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ScienceDirect

European Journal of Protistology 51 (2015) 79–85

European Journal of PROTISTOLOGY

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

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Received 21 August 2014; received in revised form 4 November 2014; accepted 18 November 2014
Available online 24 November 2014

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.
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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

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http://dx.doi.org/10.1016/j.ejop.2014.11.004
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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

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