Protist, Vol. 164, 24–36, January 2013
http://www.elsevier.de/protis
Published online date 20 January 2012

Protist

ORIGINAL PAPER

Utility of Genetic Markers and Morphology for Species Discrimination within the Order Tintinnida (Ciliophora, Spirotrichea)

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Submitted October 11, 2011; Accepted December 20, 2011
Monitoring Editor: Michael Melkonian

We evaluated the small- and large-subunit rDNA (SSU and LSU, respectively) for their ability to discriminate morphospecies of tintinnid ciliates. Multiple individuals from 29 morphospecies were identified according to microscopically-observed characteristics of the lorica, and then sequenced for both loci (21 new species for SSU and all of them new for LSU). Sequences from public databases were included in our analyses, and two hypervariable SSU regions (V4 and V9) were separately examined. Of the four regions, LSU is the most useful as a potential barcoding tool. It showed a gap in distances within and between species, and discriminated the maximum number of phylotypes (86% at 1% cut-off). SSU and V4 were less consistent, sometimes lumping together very distinctive morphospecies, even at the 1% level of sequence divergence. V9 was the least reliable marker in delimitating morphospecies. The agreement in sequences and morphology suggests that the lorica is useful for species discrimination, even in agglomerated forms. However, the observation of both genetically constant yet polymorphic groups of species, as well as similar morphospecies with divergent sequences, indicates that previous taxonomic schemes are complementary to the emerging molecular database.

Key words: Barcoding; Ciliophora; LSU rDNA; SSU rDNA; V4; V9.

Introduction

Ciliates are, in some ways, ideal models for examining diversity, biogeography, and the species concept in protists. Most ciliates have consistent observable features, and their highly amplified genomes make DNA-based methods relatively easy to apply, thus allowing for contrast of the "morphological" and "genetic" species concepts, even on single individuals (Lynn and Pinheiro 2009). In addition, most species practice sexual recombination, which makes them fit the "biological" species concept (Sonneborn 1957). Finally, many forms are amenable to cultivation, allowing for the discrimination of ecotypes and the use of an "ecological" species concept (Finlay 2004; Weisse and Montagnes 1998). Despite a long history of morphological observations, ciliates remain an undersampled group relative to

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doi:10.1016/j.protis.2011.12.002

larger organisms, and the application of genetic and ecological methods for studying their distributions has only just begun. Questions about diversity, biogeography and ecological roles of ciliates, and protists in general, can only be resolved when variations in morphology, genetics, and ecology are reconciled (Foissner 2008; McManus and Katz 2009).

A major concern for linking these aspects is finding appropriate genetic markers. The small-subunit rDNA (SSU) is the most widely used marker for genealogy and systematics, since it provides an appropriate phylogenetic signal, and can be sequenced simply, accurately and universally (Woese 1987). A growing dataset of reference SSU sequences exists for virtually all protist groups, and thus this gene frequently is the marker of choice to estimate molecular diversity in environmental samples (Medlin and Kooistra 2010). For example, short hypervariable regions of SSU, such as V4 and V9, have been recently used as targets for high-throughput sequencing in metagenomic studies (Amaral-Zettler et al. 2009; Stoeck et al. 2009, 2010). These approaches allow for the discrimination of thousands of phylotypes (or operational taxonomic units, OTUs) in a single sample, and have revealed levels of DNA diversity much higher than previously known, especially in marine planktonic microorganisms (López-García and Moreira 2008). However, environmentally-sequenced OTUs hardly ever can be linked to the few protist morphospecies already sequenced, and it is still under debate if the molecular and morphological criteria provide comparable diversity estimates (Caron et al. 2009; Medinger et al. 2010; Nebel et al. 2011). Apart from methodological causes, one of the reasons for the decoupling between both kinds of measures is that SSU, even V4 or V9, is too conservative to distinguish between closely related species within some taxa (Stoeck et al. 2010).

Another locus that has been used to characterize and classify species is the relatively fast-evolving mitochondrial cytochrome *c* oxidase subunit I (COI) gene, which is currently considered as the ideal DNA barcode (Hebert et al. 2003). COI has been useful to differentiate morphospecies in different protist phyla (Chantangsi et al. 2007; Evans et al. 2007; Heger et al. 2011; Lin et al. 2009). However, its universal utility is questionable for protists. Multi-primer approaches are usually needed for amplification and sequencing, and some taxa, for example foraminiferans (Pawlowski and Lecroq 2010) and some classes of ciliates (Strüder-Kypke and Lynn 2010), cannot be sequenced reliably yet. Furthermore, mitochondria, and hence their genome, are lacking in several endosymbiotic protists, such as ciliates of the orders Clevelandellida and Plagiopylida (Lynn 2008).

Among other loci that have been proposed as alternative or additional markers for barcoding (reviewed by Frézal and Leblois 2008), the 5′ end region of the large-subunit rDNA (LSU) is universally present, and has been adequate for morphospecies discrimination in groups such as heterotrophic flagellates (Wylezich et al. 2010), dinoflagellates (Guillou et al. 2002), and diatoms (Hamsher et al. 2011). In addition, LSU has been used to study phylogeography and cryptic species in ciliates (Finlay et al. 2006; Gentekaki and Lynn 2009; Tarcz et al. 2006), and to complement SSU-based phylogenies in several protist lineages (Hewitt et al. 2003; Marande et al. 2009; Moreira et al. 2007). However, the usefulness of LSU as a diversity marker still needs to be tested for many taxa.

The goal of this study was to compare the ability of four genetic markers (SSU, V4, V9, and LSU) to differentiate morphospecies. We used tintinnid ciliates as model. These organisms are both ecologically important within the marine microzooplankton, and morphologically conspicuous due to the presence of a resistant structure (the lorica). The lorica is the basis for species classification (Kofoid and Campbell 1929, 1939) and has provided a powerful tool to analyze patterns of diversity and biogeography (e.g., Dolan et al. 2009; Thompson and Alder 2005), although morphospecies limits are not always clear due to the polymorphism of this structure (Alder 1999; Laval-Peuto and Brownlee 1986). More reliable systematic data, such as cytology and DNA sequences (mainly SSU), are available for fewer than 5% of the approximately 1,200 described morphospecies, and show the limitations of lorica morphology for phylogenetic reconstruction (Agatha and Strüder-Kypke 2007; Gao et al. 2009; Li et al. 2009; Strüder-Kypke and Lynn 2003, 2008). However, sequence comparison between closely related species has been rare, and variability at the intraspecific level has been documented for only a few individuals within five species so far (Kim et al. 2010; Snoeyenbos-West et al. 2002), thus preventing definitive conclusions to be made about the usefulness of genes and lorica morphology for species delimitation. In this study we analyzed the genetic variability within and between morphospecies to contrast species delimitation not only by genetic markers used in environmental