Published in European Journal of Protistology 49, 222-237, 2013
which should be used for any reference to this work

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

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

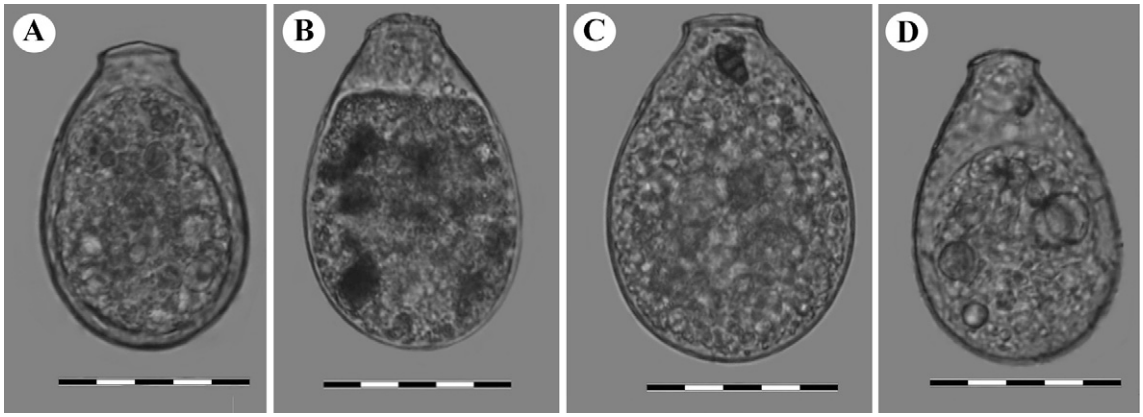

Fig. 1. Light micrographs of clade A cells (*Nebela guttata*): **A.** LC-126 from Le Cachot population, Switzerland, **B.** PE-159 from Pechora population, Russia, **C.** LC-118 from Le Cachot population, Switzerland, and **D.** LC-103 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

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

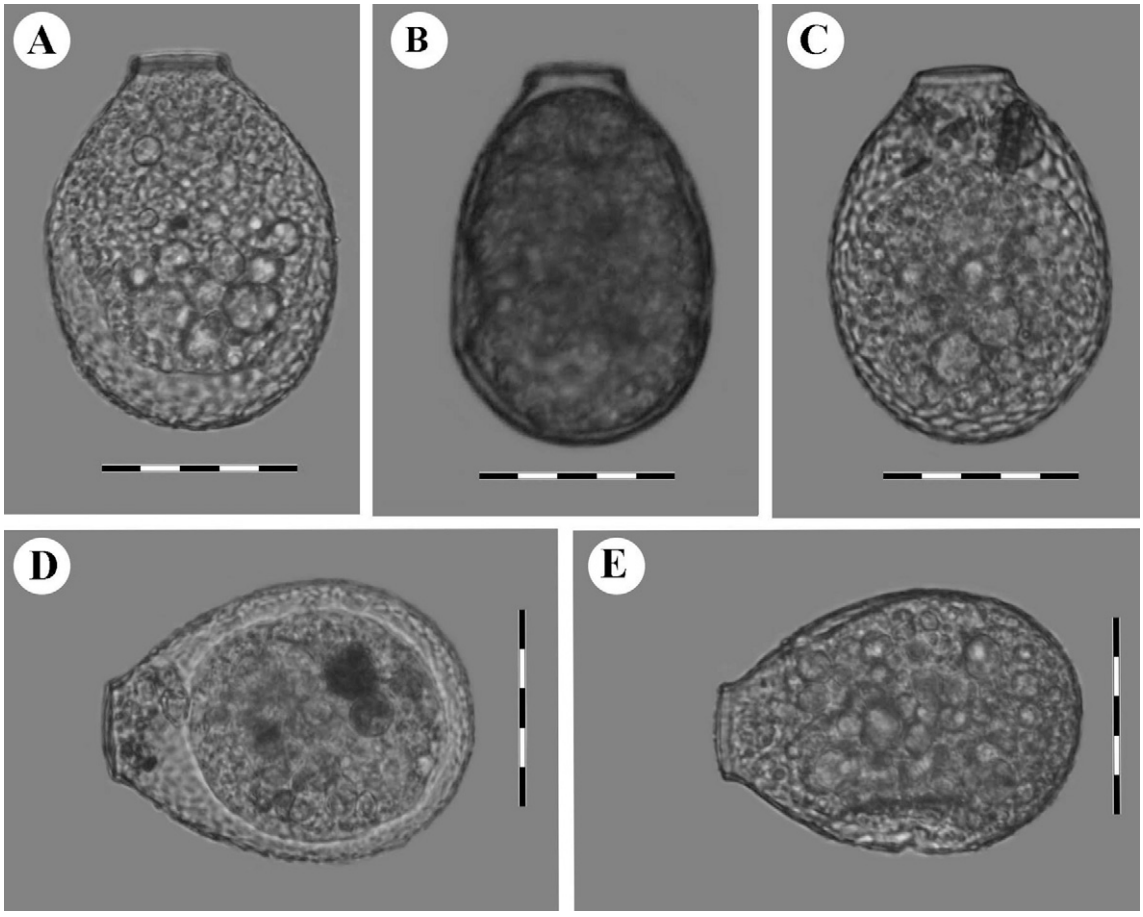

Fig. 2. Light micrographs of clade B cells (*Nebela tincta*): **A.** LC-86, **B.** LC-137, **C.** LC-117, **D.** LC-89, **E.** LC-62 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

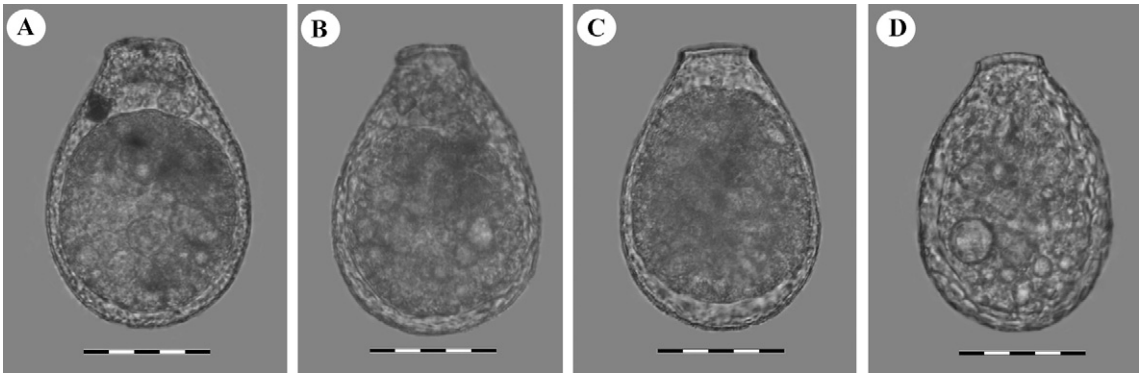

Fig. 3. Light micrographs of clade C cells (*Nebela collaris*): **A.** LC-75, **B.** LC-69, **C.** LC-64, **D.** LC-55 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

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).

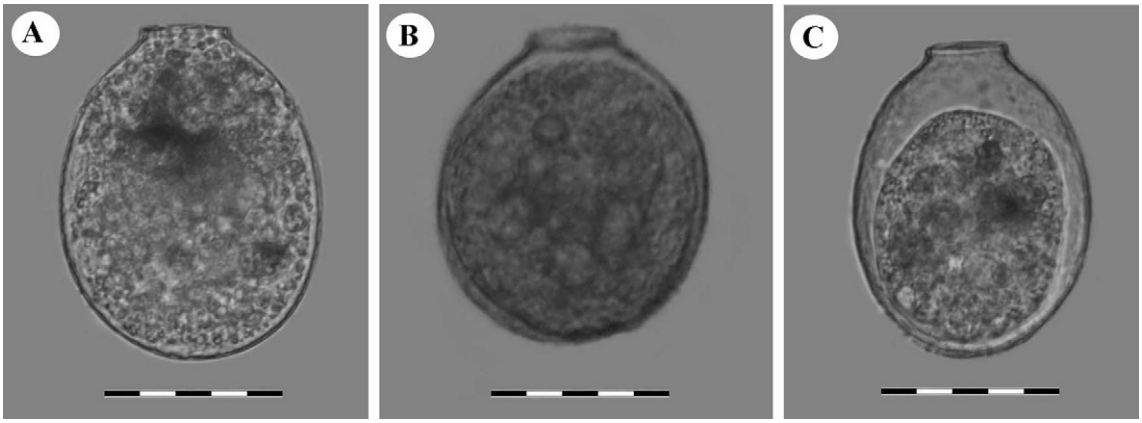

Fig. 4. Light micrographs of clade D cells (*Nebela rotunda*): **A.** LC-58, **B.** LC-71, **C.** LC-74 from Le Cachot population, Switzerland. Scale bars represent 50 μm.

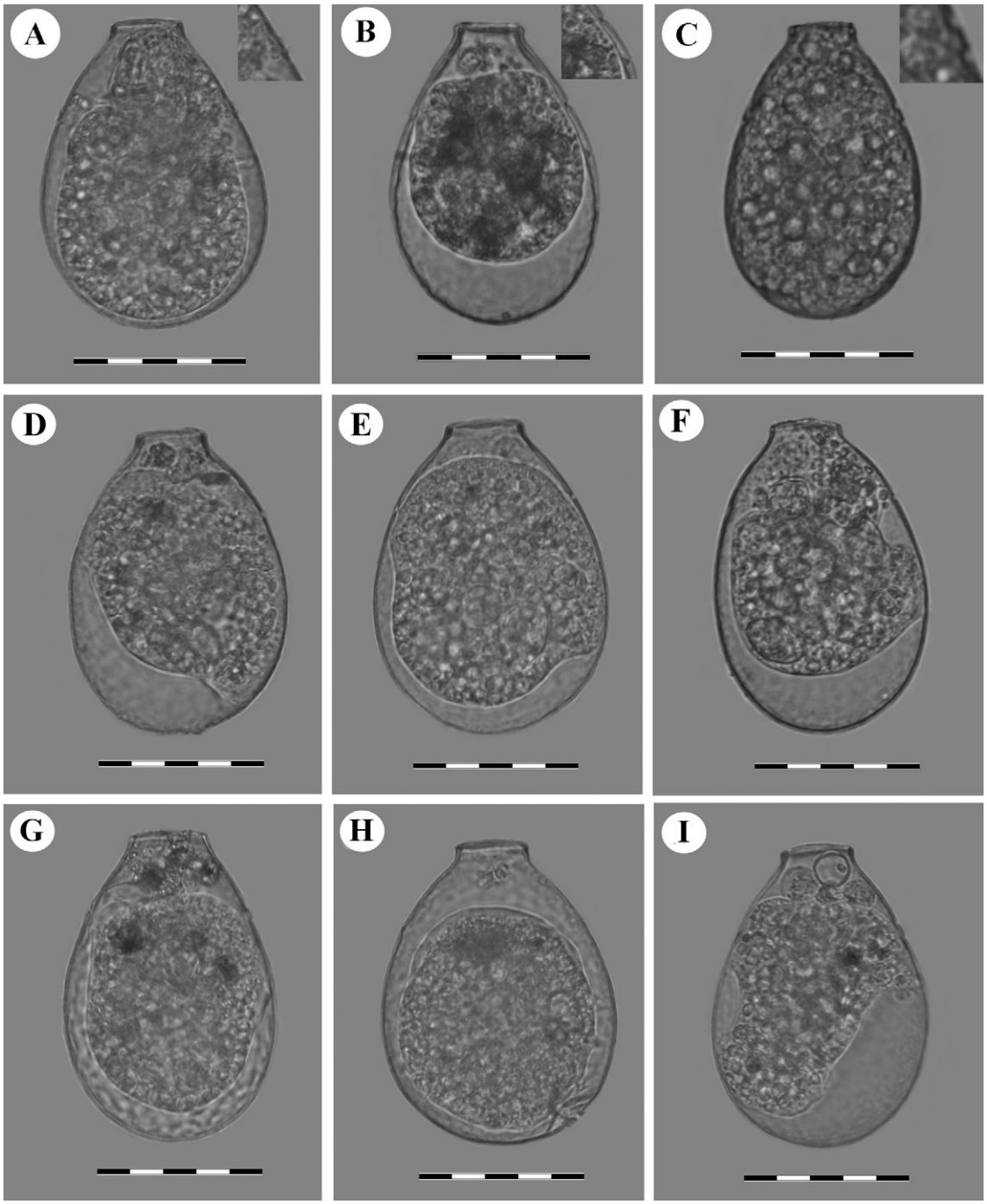

Fig. 5. Light micrographs of clade E cells (*Nebela pechorensis*): **A.** PE-149 from Le Pechora population, Russia, with its detailed picture of lateral pores, **B.** PE-151 from Pechora population, Russia, with its detailed picture of lateral pores, **C.** LC-135 from Le Cachot population, Switzerland,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

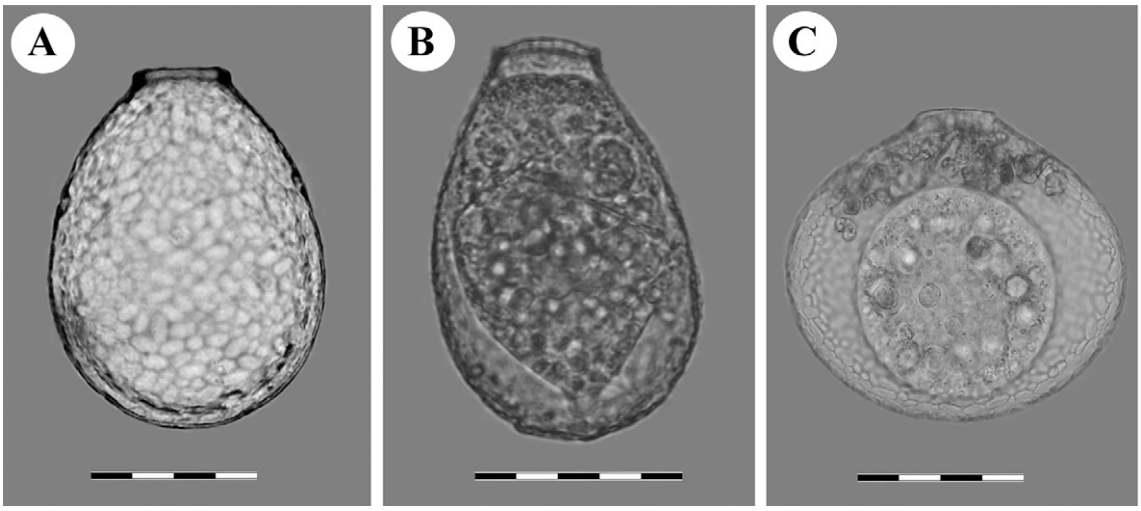

Fig. 6. Light micrographs of the remaining forms: **A**. *Nebela aliciae* n. sp. from Costa Rica, **B.** PE-155 from Pechora population, Russia, **C.** *N. flabellulum* from Canada. Scale bars represent 50 μm for A and B, and 60 μm for C.

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

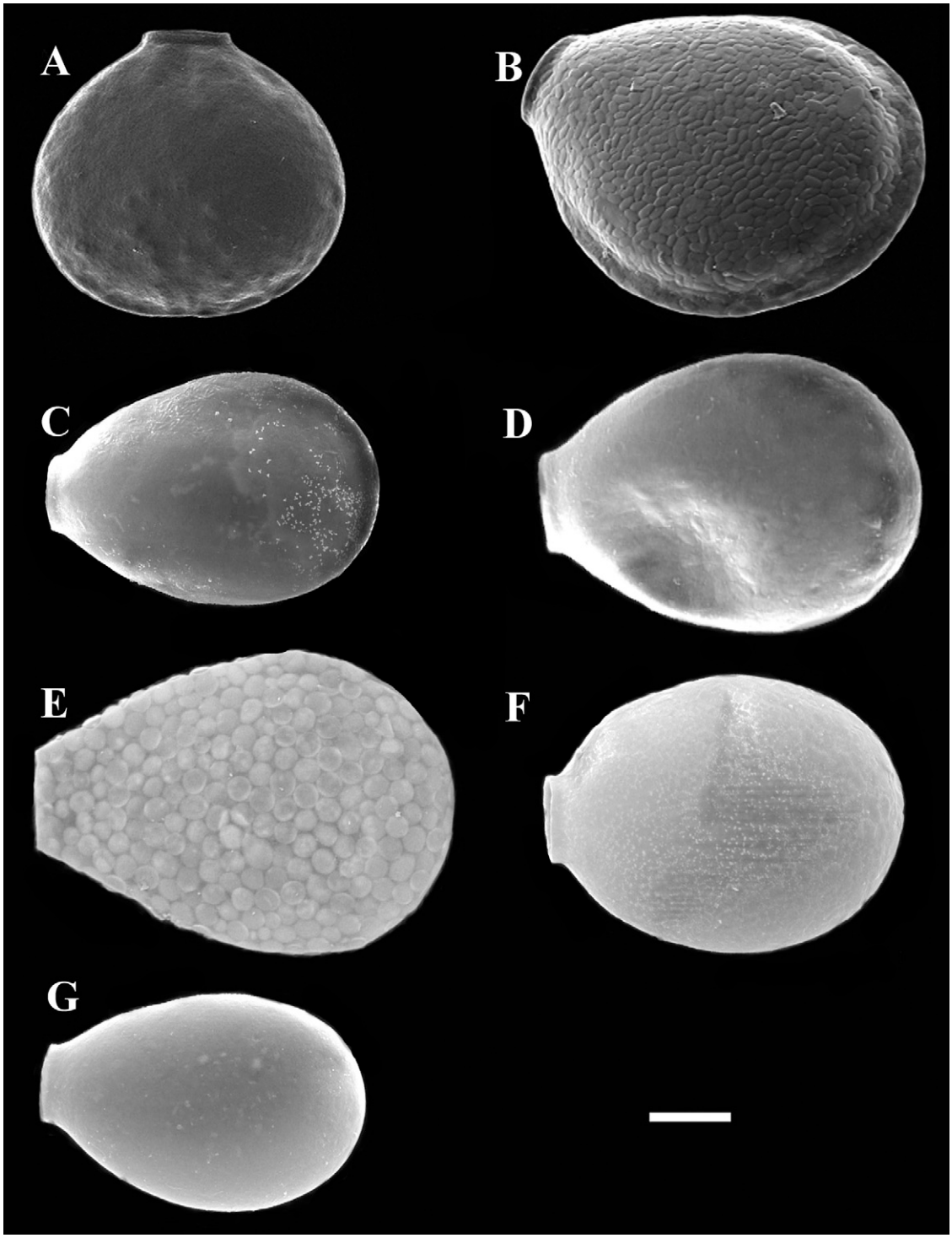

Fig. 7. Scanning electron micrographs of cells from each species-population: **A.** *Nebela flabellulum* from Lynn Peak, Canada, **B.** *N. aliciae* n. sp. from Volcán Poás, Costa Rica, **C.** *Nebela guttata* n. sp. from Le Cachot, Switzerland, **D.** *N. tincta* from Le Cachot, Switzerland, **E.**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

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

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

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

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

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

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

Discussion

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

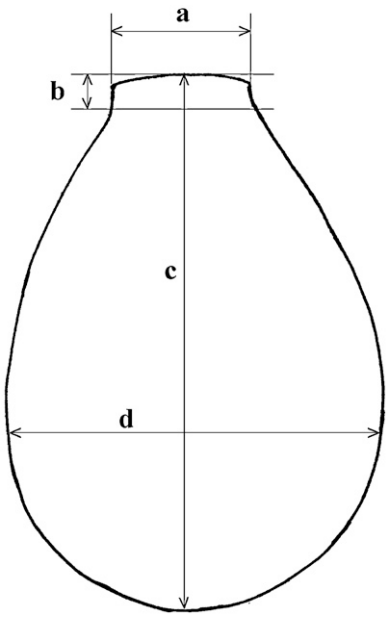

Fig. 8. Schematic sketch and position of the measured axes of the test: **a.** diameter of aperture, **b.** length of the neck, **c.** length of the test, **d.** breadth of the test.

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

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

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

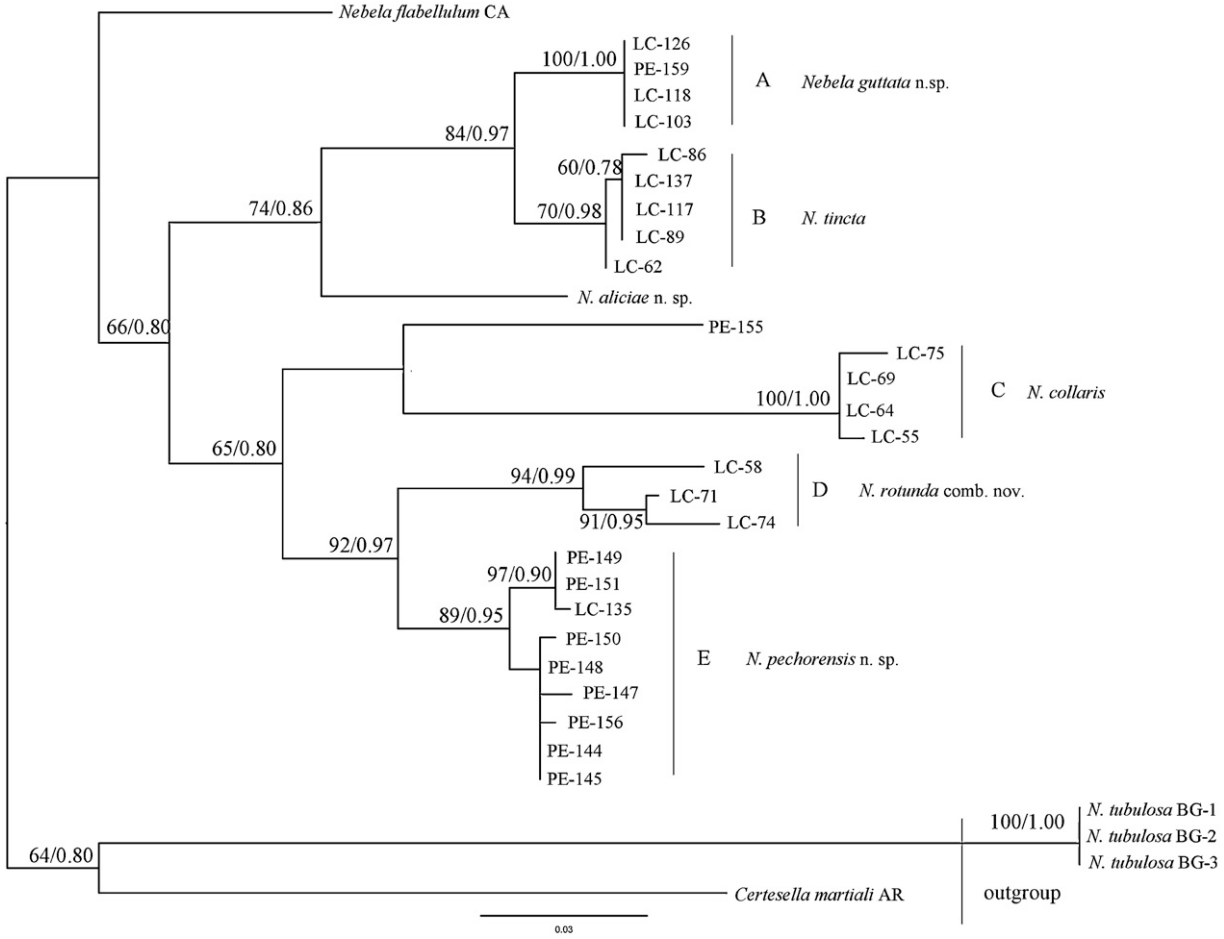

Fig. 9. Maximum likelihood bootstrap consensus tree of 31 *Nebela collaris s.l.* testate amoeba COI sequences based on 300–665 nucleotide positions. The numbers along the branches represent respectively the bootstraps obtained by maximum likelihood method and the posterior probabilities as calculated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

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

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

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

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

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

Phylogenetic analyses of *Nebela collaris s.l.*

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

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

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

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

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

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

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

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

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

Taxonomic actions

1. *Nebela tincta*

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

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

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

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

2. *Nebela collaris*

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

This work was funded by Swiss NSF projects no. 205321-109709/1 & 2 to E. Mitchell; Science and Technology Cooperation Program Switzerland – Russia grant IZLR Z3_128338 to J. Pawlowski, E. Mitchell & E. Lara; Swiss NSF Ambizione grant no. PZ00P2_122042 to E. Lara, and Swiss NSF – Scientific & Technological Cooperation Programme Switzerland – Russia: "*Protist diversity and palaeoecological reconstruction of the Petchora Biosphere reserve*." to E. Mitchell and Yuri Mazei. SEM was made possible through the Swiss Centre of Electronic and Microtechnique (CSEM) in Neuchâtel. We thank two anonymous reviewers for commenting on the manuscript.

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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