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Discrimination of Closely Related Species in Tintinnid Ciliates: New Insights on Crypticity and Polymorphism in the Genus *Helicostomella*

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This study focuses on the utility of molecular markers for the discrimination of closely related species in tintinnid ciliates. We analyzed the ecologically important genus *Helicostomella* by sequencing part of the large-subunit rDNA (LSU rDNA) and the 5.8S rDNA combined with the internally transcribed spacer regions 1 and 2 (5.8S rDNA-ITS) from forty-five individuals collected in NW and SW Atlantic waters and after culturing. Although all described *Helicostomella* species represent a continuum of morphologies, forms with shorter or longer loricae would correspond to different species according to previous molecular data. Here we observed that long forms show both crypticity (i.e. two almost identical long forms with different DNA sequences) and polymorphism (i.e. some long forms develop significantly shorter loricae after culturing). Reviewing all available tintinnid sequences, we found that 1) three *Helicostomella* clusters are consistent with different species from a molecular perspective, although these clusters are neither clearly differentiated by their loricae nor unambiguously linked to described species, 2) *Helicostomella* is closely related (probably to the family or genus level) to four "*Tintinnopsis*-like" morphospecies, and 3) if considered separately, neither LSU rDNA nor 5.8S rDNA-ITS completely discriminate closely related species, thus supporting the use of multi-gene barcodes for tintinnids.

Key words: Tintinnid ciliate; closely related species; cryptic species; polymorphic species; molecular markers; DNA barcoding.

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Introduction

Differentiating closely related species in protists is a challenge, considering both conceptual and methodological issues (Almeida and Araujo 2013; Boenigk et al. 2012). Rather than rigid limits, diffuse circumscriptions apply to protist species (Caron 2009), which still need to be discriminated if we want to understand their diversity, biogeography and ecological roles. In particular, knowing the correspondence between morphology and DNA sequences is essential to understand protists from the systematic and ecological points of view (Caron 2013). Such comparisons have proliferated during the last decade for the tintinnid ciliates, a group with classical taxonomy based mostly on the morphology of the lorica that is attached to the cell (e.g. Kofoid and Campbell 1929, 1939) and with an increasing amount of molecular information predominantly on ribosomal DNA (rDNA) sequences (e.g. Agatha and Strüder-Kypke 2007, 2012; Bachy et al. 2012; Santoferrara et al. 2012; Xu et al. 2013). The still-limited data on DNA sequences, cytology and lorica ultrastructure have revealed several inconsistencies in the lorica-based taxa and are gradually allowing for rearrangements in families and genera (see reviews by Agatha and Strüder-Kypke 2013, 2014), while only recent studies have focused on analyses at the species level (Kim et al. 2013; Santoferrara et al. 2013; Xu et al. 2012).

Among the features that are useful for species discrimination in tintinnids, phenotypic characters are either ambiguous or difficult to study. Despite providing a relatively easy means for species classification, the diagnostic value of lorica morphology is questionable due to its plasticity during the life cycle or as a consequence of environmental conditions, which complicates the discrimination of morphologically similar species. Furthermore, the difficulty in culturing these organisms has resulted in a scarcity of data on cytology, physiology and reproductive isolation. For example, the ciliary pattern, which is the gold standard for ciliate systematics, has been studied in only a few tintinnids (fewer than thirty out of the more than one thousand described species; Agatha and Strüder-Kypke 2014), and thus the significance of some cytological features for discriminating closely related species is unknown (e.g. Kim et al. 2013). Studies on sexual compatibility between distinct morphospecies have been successful in only *Favella ehrenbergii* so far (Laval-Peuto 1981).

Among the genetic markers that have been sequenced for tintinnids, the small-subunit (SSU) rDNA appears to be too conservative to differentiate closely related species. In contrast, the more variable D1-D2 region of the large-subunit (LSU) rDNA performs better for morphospecies discrimination in tintinnids (Santoferrara et al. 2013), and it has been suggested as an alternative barcode in ciliates due to the non-ideal performance of the mitochondrial cytochrome oxidase subunit I gene (Stoeck et al. 2014). Sequences of the 5.8S rDNA combined with the internally transcribed spacer regions 1 and 2 (5.8S rDNA-ITS) and/or the secondary structure of ITS2 have been used for tintinnid species discrimination (Snoeyenbos-West et al. 2002; Xu et al. 2012), although their global utility has not been tested for the order Tintinnida yet. Finding adequate molecular markers for species circumscription and identification (i.e. DNA barcodes) is important, for example, given the increasing application of genetic tools in environmental surveys of ciliates (e.g. Bachy et al. 2013; Doherty et al. 2007; Grattepanche et al. 2014; Santoferrara et al. 2014).

One example where species differentiation has been problematic is *Helicostomella* Jörgensen 1924. This genus is ecologically relevant because of its global neritic distribution (Dolan and Pierce 2013) and its occasional dominance in marine ciliate assemblages (Bojanić et al. 2006; Nakane et al. 2008; Santoferrara and Alder 2009a, 2012) or even in the entire microzooplankton community (Kim et al. 2007). For this genus, seven species have been described based on lorica morphology (see review by Santoferrara and Alder 2009b). However, these species (including also one species of *Metacylis* Jörgensen 1924) are impossible to discriminate using lorica features alone because they show constancy in oral diameter (the lorica feature of highest diagnostic value in tintinnids), continuity in length-related parameters and inconsistency in other characters used for creating them (e.g., general shape, presence of denticulation or wrinkles, shape of the aboral end) (Alder 1999; Santoferrara and Alder 2009b). Cytological data is limited to one species at present (Pierce 1996). Based on only eight *Helicostomella* individuals isolated from the Atlantic Ocean (Connecticut coast, USA), Santoferrara et al. (2013) could not establish clear differences in SSU rDNA or LSU rDNA sequences. In contrast, Xu et al. (2012) suggested that "short" and "long" forms of *Helicostomella* are different species based on 5.8S rDNA-ITS sequences and ITS2 secondary structure from five individuals from the western Pacific Ocean (Korea coast).

In this study we show that the taxonomic complexity within *Helicostomella* is greater than previously thought. We took into consideration that multiple single cells from closely related species need to be sequenced to attempt their discrimination. Furthermore, if the variability of a given molecular marker is used for species circumscription, it is necessary to evaluate the meaning of such variability in a broader, lineage-wide context. Here, by collecting multiple *Helicostomella* individuals from two coastal sites of the NW and SW Atlantic, during different dates, and after culturing, we detected three clusters according to LSU rDNA and 5.8S rDNA-ITS: two clusters that correspond to the short and long forms reported in the Pacific (Xu et al. 2012), plus a new cluster that overlaps in morphology with the long form. In addition, we found that the long form overlapped in morphology with the short form under culture conditions. We evaluated this information in the context of all available LSU rDNA and 5.8S rDNA-ITS tintinnid sequences in order to 1) gain new insights on the circumscription of *Helicostomella* species, 2) analyze the relationship between *Helicostomella* and other tintinnid genera, and 3) test these molecular markers for the discrimination of closely related species within tintinnids.

Results

Three Sequence Clusters within *Helicostomella*

We sequenced forty-five *Helicostomella* individuals from the NW and SW Atlantic (Fig. 1; Table 1 and Supplementary Material Table S1). *Helicostomella* sequences formed three clusters based on LSU rDNA (Fig. 2) and 5.8S rDNA-ITS (Fig. 3). Mean p-distance values within clusters were lower than 0.2% (Table 1) while mean values among clusters ranged from 0.4% to 1.4% for LSU rDNA, and from 1.2% to 4.5% for 5.8S rDNA-ITS (Table 2).

Cluster I was detected exclusively in NW Atlantic during late summer/ early autumn in three years and during early summer in only one year. Cluster II comprised sequences from NW Atlantic during late winter/ spring of three consecutive years (this study), from the Pacific during the same time of the year (Xu et al. 2012), and from NW and SW Atlantic in summer of only one year (this study). Cluster III included sequences from NW Atlantic (this study) and Pacific (Xu et al. 2012) in late summer (Table 1 and Supplementary Material Table S1).

Sequence Clusters and Morphospecies are Decoupled within *Helicostomella*

The three *Helicostomella* clusters are difficult to discriminate based on their corresponding loricae (Fig. 1; Table 1). Field-collected loricae from each cluster differed significantly in oral diameter and total length (*t* test, $p < 0.05$; Supplementary Material Table S2), but, in practice, such differences were difficult to establish as they either showed overlapping ranges or varied by only a few micrometers. Loricae of clusters I and II overlapped in total length (178 ± 37 μm and 128 ± 25 μm, respectively) and corresponded to "long forms" sensu Xu et al. (2012). Both clusters included individuals with or without denticulation in the oral rim and intermediate rings. The oral diameter was only 2 μm narrower for cluster I (20 ± 1 μm) than for cluster II (22 ± 3 μm). Although loricae from both clusters showed a conical aboral end usually followed by a horn of variable length, cluster II loricae sometimes showed a more marked constriction before the horn (Fig. 1B). The "short" loricae corresponding to cluster III were relatively distinct given their smaller average size (total length = 67 ± 11 μm, oral diameter = 18 ± 1 μm), thinner wall, lack of denticulation, and aboral end rounded with an infrequent, very short horn. However, as explained below, similar short loricae can be produced by "long forms".

Cluster II individuals produced both long and short loricae in cultures. After 20 days culturing, cluster II loricae decreased significantly in total length (from 168 ± 10 μm in the individuals isolated from NW Atlantic on May 2013 to 107 ± 16 μm after culturing; *t* test, $p < 0.05$) but did not vary significantly in oral diameter (23 ± 1 μm; *t* test, $p > 0.05$) (Table S2). Loricae of cultured individuals were as short as 83 μm and resembled cluster III loricae also in the lack of denticulation and general shape (Fig. 1C-D). Cultured individuals had sequences identical to cluster II, eliminating the possibility that cells from other clusters were present in the initial cultures (Table 1).

Close Relationship between *Helicostomella* and Some *Tintinnopsis* Morphospecies

A cluster of *Tintinnopsis* morphospecies (*T. parva*, *T. rapa*, *T.* sp. 5 and *T.* sp. 6, hereafter referred as "*Tintinnopsis* cluster") grouped with the three *Helicostomella* clusters based on LSU rDNA (Fig. 2) and 5.8S rDNA-ITS (Fig. 3). These *Tintinnopsis* morphospecies were found in the NW Atlantic mostly during the same months as *Helicostomella*

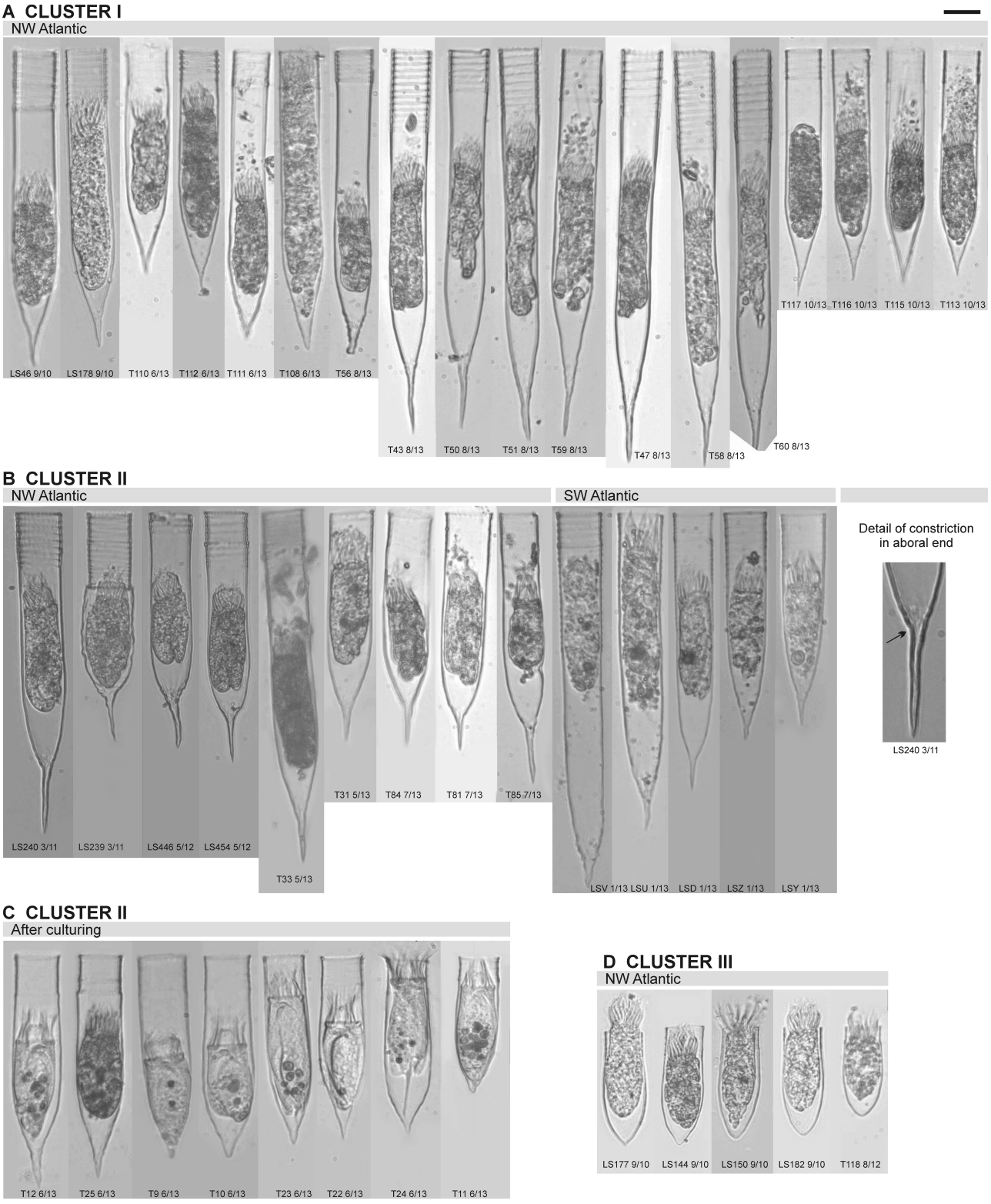

Figure 1. *Helicostomella* specimens from cluster I (part **A**), cluster II (parts **B**, **C**) and cluster III (part **D**). Each specimen sequenced is shown and labeled with isolate number and date (month/ year). Scale bar = 20 μm.

Table 1. Morphological and molecular characterization of *Helicostomella* clusters and the closely related *Tintinnopsis* cluster.

Group	Site	Period	N (n)[g]	Oral diameter[h]	Total length[h]	Mean p-distance within cluster	
						LSU rDNA	5.8S rDNA-ITS
***Helicostomella* clusters**							
Cluster I	NW Atlantic	Aug-Sept 2010[a] Sept-Oct 2012 Jun 3013 Aug-Sept-Oct 2013	70 (18)	19.9 (1.0)	177.6 (37.4)	0.06%	0%
Cluster II	NW Atlantic	Mar-Apr 2011[a] Apr-May 2012 Apr-May 2013 Jul 2013	87 (17)	23.4 (1.3)	158.5 (20.4)[d] 107.3 (15.7)[e]	0%	0%
	SW Atlantic	Jan 2013	17 (5)	23.0 (1.2)	140.1 (33.7)	0.12%	0%
	Pacific	Mar 2009[b]	30 (2)[b]	18.2 (4.2)[b]	108.0 (21.8)[b]	-	0%
	All	All	134 (24)	21.5 (2.9)	128.4 (25.1)	0.03%	0.18%
Cluster III	NW Atlantic	Sept 2010[a] Aug 2012	10 (5)	18.3 (1.0)	53.9 (4.3)	0%	0%
	Pacific	Oct 2008[b] Aug 2009[b]	65 (3)[b]	18.0 (0.5)[b] 19.0 (1.5)[b]	70.6 (3.8)[b] 75.0 (8.2)[b]	-	0%
	All	All	75 (8)	18.4 (0.5)	66.5 (11.1)	-	0%
All	All	All	279 (50)	20.0 (2.3)	111.3 (44.2)	0.69%	2.35%
***Tintinnopsis* cluster**							
T. parva	NW Atlantic	Mar 2011[a]	2 (2)	23.4 (0.1)	47.4 (4.3)	0%	0%
T. rapa	NW Atlantic	Mar 2011[a, c]	5 (2)	24.6 (2.1)	58.2 (12.4)	0%	0%
T. sp. 5	NW Atlantic	Sept 2010[a, c] Jun 2012	4 (3)	21.7 (2.1)	37.7 (1.7)	0%	0%
T. sp. 6	NW Atlantic	Apr 2011[a]	3 (2)	21.5 (0.6)	40.1 (1.0)	0.16%	-[f]
All	All	All	14 (9)	23.4 (2.5)	48.5 (12.6)	0.91%	0%
***Helicostomella* clusters + *Tintinnopsis* cluster**							
All	All	All	293 (59)	21.0 (2.4)	89.5 (48.0)	1.63%	2.47%

[a] Collections from Santoferrara et al. 2013. [b] From Xu et al. 2012. [c] Isolated in same sample than *Helicostomella*. [d] Cultured individuals excluded. [e] Value for nine cultured individuals. [f] Only one individual sequenced (but included in "All"). [g] N = number of individuals measured; n = number of individuals sequenced. [h] Lorica measures as mean (standard deviation), in μm.

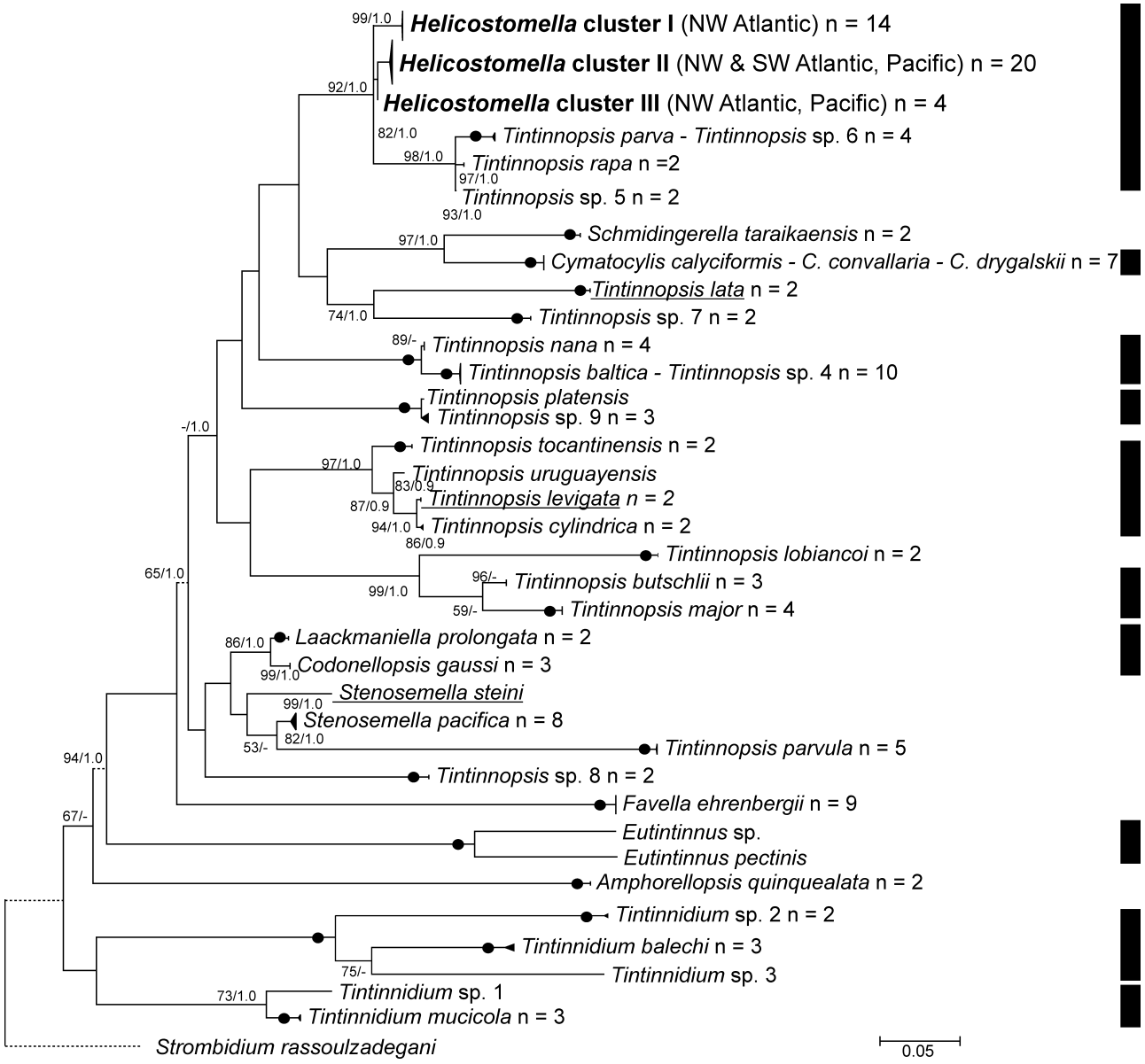

Figure 2. Maximum likelihood (ML) tree based on LSU rDNA sequences of the order Tintinnida. For morphospecies with more than one sequence available, the number of sequences collapsed in each branch (n) is shown. Different morphospecies with identical sequence were also collapsed. Numbers on each node are ML bootstrap support (MLS) and Bayesian posterior probability (BPP), respectively. Only MLS $\geq$50% and/or BPP $\geq$0.9 are shown. A black circle indicates full support in both analyses. One species from the subclass Oligotrichia was used as outgroup. The scale bar represents five substitutions per 100 nucleotides. For convenience of illustration, some long branches were shortened to one-tenth their actual length (dotted lines). Underlined morphospecies were newly sequenced in this study. GenBank accession numbers for sequences from other studies are detailed in Supplementary Material Table S4. Black bars indicate the groups of closely related species considered in Figure 4 (see Supplementary Material text 2).

clusters, but they co-occurred in only a few samples (Table 1).

The *Tintinnopsis* morphospecies showed loricae with similar oral diameter ($23 \pm 3\,\mu m$), continuous variability in total length ($49 \pm 13\,\mu m$), and slight differences in overall shape (Table 1, Supplementary Material Fig. S1, Table S3, text S1). They were identical according to 5.8S rDNA-ITS, but varied by 0.1 to 1.7% in LSU rDNA (Table 2).

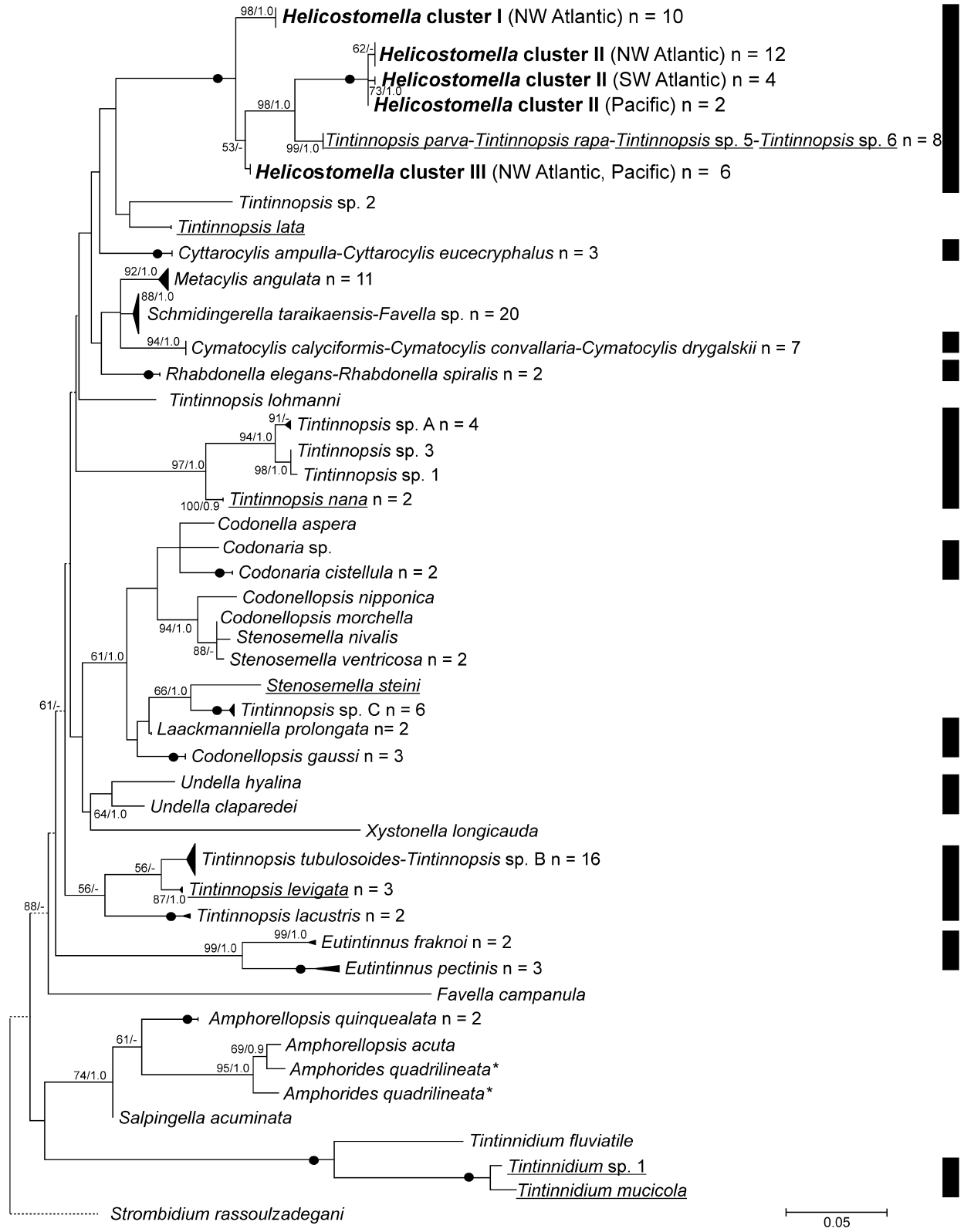

Figure 3. Maximum likelihood tree based on 5.8S rDNA-ITS sequences of the order Tintinnida. Explanations as in Figure 2.

Table 2. Mean p-distance (%) between each *Helicostomella* cluster and closely related *Tintinnopsis*.

	H. cluster I	*H.* cluster II	*H.* cluster III	*T. parva*	*T. rapa*	*T.* sp. 5	*T.* sp. 6
LSU rDNA							
H. cluster I	-	1.4	1.2	4.9	3.8	4.0	4.9
H. cluster II	-	-	0.4	4.1	3.4	3.2	4.2
H. cluster III	-	-	-	3.9	3.2	3.0	4.0
T. parva	-	-	-	-	1.6	1.4	0.1
T. rapa	-	-	-	-	-	0.3	1.7
T. sp. 5	-	-	-	-	-	-	1.4
T. sp. 6	-	-	-	-	-	-	-
5.8S rDNA-ITS							
H. cluster I	-	4.5	1.2	3.2	3.2	3.2	3.2
H. cluster II	-	-	3.8	3.2	3.2	3.2	3.2
H. cluster III	-	-	-	2.0	2.0	2.0	2.0
T. parva	-	-	-	-	0	0	0
T. rapa	-	-	-	-	-	0	0
T. sp. 5	-	-	-	-	-	-	0
T. sp. 6	-	-	-	-	-	-	-

Mean distances within *Helicostomella* or within *Helicostomella* combined with the *Tintinnopsis* cluster were very similar for LSU rDNA (0.7% and 1.6%, respectively) and 5.8S rDNA-ITS (2.4% and 2.5%, respectively). Mean distances among *Helicostomella* clusters and morphospecies in the *Tintinnopsis* cluster ranged from 3.0% to 4.9% for LSU rDNA and 2.0% to 3.2% for 5.8S rDNA-ITS (Table 2). Furthermore, *Helicostomella* clusters and the *Tintinnopsis* cluster share a 23-nucleotide deletion in the 5′ end of ITS1 that is not present in any other tintinnid sequenced so far (Supplementary Material Fig. S2). A close relationship between *Helicostomella* and the *Tintinnopsis* cluster was confirmed also by inferences based on concatenated SSU rDNA, 5.8S rDNA-ITS and LSU rDNA with full support in both Maximum Likelihood and Bayesian Inference trees (Supplementary Material Fig. S3).

Discussion

Discrimination of Closely Related Species by Genetic Markers

Here we compare morphological and molecular data to discriminate species within *Helicostomella*, a genus that cannot be unambiguously classified down to species based on lorica features alone (Santoferrara and Alder 2009b; Fig. 1) and presents at least three clusters of LSU rDNA and 5.8S rDNA-ITS sequences (Figs 2 and 3). Both to understand the meaning of genetic divergence among *Helicostomella* clusters and to evaluate the utility of those two molecular markers for differentiation of closely related species in tintinnids, we compared the distribution of distance values within and between all the morphospecies sequenced for this order (Fig. 4 A, C).

Sequence distances within morphospecies seem to have clear limits in tintinnids, with 0% as the most frequent value and scattered higher values. The highest variability within morphospecies was found for 5.8S rDNA-ITS, with values up to 1.5%. This range of variability is wider than the 0 to 1% values detected in tintinnids using either a partial 5.8S rDNA-ITS sequence (comparing only 23 sequences of 232 bp; Bachy et al. 2013) or SSU rDNA (Gong et al. 2013; Santoferrara et al. 2013). The lowest variability within morphospecies corresponded to LSU rDNA, with values not higher than 0.6%. This figure is slightly lower than previously estimated for tintinnids (because eight *Helicostomella* sequences had been lumped at that time; Santoferrara et al. 2013) and identical to the maximum variability found within each of twenty-four mating species of *Paramecium* using the same marker (Stoeck et al. 2014).

Analyzing variability between morphospecies is more problematic due to current uncertainties in tintinnid systematics. We focused on closely related species, which a priori would be those from the same genus. However, given known inconsistencies (especially in *Tintinnopsis*), we used the trees in Figures 2 and 3 to define close relationships (black bars; see also Supplementary Material text 2). Distances between closely related species

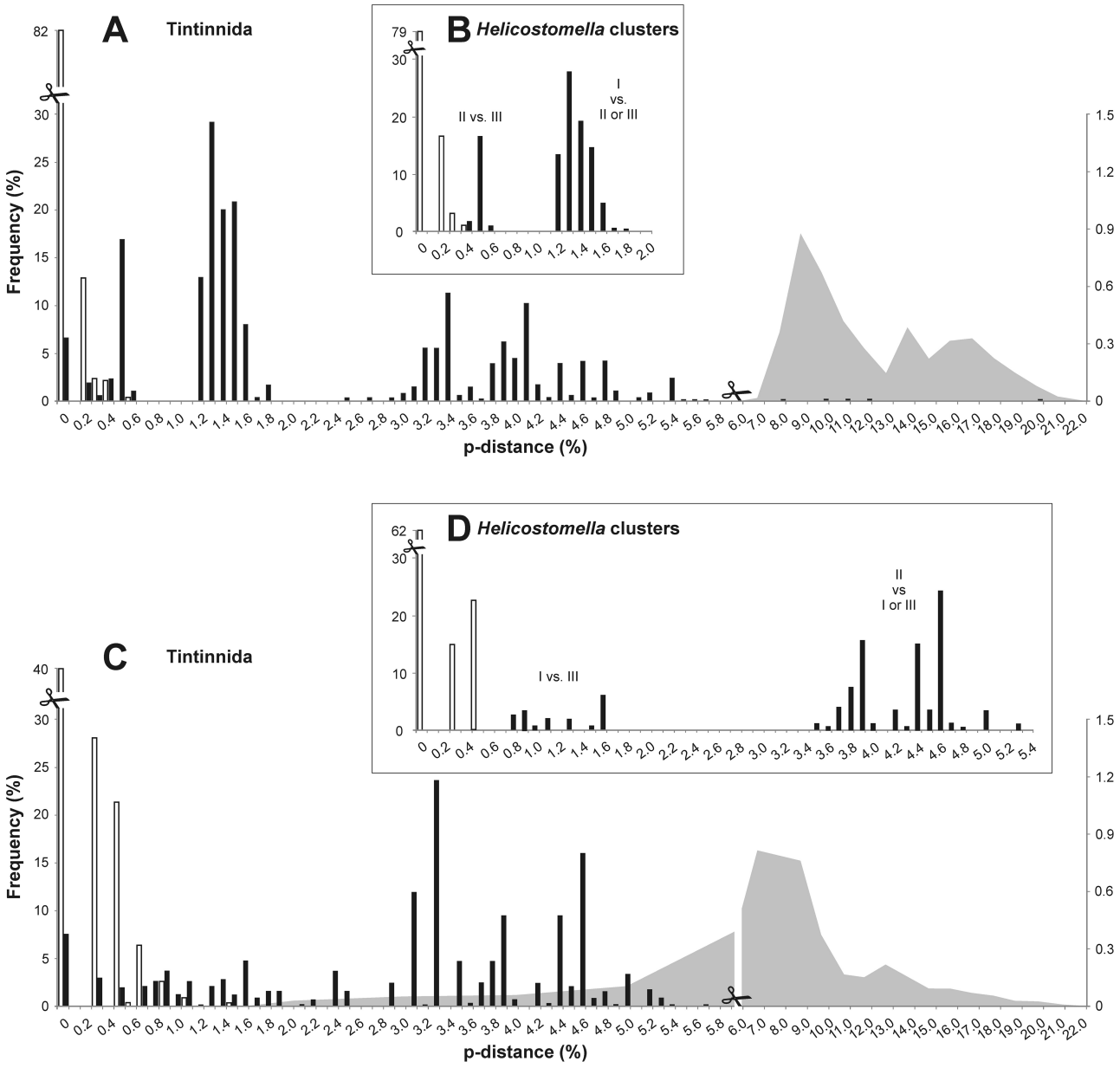

Figure 4. Frequency distribution of p-distance values within and between tintinnid morphospecies according to LSU rDNA (A-B) and 5.8S rDNA-ITS (C-D). A and C include all tintinnids: pairwise p-distance within morphospecies (white bars, primary *y* axis), between closely related morphospecies according to Figures 2 and 3 (black bars, secondary *y* axis), and between all the remaining morphospecies (gray area, primary *y* axis). B and D consider only *Helicostomella* clusters: pairwise p-distance within and between clusters (white and black bars, respectively). Number of pairwise sequence comparisons: A [within (457), between close (901), between non-close (8,045)], B [within (307), between (434)], C [within (580), between close (831), between non-close (10,492)], and D [within (213), between (348)].

generally ranged from 0 to 6%, both for LSU rDNA and 5.8S rDNA-ITS (the few exceptions corresponded to *Eutintinnus* spp. and *Tintinnidium* spp., with distances between species up to 8% and 16%, respectively). Surprisingly, even distances between each *Helicostomella* cluster and a morphologically distinct *Tintinnopsis* cluster (Supplementary Material Fig. S1) fall within the range of "closely related species" according to LSU rDNA and 5.8S rDNA-ITS (Fig. 4, Table 2). On the other hand, distances

Table 3. Discrimination of tintinnid morphospecies by molecular markers.

	LSU rDNA	5.8S rDNA-ITS
Comparisons within morphospecies		
Number of morphospecies analyzed	32	25
Number of individuals analyzed	113	129
Number of pairwise comparisons	457	580
Proportion of pairwise comparisons resulting in p-distance = 0%	82%	40%
Maximum p-distance value (cut-off)	0.6%	1.5%
Comparisons between morphospecies		
Number of morphospecies analyzed	40	52
Number of pairs of morphospecies compared	780	1326
Number of pairs of closely related morphospecies compared	41	43
Proportion of pairs of morphospecies with p-distance $\leq$ cut-off	1%	1%
Proportion of pairs of closely related morphospecies with p-distance $\leq$ cut-off	22%	30%[a]

[a]Pairs among *Amphorides-Amphorellopsis* and *Stenosemella-Codonellopsis* excluded.

between non-closely related species ranged from 6 to 22% for LSU rDNA and from 2 to 21% for 5.8S rDNA-ITS. In this case, the lower limit in variability for 5.8S rDNA-ITS is because, for example, *Cymatocylis* and *Schmidingerella* differed by only 2.6% in this marker but 6.8% in LSU rDNA.

Both LSU rDNA and 5.8S rDNA-ITS have some degree of limitation for the discrimination of closely related species in tintinnids, as indicated by the overlap in distance distributions within and between morphospecies (Fig. 4). Of the paired morphospecies compared, 22 and 30% of the closely related species could not be discriminated by LSU rDNA and 5.8S rDNA-ITS using cutoff values of 0.6 and 1.5%, respectively (Table 3). This is influenced by limitations in both the use of the lorica for morphospecies discrimination and the resolution power of the molecular markers. First, polymorphic loricae may belong to the same species. For example, three *Cymatocylis* morphospecies (*C. convallaria*, *C. calyciformis* and *C. drygalskii*) have identical LSU rDNA and 5.8S rDNA-ITS, which supports synonymy (Kim et al., 2013) and the idea that species in this genus develop polymorphic loricae as a response to environmental conditions (Alder 1999; Boltovskoy et al. 1990). Second, in some cases a molecular marker may be too conservative to discriminate species, with additional markers required. For example, 5.8S rDNA-ITS failed to discriminate the morphospecies included in the "*Tintinnopsis* cluster", but they represent at least two pairs of morphospecies up to 1.7% different according to LSU rDNA (Table 2). The proportion of morphospecies not discriminated by LSU rDNA and 5.8S rDNA-ITS dropped to only 1% if we consider the total data available (Table 3), although this conclusion is preliminary given that most described tintinnid morphospecies still need sequencing.

Species Differentiation in *Helicostomella*

Our molecular data suggest that the three clusters of *Helicostomella* sequences correspond to different species. There was no overlap in the variability of LSU rDNA and 5.8S rDNA-ITS within and between each *Helicostomella* cluster (Fig. 4 B, D), although values among *Helicostomella* clusters (0.4-1.8% for LSU rDNA and 0.8-5.3% for 5.8S rDNA-ITS) slightly overlapped the global variability within tintinnid morphospecies (Fig. 4 A, C). Thus, the combination of two molecular markers was crucial, as LSU rDNA clearly discriminated cluster I versus II and III, while 5.8S rDNA-ITS differentiated better cluster II versus I and III (Fig. 4, Table 2). Also, 5.8S rDNA-ITS sequences from cluster II seem to show structure among populations of different geographic origin (mean distances <0.2%, Table 1; Fig. 3), a matter of future research provided the amount of data is enough for statistical tests on population genetics.

In contrast to LSU rDNA and 5.8S rDNA-ITS sequences, predictions of ITS2 secondary structure did not help to discriminate the three *Helicostomella* clusters (Supplementary Material Fig. S4). The presence of at least one compensatory base change (CBC) in the most conserved region of ITS2 (the 30 bp on the 5′ side of helix III) has been shown to correlate with sexual incompatibility among eukaryotes, and thus it has been proposed as tool for distinguishing biological species (Coleman 2009). More relaxed criteria suggest that one CBC anywhere in ITS2 is indicative of different biological species (Müller et al. 2007).

For *Helicostomella*, a single CBC between cluster II and either cluster III or cluster I is found on a less conserved region of ITS2 (3′ side of helix B, Supplementary Material Fig. S4; Xu et al. 2012). In addition, no CBCs were detected between *Helicostomella* clusters I and III or between *Helicostomella* cluster II and the *Tintinnopsis* cluster, which is not surprising because the absence of CBCs does not prove conspecificity (Ruhl et al. 2010). The correct interpretation and power of ITS2 secondary structure for species differentiation is currently debated (Caisová et al. 2011, 2013) and errors in data analysis can be found in the literature. For these reasons, and given that this criterion has been explored in very few tintinnids so far, the ITS2 secondary structure cannot be accepted at present as a useful means for the discrimination of species in these ciliates.

Crypticity and Polymorphism

Lorica morphology and molecular markers correlate relatively well in tintinnid species (Santoferrara et al. 2013), with *Helicostomella* being a clear exception. Although our molecular analyses support the existence of at least three species in *Helicostomella*, they also confirm that lorica morphology is not useful for species delimitation in this genus. In addition to the wide and overlapping ranges in lorica characters (Fig. 1), which support doubts about the discrimination power of this structure within *Helicostomella* (and even between some *Helicostomella* and *Metacylis* species; Alder 1999; Jörgensen 1924; Margalef and Durand 1953; Santoferrara and Alder 2009b, Xu et al. 2012), DNA sequences helped to confirm crypticity and polymorphism in this genus.

Helicostomella clusters I and II are cryptic from the lorica point of view, as their slight differences are insufficient to discriminate them or to assign them to different described morphospecies (Fig. 1A-B). Both clusters correspond to long loricae compatible with the type species of the genus, *Helicostomella subulata* (Ehrenberg 1833) Jörgensen 1924 (cylindrical lorica with anterior part "annulated", less than 120 μm to 500 μm long, 20-26 μm wide). In contrast, loricae of cluster III (Fig. 1D) seem easier to differentiate (but see next paragraph) and may correspond to *Helicostomella longa* (Brandt 1906) Kofoid and Campbell, 1929 (50-80 μm long and aboral horn absent or scarcely differentiated, but with a greater width of 22-26 μm) or *Metacylis annulifera* (Ostenfeld and Schmidt 1901) Kofoid and Campbell 1929 (52-73 μm long, 18 μm wide, acute or obtuse aboral end).

Helicostomella cluster II is polymorphic, as it corresponded to long loricae in the field samples but produced some short loricae under culture conditions (Fig. 1B-C). Only a few examples of lorica polymorphism have been confirmed by culturing, the most conclusive of them for *Favella ehrenbergii*, which produces very dissimilar loricae in different stages of its cell cycle (Laval-Peuto 1981). Given that these phenotypic variations can also occur in nature (Laval-Peuto 1994), then in some cases long and short forms of *Helicostomella* may still belong to the same genetic species, thus complicating ecological conclusions if they are detected together (Margalef and Durand 1953; Santoferrara and Alder 2009b). Therefore, the division of *Helicostomella* into short and long forms is ambiguous and we recommend rDNA sequencing for the correct identification of clusters I, II and III.

Apart from crypticity and polymorphism, we also detected inconsistencies in the use of the lorica for classification at higher taxonomic levels. The morphospecies that we identified as *Tintinnopsis parva* and *Tintinnopsis rapa* (as well as the closely related *Tintinnopsis* sp. 5 and *Tintinnopsis* sp. 6) clustered apart from the GenBank sequence labeled as *Tintinnopsis beroidea*, the type of the genus, according to SSU rDNA phylogenetic inferences (Santoferrara et al. 2012). Although any revision is premature due to the lack of cytological information for most tintinnid species, we suggest that these morphospecies do not belong to *Tintinnopsis* Stein, 1867 and they probably share a family (or even a genus, see above) with *Helicostomella*, given their close and strong genetic relationship (Figs 2, 3; Supplementary Material Figs S2 and S3). In addition, *Helicostomella* may not belong to the family Metacylididae Kofoid and Campbell, 1929, as it clustered apart from the type genus, *Metacylis* Jörgensen, 1924, according to both SSU rDNA (Santoferrara et al. 2012) and 5.8S rDNA-ITS (Fig. 3). Additional work on cytology and lorica ultrastructure is needed to confirm the affiliation of these taxa.

Seasonality

The seasonality of three genetic clusters within morphologically-indistinguishable *Helicostomella* species supports the view that some morphospecies are actually monophyletic assemblages of sibling species restricted temporally (and very likely, spatially) due to adaptation to different ecological niches, as found in other protists with hard shells (de Vargas et al. 2004). The *Helicostomella* clusters consistently appeared (and disappeared)

during the same seasons from our NW Atlantic samples (Table 1). However, our sequencing based on a high number of individuals showed that the clusters were not completely segregated by season (Supplementary Material Table S1), in contrast to the seasonal isolation previously reported for short and long forms of *Helicostomella* (Xu et al. 2012). A review of ecological works combined with field data from the SW Atlantic suggested that alternation of these forms is related to seasonally-influenced environmental factors such as temperature, structure of the water column and food quantity (Santoferrara and Alder 2009b), excystment/ encystment being one of the mechanisms that leads to the periodic appearance/disappearance of *Helicostomella* from the plankton (Kamiyama 2013; Paranjape 1980).

While closely related species probably experience ecological partitioning, they may still share some niche parameters, such as food quality. *Helicostomella* clusters were rarely found together, or in combination with the closely related *Tintinnopsis* morphospecies (Table 1 and Supplementary Material Table S1). This is consistent with the idea that closely related species use similar niches and that greater niche similarity causes more frequent competitive exclusion and less frequent co-occurrence (reviewed in Cavender-Bares et al. 2009; Wiens et al. 2010). For example, Violle et al. (2011) demonstrated that the frequency of competitive exclusion was significantly greater between ciliate species that were more similar not only phylogenetically but also in mouth size, which influences prey selection. Considering that *Helicostomella* and closely related *Tintinnopsis* share an oral diameter of about 20 μm (Table 1), and that this feature correlates with preferred prey size in tintinnids (Dolan 2010), one potential explanation for the low frequency of co-occurrence among these species is that they use the same food resources, thus resulting in competitive exclusion.

Conclusions

Analyzing all available LSU rDNA and 5.8S rDNA-ITS sequences for tintinnids, we have shown that three *Helicostomella* clusters are consistent with different species based on a molecular point of view. Lorica features are not enough to differentiate species according to a morphological criterion, while cytology and sexual isolation remain to be studied in order to confirm species circumscriptions within *Helicostomella*. We have found that increasing the number of DNA markers and individuals sequenced can reveal patterns more complex than previously thought and that it is important to evaluate the discrimination power of genetic markers before using them for species circumscription. Unresolved taxonomy can cause misinterpretation of barcoding power and, in some cases, the combination of different markers is key, thus supporting the idea that multi-locus approaches will provide a better barcoding strategy. Next-generation sequencing, which now enables targeting up to thousands of genetic markers simultaneously (Davey et al. 2011), will help to clarify relationships between closely related species and permit more informative studies on population genetics. Ultimately, a combination of morphology, DNA sequences, physiology and ecology is needed to discriminate closely related species and to elucidate their ecological significance.

Methods

Sample collection, microcopy and single cell sequencing: Samples were collected with a net of 20 μm mesh. Tows were done in shallow (2 m depth), coastal waters from the dock of the University of Connecticut (Groton, CT, USA) on multiple dates between August 2010 and December 2013 (Supplementary Material Table S1) and in a coastal location near Staten Island (Argentina) on January 1st 2013. These samples were preserved with non-acidic Lugol's solution (2% final concentration). Culture of the NW Atlantic populations was attempted in different periods (May, August and October 2013) and under different conditions, but only the May attempt was successful. Cells isolated on May 20, 2013 were cultured until June 10, 2013 in an incubator at 19 °C and under a14 h light/ 10 h dark cycle. The growth medium consisted of a 1:1 mixture of filtered seawater from the same sampling site and supplemental mineral nutrients solution (salinity 30 and nutrients added as f/2; Guillard and Ryther 1962). The algae *Isochrysis* sp. strain T-Iso (10^4 cells mL^{-1}) and *Dunaliella tertiolecta* strain DT (10^4 cells mL^{-1}), obtained from the NMFS laboratory (Milford, CT, USA), were used as food.

Presence/absence and lorica morphology of *Helicostomella* were documented in the microscope for each Lugol sample and for the initial and final culture samples (Supplementary Material Table S1). Since a detailed morphological study of *Helicostomella* showed limited value of lorica features for species differentiation (Santoferrara and Alder 2009b), we evaluated only the general morphology, oral diameter and total length. Loricae were measured using a total magnification of 400x and a calibrated digital system.

Single individuals were isolated for DNA extraction, PCR amplification and sequencing according to Santoferrara et al. (2013). Apart from *Helicostomella*, ten morphospecies were newly sequenced (three for LSU rDNA, ten for 5.8S rDNA-ITS and two for SSU rDNA; Supplementary Material Fig. S1, Table S3, text S1). Primer sequences are included in Supplementary Material Table S5. The D1-D2 region of LSU rDNA and 5.8S rDNA-ITS amplification and sequencing were based on primers reported by Ortman (2008) and Snoeyenbos-West et al. (2002), respectively. Two overlapping fragments of SSU rDNA were amplified by combining primers reported by Zhang et al.

(2005) and Santoferrara et al. (2013). Sequencing was done in a capillary DNA sequencer (ABI 3730, Applied Biosystems Inc.) and chromatograms were checked for ambiguous peaks. Two variable sites at the intraindividual level were detected in 5.8S rDNA-ITS sequences from cluster II (double-peaks of the same intensity at positions 16 and 77), although no further analyses were done due to the low number of observations (five and two sequences, respectively). All new sequences were uploaded to NCBI GenBank (accession numbers KM982810-KM982897). Following previous studies (Santoferrara and Alder 2009; Xu et al. 2012), and given current uncertainties about the link between clusters I, II and III and described *Helicostomella* species (see Discussion), all records have been labeled as *Helicostomella subulata* (cluster I, II or III) until the taxonomy of this genus is clearer.

Sequence analysis: All of our LSU rDNA and 5.8S rDNA-ITS sequences and those available in GenBank for tintinnid morphospecies (Supplementary Material Table S4) were combined and aligned with Muscle. The oligotrich ciliate *Strombidium rassoulzadegani* was added as outgroup. Alignments were refined manually and resulted in 139 and 157 sequences with 789 and 527 sites for LSU rDNA and 5.8S rDNA-ITS, respectively. Morphospecies with known SSU rDNA, 5.8S rDNA-ITS and LSU rDNA sequences were included in a concatenated alignment of 20 sequences and 2868 sites. Sequence JQ408165 (*Cyttarocylis ampulla*; Bachy et al. 2012) was trimmed at 391 bp because of its high divergence in the terminal end compared to other tintinnids. Sequence AF399032 (*Tintinnopsis* sp. B; Snoeyenbos-West et al. 2002) was excluded because of its divergence within the same morphospecies, and two SSU rDNA sequences labeled as *Helicostomella* (AB640639 and AB640640; Kazama et al. 2012) were disregarded due to potential inadequacy (see Agatha and Strüder-Kypke 2014). We updated GenBank records for sequences previously labeled as *Favella taraikaensis* (Santoferrara et al. 2012, 2013) and *Favella* sp. (Snoeyenbos-West et al. 2002), now re-labeled as *Schmidingerella* species (see Agatha and Strüder-Kypke 2012).

LSU rDNA, 5.8S rDNA-ITS and concatenated alignments were used for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. ML analyses were done with RAxML (Stamatakis et al. 2007), setting 5,000 bootstrap replicates and a random starting tree. The ML bootstrap support (MLS) for each node and the Best-Known Likelihood Tree were estimated. BI analyses were done using MrBayes (Ronquist and Huelsenbeck 2003). Five million generations were run 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 estimate the Bayesian Posterior Probabilities (BPP). For each analysis, the GTR model with a Γ model of rate heterogeneity and a proportion of invariable sites was used, as previously identified with MrModeltest under the Akaike Information Criterion (Nylander 2004). LSU rDNA and 5.8S rDNA-ITS alignments were used for estimating pairwise uncorrected p-distances with MEGA v5 (Tamura et al. 2011).

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2014.11.005.

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Discrimination of Closely Related Species in Tintinnid Ciliates: New Insights on Crypticity and Polymorphism in the Genus *Helicostomella*

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Supplementary data

Supplementary text 1. Discussion of species identifications

We focused on *Helicostomella* species, which are characterized in the main text of this paper, Fig. 1, Tables 1 and S1. To contribute increasing the amount of molecular data on tintinnid species, we newly sequenced *Stenosemella steini*, *Tintinnopsis lata* and *Tintinnopsis levigata*, which are characterized below (Fig. S1; Table S3). We also obtained the first 5.8S rDNA-ITS sequences for *Tintinnidium mucicola, Tintinnopsis nana, Tintinnopsis parva, Tintinnopsis rapa* and three unidentified morphospecies (*Tintinnidium* sp. 1, *Tintinnopsis* sp. 5 and *Tintinnopsis* sp. 6), that were previously characterized and sequenced for LSU rDNA by Santoferrara et al (2013), and are further characterized in Fig. S1, Tables 1 and S3.

Stenosemella steini (Jörgensen 1912) Jörgensen 1924 (Fig. S1 G) presented a densely agglomerated body and a low, narrow collar with smaller particles. The body was sharply expanded bellow the collar. This species is very similar to *Stenosemella ventricosa* (Claparède and Lachmann 1858) Jörgensen 1924, which instead presents a more rounded transition between the body and the collar (Jörgensen 1924). Our specimens had a slightly wider oral diameter and were longer (Table S3) than the type population (43-45 µm in oral diameter, 71-74 µm in maximum diameter and 86-88 µm in length; Jörgensen 1912).

Individuals identified as *Tintinnopsis lata* Meunier 1910 (Fig. S1 F) matched its original description in shape (maximum diameter wider than oral diameter, pointed aboral end, agglomerated with very small particles, and straight oral end, although the lorica illustrated in Fig. S1, isolate LS460, showed a slightly expanded oral end). Meunier (1910) did not provide measurements, but the size of our specimens (Table S3) matched the ones provided in Marshal's compilation (1969): 38-42 µm in oral diameter and 62-70 µm in total length.

Tintinnopsis levigata Wailes 1925 (Fig. S1 E) has a cylindrical lorica, with a truncated, cylindrical-conical appendix. Our specimens (Table S3) matched the sizes provided in the original description (20-30 µm in oral diameter and 50-70 µm in total length; Wailes 1925 as *Tintinnopsis davidoffi* var. *laevis*), although some of them are slightly longer. Loricae were also very similar to previous specimens reported in Long Island Sound (NW Atlantic) by Gold and Morales (1975).

Supplementary text 2. Explanation for groups of morphospecies considered as more closely related in Fig. 4 (black bars in Fig. 2 and 3)

Within the genus *Tintinnidium*, that includes more than one supraspecific taxon (Agatha and Strüder-Kypke 2007; Santoferrara et al. 2012), we separated two groups of morphospecies. For the paraphyletic genus *Tintinnopsis* (Agatha and Strüder-Kypke 2014), we considered as more closely related those morphospecies that shared clusters supported by phylogenetic inferences (excluding *T. lobiancoi*, *T. lata*, *Tintinnopsis* sp. 2 and *Tintinnopsis* sp. 7 because they form long branches and/ or unclear clusters). *Helicostomella* and the "*Tintinnopsis* cluster" were grouped together due to their high molecular similarity (see Results in main text). *Codonellopsis gaussi* and *Laackmanniella prolongata* were also grouped together because of their similarity in sequences and cytology, which suggest that they belong to the same genus (Kim et al. 2013). Distances among other *Codonellopsis* spp., *Stenosemella* spp. and *Tintinnopsis* sp. C were excluded from the analysis in Fig. 4 due to potential misidentifications (*Tintinnopsis* sp. C may actually correspond to the genus *Codonellopsis* according to the published micrograph; Snoeyenbos-West et al. 2002) and unclear relationships among these genera (see Zhao et al. 2012; Agatha and Strüder-Kypke 2014). In the same way, distances among *Amphorides* and *Amphorellopsis* morphospecies were excluded from this analysis given possible misidentifications (*Amphorides quadrilineata* isolates FG293-FG295 and FG1141 differ in published micrographs and SSU rDNA sequences between them and respect to the apparently well identified *A. quadrilineata* isolate FG618; Bachy et al. 2012) and incomplete understanding of their relationship (e.g. Xu et al. 2013).

Table S1. Presence/ absence of *Helicostomella* clusters in samples from NW Atlantic collected between August 2010 and December 2013.

Date	Cluster	N (n)	Oral Diameter (µm)	Total length (µm)
8/2/2010	-			
8/13/2010	-			
8/18/2010[a]	I	1 (1)	22.2	160.2
8/20/2010	-			
8/23/2010	-			
8/25/2010	-			
9/8/2010	-			
9/22/2010[a, b]	I	6 (1)	21.6 (1.3)	150.0 (14.9)
9/22/2010[a, b]	III	4 (4)	19.4 (0.1)	58.2 (2.4)
3/28/2011[a]	II	2 (2)	26.2 (0.4)	166.6 (37.2)
4/9/2011	II	1 (0)	22.7	128.1
3/8/2012	II	0 (0)		
4/6/2012	II	0 (0)		
4/16/2012	-			
4/24/2012	II	1 (0)	24.1	202.9
5/4/2012	II	3 (0)	22.7 (0.6)	156.6 (13.4)
5/7/2012	II	4 (0)	24.8 (1.5)	171.8 (31.2)
5/14/2012	II	3 (0)	23.8 (1.3)	148.2 (22.4)
5/21/2012	II	3 (2)	22.6 (0.6)	136.2 (13.6)
5/30/2012	-			
6/12/2012	-			
6/20/2012	-			
6/26/2012	-			
7/25/2012	-			
8/8/2012	III	6(1)	17.6 (0.3)	51.1 (2.4)
8/16/2012	-			
8/29/2012	-			
9/5/2012	-			
9/15/2012	I	5 (0)	19.8 (0.6)	133.7 (7.7)
9/18/2012	-			
9/26/2012	-			
10/12/2012	I	0 (0)		
1/15/2013	-			
2/14/2013	II	1 (0)	23.4	166.1
3/4/2013	-			
4/15/2013	-			
5/13/2013	II	3 (0)	23.5 (0.4)	183.6 (15.0)
5/20/2013	II	18 (2)	23.1 (0.8)	168.1 (9.9)
5/20/2013	II[c]	9 (8)	23.5 (1.0)	107.3 (15.6)
5/29/2013	II	16 (0)	23.4 (1.0)	154.5 (24.7)
6/19/2013	I	6 (3)	20.7 (1.1)	160.0 (18.6)
6/26/2013	I	3 (1)	19.2 (1.1)	142.9 (6.1)
6/28/2013	I	2 (0)	19.9 (0.1)	136.3 (12.7)
7/8/2013	II	23 (3)	23.4 (1.6)	151.2 (13.9)
7/10/2013	-			
7/17/2013	-			
8/14/2013	I	21 (8)	20.0 (0.4)	204.9 (24.7)
8/16/2013	I	10 (0)	19.0 (0.6)	226.3 (14.5)
8/21/2013	-			
8/30/2013	I	10 (0)	19.7 (0.6)	167.6 (19.4)
9/4/2013	I	0 (0)		
9/11/2013	-			
9/27/2013	I	1 (0)	20.4	127.7
10/8/2013	I	5 (4)	19.9 (0.8)	134.3 (8.6)
11/8/2013	-			
12/13/2013	-			

[a] Collections from Santoferrara et al. 2013. [b] Isolated in the same sample. [c] Cultured until 6/10/2013. N = number of individuals measured; n = number of individuals sequenced. Lorica measures as mean (standard deviation).

Table S2. Comparison of oral diameter and total length in *Helicostomella* specimens from NW and SW Atlantic. Differences between paired clusters and in cluster II before and after culturing were tested by the *t* test assuming unequal variances (alpha = 0.05).

	Differences between clusters			
	Oral diameter		Total length	
	Cluster I	Cluster II[a]	Cluster I	Cluster II[a]
Mean	19.98	23.35	177.58	155.41
Variance	1.01	1.64	1400.72	577.48
Observations	70	94	70	94
df	162		110	
t Stat	-18.94		4.34	
p one-tail	$3.06E^{-43}$		$1.61E^{-05}$	
p two-tail	$6.13E^{-43}$		$3.22E^{-05}$	
	Cluster II[a]	Cluster III	Cluster II[a]	Cluster III
Mean	23.35	18.30	155.41	53.91
Variance	1.64	0.97	577.48	18.57
Observations	94	10	94	10
df	12		81	
t Stat	14.95		35.88	
p one-tail	$2.01E^{-09}$		$8.57E^{-52}$	
p two-tail	$4.03E^{-09}$		$1.71E^{-51}$	
	Cluster I	Cluster III	Cluster I	Cluster III
Mean	18.30	19.98	177.58	53.91
Variance	0.97	1.01	1400.72	18.57
Observations	10	70	70	10
df	12		77	
t Stat	-5.02		26.45	
p one-tail	0.000149		$1.1E^{-40}$	
p two-tail	0.000298		$2.19E^{-40}$	
	Differences in cluster II before and after culture			
	Oral diameter		Total length	
	before	after	before	after
Mean	23.09	23.47	168.07	107.34
Variance	0.67	1.07	98.54	244.38
Observations	18	9	18	9
df	13		11	
t Stat	-0.94		10.63	
p one-tail	0.18		$2E^{-07}$	
p two-tail	0.36		$4E^{-07}$	

[a]Nine cultured individuals excluded.

Table S3. Other tintinnids from NW Atlantic newly sequenced.

Morphospecies	References	Isolation dates	N(n)	Oral/ Maximum Diameter (µm)	Total length (µm)
Stenosemella steini (Jörgensen 1912) Jörgensen 1924	Jörgensen 1912	4/6/2012-5/30/2012	10 (1)	35.7 (1.9)/ 70.5 (4.4)	101.5 (5.2)
Tintinnopsis lata Meunier 1910	Meunier 1910; Marshal 1969	5/30/2012	3 (2)	41.8 (3.6)/ 48.1 (2.5)	69.8 (8.6)
Tintinnopsis levigata Wailes 1925	Wailes 1925; Gold and Morales 1975	5/30/2012-6/12/2012	4 (3)	22.5 (2.1)/ na	77.4 (9.3)
Tintinnopsis nana Lohmann 1908	Lohmann 1908; Kofoid and Cambell 1929	8/30/2013	2 (2)	19.1 (2.3)/ na	42.6 (2.1)
Tintinnopsis parva Merkle 1909	Merkle 1909; Kofoid and Cambell 1929	3/17/2011[a]	2 (2)	23.4 (0.1)/ 33.3 (0.9)	47.4 (4.3)
Tintinnopsis rapa Meunier 1910	Meunier 1910 ; Kofoid and Cambell 1929	3/17-28/2011[a]	5 (2)	24.6 (2.1)/ 29.7 (2.8)	58.2 (12.4)
Tintinnopsis sp. 5	-	9/22/2010[a] 6/20/2012	4 (3)	21.7 (2.1)/ 26.0 (2.3)	37.7 (1.7)
Tintinnopsis sp. 6	-	4/26/2011[a]	3 (1)	21.5 (0.6)/ na	40.1 (1.0)
Tintinnidium mucicola (Claparède and Lachmann 1858) Daday 1887	Claparède and Lachmann 1858; Kofoid and Cambell 1929	6/12/2012	2 (1)	48.6 (5.6)/ na	68.5 (9.4)
Tintinnidium sp. 1	-	8/20/2010[a]	4 (1)	31.6 (1.5)/ na	87.3 (10.1)

[a]Collections from Santoferrara et al. 2013. N = number of individuals measured; n = number of individuals sequenced. Lorica measures as mean (standard deviation).

Table S4. Tintinnid sequences from GenBank.

Morphospecies	Acc. number	Reference
	LSU rDNA	
Eutintinnus pectinis	JN831856	Santoferrara et al. 2012, 2013
Eutintinnus sp.	JN831857	Santoferrara et al. 2012, 2013
Favella ehrenbergii	JN831858-66	Santoferrara et al. 2012, 2013
Helicostomella subulata	JN831869-76	Santoferrara et al. 2012, 2013
Schmidingerella taraikaensis	JN831867-68	Santoferrara et al. 2012, 2013
Stenosemella pacifica	JN831877-84	Santoferrara et al. 2012, 2013
Strombidium rassoulzadegani	JQ028733	Santoferrara et al. 2012, 2013
Tintinnidium balechi	JN831885-87	Santoferrara et al. 2012, 2013
Tintinnidium mucicola	JN831888-90	Santoferrara et al. 2012, 2013
Tintinnidium sp._1	JN831891	Santoferrara et al. 2012, 2013
Tintinnidium sp._2	JN831892-93	Santoferrara et al. 2012, 2013
Tintinnidium sp._3	JN831894	Santoferrara et al. 2012, 2013
Tintinnopsis baltica	JN831895-97	Santoferrara et al. 2012, 2013
Tintinnopsis butschlii	JN831898-900	Santoferrara et al. 2012, 2013
Tintinnopsis cylindrica	JN831901-02	Santoferrara et al. 2012, 2013
Tintinnopsis lobiancoi	JN831903-04	Santoferrara et al. 2012, 2013
Tintinnopsis major	JN831905-08	Santoferrara et al. 2012, 2013
Tintinnopsis nana	JN831909-10	Santoferrara et al. 2012, 2013
Tintinnopsis parva	JN831911-12	Santoferrara et al. 2012, 2013
Tintinnopsis parvula	JN831913-17	Santoferrara et al. 2012, 2013
Tintinnopsis platensis	JN831918	Santoferrara et al. 2012, 2013
Tintinnopsis rapa	JN831919-20	Santoferrara et al. 2012, 2013
Tintinnopsis tocatinensis	JN831921-22	Santoferrara et al. 2012, 2013
Tintinnopsis uruguayensis	JN831923	Santoferrara et al. 2012, 2013
Tintinnopsis sp._4	JN831924-30	Santoferrara et al. 2012, 2013
Tintinnopsis sp._5	JN831931	Santoferrara et al. 2012, 2013
Tintinnopsis sp._6	JN831932-33	Santoferrara et al. 2012, 2013
Tintinnopsis sp._7	JN831934-35	Santoferrara et al. 2012, 2013
Tintinnopsis sp._8	JN831936-37	Santoferrara et al. 2012, 2013
Tintinnopsis sp._9	JN831938-40	Santoferrara et al. 2012, 2013
Amphorellopsis quinquealata	JQ924058-59	Kim et al. 2013
Codonellopsis gaussi	JQ924053-55	Kim et al. 2013
Cymatocylis calyciformis	JQ924046-49	Kim et al. 2013
Cymatocylis convallaria	JQ924050-51	Kim et al. 2013
Cymatocylis drygalskii	JQ924052	Kim et al. 2013
Laackmanniella prolongata	JQ924056-57	Kim et al. 2013
	5.8S rDNA-ITS	
Eutintinnus pectinis	AF399105-07	Snoeyenbos-West et al. 2002
Metacylis angulata	AF399068-78	Snoeyenbos-West et al. 2002
Schimidingerella sp.	AF399086-104	Snoeyenbos-West et al. 2002
Tintinnopsis tubulosoides	AF399017-20	Snoeyenbos-West et al. 2002
Tintinnopsis sp._clone_A	AF399013-16	Snoeyenbos-West et al. 2002
Tintinnopsis sp._clone_B	AF399021-33	Snoeyenbos-West et al. 2002
Tintinnopsis sp._clone_C	AF399034-39	Snoeyenbos-West et al. 2002
Amphorides quadrilineata	JQ408156, 76	Bachy et al. 2012
Codonaria cistellula	JQ408154, 67	Bachy et al. 2012
Codonaria sp.	JQ408172	Bachy et al. 2012
Codonella aspera	JQ408166	Bachy et al. 2012
Codonellopsis morchella	JQ408173	Bachy et al. 2012
Cyttarocylis ampulla	JQ408165, 68	Bachy et al. 2012
Cyttarocylis eucecryphalus	JQ408169	Bachy et al. 2012
Eutintinnus fraknoi	JQ408157, 59	Bachy et al. 2012
Rhabdonella elegans	JQ408175	Bachy et al. 2012
Rhabdonella spiralis	JQ408158	Bachy et al. 2012
Salpingella acuminata	JQ408155	Bachy et al. 2012
Stenosemella ventricosa	JQ408170, 74	Bachy et al. 2012
Tintinnidium fluviatile	JQ408163	Bachy et al. 2012
Tintinnopsis lacustris	JQ408161-62	Bachy et al. 2012
Undella claparedei	JQ408164	Bachy et al. 2012
Undella hyalina	JQ408171	Bachy et al. 2012
Xystonella longicauda	JQ408160	Bachy et al. 2012
Helicostomella subulata	JX000465-69	Xu et al. 2012
Amphorellopsis acuta	JN033241	Zhao et al. 2012
Codonellopsis nipponica	JN033239	Zhao et al. 2012

Favella campanula	JN033238	Zhao et al. 2012
Schmidingerella (as *Favella*) *taraikaensis*	JN033237	Zhao et al. 2012
Stenosemella nivalis	JN033240	Zhao et al. 2012
Tintinnopsis lohmanni	JN033233	Zhao et al. 2012
Tintinnopsis sp._1	JN033234	Zhao et al. 2012
Tintinnopsis sp._2	JN033235	Zhao et al. 2012
Tintinnopsis sp._3	JN033236	Zhao et al. 2012
Amphorellopsis quinquealata	JQ924058-59	Kim et al. 2013
Codonellopsis gaussi	JQ924053-55	Kim et al. 2013
Cymatocylis calyciformis	JQ924046-49	Kim et al. 2013
Cymatocylis convallaria	JQ924050-51	Kim et al. 2013
Cymatocylis drygalskii	JQ924052	Kim et al. 2013
Laackmanniella prolongata	JQ924056-57	Kim et al. 2013
	SSU rDNA	
Strombidium rassoulzadegani	AY257125	McManus et al. 2010
Helicostomella subulata	JN831780-81, 84	Santoferrara et al. 2012, 2013
Schmidingerella taraikaensis	JN831778	Santoferrara et al. 2012, 2013
Tintinnidium mucicola	JN831800	Santoferrara et al. 2012, 2013
Tintinnidium sp._1	JN831801	Santoferrara et al. 2012, 2013
Tintinnopsis cylindrica	JN831811	Santoferrara et al. 2012, 2013
Tintinnopsis nana	JN831821	Santoferrara et al. 2012, 2013
Tintinnopsis parva	JN831823	Santoferrara et al. 2012, 2013
Tintinnopsis rapa	JN831834	Santoferrara et al. 2012, 2013
Tintinnopsis sp. 5	JN831846	Santoferrara et al. 2012, 2013
Tintinnopsis sp. 6	JN831848	Santoferrara et al. 2012, 2013
Codonellopsis gaussi	JQ924053	Kim et al. 2013
Cymatocylis calyciformis	JQ924046	Kim et al. 2013
Cymatocylis convallaria	JQ924050	Kim et al. 2013
Cymatocylis drygalskii	JQ924052	Kim et al. 2013
Laackmanniella prolongata	JQ924056	Kim et al. 2013

Table S5. Primers used for PCR amplification and sequencing of SSU rDNA, LSU rDNA and 5.8S rDNA-ITS.

Primer	Sequence (5’ - 3’)	Reference
18Scom-F1	GCTTGTCTCAAAGATTAAGCCATGC	Zhang et al. 2005
18Scom-R1	CACCTACGGAAACCTTGTTACGAC	Zhang et al. 2005
Tin18S-F	ATTAGTACTTAACTGTCAGAGGTG	Santoferrara et al. 2013
Tin18S-R1	TTCAGCCTTGCGACCATACTC	Santoferrara et al. 2013
Tin18S-R2	CGGCATAGTTTATGGTTAAGACT	Santoferrara et al. 2013
28S-F1a	GCGGAGGAAAAGAAACTAAC	Ortman 2008
28S-R1a	GCATAGTTTCACCATCTTTCGGG	Ortman 2008
ITS-F	AAGGTWTCCGTAGGTGAACCTG	Snoeyenbos-West et al. 2002
ITS-R	TAKTRAYATGCTTAAGTYCAGCG	Snoeyenbos-West et al. 2002

Figure S1. *Tintinnopsis* morphospecies closely related to *Helicostomella* (A-D) and other tintinnids newly sequenced (E-J). Scale bar = 20 µm.

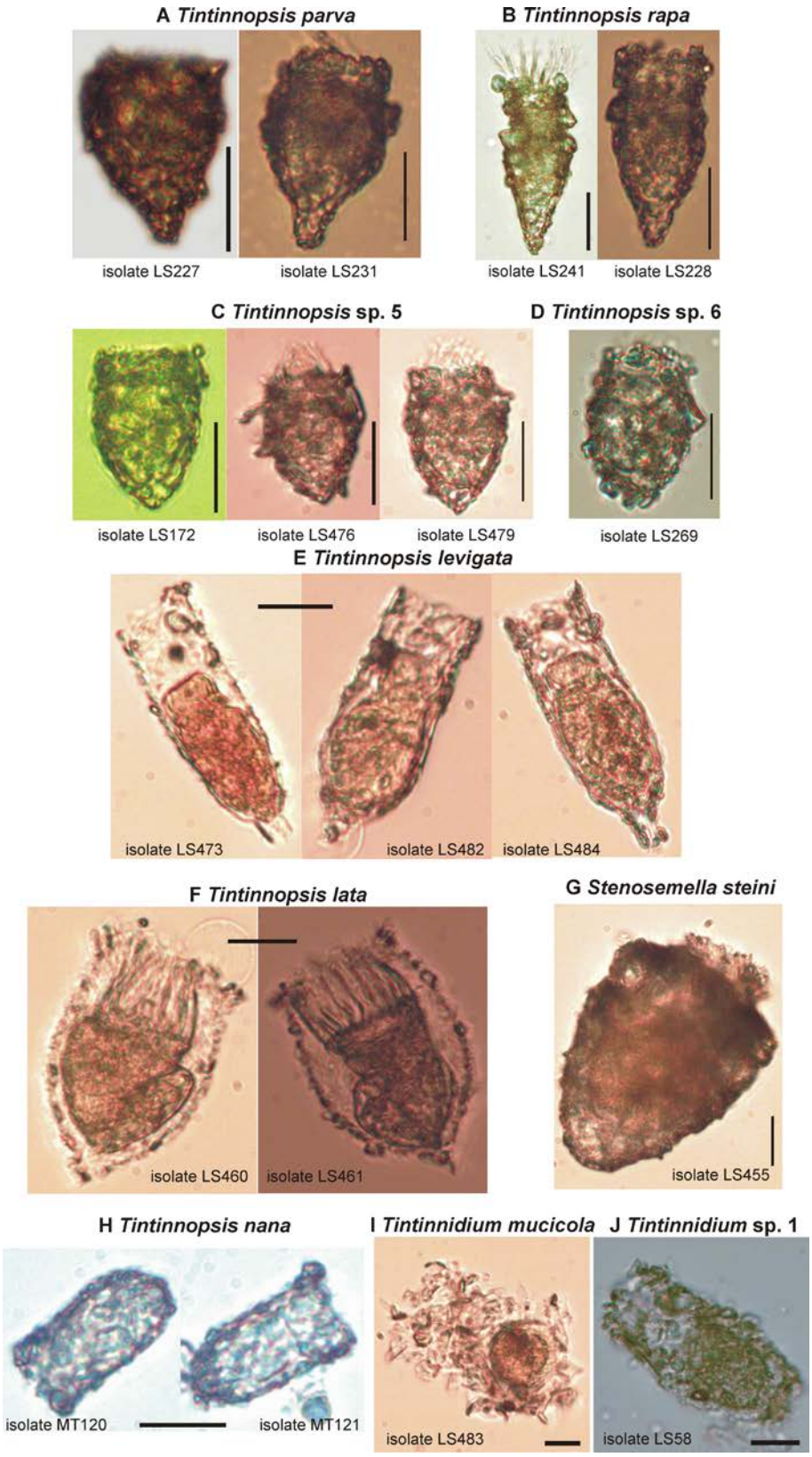


Figure S2. Partial alignment of ITS1 in representative tintinnid morphospecies. A 23-nucleotide deletion from position 43 to 65 is shared between *Helicostomella* and closely related *Tintinnopsis*, but is absent in all other tintinnids sequenced so far.

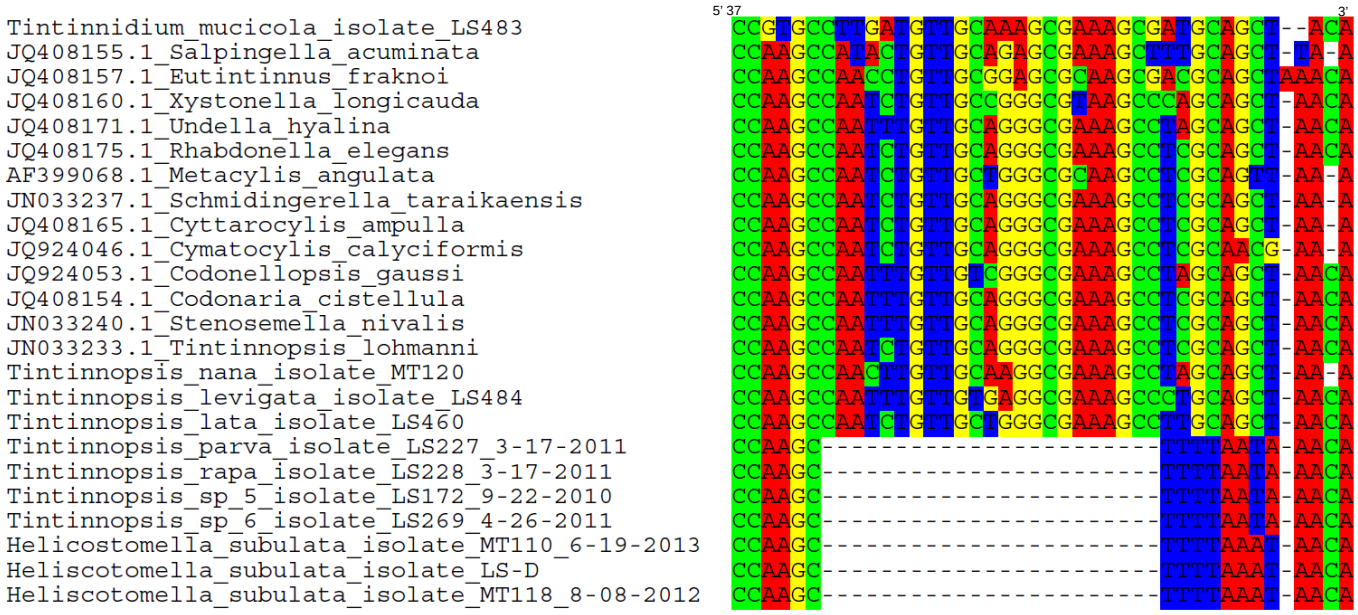


Figure S3. Phylogenetic analysis of the order Tintinnida based on concatenated SSU rDNA, 5.8S rDNA-ITS and LSU rDNA sequences. *Helicostomella* and some *Tintinnopsis* morphospecies form a monophyletic cluster with full support (in bold). Tree topology corresponds to the maximum likelihood (ML) analysis. Numbers on each node are ML bootstrap support (MLS) and Bayesian posterior probability (BPP), respectively. Only MLS ≥50% and/or BPP ≥0.5 are shown. Underlined morphospecies were newly sequenced. Other explanations as in Fig. 2.

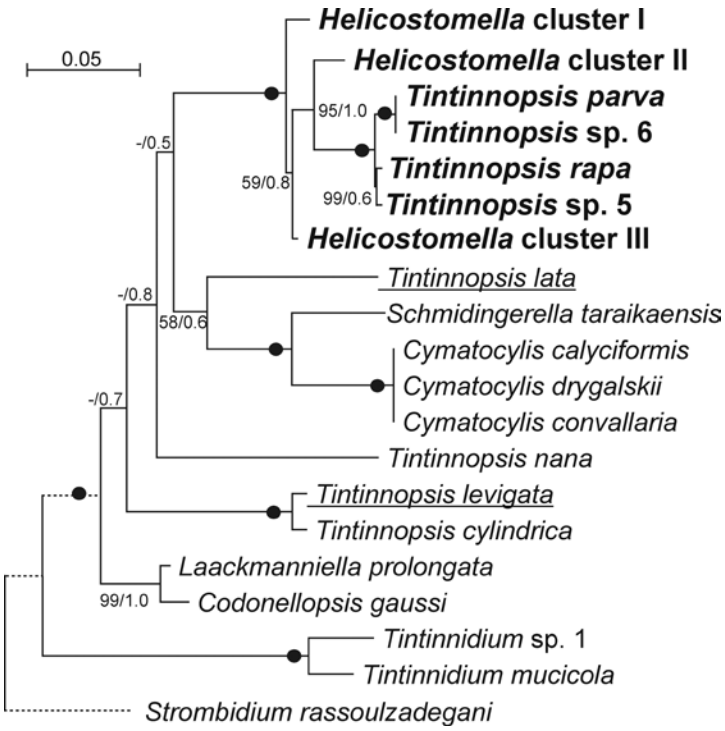
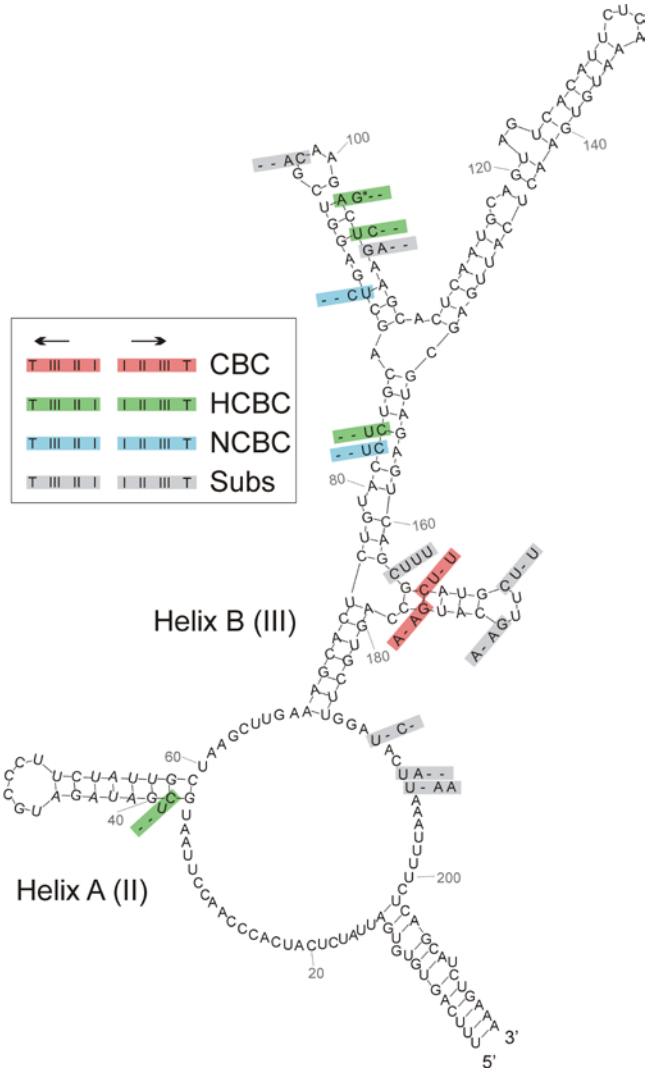


Fig. S4. Secondary structure of ITS2 in *Helicostomella* and closely related *Tintinnopsis*. The ITS2 secondary structure in in these species includes two helices, in agreement with previous models for six tintinnid genera (Zhao et al. 2012) and other spirotrichs (Coleman 2005; Li et al. 2013). Helices A and B are parallel to helices II and III, respectively, out of the four helices generally found in eukaryotes (Coleman 2005). There is one compensatory base change (CBC) on the 3' side of helix B when comparing *Helicostomella* clusters I and III against *Helicostomella* cluster II or the *Tintinnopsis* cluster. Comparison of *Helicostomella* clusters I and III and the *Tintinnopsis* cluster against *Helicostomella* cluster II indicates four hemi-CBCs (one of them only for the individuals from SW Atlantic) and two non-CBCs. The ITS2 region was trimmed from the 5.8S rDNA-ITS alignment and its secondary structure was predicted using Mfold v. 3.6 (Zuker 2003) under default parameters. CBCs (i.e. base changes in two positions that retain pairing by complementarity), hemi-CBCs (i.e. base changes in one position that retain pairing) and non-CBCs (i.e. pair ↔ non-pair) were defined as suggested by Caisová et al. (2011). Diagram corresponds to *Helicostomella* cluster I. CBC, HCBC, NCBC other substitutions (Subs) respect to *Helicostomella* cluster II, *Helicostomella* cluster III and *Tintinnopsis* cluster (T), respectively, are shown in colored panels (note the palindromic order on the panels orientated to the right). The dash (-) indicates no change. The asterisk (*) indicates change only in individuals from SW Atlantic.



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