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

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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.

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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

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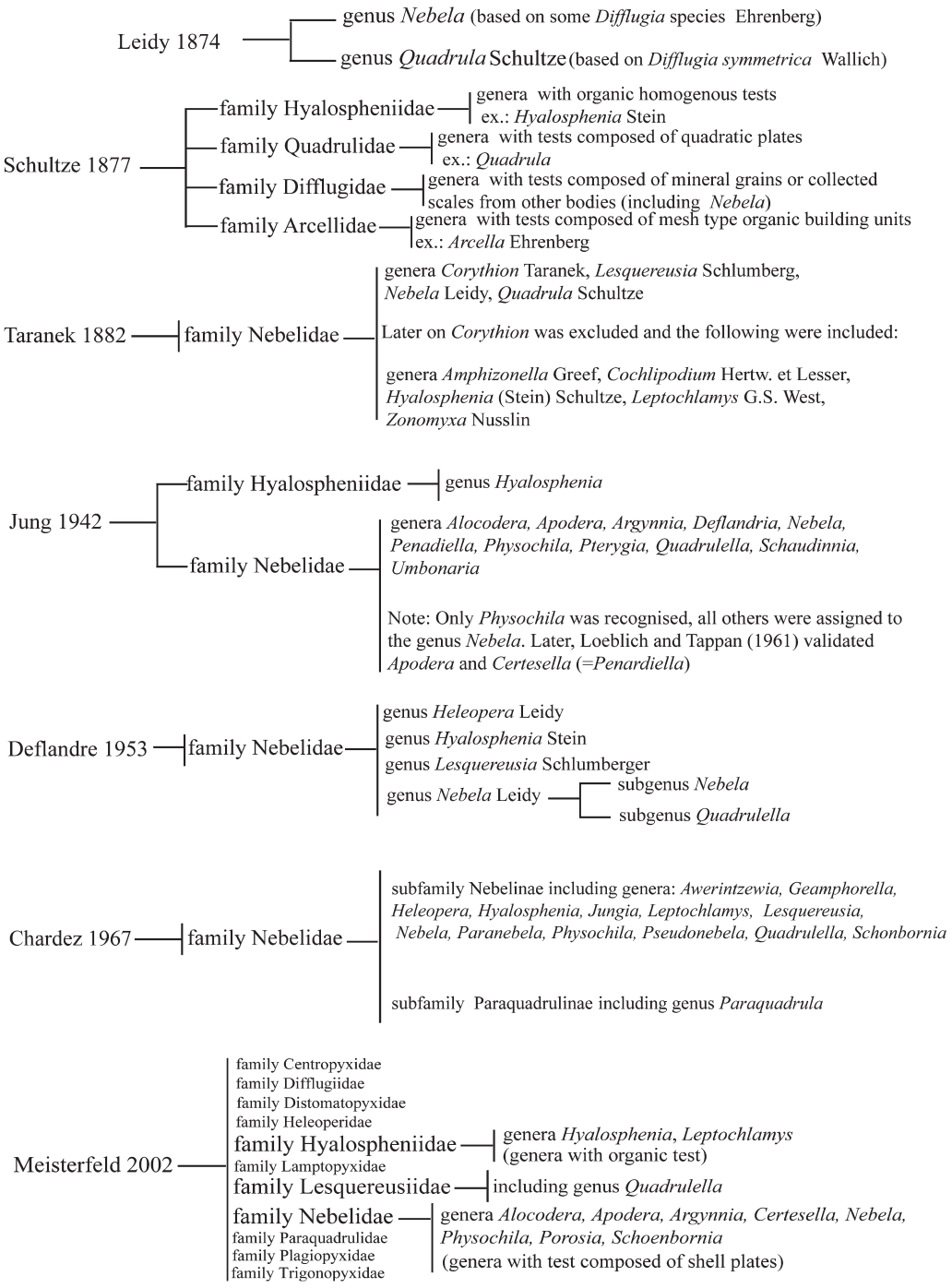

Figure 1. Summarised history of classification of genus *Nebela* and related taxa.

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

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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

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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

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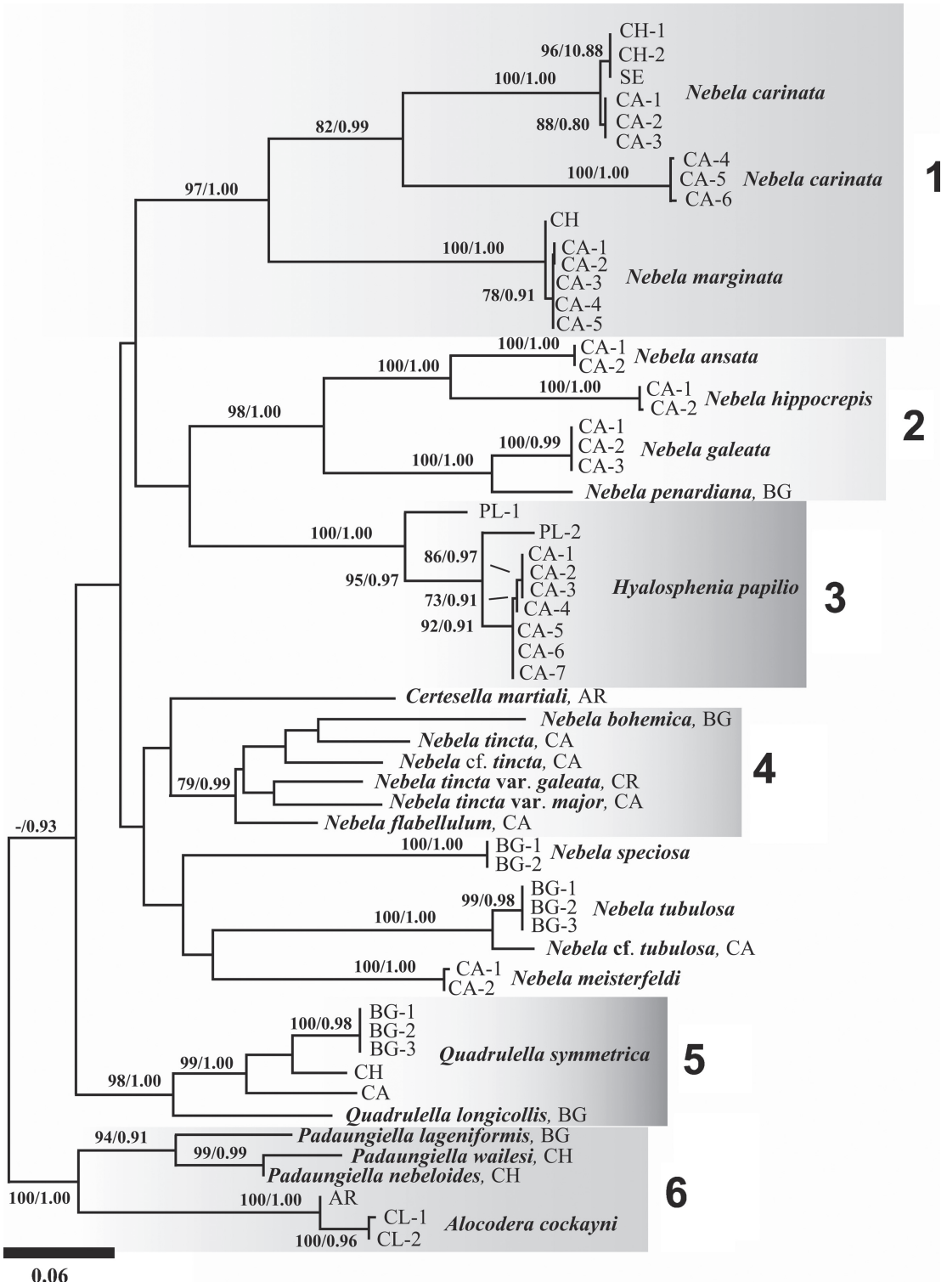

Figure 2. Maximum likelihood bootstrap consensus tree of 59 *Nebela* s.str. and related taxa testate amoebae COI sequences based on 677 nucleotide positions. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated 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*).

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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, Hyalospheniidae, 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

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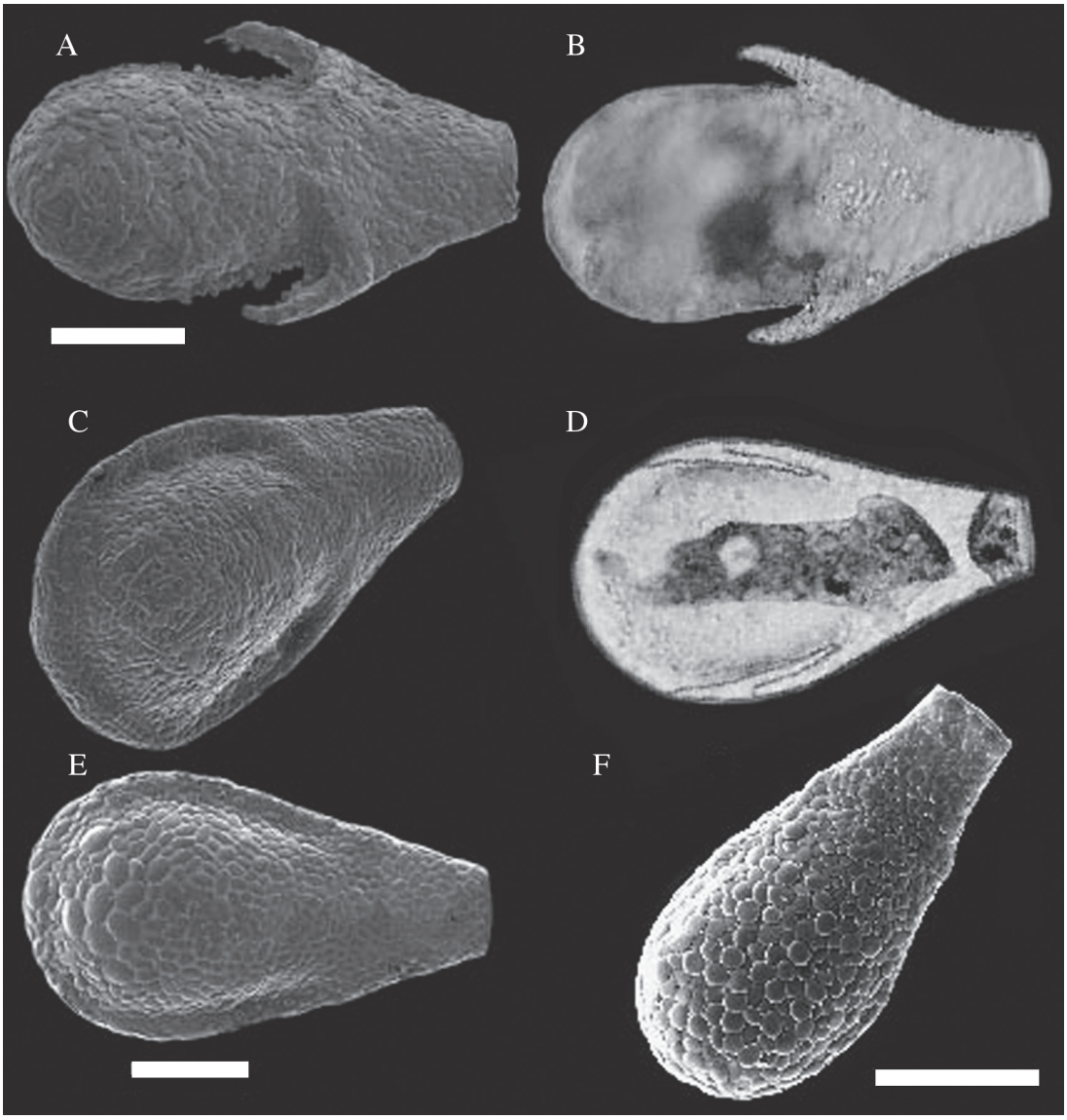

Figure 3. Scanning electron and light micrographs of group 2 morphospecies: **A**. and **B**. *Nebela ansata* from Peggy's Cove, Canada (picture **A** from Heger et al. 2011b). **C**. and **D**. *N. hippocrepis* from Cape Breton, Canada. **E**. *N. galeata* from Cape Breton, Canada. **F**. *N. penardiana*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

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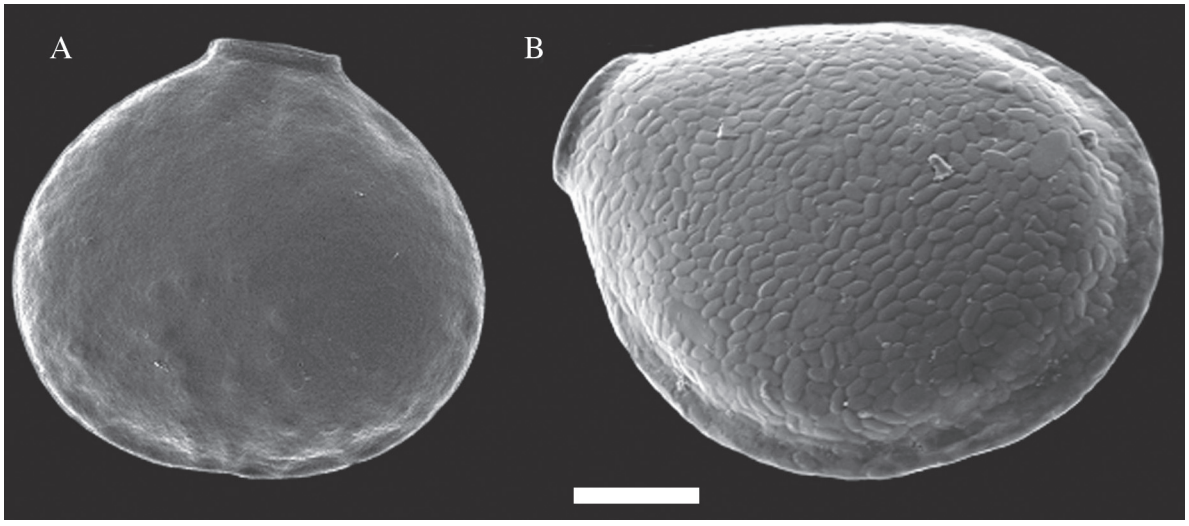

Figure 4. Scanning electron micrographs of *Nebela* morphospecies: **A**. *Nebela flabellulum* from Lynn Peak, Canada. **B**. *N. tincta* ssp. *galeata* from Volcán Poás, Costa Rica. Scale bar represent 20 μm.

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

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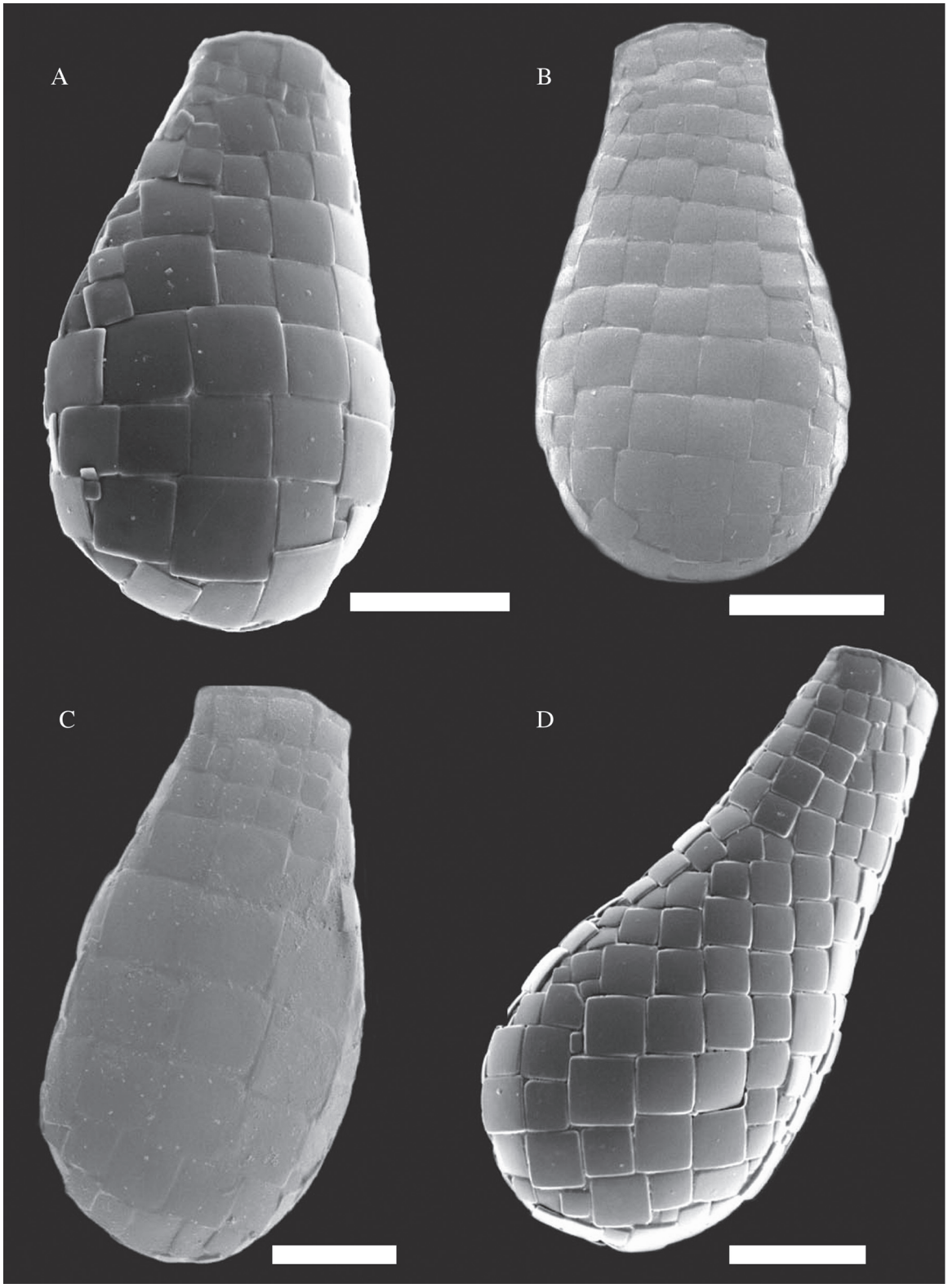

Figure 5. Scanning electron micrographs illustrating morphological variations within the *Quadrulella symmetrica* (*sensu lato*) morphospecies: **A**. *Q. symmetrica* from Bulgaria. **B**. *Q. symmetrica* from Canada. **C**. *Q. symmetrica* from Switzerland. **D**. *Q. longicollis* from Bulgaria. Scale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

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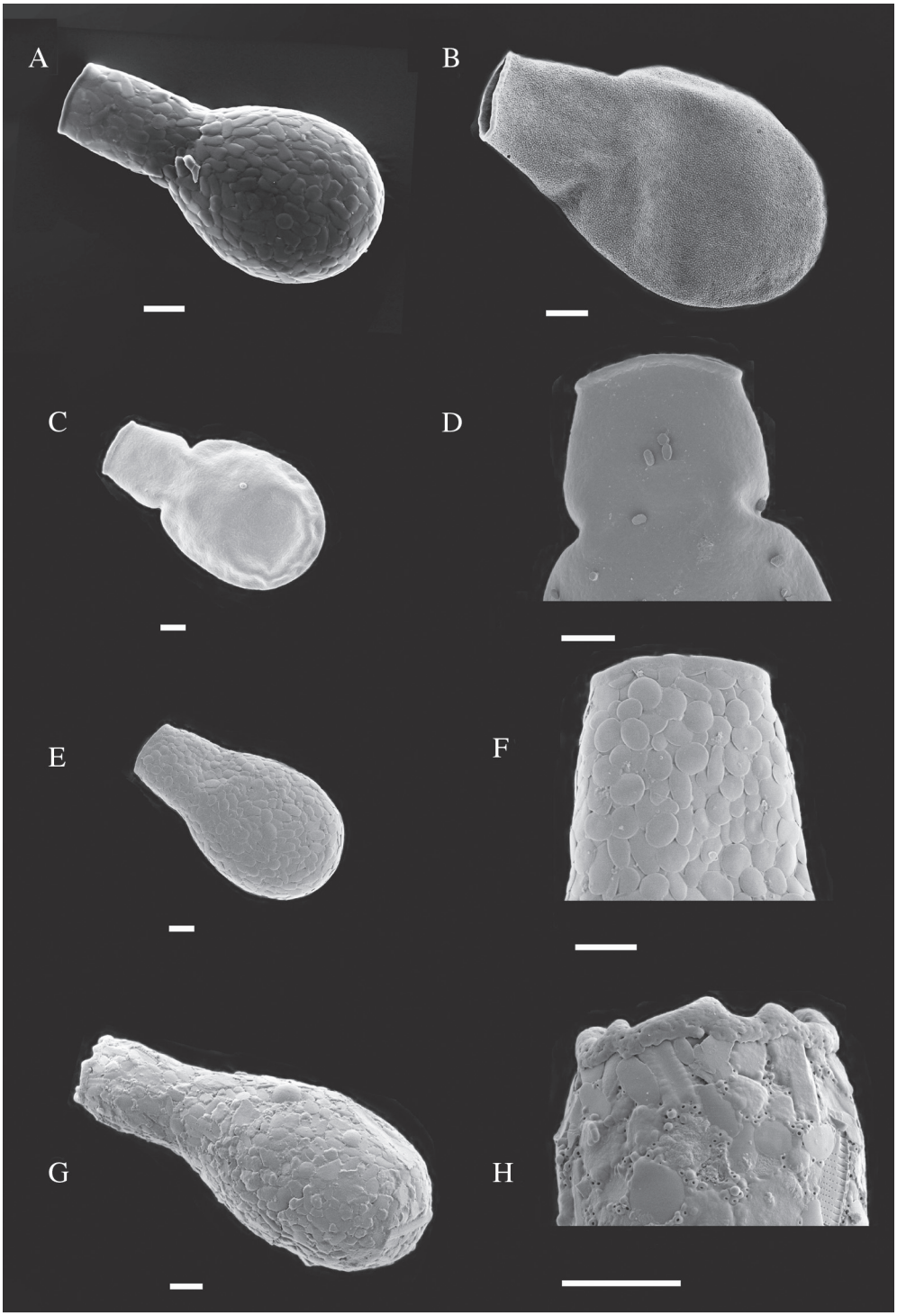

Figure 6. Scanning electron micrographs of outgroup species. **A**. *Padaungiella lageniformis* from Bulgaria. **B**. *Alocodera cockayni* from Argentina. **C-D**. *A. cockayni* from Chile and close view of its aperture respectively. **E-F**. *P. wailesi* from Switzerland and close view of its aperture.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

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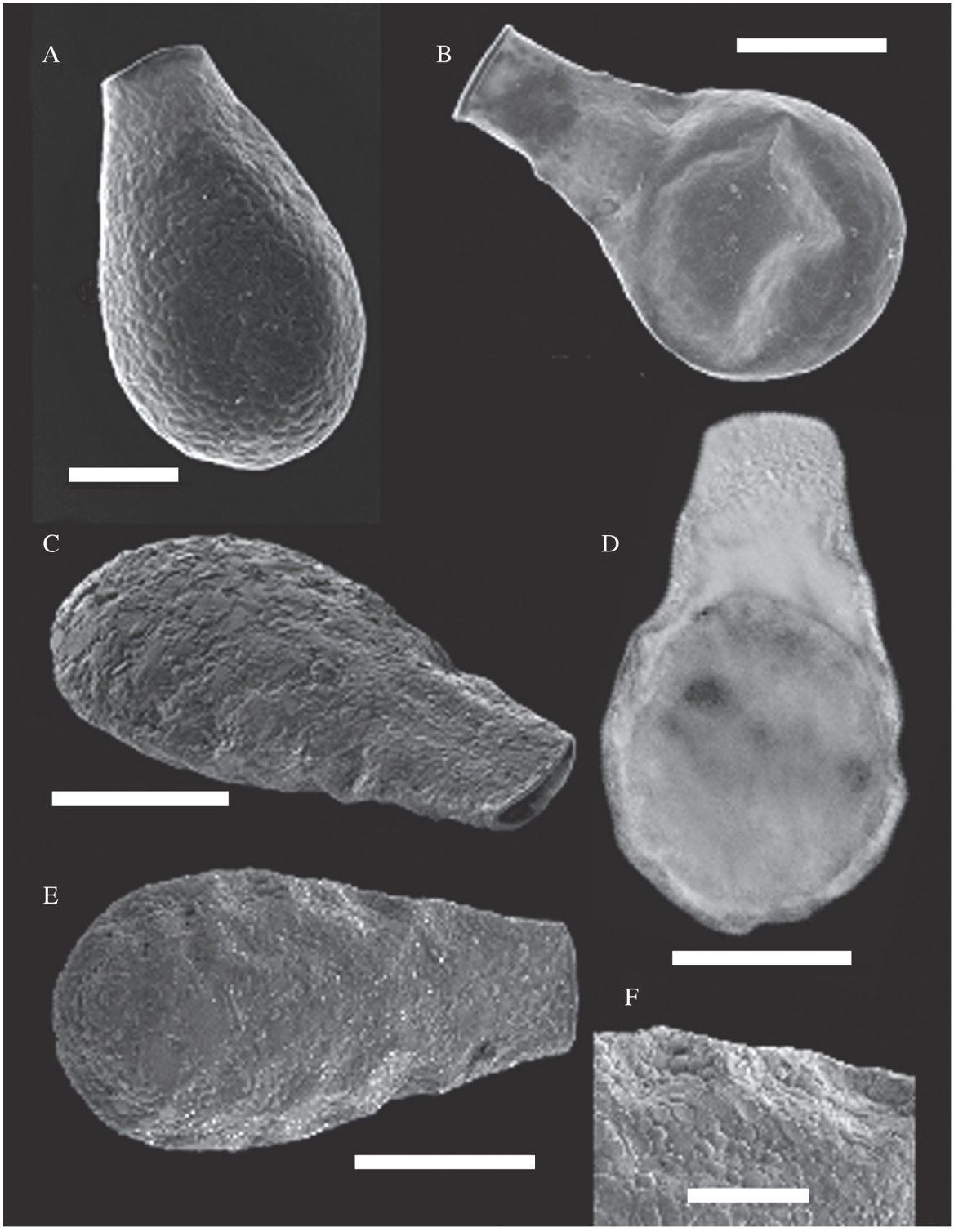

Figure 7. Scanning electron and light micrographs of species having uncertain phylogenetic position: **A**. *Nebela tubulosa* from Bulgaria. **B**. *Certesella martiali* from Tierra del Fuego Province, Argentina. **C**. *Nebela meisterfeldi* from Strathcona Park, Canada. **D**. *N. meisterfeldi* from 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.

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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).

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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

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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

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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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