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ORIGINAL PAPER

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

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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.
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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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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

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sequencing and as potential barcodes, but also by lorica morphology.

Results

Twenty-nine tintinnid morphospecies from six genera were classified based on the lorica (Fig. 1, Supplementary Tables S1 and S2). Within morphospecies, loricae were either relatively constant (e.g., *Tintinnopsis cylindrica*, *Tintinnopsis lobiancoi*) or gradually variable (e.g., *Favella ehrenbergii*, *Helicostomella subulata*, *Stenosemella pacifica*, *Tintinnopsis* sp. 4). Most morphospecies were clearly different among them, while some morphospecies showed subtly different loricae (*Tintinnidium balechi* and *Tintinnidium* sp. 2; *Tintinnopsis butschlii* and *Tintinnopsis major*; *Tintinnopsis parvula* and *Tintinnopsis rapa*).

All the individuals illustrated in Figure 1 (up to nine per morphospecies) were sequenced, with a total of 90 and 85 sequences for SSU and LSU, respectively (GenBank Accession number

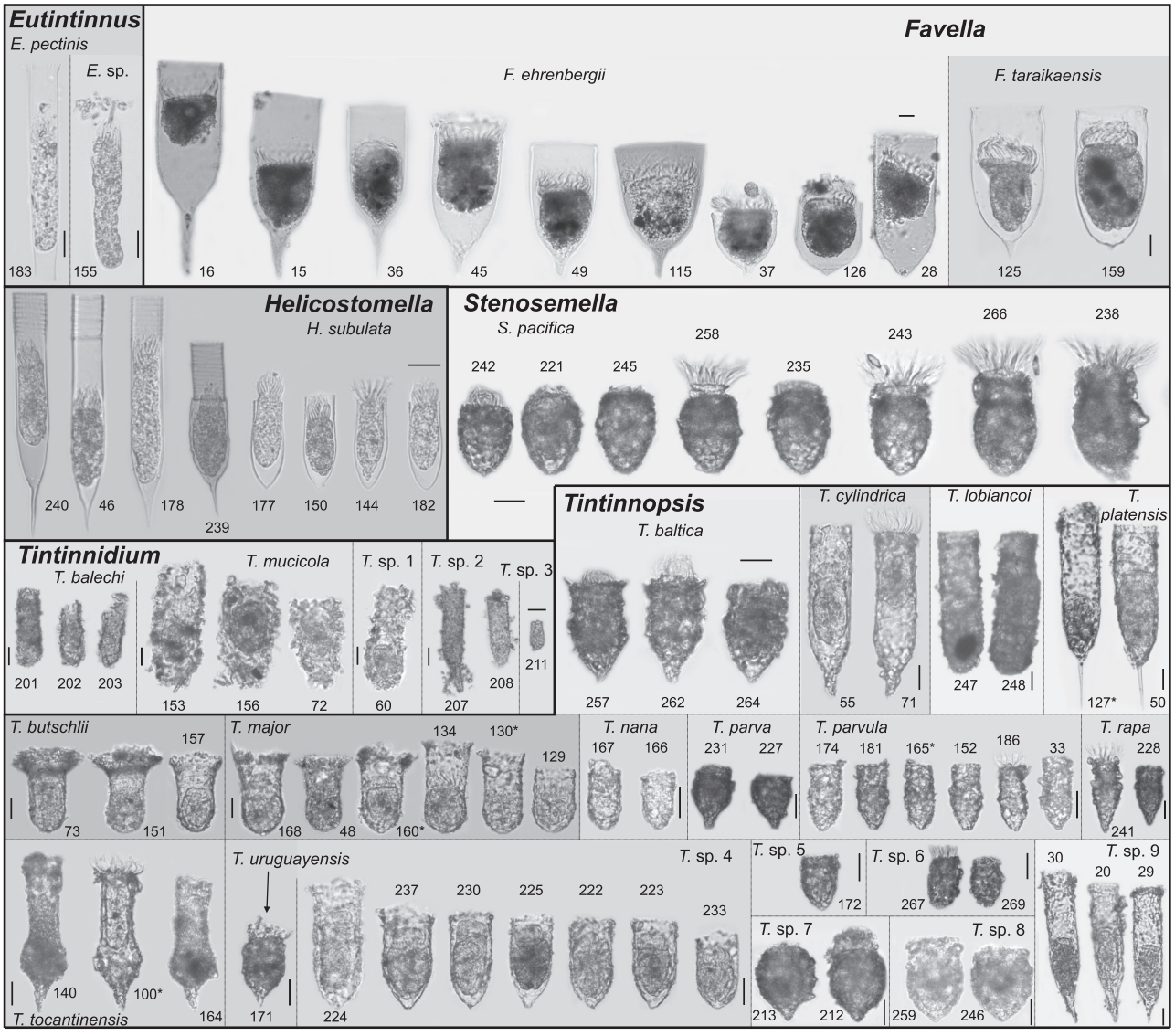

Figure 1. Morphological variability of tintinnid species. To avoid confusion, the unclassified morphospecies of *Tintinnopsis* (*T.* sp 4 to *T.* sp 9) were numbered consecutively to those already reported in GenBank (see Supplementary Table S3). Numbers on each image are individual labels. * Large-subunit rDNA sequence not available. Scale bar = 20 μm.

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JN831766 to JN831940). Twenty-one of these morphospecies were newly sequenced for SSU (see Supplementary Table S1), while tintinnid sequences were obtained for the first time for LSU. The SSU data were pooled with tintinnid sequences previously reported in GenBank (Supplementary Table S3), and additional datasets were created for just the V4 and V9 regions of the SSU sequences. Table 1 summarizes all the genetic data analyzed. Note that, to include as much data as possible, some relatively short SSU and LSU sequences (not more than 12% shorter than those with the maximum length) were also considered.

To find a cut-off value in the genetic divergences within and between morphospecies, the frequency distribution of pairwise p-distances was examined for each marker (Fig. 2). In all cases, the modal distance within morphospecies ranged from 0% to 1%. The scattered values between 1 and 2% (1.1-1.4%) shown by V4 and LSU were caused by single morphospecies (two individuals of *Amphorellopsis acuta* and individuals #46 and 178 of *Helicostomella subulata* in Fig. 1, respectively), while values up to 6% for V9 were caused by the same two morphospecies plus two other individuals (*Tintinnopsis major* #160 and *Tintinnopsis parvula* #165 in Fig. 1). Yet, the mean distance within morphospecies was <1% in almost all cases (Supplementary Table S4). Figure 2 also shows that the distance between morphospecies was, in general, higher than 1% (for V9), 2% (for SSU and V4), or 8% (for LSU), but in all cases it had a minimum value of 0%, thus causing an overlap between both frequency distributions (grey zones in the plots). The overlap was caused by a variable number of morphospecies (see below). The only marker that showed a complete gap between the two distributions was LSU, which did not have any value between 6 and 8%.

The contrast in the proportion of phylotypes differentiated by each genetic marker showed three aspects (Table 2). First, for SSU, V4 and V9, such proportions were 1 to 21% higher when only sequences from this study were considered, compared to the same analysis when also sequences from other studies were included. Second, with a cut-off value of 1%, the proportion of phylotypes differentiated was maximum for LSU (86%) and identical for SSU, V4 and V9 (72%; only sequences from this study). Finally, increasing the cut-off value up to 3% caused 10-20% fewer phylotypes to be differentiated by each of the four markers (only sequences from this study).

To show how sequences are related within and among morphospecies, distance trees were obtained for each genetic marker (Figs 3–6). Individual sequences were grouped or separated based on pairwise p-distances, using the cut-off value that included almost all the "intramorphospecies" variability (1%, see above). Neighbor-Joining, Maximum Likelihood and Bayesian Inference analyses provided similar results, and thus topologies from only the Neighbor-Joining method are illustrated. In the SSU tree, 28 morphospecies were discriminated, but 11 clusters grouping two to five morphospecies were also found (Fig. 3). V4 differentiated 30 morphospecies, but grouped two to five morphospecies in 10 clusters, and separated the individuals of the distinctive morphospecies *A. acuta* (Fig. 4). The tree based on V9 was less consistent, and, even considering some nodes with low support, it differentiated only 23 morphospecies and grouped two to seven morphospecies in 12 clusters, while some individuals from three distinctive morphospecies were divided (Fig. 5). LSU discriminated 21 morphospecies and revealed only four groups of two morphospecies (Fig. 6).

Table 1. Datasets of tintinnid sequences analyzed for small-subunit rDNA (SSU), V4 region of SSU (V4), V9 region of SSU (V9), and large-subunit rDNA (LSU).

Marker	Length (bp)	N morphospecies			N individuals		
		This study	Other studies	Total	This study	Other studies	Total
SSU	1597–1673[a]	29 (21 new)	37	58	90	53	143
V4	367–374	29 (21 new)	37	58	90	53	143
V9	97–103[b]	29 (21 new)	37	58	81	51	132
LSU	618–734[c]	29 (29 new)	0	29	85	0	85

[a]Differences were caused by 18 sequences which were 1 to 71 bases shorter than those with maximum length.
[b]Sequences shorter than 100 bp were excluded, except for the only sequence of *Tintinnidium* sp. 3 (97 bp).
[c]Differences were caused by 19 sequences which were 2 to 87 bases shorter than those with maximum length.
N = number; bp = base pairs.

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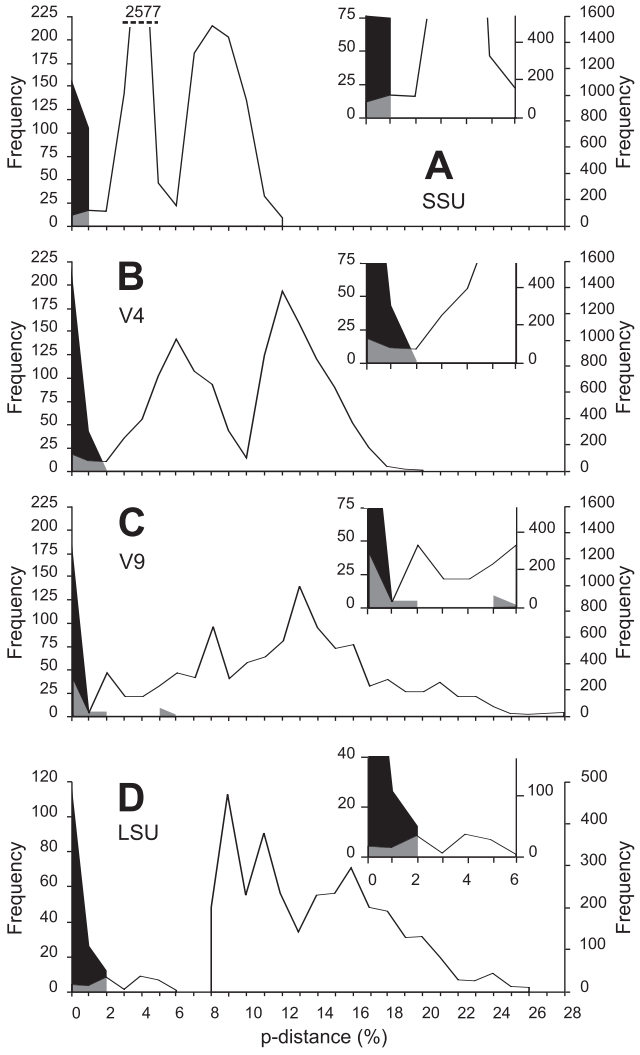

Figure 2. Frequency distribution of pairwise uncorrected p-distances within and between morphospecies (black and white areas, left and right axes, respectively). The region between 1 and 6% was expanded for better resolution. **A** Small-subunit rDNA (SSU); **B** V4 region of SSU (V4); **C** V9 region of SSU (V9); **D** large-subunit rDNA (LSU).

Discussion

The Agreement between Genetic Markers and Morphology

It is still under debate if morphological and molecular characters are comparable for species delimitation, although knowing the degree of correlation between these criteria is needed to understand the diversity, biogeography, and ecological roles of protists (Foissner 2008; McManus and Katz 2009). Classical approaches need testing for cryptic or redundant species, while modern methods, such as metagenomics and barcoding, require adequate genetic markers and cut-off values for taxa discrimination. We compared the morphological and genetic variability in tintinnids to test the ability of lorica morphology and different genetic markers to differentiate species. By sequencing multiple individuals and morphospecies, we were able to quantify intraspecific and interspecific variability. This is crucial for evaluating characters for their usefulness in species discrimination.

The number of taxa discriminated by morphology and genetic markers agreed up to 86% of the time, although it depended of the genetic marker and cut-off used (Table 2). From the morphological point of view, we attribute this high level of agreement to the fact that tintinnid species can be categorized accurately by a careful study of the morphology and morphometrics of the lorica, despite discrepancies in taxonomic schemes (see below) and limited usefulness of the lorica for phylogenetic reconstruction (Agatha and Strüder-Kypke 2007). In contrast, most protists require sophisticated taxonomic techniques for morphospecies differentiation, such as cytological staining to study the infraciliature in aloricate ciliates (e.g., oligotrichs and non-tintinnid choreotrichs). This is consistent with comparisons of morphological and molecular estimates of species richness in coastal planktonic ciliates, where choreotrichs (mainly tintinnids) have shown similar numbers of both morphospecies in

Table 2. Proportion of phylotypes discriminated with different genetic markers and cut-off values. The number of phylotypes differentiated by each marker was based on pairwise p-distances, and proportions were referred to the number of morphospecies sequenced in this study (29) or the total number of morphospecies sequenced up to now (58, except for LSU as only sequences from this study are available).

	SSU		V4		V9		LSU
Cut-off	This study	Total	This study	Total	This study	Total	This study
1%	72%	67%	72%	71%	72%	66%	86%
2%	59%	50%	69%	60%	66%	62%	76%
3%	52%	36%	62%	41%	62%	50%	69%

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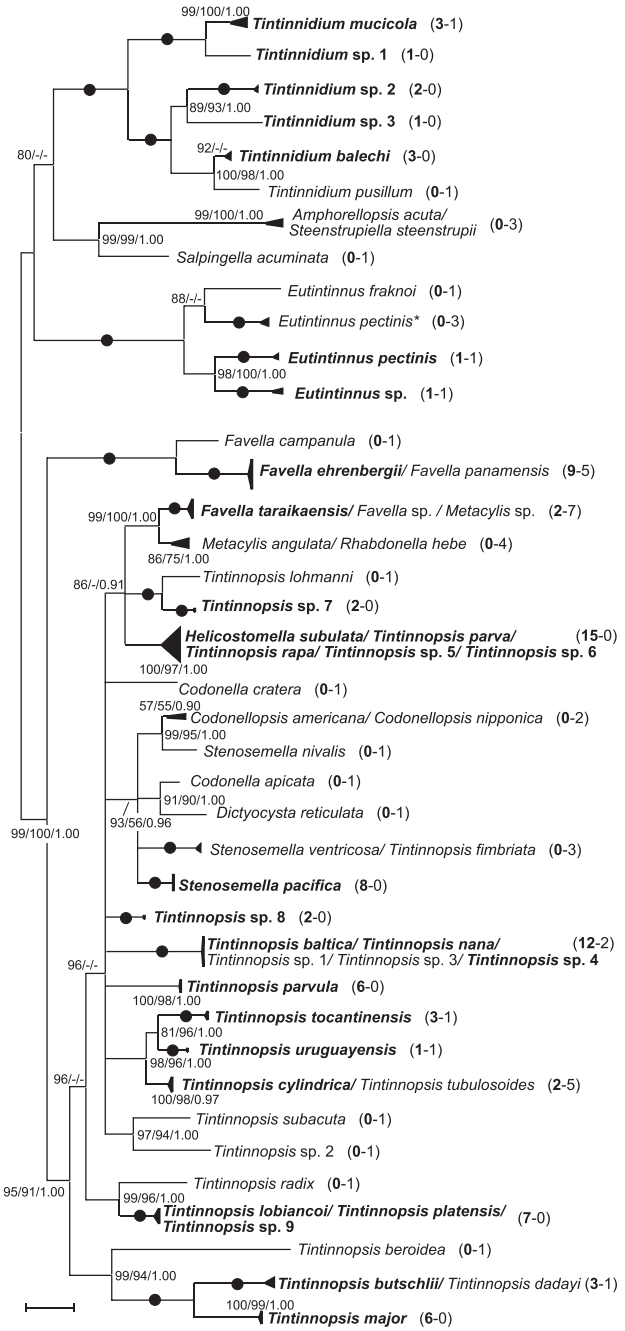

Figure 3. Distance tree based on small-subunit rDNA (SSU). In brackets is shown the number of individual sequences (bold = from this study; regular = from previous studies) that were included within each terminal taxon (1% cut-off). Numbers on each node are bootstrap support values for Neighbor-Joining and Maximum Likelihood (%), and Bayesian Posterior Probability (BPP), respectively. Only nodes with bootstrap support $\geq$ 50% and BPP $\geq$ 0.90 are shown. Black circles indicate fully-supported nodes. Scale bar = 1% distance. *Species classification not coincident with this study.

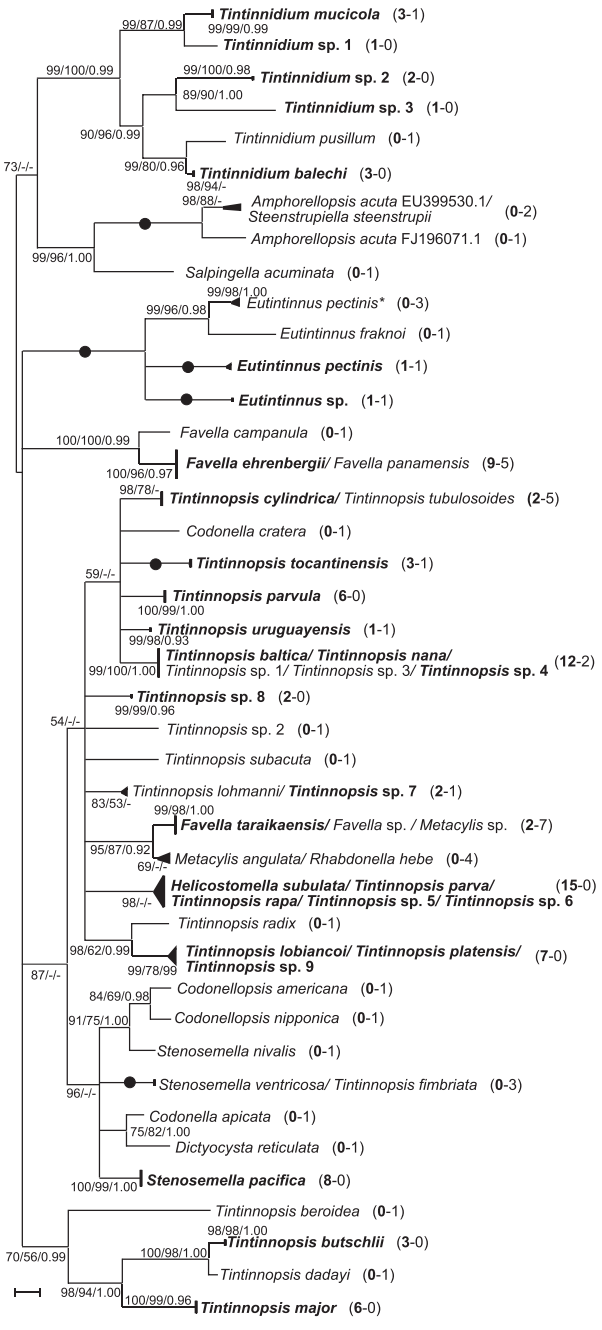

Figure 4. Distance tree based on the V4 region of the SSU. In brackets is shown the number of individual sequences (bold = from this study; regular = from previous studies) that were included within each terminal taxon (1% cut-off). Numbers on each node are bootstrap support values for Neighbor-Joining and Maximum Likelihood (%), and Bayesian Posterior Probability (BPP), respectively. Only nodes with bootstrap support $\geq$ 50% and BPP $\geq$ 0.90 are shown. Black circles indicate fully-supported nodes. Scale bar = 1% distance. *Species classification not coincident with this study.

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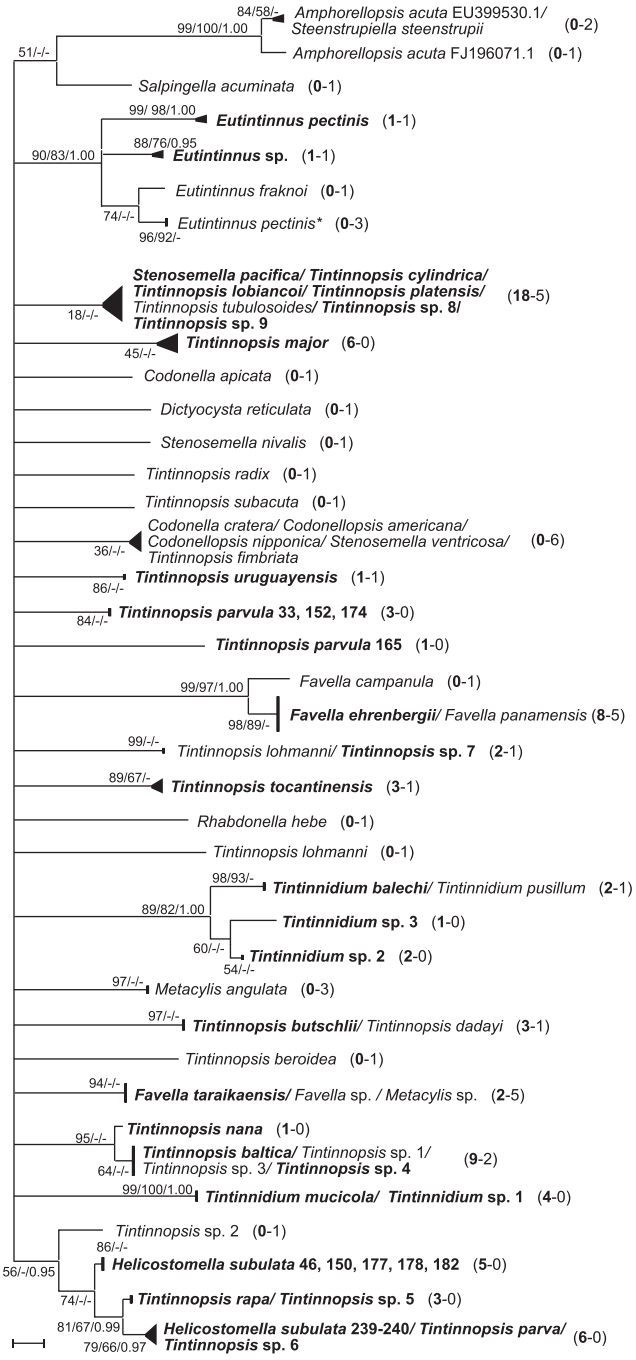

Figure 5. Distance tree based on the V9 region of the SSU. In brackets is shown the number of individual sequences (bold = from this study; regular = from previous studies) that were included within each terminal taxon (1% cut-off). Numbers on each node are bootstrap support values for Neighbor-Joining and Maximum Likelihood (%), and Bayesian Posterior Probability (BPP), respectively. Except three nodes, only those with bootstrap support $\geq$ 50% and BPP $\geq$ 0.90 are shown. Scale bar = 1% distance. *Species classification not coincident with this study.

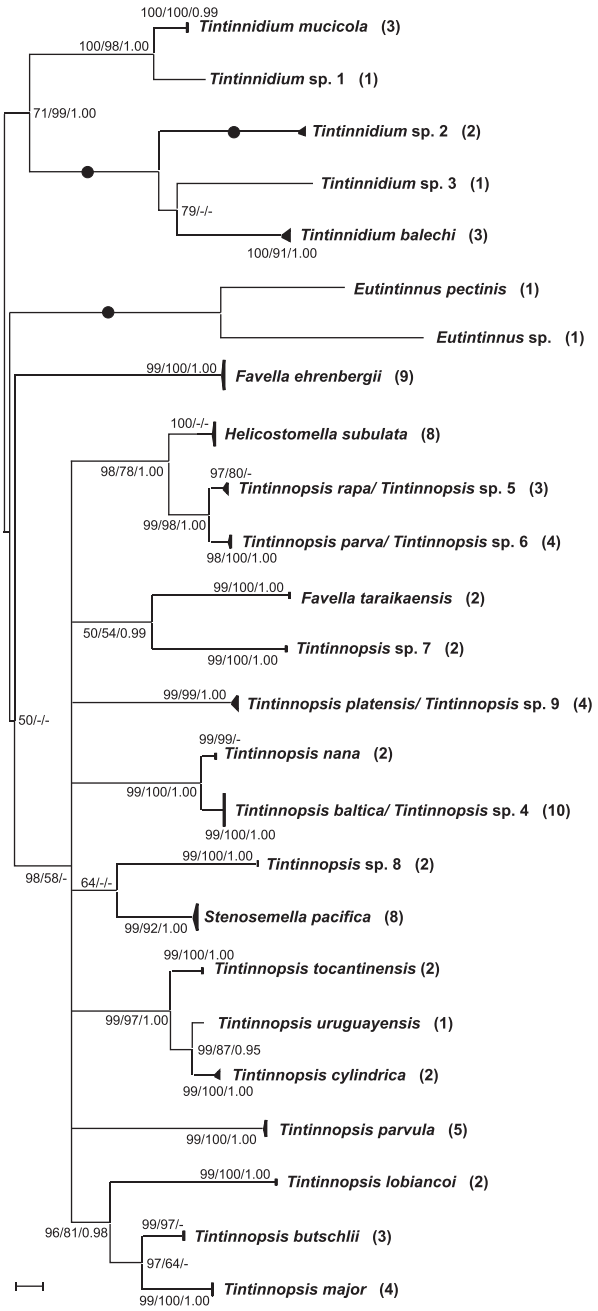

Figure 6. Distance tree based on large-subunit rDNA (LSU). In brackets is shown the number of individual sequences (all from this study) that were included within each terminal taxon (1% cut-off). Numbers on each node are bootstrap support values for Neighbor-Joining and Maximum Likelihood (%), and Bayesian Posterior Probability (BPP), respectively. Only nodes with bootstrap support $\geq$ 50% and BPP $\geq$ 0.90 are shown. Black circles indicate fully-supported nodes. Scale bar = 1% distance.

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fixed samples and OTUs in SSU clone libraries, while oligotrichs have revealed a much higher diversity in DNA sequences than in gross morphology (Doherty et al. 2007).

Genetic Markers

Morphospecies and phylotypes do not necessarily provide comparable diversity estimates, as they are quantified by different methods (Nebel et al. 2011). However, for the markers generally used to analyze diversity in environmental samples, we found that SSU, V4 and V9 provided similar levels of phylotype discrimination (Table 2), although V9 was less effective to assign sequences to the correct morphospecies (Fig. 5). In addition, V9 discriminated 5% fewer phylotype than V4 if sequences from previous studies and a 1% cut-off are considered (Table 2). Even though we probably underestimated the suitability of V9, as its length in tintinnid sequences (ca. 100 base pairs, Table 1) was at the lower limit of that considered in previous studies, V4 is the more variable SSU region for several groups of planktonic protists, and thus inherently provides higher diversity estimates (Amaral-Zettler et al. 2009; Stoeck et al. 2010).

For SSU and its hypervariable regions, we found that a 1% cut-off included all (SSU) or almost all (V4 and V9) the variability within tintinnid morphospecies (Fig. 2 A-C), and discriminated up to 17% or 31% more taxa than a 2% or 3% cut-off, respectively (Table 2). A 1% divergence agrees with the level of SSU-based OTU delimitation used by others for planktonic oligotrichs and choreotrichs (including tintinnids) (Doherty et al. 2007, 2010; Tamura et al., 2011b), although there may not be a single value that can be applied across all ciliates (Nebel et al. 2011). Because ciliates are sexually-reproducing organisms, direct observation of conjugation is needed for including different strains into a species. Within known biological species, e.g. *Tetrahymena thermophila,* the intraspecific divergence in SSU sequences has been estimated to be lower than 0.2% (Katz et al. 2006). When observation of conjugation is lacking, evidence of recombination should be the best measure for including or excluding sequences in a taxon. For that, one would need a large number of sequences from two putative taxa so that the presence or lack of recombinants could be established. This is precluded by our approach of sequencing single individuals, but a larger survey of environmental DNA, especially using next-generation methods to produce a large sequence library, would provide such data.

LSU showed the best performance for phylotype differentiation (Table 2). However, in contrast to SSU and its hypervariable regions, the utility of LSU for diversity estimates by environmental sequencing is currently limited for two main reasons. First, there is a lack of LSU sequences of known morphospecies in GenBank for comparisons. In addition, the usefulness of LSU primers to amplify just ciliates from environmental genomic DNA still needs to be tested.

In spite of this, the usefulness of LSU for phylotype discrimination and identification in tintinnids (Table 2, Fig. 6) makes this marker a promising tool for barcoding, as has been proposed for other protist groups (Hamsher et al. 2011; Wylezich et al. 2010). LSU has an evolutionary rate fast enough to provide higher diversity resolution than SSU, but also to classify sequences correctly. In most cases, LSU-based distances within and between tintinnid morphospecies were $\leq$1% and $\geq$8%, respectively (Fig. 2 D), thus showing a potential "barcoding gap" (Hebert et al. 2003). The partial overlap in distance distributions resulted in four pairs of morphospecies not being discriminated with a 1% cut-off (Fig. 6), although we cannot confirm if it is an artifact due to the use of the lorica for morphospecies classification (see below), or if it is caused by the fact that even LSU may be too conservative to differentiate some highly related species (Hamsher et al. 2011). Low genetic divergence between different tintinnid morphospecies occurs also for other fast-evolving nuclear loci, the internally transcribed spacer regions (ITS) (Snoeyenbos-West et al. 2002), and the lack of a complete barcode gap is known even for COI in some groups of metazoans (Bucklin et al. 2011). Nevertheless, LSU has some advantages over ITS and COI for barcoding. Problems of intraclonal and intraindividual variability seem to be less frequent for LSU (Beszteri et al. 2005) than for ITS (Behnke et al. 2004; Vollmer and Palumbi 2004; Windsor et al. 2006). In addition, LSU has considerably higher universality than COI (Hamsher et al. 2011). COI has not been sequenced reliably for the class Spirotrichea yet (Strüder-Kypke and Lynn 2010), and the scarce COI sequences labeled as tintinnids in GenBank cluster with organisms other than ciliates in BLAST searches (e.g., accession numbers HM880462, HM880464-6, and HM880468).

Morphology

Tintinnid species limits based on lorica morphology has been widely discussed. While some authors (the "splitters") have described hundreds of species

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based on minute differences in lorica shape (Kofoid and Campbell 1929, 1939), others (the "lumpers") have unified taxa based on continuous variations in morphology and size (Alder 1999; Bakker and Phaff 1976; Laval-Peuto and Brownlee 1986). Finding out if the lorica is useful for species discrimination is important because of the methodological advantages offered by this structure (it is easy to collect, preserve and identify), which make it the basis for quantification and species classification in ecologic surveys, and have resulted in extensive publications on the distribution, diversity and biogeography of tintinnids. Our results show that, in most cases, similarities and differences in both morphology (Fig. 1, Supplementary Table S1) and sequences (Figs 3–6) were consistent within and between species, respectively. However, deviations from this general trend indicate that the two main taxonomic currents that have focused on the lorica are complementary.

On the one hand, subtle morphological difference but high genetic divergence was found between some morphospecies. For example, the maximum diameter and a more or less acute posterior end are the only differences between loricae of *Tintinnopsis parvula* and *Tintinnopsis rapa* (Fig. 1). In addition, the shape of the posterior end is the only difference between loricae of *Tintinnopsis butschlii* and *Tintinnopsis major*, as well as *Tintinnidium balechi* and *Tintinnidium* sp. 2. Yet SSU and LSU were able to separate all of them as distinctive genetic species at the 1% level of divergence (Figs 3 and 6). These morphospecies thus represent examples of "pseudocryptic" species from the lorica point of view (lack of lorica distinctiveness would suggest they be lumped), as is found for other protists with shell-based taxonomies (Heger et al. 2011). Therefore, these results support a criterion that has been used to create hundreds of species based on small differences in the lorica (Kofoid and Campbell 1929, 1939).

In contrast, variation in morphology but constancy in DNA sequences was found for some species. For example, continuous polymorphism coincided with a lack in genetic divergence for *Tintinnopsis* sp. 4 (Fig. 1, Supplementary Table S4). In addition, genetic similarity despite morphological variability is consistent with previous culture, cytological and/ or molecular data for *Favella ehrenbergii* (Kim et al. 2010; Laval-Peuto 1981) and *Stenosemella pacifica* (Agatha and Tsai 2008). Finally, *Helicostomella subulata* varied mainly in length, but the constancy in the oral diameter, which is the only lorica-based character with diagnostic value in this morphospecies (Santoferrara and Alder 2009), agreed with mean distances lower than 1% in both SSU and LSU sequences (Supplementary Table S4). Even if two out of eight individuals within *H. subulata* diverged by 1.1 and 1.4% in LSU, these values agree with LSU intraspecific variability within known biological species, e.g. *Paramecium pentaurelia* (1.1%; Przybós et al. 2011) and *Paramecium dodecaurelia* (1.3%; Przybós et al. 2008). These results support a taxonomic view in which small or continuous variations in the lorica lack value for species description, and the oral diameter is the only unambiguous lorica-based character for species diagnosis (Alder 1999; Laval-Peuto and Brownlee 1986).

Despite its utility for morphospecies discrimination, taxonomic assignment based on the lorica may be ambiguous in some cases. Phylotype discrimination was higher when only sequences from this study were considered (Table 2), possibly because GenBank data results from different criteria for sequence assignment to morphospecies. Thus, some taxa similar in morphology and sequences may be synonyms, for example *Tintinnopsis butschlii* and *Tintinnopsis dadayi*, as well as *Favella ehrenbergii* and *Favella panamensis* (already suggested by Kim et al. 2010), while dissimilar sequences assigned to *Eutintinnus pectinis* may correspond to two different morphospecies (Fig. 3). On the other hand, in this study the lorica did not provide enough information to link some *Eutintinnus*, *Tintinnidium*, and *Tintinnopsis* morphospecies to previous descriptions (Fig. 1, Supplementary Table S1). Of them, four *Tintinnopsis* spp. clustered with other morphospecies even using LSU and a 1% cut-off (Fig. 6). *Tintinnopsis* sp. 6 and *Tintinnopsis* sp. 9 clustered with *Tintinnopsis parva* and *Tintinnopsis platensis*, respectively, but differences in morphology and morphometrics suggest that they may be different species. In contrast, *Tintinnopsis* sp. 4 and *Tintinnopsis* sp. 5 clustered with *Tintinnopsis baltica* and *Tintinnopsis rapa*, respectively, but their morphological discrimination is less clear. More individual samplings, additional genetic markers, and cytological data are needed to resolve these ambiguous classifications.

Conclusions

The congruence in morphology and DNA sequences for species discrimination depends on taxonomic criteria, genetic markers, and cut-off values. In tintinnids, taxon discrimination by lorica morphology (complementing the "lumping" and

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"splitting" taxonomic criteria) and LSU sequences (using a 1% cut-off) agree 86% of the time. These observations, the presence of a "barcoding gap", and the universality of LSU, make this marker a promising tool for diversity studies and barcoding in tintinnids. Among markers currently used to estimate diversity in environmental samples, SSU, V4 and V9 provide similar taxon discrimination, although V9 was less effective for identification. Finally, the general concordance in our study between phylotypes and morphologically-identified species suggests that a practical taxonomy of tintinnids that is useful for ecological studies can be constructed using sequence data and the rich body of morphospecies descriptions available in the literature. We recommend continued efforts to include more tintinnids in genetic databases, to perform more analyses to test the utility of genetic markers in environmental samples, and to develop LSU barcoding procedures to expand studies of tintinnid biogeography and ecology.

Methods

Sampling and single-cell sequencing: Tintinnids were sampled in coastal waters of the NW and SW Atlantic during summer and autumn 2010 and spring 2011 (Supplementary Table S1). Samples were collected with a 20 μm-mesh plankton net and preserved with non-acidic Lugol Solution (2% f.c.). Single individuals (lorica plus cell) were picked up with a capillary pipet under an inverted microscope, placed in a drop of water on a slide, measured, and photographed to document morphology. Each individual was classified based on lorica morphology, and the primary identifications were not changed in light of genetic evidence. Original descriptions and illustrations reproduced in the revisions by Kofoid and Cambell (1929, 1939) and Alder (1999) were used for identification. In addition, studies based on culture, cytology or detailed lorica morphometrics that have demonstrated or suggested intraspecific polymorphism (e.g., Agatha and Tsai 2008, for *S. pacifica*; Kim et al. 2010, Laval-Peuto 1981, for *F. ehrenbergii*; Santoferrara and Alder 2009, for *H. subulata*), as well as works by Agatha (2010) and Agatha and Riedel-Lorjé (2006), were considered (see Supplementary Table S1).

For DNA extraction, each individual was transferred to a microcentrifuge tube with 50 μL of lysis buffer (1% SDS, 0.1 M EDTA, pH = 8), mixed with 1 μL of Proteinase K (20 mg mL^{-1}), and incubated at 55 °C for 12 h. Then, DNA was purified using DNA Clean & concentrator-25 (Zymo Research, Orange, CA) and eluted with 20 μL of 10 mM Tris.Cl, pH = 8. For PCR amplification of SSU and LSU, the primers listed in Table 3 were used. In some cases, nested-PCR combining the universal and tintinnid-specific primers was used for SSU amplification. PCR reactions were carried out in a final volume of 25 μL, which included 2.5 μL of Takara® buffer (10×), 2 μL of dNTPs (2.5 mM each), 0.5 μL of Mg Cl_2 (25 mM), 1 μL of each primer (5 μM), 0.625 units of Takara® DNA polymerase, and 1 μL of template DNA. PCR conditions were: for SSU amplification, 95 °C for 1 min, 35 cycles of 15 s at 94 °C, 30 s at 55 or 56 °C and 40 s at 72 °C, and 72 °C for 10 min; and for LSU amplification,

Table 3. Primers used for PCR amplification and sequencing of small-subunit rDNA (SSU) and large-subunit rDNA (LSU), and for delimiting the V4 and V9 regions of SSU.

Primer	Target[a]	Use	Sequence (5′ - 3′)	Reference
18Scom-F1	Universal, SSU, 5′ end (30-54)	Amplification/ sequencing	GCTTGTCTCAAAGATTAAGCCATGC	Zhang et al. 2005
18Scom-R1	Universal, SSU, 3′ end (1736-1760)	Amplification/ sequencing	CACCTACGGAAACCTTGTTACGAC	Zhang et al. 2005
Tin18S-F	Tintinnids, SSU, internal (873-897)	Amplification/ sequencing	ATTAGTACTTAACTGTCAGAGGTG	This study
Tin18S-R1	Tintinnids, SSU, internal (1100-1120)	Amplification	TTCAGCCTTGCGACCATACTC	This study
Tin18S-R2	Tintinnids, SSU, internal (1105-1127)	Sequencing	CGGCATAGTTTATGGTTAAGACT	This study
28S-F1a	Universal, LSU, 5′ end (50-70)	Amplification/ sequencing	GCGGAGGAAAAGAAACTAAC	Ortman 2008
28S-R1a	Universal, LSU, 5′ end (832-855)	Amplification/ sequencing	GCATAGTTTCACCATCTTTCGGG	Ortman 2008
TAReuk454FWD1	Eukaryotes, V4, 5′ end (560-580)	Trimming of V4	CCAGCA(G/C)C(C/T)GCGGTAATTCC	Stoeck et al. 2010
TAReukREV3	Eukaryotes, V4, 3′ end (977-995)	Trimming of V4	ACTTTCGTTCTTGAT(C/T)(A/G)A	Stoeck et al. 2010
1391F	Universal, V9, 5′ end (1617-1633)	Trimming of V9	GTACACACCGCCCGTC	Lane 1991

[a]Numbers in brackets correspond to the annealing position based on *Halteria grandinella* GenBank accession number AF508759.

94 °C for 3 min, 35 cycles of 45 s at 94 °C, 40 s at 50 °C and 90 s at 72 °C, and 72 °C for 10 min. Products were isolated by electrophoresis in 1.2% agarose/ TAE gel, and purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). For SSU sequencing, the tintinnid-specific primers were used in all cases, and the universal primers were also utilized in some cases to get longer sequences; for LSU, both strands were sequenced for all samples (Table 3). Big Dye Terminator v3.1 and a capillary DNA sequencer (ABI 3730 or ABI 3130, Applied Biosystems Inc.) were used.

Data analysis: The four datasets analyzed (Table 1) were edited using MEGA v5 (Tamura et al., 2011a). Alignments were done with ClustalW (Thompson et al. 1994) and refined manually. The datasets of V4 and V9 were trimmed using the primer sequences detailed in Table 3; the 3' end of V9 was delimited by the use of the primer 18Scom-R1.

For each dataset, pairwise uncorrected p-distances within and between morphospecies were estimated using MEGA v5. In addition, trees based on p-distance were constructed using the Neighbor-Joining algorithm (Saitou and Nei 1987) and setting 10,000 bootstrap replicates in MEGA v5. To check the topology of the distance trees, additional analyses were performed using Maximum Likelihood and Bayesian Inference. The Maximum Likelihood analyses were carried out using the MPI version of RAxML (Stamatakis et al. 2007), setting 10,000 bootstrap replicates, the GTR model of nucleotide substitution with the Γ model of rate heterogeneity (GTR-GAMMA option), and a random starting tree. Additional searches for the Best-Known Likelihood tree were done (200 inferences). The Bayesian Inference analyses were performed using the serial version of MrBayes (Ronquist and Huelsenbeck 2003). The models of sequence evolution for each dataset were identified with MrModeltest v2.3 under the AIC criterion (Nylander 2004). Analyses were run for 5,000,000 generations, and trees were sampled each 1,000 cycles. The initial 1,000 trees were discarded as burn-in, and the remaining 4,000 trees were used to make a consensus tree and estimate the posterior probabilities at each node.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2011.12.002.

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