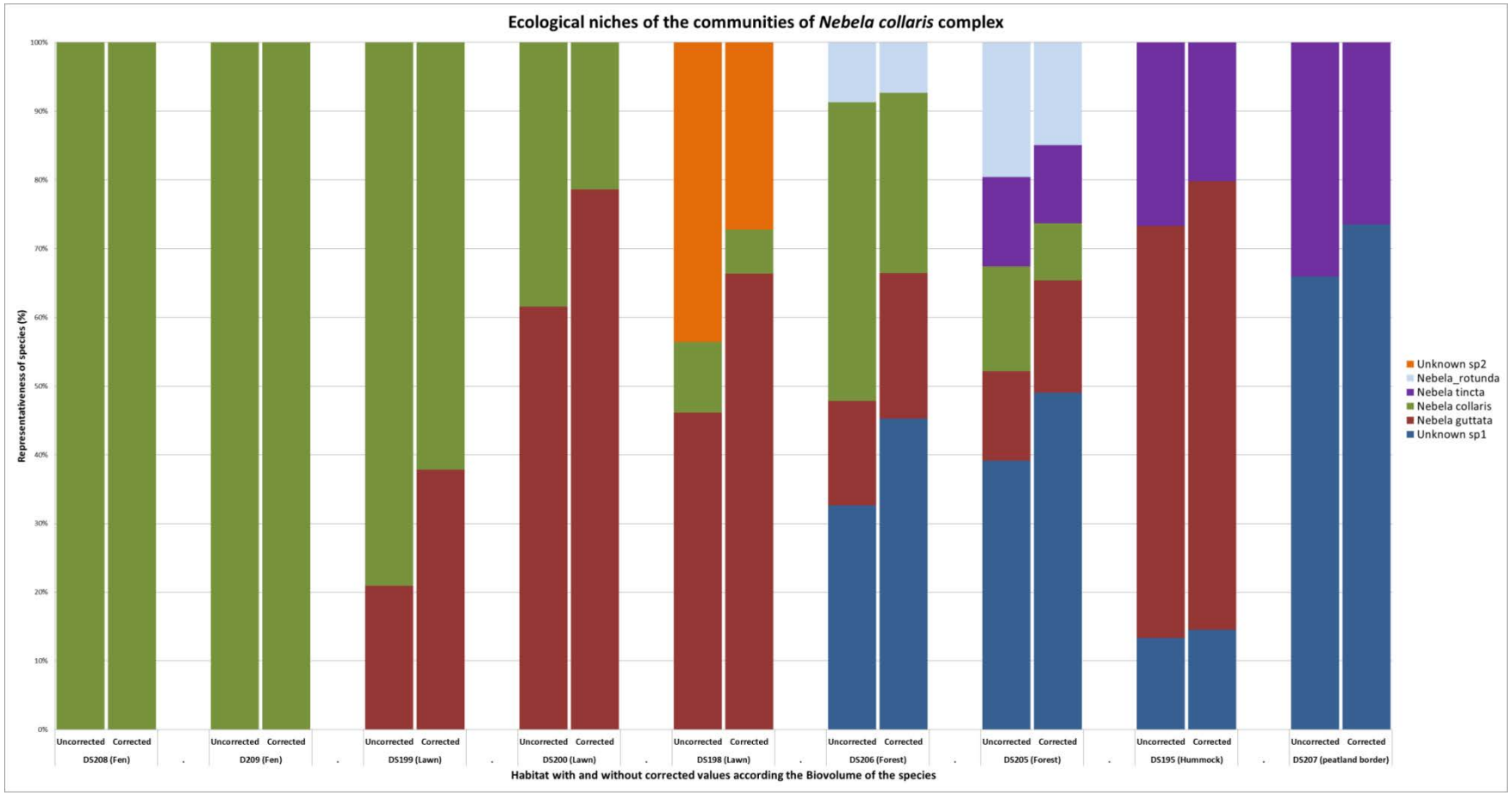

Figure 3. Ecological niches of *Nebela collaris* complex S.l. The uncorrected column correspond at the relative ratio of the numbers of species sequences. The corrected column corresponds to the relative ratio corrected with the biovolume of the cells (kosakyan, in press). The unknown sp correspond to the new clade describe by Kosakyan (2012)

Discussion and further developments

Our results confirm that the different haplotypes (corresponding to molecular species) are distributed unevenly in the microhabitats, seem to show different optima with respect to water table depth. This concerns identified species as well as the two unidentified (and most likely cryptic) ones; Undetermined species 1 seems to inhabit preferentially drier habitats, following the trend of *Nebela tincta*; it has been suggested that it is morphologically similar to *N. guttata* (Chapter 4).

Other factors than water table depth seem to play also a role: *N. rotunda* was found indeed only in the forest, and was present in both samples. It might be that this species is more abundant there, as the presence of trees changes the conditions locally as they mitigate UV radiation and high temperatures, and release needles. Forest environments also host a higher richness, as it has an intermediate position in the wetness gradient, thus hosting species that are found in both extremes of the gradient (i.e. *N. tincta* and *N. collaris*). However, we are only witnessing realized niches and not effective ecological optima of the different species. Competition can also be a factor that plays a major role in species distribution. In order to confirm our hypotheses that are built on a single peat bog and on a limited number of samples, we plan to add data from another site from the Swiss Jura Mountains: Praz-Rodet, (46.33°N, 6.10°E). Likewise, in order to better understand other potential environmental variables influence, we will add the C/N ratio, a measure of nutrient turnover efficiency and integrate them into our models.

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Appendix II

One alga to rule them all: Unrelated mixotrophic testate amoebae (Amoebozoa, Rhizaria and Stramenophiles) 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)

Fatma Gomaa[a,b,1], Anush Kosakyan[a], Thierry J. Heger[c], Daniele Corsaro[a], Edward A.D. Mitchell[a], and Enrique Lara[a,1]

[a]Laboratory of Soil Biology, University of Neuchâtel, Rue Emile-Argand 11, CH-2000, Switzerland
[b]Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt
[c]Departments of Botany and Zoology, University of British Columbia, Vancouver, BC, Canada

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 green endosymbionts in four obligate mixotrophic testate amoeba species belonging to three major eukaryotic clades, *Hyalosphenia papilio* and *Heleopera sphagni* (Amoebozoa: Arcellinida), *Placocista spinosa* (Rhizaria: Euglyphida), and *Archerella flavum* (Stramenopiles: Labyrinthulomycetes) based on *rbcL* (ribulose-1,5-diphosphate carboxylase/oxygenase large subunit) gene sequences. We further investigated whether there were different phylotypes of algal endosymbionts within single *H. papilio* cells and the degree of host-symbiont specificity by amplifying two genes: COI (mitochondrial cytochrome oxydase subunit 1) 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 several free-living algae. Most *rbcL* gene sequences derived from symbionts from all testate amoeba species were almost identical (at most 3 silent nucleotides difference out of 780 bp) and were assigned to a new Trebouxiophyceae taxon we named TACS (Testate Amoeba *Chlorella* Symbionts). This "one alga fits all mixotrophic testate amoeba" pattern suggests that photosynthetic symbionts have pre-adaptations to endosymbiosis and colonise diverse hosts from a free-living stage.

Key words: Secondary endosymbiosis; peatlands; *Hyalosphenia papilio*; *rbcL* gene; COI gene; co-evolution; *Chlorella*.

[1]Corresponding authors; fax +41 32 718 3001
e-mail fatma.gomaa@gmail.com (F. Gomaa), enrique.lara@unine.ch (E. Lara).

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 and 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 glaucophyte 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 independently 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 nucleus (Figueroa et al. 2009; Nowack and 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, for example in *P.bursaria* (Summerer et al. 2007, 2008).

In a further step, these consortia can become obligatory for the host at least, which can no longer survive in the long run 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 harbour a broad range of algae such as dinoflagellates, diatoms, chlorophytes, chrysophytes, and haptophytes.

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 and Caron 1996, 2001; Gast et al. 2000; Santos et al. 2004; Zoller and Lutzoni 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 environment through a highly selective recognition mechanism (Okamoto and 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 diatom 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 (dinoflagellate) nucleus (Figueroa et al. 2009; Nowack and Melko-nian 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

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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	–	(KJ446794/KJ446843)
2- *Hyalosphenia papilio* (PR)	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 06°10′E	6	(KJ446795/KJ446844)
3- *Hyalosphenia papilio* (PR)	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 06°10′E	8	(KJ446796/KJ446845)
4- *Hyalosphenia papilio* (PR)	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 06°10′E	5	(KJ446797/KJ446846)
5- *Hyalosphenia papilio* (PR)	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 06°10′E	–	(KJ446798/KJ446847)
6- *Hyalosphenia papilio* (PR)	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 06°10′E	5	(KJ446799/KJ446848)
7- *Hyalosphenia papilio* (PR)	Poor fen, small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 06°10′E	–	(KJ446800/KJ446849)
8- *Hyalosphenia papilio* (BD-A)	Submerged *Sphagnum*, Bois-des-lattes bog	Switzerland	46°58′N 06°42′E	8	(KJ446801/KJ446850)
9- *Hyalosphenia papilio* (BD-A)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	–	(KJ446802/KJ446851)
10- *Hyalosphenia papilio* (BD-A)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	6	(KJ446803/KJ446852)

11- *Hyalosphenia papilio* (BD-A)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	5	(KJ446804/KJ446853)
12- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	6	(KJ446805/KJ446854)
13- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	5	(KJ446806/KJ446855)
14- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	–	(KJ446807/KJ446856)
15- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	6	(KJ446808/KJ446857)
16- *Hyalosphenia papilio* (BD-B)	Submerged *Sphagnum*, Bois-des-Lattes bog	Switzerland	46°58′N 06°42′E	–	(KJ446809/KJ446858)
17- *Hyalosphenia papilio* (LC)	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47°05′N 06°04′E	10	(KJ446810/KJ446859)
18- *Hyalosphenia papilio* (LC)	*Sphagnum* mosses, Le Cachot bog, Jura Mountains	Switzerland	47°05′N 06°04′E	10	(KJ446811/KJ446860)
19- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	5	(KJ446812/KJ446861)
20- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	–	(KJ446813/KJ446862)
21- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N; 45°24′E	5	(KJ446814/KJ446863)

Table 1 (*Continued*)

Taxa	Sampling site	Country	Coordinates	No. of *rbcL* clones	GenBank accession number *rbcL*/COI
22- *Hyalosphenia papilio* (PZ-A)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	7	(KJ446815/KJ446864)
23- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	–	(KJ446816/KJ446865)
24- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	–	(KJ446817/KJ446866)
25- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	–	(KJ446818/KJ446867)
26- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	7	(KJ446819/KJ446868)
27- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	6	(KJ446820/KJ446869)
28- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	6	(KJ446821/KJ446870)
29- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	–	(KJ446822/KJ446871)
30- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37′N 45°24′E	–	(KJ446823/KJ446872)

31- *Hyalosphenia papilio* (PZ-B)	Wet *Sphagnum*, Bezimyanoe bog, 15 Km North- East Penza city	Russia	53°37'N 45°24'E	–	(KJ446824/KJ446873)
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	(KJ446825/KJ446874)
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	–	(KJ446826/KJ446875)
34- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	–	(KJ446827/KJ446876)
35- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	6	(KJ446828/KJ446877)
36- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	–	(KJ446829/KJ446878)
37- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	8	(KJ446830/KJ446879)
38- *Hyalosphenia papilio* (AK-C)	*Sphagnum*, rich fen on side of road, *Sphagnum* hummock, Alaska	USA	61°24'N 143°03'W	6	(KJ446831/KJ446880)
39- *Hyalosphenia papilio* (BC)	Wet *Sphagnum*, Allan Creek, South central British Columbia	Canada	52°32'N 119°06'W	6	(KJ446832/KJ446881)

Table 1 (*Continued*)

Taxa	Sampling site	Country	Coordinates	No. of *rbcL* clones	GenBank accession number *rbcL*/COI
40- *Hyalosphenia papilio* (BC)	Wet *Sphagnum*, Allan Creek, South central British Columbia	Canada	52°32′N 119°06′W	5	(KJ446833/KJ446882)
41- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 6°10′E	6	(KJ446834/—)
42- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 6°10′E	5	(KJ446835/—)
43- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 6°10′E	–	(KJ446836/—)
44- *Heleopera sphagni* (PR)	Small pool, submerged *Sphagnum*, Praz-Rodet bog	Switzerland	46°34′N 6°10′E	–	(KJ446837/—)
45- *Placocista spinosa* (PT)	*Sphagnum* mosses, poor fen on the side of Pechora River	Russia	61°55′N 57°54′E	7	(KJ446838/—)

46- *Placocista spinosa* (PT)	*Sphagnum* mosses, poor fen on the side of Pechora River	Russia	61°55′N 57°54′E	5	(KJ446839/—)
47- *Archerella flavum* (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23′N 122°27′W	4	(KJ446840/—)
48- *Archerella flavum* (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23′N 122°27′W	7	(KJ446841/—)
49- *Archerella flavum* (BC)	Brown submerged mosses, West of Duffey Lake, South central British Columbia	Canada	50°23′N, 122°27′W	–	(KJ446842/—)

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

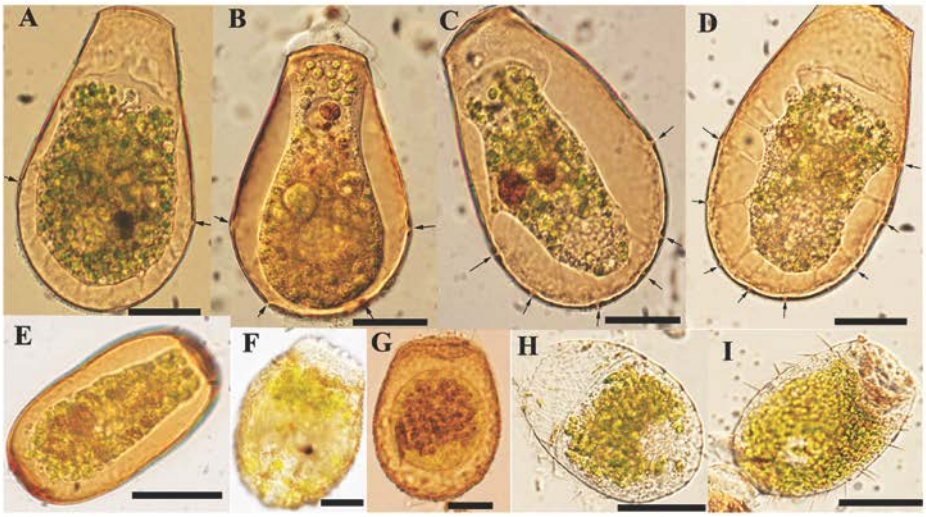

Figure 1. Light microscopy photographs of (**A-D**) *Hyalosphenia papilio*, arrows indicate the number of pores present at the shell edge, (**E**) *Archerella flavum*, (**F**, **G**) *Heleopera sphagni* and (**H**, **I**) *Placocista spinosa*. For all photographs scale bar is 20 μm.

sequences of the clones (5-10 in each cell) were strictly identical within each host cell.

Genetic Characterization of One Host: *Hyalosphenia papilio*

In contrast to the algal symbiont, we observed clear genetic diversity in the studied host (*Hyalosphe-nia papilio* morphospecies): the 40 analysed cells formed five different lineages (here designated as A, C, D, G, and J) as described in Heger et al. (2013) (Fig. 3). Inter-clade genetic variabil-ity ranged between 2.5 and 7%, and the intra-clade variability was below 1%. We did not detect any morphological feature concomitant with assign-ment to one or another clade. For example, the number of pores along the shell margin (Fig. 1, var-ied independently from genetic affiliation. However, the phylogenetic tree suggested a possible pattern of geographical origin (Fig. 3).

Discussion

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

Almost all symbiont's *rbcL* sequences fall within the TACS (Testate Amoeba *Chlorella* Symbionts) clade (Fig. 2). Diversity within TACS (0.3%) is far below the level of genetic divergence usu-ally observed between plant species (i.e. a 1-2% threshold is generally accepted for specific dis-crimination in higher plants (CBOL Plant Working Group 2009; Burgess et al. 2011)). This sug-gests that TACSactually correspond to a single

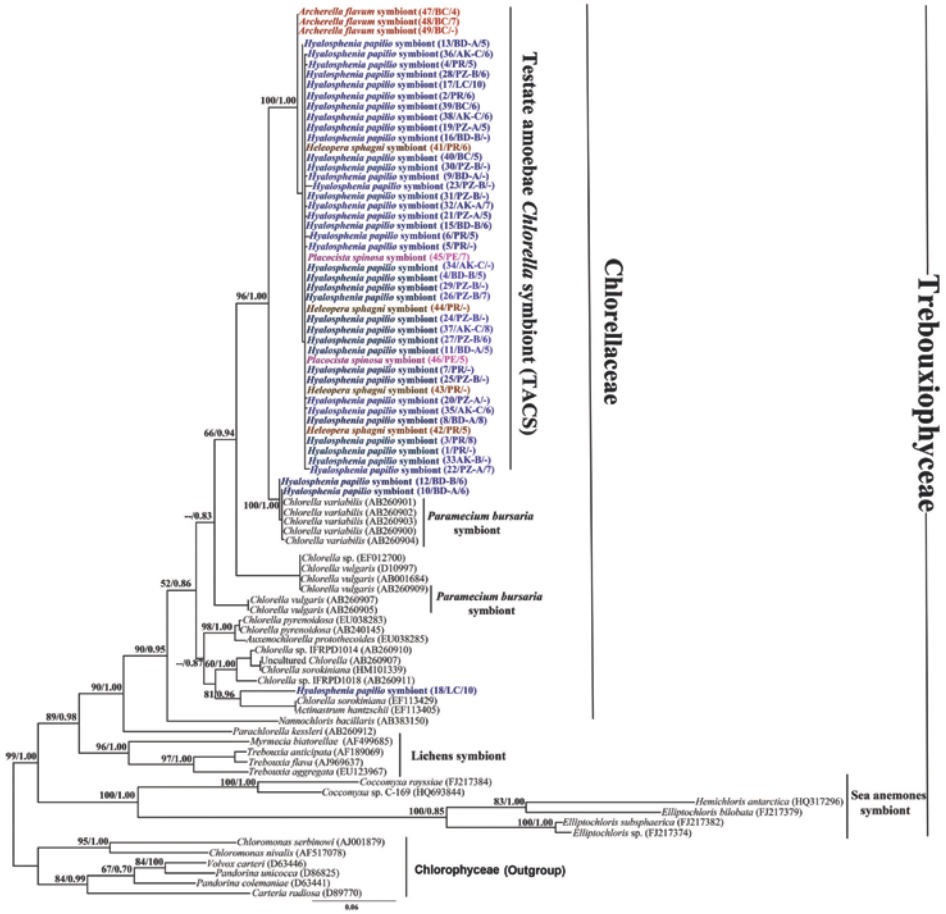

Figure 2. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on the large subunit of the ribulose-bisphosphate carboxylase (*rbcL*) gene sequences obtained from the endosymbionts of four mixotrophic testate amoebae species *Hyalosphenia papilio* (blue), *Archerella flavum* (red), *Heleopera sphagni* (brown) and *Placocista spinosa* (violet). For each sequence we indicated the (number of sample / geographical origin - code of the *Sphagnum* samples where applicable / number of *rbcL* 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.

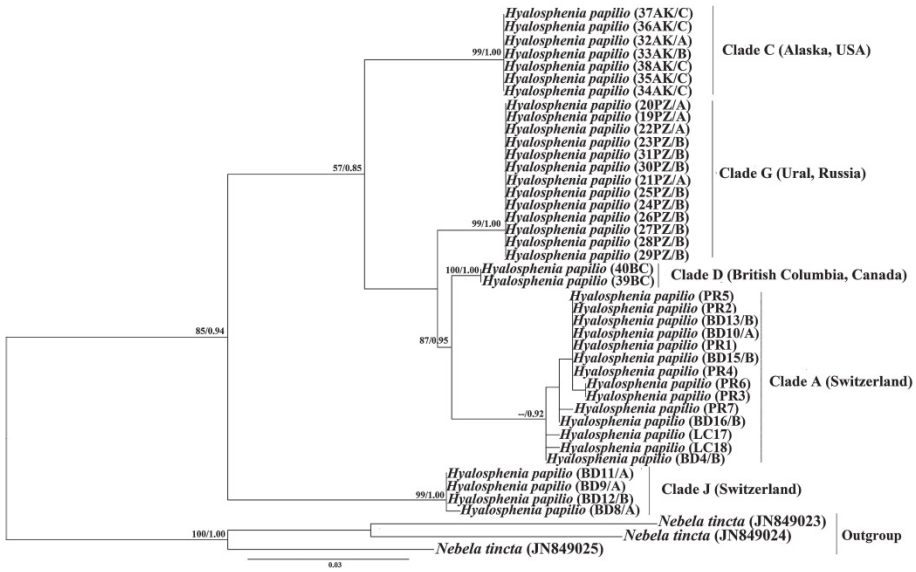

Figure 3. Molecular phylogenetic tree inferred from both maximum likelihood and Bayesian analysis based on mitochondrial cytochrome oxydase first subunit (COI) gene sequences obtained from 40 individual cells of *Hyalosphenia papilio*. The numbers along the branches represent respectively the bootstraps 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.

species. In contrast, testate amoebae hosts belong to three different major eukaryotic clades (Amoebozoa, Rhizaria and Stramenopiles) (Bhattacharya et al. 1995; Gomaa et al. 2013; Nikolaev et al. 2005) whose roots dive deeply into the origins of the domain. The likelihood that this pattern results from a single endosymbiosis which would have taken place in the common ancestor of these three clades, close to the base of the eukaryotic tree, is highly unlikely because traces of this event would have been detectable in most present day eukaryotic taxa. The most likely explanation that is suggested by our data is that symbiosis occurred relatively recently and independently in the different lineages in species sharing the same lifestyle and evolutionary pressures. Indeed, mixotrophy is favoured in nutrient-depleted environments such as peat bogs (Gerea et al., 2013; Johnson 2011). Not only does it constitute a “starvation insurance” for the host, allowing it to survive periods of low food availability in light conditions and outcompete heterotrophs (Rothhaupt 1996), it also allows it to exploit prey sources beyond thresholds allowing sustainable growth of potential competitor (Tittel et al. 2003). However, to clearly determine whether or not the TACS clade is exclusive to mixotrophic testate amoebae, further research ideally targeting multiple genetic markers and a broader taxonomic sampling particularly from different mixotrophic organisms would be required.

Furthermore, in contrast to the low diversity observed in the algal symbionts derived from different geographical locations, we found a large genetic diversity within the amoeba *H. papilio* at the COI level. This is consistent with previous results on *H. papilio* and other Hyalospheniidae (Heger et al. 2013), 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 the Ural, 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 prob-ably have an independent life-stage, as in the case of *Chlorella variabilis*, which can live inde-pendently from *Paramecium bursaria* (Blanc et al. 2010; Summerer et al. 2007). Thus, the symbionts do not appear to be strictly "maternally" trans-ferred 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 organ-isms 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) We show here that *C. variabilis* can also live within *H. papilio* (as exemplified by the sequences 10/BD-A/6 and 12/BD-B/6, Table 1). In addition, a recent experimental study showed that the digestive vacuoles of *Paramecium bur-saria* have the ability to recognize and differentiate symbiotic algae from other ingested food parti-cles based on their size and shape, allowing the *Chlorella* symbiont to escape from the digestive vacuole by budding from the vacuole membrane (Kodama and Fujishima 2012). The ability of *Chlorohydra* and some marine invertebrates to recognize symbiotic algae is believed to depend on algal cell wall associated proteins / proteo-glycans (Huss et al. 1993; Robert 1989). It is therefore most likely that the TACS clade mem-bers also have pre-adaptations for endosymbiotic life; a pre-adaptation of testate amoebae sym-bionts would agree with Law's (1985) hypothesis that "*through natural selection genotypes of sym-bionts are produced that are so accommodating they could be transferred even among unrelated hosts*" (Piercey-Normore and Depriest 2001). It is probable that TACShave similar adaptations to intracellular life as *C. variabilis*. Furthermore, the existence of the distantly related strain 18/LC/10 (Table 1) 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. 2013), it seems that it is not able to survive for long periods (i.e. three months in Schönborn's in-situ shading experiment) in the absence of light (Schönborn 1965). Half-digested *Chlorella* cells were observed in the cytoplasm of *A. flavum*, suggesting that it uses 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 (Keeling 2009; Lane and Archibald 2008).

Methods

Samples collections and documentation: Four species of mixotrophic testate amoebae (*Hyalosphenia papilio, Heleopera sphagni, Placocista spinosa* and *Archerella flavum*) (Fig. 1) were collected from their natural environments in *Sphagnum* peatlands (Table 1). Testate amoebae were isolated from *Sphagnum* mosses through serial filtrations, then washed sev-eral 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 extractions of *Heleopera sphagni* and *Placocista spinosa* contained from 4 to 10 different cells, and for *Archerella flavum* from 50 to 70 cells. 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 and Sacchi 1987). The extracted DNA was

pelleted, and re-suspended in 15 μL of Trisbuffer (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 con-ducted 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 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a final elongation of 10 min at 72 °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 sub-unit 1 (COI) of the host. The COI fragments were amplified in two steps. The first amplification was performed using uni-versal 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 vol-ume of 30 μ. l with amplification profile consisting of (5 minutes at 95 °C followed by 40 cycles 15 sec at 94 °C, 15 sec at 55 °C and 1 min 30 sec at 72 °C with a final elongation of 10 min at 72 °C). 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 *Hele-opera 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 (Invitro-gen) according to the manufacturer's instructions. Clone inserts were amplified with vector T7 (5′-TAATACGACTCACTATAGG-3′), SP6 (5′-GATTTAGGTGACACTATAG-3′) Primers (Promega, Switzerland). From five to ten 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 (Hall 1999) together with sequences retrieved from GenBank that are highly similar to our obtained sequences. Our align-ments had respectively 480 bp for COI and 780 by for *rbcL*, and we used respectively members of the *Nebela tincta-collaris-bohemica* complex (Kosakyan et al. 2012) as the outgroup for the COI alignment and members of Ulvophyceae as the out-group 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. The reliability of internal nodes was esti-mated by bootstrapping (1000 replicates).

The obtained tree was compared to a phylogeny gener-ated through Bayesian analysis 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 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 diver-gences between sequences were calculated using the program BioEdit software (Hall 1999).

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Anush Kosakyan

Institute de Biologie, Emile-Argand11. CH-2000
Neuchâtel, Switzerland
Tel: 0041-0767742980
Email: anna.kosakyan@gmail.com

PERSONAL DETAILS

Date of Birth: 13 June 1981
Place of Birth: Yerevan, Republic of Armenia
Citizenship: Armenian

EDUCATION

2009 October- till now (finalizing date August 2014): Laboratory of Soil Biology, Institute of Biology, University of Neuchâtel, Switzeland
PhD assistant
Research: Phylogeny, systematic and biogeography of free living microorganism using testate amoebae as model

2006 September- 2009 September:
Department of Evolutionary & Environmental Biology, Institute of Evolution, Faculty of Science & Science Education, University of Haifa, Israel
PhD in Mycology
Research: Taxonomy, Biology, and Phylogeny of Some Taxa of Agaricales s.l. of Israel with Special Attention to Biotechnologically Important Species

2004 September-2005 August: Department of Sustainable Agriculture, Mediterranean Agronomic Institute of Chania (MAICh), Create, Greece
Certificate of Specialist Post-Graduate Studies

2002 September-2004 June: Department of Botany, Faculty of Biology, Yerevan State University, Yerevan, Armenia
Master's Degree in Biology
Thesis title: Examination of Mycophilous fungi of Armenia

1998 September-2002 June: Department of Plant Protection, Faculty of Agronomy, Armenia Agricultural Academy, Yerevan, Armenia

Bachelor's Degree in Agronomy, Phytopathology

WORK EXPERIENCE

2002 September-2003 July: Laboratory assistant in private mycological laboratory (mushroom cultivation), Yerevan, Armenia

2003 September-2004 March: Operator in international company "Project Harmony", Yerevan, Armenia

2003 September-2004 August: Research assistant in private mycological company TNUPI (mycelium production), Yerevan, Armenia

2008 November - 2009 April: Experimental research (Multigene phylogeny of some taxa of Agaricaceae) at the Department of Natural Resources and Environmental Design, North Carolina Agricultural and Technical State University, Greensboro, NC, USA

SKILLS

-Good knowledge of molecular biology methods (DNA extraction, amplification, cloning, sequencing, molecular data analysing)
-Good knowledge of mycological culturing methods
-Good knowledge of Microscopy (LM, SEM)
-Good organisation and administrative skills
-Good written and verbal communication skills
-Experience in teaching and preparation of seminars

LANGUAGES

Armenian-mother tongue
Russian-fluent
English-fluent
Italian-well spoken
French-intermediate

PUBLICATIONS

Research articles

Gomaa F, **Kosakyan A**, Heger TJ, Corsaro D, Mitchell EAD, Lara E. 2013. EOne alga to rule them all: Unrelated mixotrophic testate amoebae (Amoebozoa, Rhizaria and Stramenopiles) share the same symbiont (Trebouxiophyceae). Protist, http://dx.doi.org/10.1016/j.protis.2014.01.002

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Book Chapters

Barseghyan GS, **Kosakyan A**, Didukh M, Wasser SP, Nevo E, Isikhuemhen OS. 2011. Phylogenetic analysis within genera Morchella (Ascomycota, Pezizales) and Macrolepiota (Basidiomycota, Agaricales) inferred from rDNA ITS and EF-1α sequences. Systematics and Evolution of Fungi. Edit. J.K. Misra & S.K. Deshmukh, Science Publishers, NH, USA. 426 p.

Books

Kosakyan A, Zmitrovich I, Didukh M, Wasser SP, Nevo E. 2013. Agaricomycetes of Israel. Koeltz Scientific Books, Germany, 375pp.

Submitted articles

Kosakyan A, Mulot M, Mitchell EAD, Lara E. 2013. Environmental DNA COI barcoding for quantitative analysis of protists communities at fine taxonomic level: a test using the *Nebela collaris* species complex (Amoebozoa; Arcellinida; Hyalospheniidae). Submitted to Microbial Ecology

Biketova A, **Kosakyan A**, Wasser SP, Nevo E. 2013. Some noteworthy and rare species of genus Boletus in Israel.I. Submitted to Cryptogamie, Mycologie

PRESENTATIONS TO SCIENTIFIC MEETINGS

2010 June - oral presentation-Kosakyan A. Genetically modified organism (GMO). Problems or Solutions? In: SPSW summer school: the global food crisis - how can plant sciences contribute. (Mürren, Switzerland)

2010 June - oral presentation-Kosakyan A, Heger TJ, Todorov M, Mitchell EAD, Lara E. Barcoding of "core Nebelas" inferred from mt DNA COI gene sequences. In: Joint meeting of the ISOP/BSBP. (Canterbury, UK)

2011 July - poster-Kosakyan A, Gomaa F, Mitchell EAD, Verrecchia E, Lara E. DOES morphology correlate with

Molecular data? Case study of Nebelid testate amobeae (Amoebozoa, Arcellinida). In: European congress of protistology. (Berlin, Germany)

2011 September - oral presentation-Kosakyan A. Soil protozoology at the University of Neuchâtel. In: School of protistology for young scientists. (Penza, Russia)

2012 March - oral presentation-Kosakyan A, Gomaa F, Heger TJ, Mitchell EAD, Lara E. Barcoding in protist species complexes. Case study N. collaris s.l. species complex(Arcellinida, Hyalospheniidae). In: 2nd Protist workshop at Eawag. (Dübendorf, Switzerland)

2013 February - oral presentation-Kosakyan A, Mitchell EAD, Lara E. Estimation of the cloning biases in the evaluation of diversity in microbial eukaryotes: the case of the Nebela tincta-bohemica-collaris complex. In: SME 2013, 5th Swiss microbial ecology meeting (Murten, Switzerland)

2013 July - oral presentation-Kosakyan A, Mitchell EAD, Lara E. A culture-independent method to screen fine-level taxonomic diversity in protists: the case of the Nebela tincta-bohemica-collaris complex (Amoebozoa; Arcellinida; Hyalospheniidae). In: ICOP XIV, international congress of protistology. (Vancouver, Canada)

2013 December - oral presentation-Kosakyan A, Mulot M, Mitchell EAD, Lara E. Environmental DNA COI barcoding for quantitative analysis of protists communities at fine taxonomic level: a test using the Nebela collaris species complex (Amoebozoa; Arcellinida; Hyalospheniidae). In: DNA WATCH meeting. (Frasne, France).

2014 February - oral presentation-Kosakyan A, Lara E, Mitchell EAD. Exploring the true diversity of protist groups using DNA-barcoding: A case study of genus *Quadrulella* (Amoebozoa, Arcellinida, Hyalospheniidae). In 33rd Annual DGP meeting (Essen, Germany).

2014 February - poster-Kosakyan A, Lara E, Meisterfeld R, Mitchell EAD. Presentation of monograph "Family Hyalospheniidae": A case study of genus *Quadrulella* (Amoebozoa, Arcellinida, Hyalospheniidae). In 33rd Annual DGP meeting (Essen, Germany).

AWARDS AND COMPETITIVE GRANTS

2010- ISOP/BSPB meeting travel award. Funding: 1500 USD

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2013-Swiss Barcoding Of Life initiative "Do cryptic species of protists share a similar ecology? The case of the genus *Quadrulella* (Hyalospheniidae, Arcellinida)" PI: E. Lara, A. Kosakyan. Funding: 3000 CHF

EVALUATION OF PAPERS AND PROJECTS

European Journal of Protistoloy
Annals of Public Health and Research
African Journal of Microbiology Research
African Journal of Food Science
Scientific Expertise Activity (Republic of Armenia)