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Eight species in the *Nebela collaris* complex: *Nebela gimlii* (Arcellinida, Hyalospheniidae), a new species described from a Swiss raised bog

David Singer[a,*], Anush Kosakyan[a], Amandine Pillonel[a], Edward A.D. Mitchell[a,b], Enrique Lara[a]

[a] *Laboratory of Soil Biology, University of Neuchâtel, Rue Emile-Argand 11, Neuchâtel CH-2000, Switzerland*
[b] *Jardin Botanique de Neuchâtel, Chemin du Perthuis-du-Sault 58, CH-2000 Neuchâtel, Switzerland*

Abstract

We describe here a new species of sphagnicolous testate amoeba found abundantly in the forested part of the Le Cachot peatland (Jura Mountains, Neuchâtel, Switzerland) based on microscopical observations (LM, SEM). The new species, called *Nebela gimlii* was placed in a phylogenetic tree based on mitochondrial cytochrome oxidase sequences (COI), and branched robustly within the *N. collaris* complex next to the morphologically similar *N. guttata* and *N. tincta*. It is however genetically clearly distinct from these two species, and differs morphologically from them by its smaller size and stouter shape of the shell. This new species completes the phylogeny of the *Nebela collaris* species complex, with now eight species described, mostly from peatlands and acidic forest litter, and further demonstrates the existence of an unknown diversity within testate amoebae. Improving the taxonomy of testate amoebae in peatlands and clarifying the ecology of newly discovered species should make these organisms even more valuable as bioindicators and for palaeoecological reconstruction.

Keywords: Arcellinida; Amoebozoa; Cytochrome oxidase gene (COI); Peatland; Protist; Testate amoeba

Introduction

Arcellinid testate amoebae are common and diverse in peatlands, where they constitute a large part (typically 10–30%) of microbial biomass (Gilbert, 1998; Gilbert et al., 1998; Mitchell et al., 2003). Their sensitivity to environmental changes and the good preservation of their shells in peat has led to their use as indicators of past environmental changes (Charman, 2001; Mitchell et al., 2008). However, their taxonomy is still far from being resolved in a satisfactory way, and recent studies have revealed a high diversity within individual morphospecies, sometimes referred to as cryptic or pseudocryptic (Kosakyan et al., 2012). A thorough morphological analysis and the application of a single-cell barcoding approach (based on the COI gene) revealed the existence of several morphologically and genetically distinct taxa within the *Nebela collaris* complex (Kosakyan et al., 2013).

Members of the *Nebela collaris* species complex (or *N. tincta* complex) are the second most common group of testate

*Corresponding author. Tel.: +41 79 218 24 26; fax: +41 32 718 30 01.
E-mail address: david.singer@unine.ch (D. Singer).

amoebae in peatlands. They were found to occur in 72.6% of all samples in a review of European and North American data and are the most dominant taxa in communities (10.8% of the community on average) (Gilbert and Mitchell, 2006). They are found through the Northern Hemisphere, but also in South America (Zapata et al., 2008).

Species discrimination has often been cited as problematic, and palaeoecologists have often lumped the different forms into a couple of species or varieties (Charman et al., 2000). This lumping may well have prevented ecologists from distinguishing forms that occupy different niches. The distribution of "*N. collaris*" along a wetness gradient indeed showed a multimodal distribution (Valiranta et al., 2012). This suggested the existence of several taxa differing in their ecological optima and several distinct species were indeed described based on COI gene sequences and morphology (Kosakyan et al., 2013). Further evidence that genetically closely-related and morphologically similar forms may occupy different ecological niches and/or have contrasted geographic distributions was found in a broad scale study of the mixotrophic (morpho)species *Hyalosphenia papilio* (Heger et al., 2013). If such closely related forms are proved to differ in their ecological preferences and if they can be securely identified, then testate amoeba-based bioindication and palaeoecological tools could potentially be improved.

In order to improve the taxonomic framework for the *N. collaris* group, and allow sound ecological work and subsequent application in bioindication, it is essential to clarify the true diversity of testate amoebae using a combination of molecular and morphological approaches. We therefore describe *Nebela gimlii*, a new species of the *Nebela collaris* group from the Le Cachot peatland in the Swiss Jura Mountains.

Material and Methods

Sample collection and identification

Sphagnum sp. mosses were collected from the forested area (*Pinus mugo uncinata*) of "le Cachot" peatland, in the Swiss Jura Mountains (47°00′15.23″N, 6°39′52.83″E). Samples were visualised under light microscopy and contained, besides *N. gimlii*, specimens of *N. collaris*, another species of the complex which cannot be mistaken morphologically as cells are almost twice as long. Cells were isolated individually with a narrow diameter pipette under an inverted microscope and rinsed several times with tap water. Measurements of 14 cells were taken under an inverted microscope (Olympus IX81) at magnifications of 100× and of 400×. Photographs were taken at magnification of 400× (Fig. 1A–D). We measured the following morphometric traits on the test: length, breadth, depth, and aperture width as described in Kosakyan et al. (2013) and calculated the width/length ratio. The biovolume was calculated according to Charrière et al. (2006).

Scanning electron microscopy

Three *Nebela gimlii* tests out of the 14 analysed cells were mounted on stubs and then kept during one week in a desiccator. The tests were coated with gold in a 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 (Fig. 2A–E).

DNA amplification

DNA from two single cells was extracted using a guanidine thiocyanate-based protocol (Chomczynski and Sacchi, 1987), adapted after (Gomaa et al., 2013). The COI sequences were obtained by polymerase chain reaction (PCR) using the broad spectrum primer LCO (Folmer et al., 1994) and a *Nebela collaris*-complex specific primer and PCR conditions as in (Kosakyan et al., 2013). The amplicons were cloned into a PCR2.1 Topo TA cloning vector and transformed into *E. coli* TOP10′ One Shots cells (Invitrogen kit) according to the manufacturer's instructions. Two inserts per PCR product were amplified with M13F, M13R primers. Sequencing was carried out using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed with a ABI-3130XL DNA sequencer (Applied Biosystems). Sequences were deposited in GenBank with the following accession numbers: KP083297 and KP083298. Light microscopy pictures of the two extracted cells are shown in Fig. 1A and B.

Phylogenetic analysis

We build an exhaustive reference database containing 31 different sequences of the COI of the *Nebela collaris* species complex present in the GenBank database (Heger et al., 2011; Kosakyan et al., 2012, 2013) plus three sequences of *Nebela tubulosa* and one sequence of *Certesella martiali* used as outgroup. We aligned the sequences manually using the BioEdit programme (Hall, 1999). The alignment is available from the authors upon request. Programmes and parameters used to build the trees are the same as described in Kosakyan et al. (2013) (Fig. 3).

Results

Description of the species

Arcellinida Kent 1880.

Hyalospheniidae (Schulze) Kosakyan and Lara.

Nebela gimlii n.sp. Singer and Lara.

The test is wide pyriform or drop-shaped, laterally compressed, with a protruding narrow neck. Two lateral pores are present ca. 1/4 from the distance from the pseudostome to the fundus of the test (Fig. 1A and B). A variable

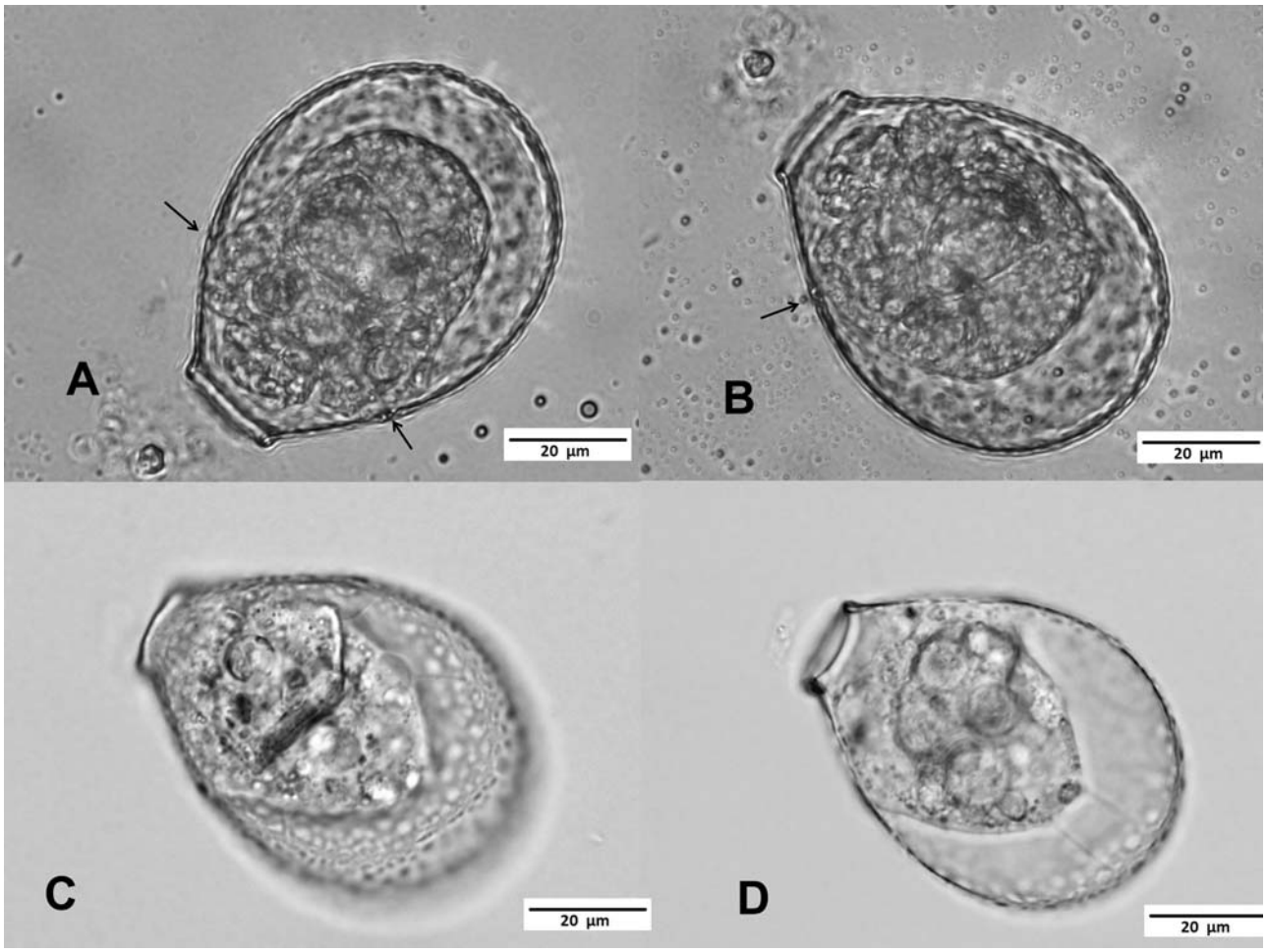

Fig. 1. (A–D) Light microscopy images of *Nebela gimlii* test. (A) test corresponding to Nebela_gimlii_el3_1 (GenBank: KP083297) – note the presence of two lateral pores (arrows) ca. 1/4 from the distance from the pseudostome to the fundus of the test, magnification of 400× (B) test corresponding to Nebela_gimlii_el3_4 (GenBank: KP083298), magnification of 400×, (C) example of *Nebela gimlii* test showing the scales, magnification of 400×, (D) example of *Nebela gimlii* with focus on the aperture, magnification of 400×.

number of small pores can be seen on SEM images in apparently random positions from near the aperture to the fundus of the test (Fig. 2A, B, E and F). The test is colourless or slightly brownish, composed of small particles (likely obtained from preys, e.g. euglyphid testate amoebae), which are covered by a thin layer of organic cement. The aperture is oval in frontal view, slightly curved in lateral view, surrounded by a thin organic collar characteristic of family Hyalospheniidae (Kosakyan et al., 2012). Dimensions based on 14 individuals: length: 67.7–77.6 µm (mean = 72.8 µm), breadth: 49.7–61.64 µm (mean = 53.9 µm), width of aperture: 17.5–21 µm (mean = 19 µm).

Illustrations and morphological data of the new species are given in Figs. 1 and 2 and Tables 1 and 2.

Ecology

Nebela gimlii was found exclusively in the relatively dry *Sphagnum* mosses in the forested part of a peat bog (influenced by lateral drainage) and the drained peatland margin a few meters from the base of old peat extraction walls. These habitats are characterised by low pH, moderate moisture and low nutrient content.

Hapantotype

The tests were collected from a *Sphagnum* sample in a peatland in Le Cachot, Jura Mountains, Switzerland (47°00′15.23″N, 6°39′52.83″E). One SEM stub with several specimens is deposited at the Natural History Museum of Neuchâtel (Ref. Nr.: UniNe-EM-5). COI gene sequences of *Nebela gimlii* el3_1 (499 bp) and *Nebela gimlii* el3_4 (499 bp) were deposited in GenBank (Nebela_gimlii_el3_1 KP083297 and, respectively, Nebela_gimlii_el3_4 KP083298).

Etymology

The name of this species refers to the name of Gimli, one of the dwarfs in J.R.R. Tolkien's masterpiece "The Lord of

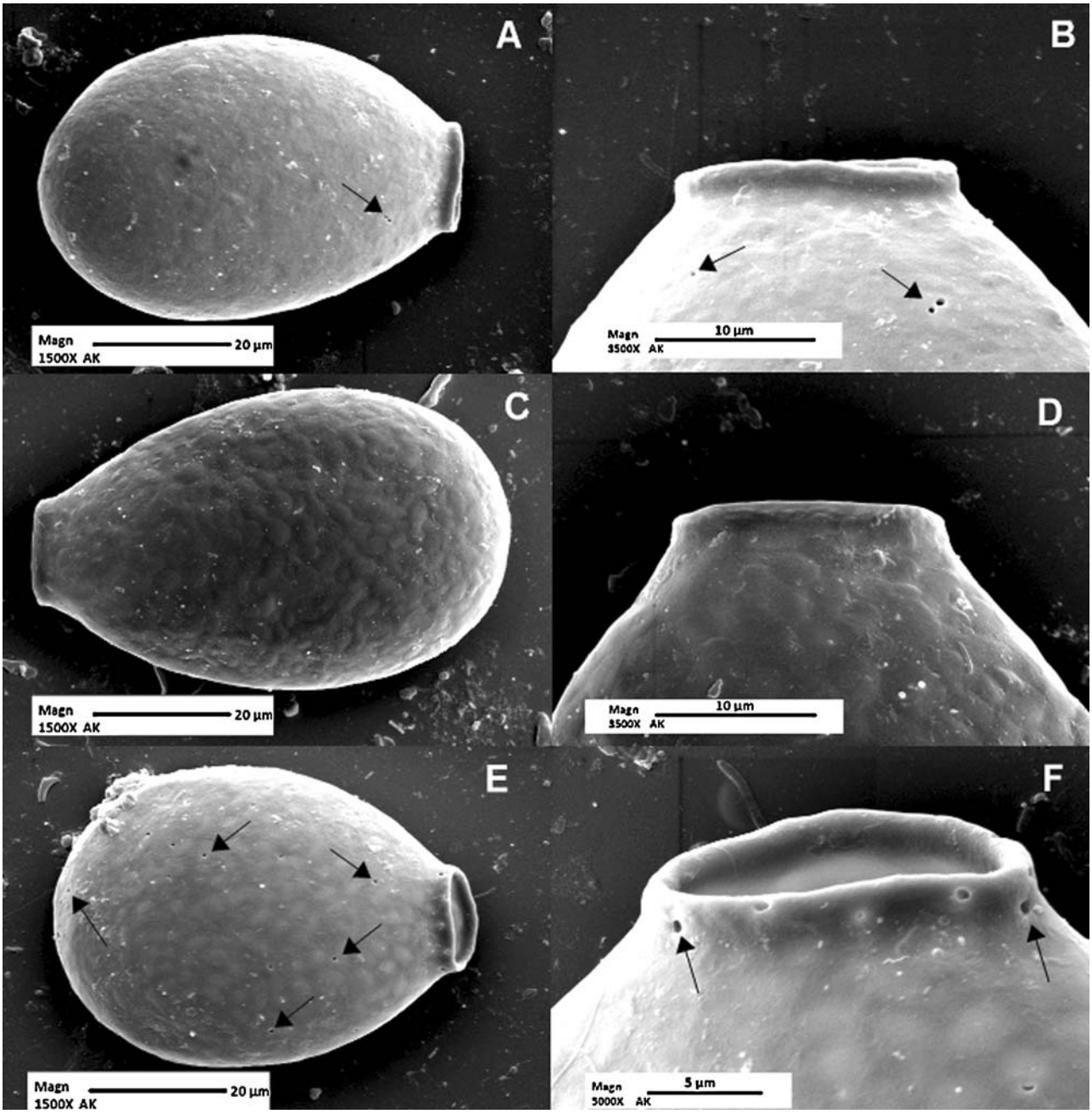

Fig. 2. (A–E) SEM micrographs of three individuals of *Nebela gimlii*. (A, C and E) – illustrating the shell general shape and composition; and (B, D and F) – detail view of the aperture of the same individuals. Arrows show some pores on the shell.

Table 1. Biometrical characteristics of *Nebela gimlii*: M – median, SD – standard deviation, SE – standard error of the mean, CV –coefficient of variation, Min – minimum, Max – maximum, *n* – number of individuals examined (measurements in µm).

Characters	Mean	M	SD	SE	CV	Min	Max	*n*
Length	72.8	73.23	2.92	0.78	4	67.71	77.61	14
Breadth	53.9	53.12	3.67	0.98	6.81	49.68	61.64	14
Depth	31.4	30.7	1.4	0.37	4.45	30.5	35	14
Aperture width	19	18.71	1.03	0.28	5.45	17.5	21.06	14
Ratio (L/B)	1.4	1.35	0.07	0.02	5.45	1.22	1.5	14
Biovolume (10^4 µm^3)	8.25	7.82	1.08	0.28	13.12	6.94	10.47	14

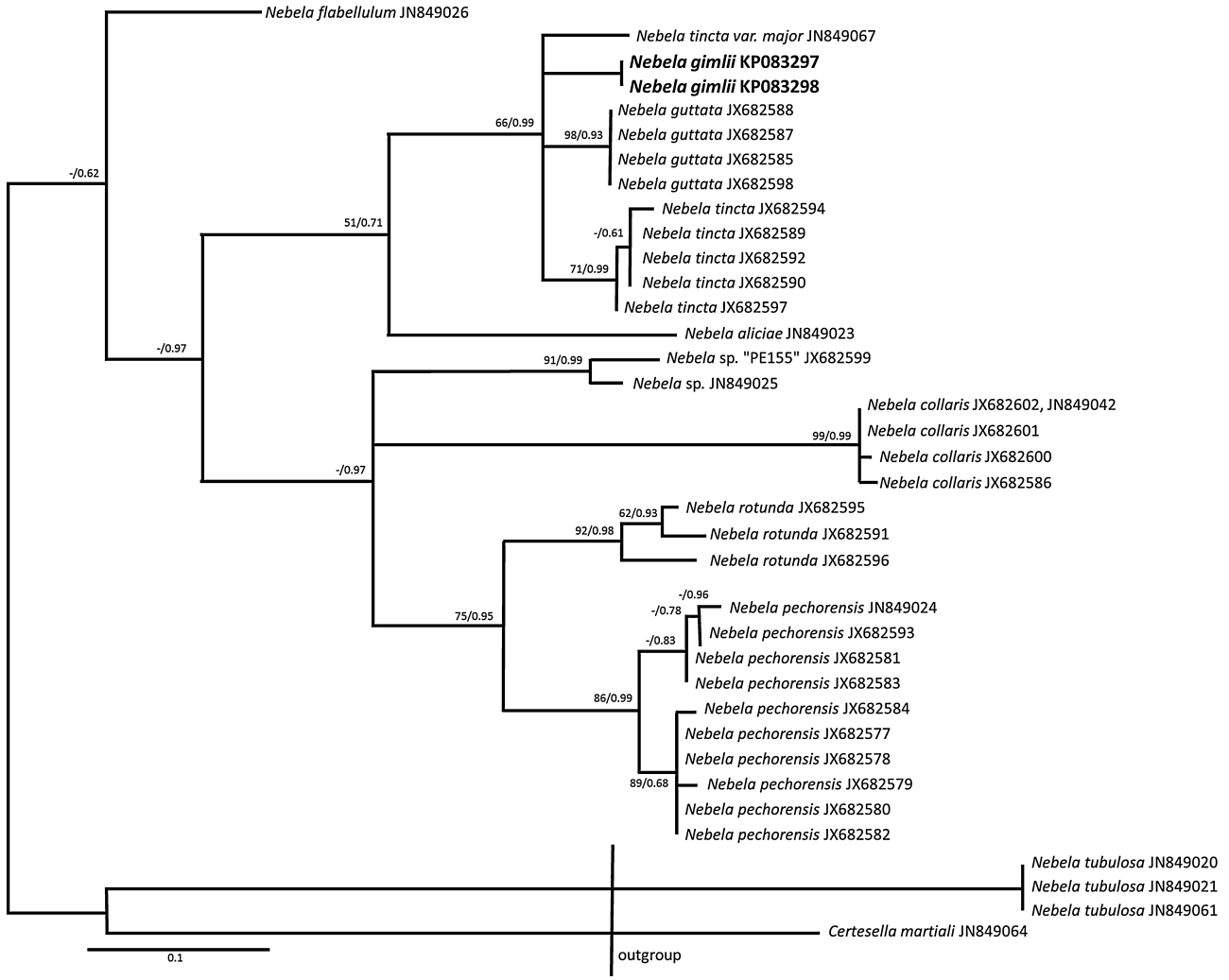

Fig. 3. Bayesian Markov Chain Monte Carlo consensus tree of 31 *Nebela collaris* s.I. testate amoeba. Based on mitochondrial cytochrome oxidase, subunit I (COI) gene. *Nebela gimlii* is denoted in bold. The numbers along the branches represent, respectively, the bootstraps obtained by maximum likelihood 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 Rings", because of its small size (the smallest known member of the *Nebela collaris* complex) and stout shape. In addition, it has been found abundantly in a forest, and Gimli was unique among his kind to have been travelling in the woods.

Table 2. Comparison of morphological characters between *Nebela gimlii* and *N. guttata*. Min – minimum, Max – maximum, *n* – number of individuals examined (measurements in μm).

Characters	*Nebela gimlii* (Min/Max) (*n* = 14)	*Nebela guttata** (Min/Max) (*n* = 4)
Length	68/78	80/89
Breadth	50/62	53/63
Aperture width	17.5/21	20/21
Ratio (L/B)	1.2/1.4	1.4/1.5

*Data from Kosakyan et al. (2013).

Note

Nebela gimlii can be confused with *Nebela guttata*, but clearly differs in the wider shape of the shell and the smaller dimensions of the test (Table 2). Both species are closely related and differ by 3.7% on the considered COI fragment. The two sequences of *N. gimlii* obtained from two different cells share 99.8% of similarity.

Discussion

Phylogeny of the *Nebela collaris* complex and position of *Nebela gimlii*

Our phylogenetic analysis places *N. gimlii* unsurprisingly within the *N. collaris* complex, as its morphology would predict. Within this group, it branches in a clade together with

N. guttata and *N. tincta*, and (although less robustly) with the non-sphagnicolous *N. aliciae*. It shares a curved aperture with *N. guttata*.

N. gimlii resembles strongly *N. guttata*, but both forms do not overlap in size and have a slightly different shape. In addition, if we consider a barcoding gap of around 4% that has been suggested for hyalospheniids (Kosakyan et al., 2012, 2013), and also vannellid naked amoebae (Nassonova et al., 2010), *N. gimlii* can be considered as a new species.

Testate amoeba taxonomy has always been based on the observation of morphology. However, the degree to which arcellinids are phenotypically plastic is debated and has caused much confusion in taxonomy. Most Arcellinids do not secrete the mineral parts of their tests and therefore lack of the geometrically well-defined ornamented plates present in euglyphids. Several species have been shown to fall within a continuum of shapes (Lahr et al., 2008). This taxonomic confusion allows endless debates between "lumpers" and "splitters" and undermines the interpretation of species' biogeography (Heger et al., 2011). DNA barcoding is thus an invaluable tool that can be used to assess the true diversity within species complexes and more generally the taxonomic significance of seemingly minor morphological differences. A possible distinctive ecological niche would corroborate the specific status of these forms, as a triple species concept would then be applied: molecular, morphological and ecological. In the case of *N. gimlii*, it can be suggested that its optimum is located in the driest parts of the peatland. Indeed, we found it only in the forested part of the bog and the drained peatland margins where the water table was low, while it was apparently absent in the wetter parts. The presence of this small species in these comparatively dry habitats is consistent with the observation that smaller sized testate amoebae are favoured in drier environments (Jassey et al., 2011). However more work is clearly required to clarify the ecological optima of individual species within the *N. collaris* complex.

Diversity of the *N. collaris* complex

Generally, peatland ecologists usually consider three or four members of the *Nebela collaris* complex: a small species (*N. tincta*), one of two larger species (*N. collaris* and/or *N. tincta* var. *major*) and the conspicuous wider than long *N. flabellulum* (Charman, 2001). With this study, we raise to eight the number of barcoded species. But the picture is most likely still incomplete, as other sequences in GenBank derived from organisms that have not been documented morphologically yet (JN849067, JN849025, JX682599 GenBank entries) do not fit within these eight species. In addition, most sequences are originated from Europe, and it is likely that some species at least have a restricted geographical distribution, as shown in *Hyalosphenia papilio* (Heger et al., 2013). The case of the *Nebela collaris* complex therefore illustrates well the current under-estimation of protist diversity. The fact that so many new species can be found in well-studied ecosystems within relatively large and conspicuous groups of protists is in line with the idea that protists dominate eukaryotic taxonomic diversity (Pawlowski et al., 2012).

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