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Environmental DNA COI barcoding for quantitative analysis of protists communities: A test using the *Nebela collaris* complex (Amoebozoa; Arcellinida; Hyalospheniidae)

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Abstract

Environmental DNA surveys are used for screening eukaryotic diversity. However, it is unclear how quantitative this approach is and to what extent results from environmental DNA studies can be used for ecological studies requiring quantitative data. Mitochondrial cytochrome oxidase (COI) is used for species-level taxonomic studies of testate amoebae and should allow assessing the community composition from environmental samples, thus bypassing biases due to morphological identification. We tested this using a COI clone library approach and focusing on the *Nebela collaris* complex. Comparisons with direct microscopy counts showed that the COI clone library diversity data matched the morphologically identified taxa, and that community composition estimates using the two approaches were similar. However, this correlation was improved when microscopy counts were corrected for biovolume. Higher correlation with biovolume-corrected community data suggests that COI clone library data matches the ratio of mitochondria and that within closely-related taxa the density of mitochondria per unit biovolume is approximately constant. Further developments of this metabarcoding approach including quantifying the mitochondrial density among closely-related taxa, experiments on other taxonomic groups and using high throughput sequencing should make if possible to quantitatively estimate community composition of different groups, which would be invaluable for microbial food webs studies.

Keywords: Community ecology; Environmental DNA; Metabarcoding; Protist diversity; *Sphagnum* peatlands; Testate amoebae

Introduction

Environmental DNA surveys are revealing a huge unknown diversity of microbial eukaryotes both globally and within individual samples (Epstein and Lopez-Garcia 2008; Lopez-Garcia et al. 2001; Pawlowski et al. 2012). This high diversity in turn suggests the existence of extremely complex, but mostly undocumented ecological interactions. To understand the ecological roles played by species quantitative estimates of their abundance and biomass are required. For soil protozoan groups such as ciliates and testate amoebae, this is currently done using highly time-consuming direct

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counting of known taxa (Foissner et al. 2002; Jassey et al. 2010; Payne and Mitchell 2009; Schwarz and Frenzel 2003) rather than environmental DNA approaches.

Assessment of environmental micro-eukaryotic diversity is almost exclusively done by sequencing partial or entire ribosomal genes (Pawlowski et al. 2012). Besides biases in DNA extraction, PCR and possibly cloning, copy numbers of ribosomal genes in eukaryotes are known to vary over more than four ranges of magnitude, from one in the picoplanctonic *Nannochloropsis salina* to 12,000 in the large dinoflagellate *Akashiwo sanguinea* (Zhu et al. 2005). Such biases, already known and documented in prokaryotes (Von Wintzingerode et al. 1997), can be expected to be even higher in protists given their larger, more complex and more variable genomes. Thus, in many cases the community structure or biomass evaluation inferred from rDNA studies are not always reliable (Crosby and Criddle 2003; Morgan et al. 2010; Weber and Pawlowski 2013; Von Wintzingerode et al. 1997). Selecting the optimal barcoding marker is not trivial either; ribosomal genes (SSU and LSU rRNA) are most useful for coarse taxon discrimination, while ITS has been shown to present intra-genomic variation in some groups and is therefore not suited as a barcoding gene for all eukaryotic groups (Pawlowski et al. 2012).

The analysis of protist communities by light microscopy is time-consuming. Precise identification of taxa is often hindered by poor taxonomy, and the existence of morphologically similar, but genetically distinct species, which may have different ecological niches. As the identification of many putative taxa is highly dependent on the observer, results are often difficult to compare among studies. This problem, often referred to as cryptic diversity, is recurrent in eukaryotic micro-organisms (Beszteri et al. 2005; Heger et al. 2010; Koch and Ekelund 2005; Kosakyan et al. 2012; Kucera and Darling 2002).

An alternative to existing approaches would be to apply molecular methods but to focus on small groups that are genetically and morphologically well-characterised. In such a context, the use of a variable marker is possible and even required to reach a higher level of taxonomic accuracy. Candidate markers have to be tested and validated for DNA barcoding before being applied to environmental DNA samples. A good candidate marker for such an approach is the mitochondrial cytochrome oxidase subunit 1 (COI) first used for Amoebozoa in species-level taxonomical studies of vannelids Nassonova et al. 2010, and later in Arcellinida (Heger et al. 2013; Kosakyan et al. 2012, 2013).

With the aim of both overcoming limitations of both direct microscopy and environmental rRNA gene surveys, we developed and tested an environmental DNA based method to screen protist diversity, using a cloning-sequencing approach and COI as marker gene. This method allows the assessment of taxonomic diversity within a selected group of closely related protist species. We focussed here on the *Nebela collaris s.l.* species complex (Amoebozoa; Arcellinida; Hyalospheniidae), a common group of testate amoebae in Northern Hemisphere *Sphagnum* peatlands and acidic forest humus.

COI is a mitochondrial marker and, in contrast to ribosomal genes, number of copies should depend directly on mitochondrial density within the organisms. In multicellular organisms (including humans), this parameter can vary depending on the physiological activity of the tissues. For instance, intense muscular activity promotes mitochondrial division, as an adaptation to higher respiration rates required (Hoppeler et al., 1985). In Hyalospheniid cells, metabolism is not expected to vary as much as in the different tissues present in multicellular organisms. Indeed, these testate amoebae have, to our knowledge, never been found alive under anoxic or microaerophilic conditions. Living testate amoebae are only found in the uppermost, photosynthetic section of *Sphagnum* mosses and the young litter immediately below (Mitchell and Gilbert, 2004), where levels of dissolved oxygen can be expected to be high. In addition, while mass-specific activity (a parameter related to mitochondrial density) decreases linearly with body mass in Metazoa, it has been shown to remain constant in plants and protists (Makarieva et al. 2008). It can therefore be reasonably expected that mitochondrial density does not vary much during the life cycle of Hyalospheniid testate amoebae. Therefore, mitochondria numbers can be expected to increase proportionally with cell biovolume. If true, quantitative estimates of community structure from environmental samples would reflect relative biovolume rather than relative cell numbers and this discrepancy would increase with increasing contrast in cell size among taxa. Species of the *Nebela collaris* group show contrasted biovolumes that vary by as much as a factor 3 and thus represent a good model to test if the method can be used to obtain quantitative estimates of community composition from environmental samples. We tested if such a bias could exist within the *Nebela collaris s.l.* species complex by comparing the relative abundance of clones vs. known community composition using artificial communities (i.e. of known composition) and environmental samples. We assessed if the accuracy of COI-based quantitative community structure estimates increased with correction for cell biovolume and thus if COI-based environmental studies could provide quantitative estimated of community structure for the *Nebela collaris* group.

Material and Methods

Analyses of natural and artificial communities

Testate amoebae were extracted from *Sphagnum* samples collected from four peatlands in the Jura Mountains of Switzerland and France (Table 1). Testate amoeba cells were extracted and concentrated by sieving (150 μm) and back sieving (20 μm). This material was used for two complementary sets of experiments schematically shown in Fig. 1 and detailed hereafter: