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

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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

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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