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# Phylogeny, classification and diversity of Choreotrichia and Oligotrichia (Ciliophora, Spirotrichea)

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ABSTRACT

Ciliated protists in the subclasses Choreotrichia and Oligotrichia are major components of marine plankton. Despite their ecological relevance, there are uncertainties in their systematics and diversity. We retrieved and curated all the GenBank ribosomal DNA (rDNA) sequences from these groups, which were analyzed in two ways. The first approach was based on morphologically-identified sequences (including those of two families and six genera newly studied here by single-cell sequencing), and aimed at improving phylogenetic inferences using concatenated sequences of three rDNA loci. Based on phylogenetic and morphological support, we update the taxonomic classification of these subclasses into 23 families, including the re-established Favellidae. We also propose an informal naming system for *incertae sedis* taxa, namely *Tintinnopsis* and five related genera that are spread among eleven lineages. The second approach included unidentified environmental sequences, and was used to explore potentially novel diversity in these subclasses. Our results support high proportions of both synonyms in tintinnids and uncharacterized taxa in choreotrichids and oligotrichs. One previously unidentified, environmental clade is here linked to our new Leegaardiellidae sequences. Our curation of almost 4000 rDNA sequences exemplifies known issues of public repositories, and suggests caution in both the use and contribution to these unique resources for evolutionary and diversity studies.



## 1. Introduction

Despite their importance in evolution of life and ecosystem functioning, major protist taxa remain poorly understood in terms of diversity and systematics (Corliss, 2002; Cotterill et al., 2008). Here we focus on two ecologically important groups of ciliated protists, the sister subclasses Choreotrichia Small and Lynn, 1985 and Oligotrichia Bütschli, 1887/1889. Although they are present in varied environments (including freshwater plankton, benthos, and even as endocommensals in sea urchins), these groups thrive in marine plankton, where they are usually species-rich and abundant (Lynn, 2008). They include heterotrophs and mixotrophs generally in a size spectrum of 10–200 μm, and thus play diverse trophic roles as algae and bacteria consumers, primary producers, and prey for small metazoans (Calbet and Saiz, 2005; McManus and Santoferrara, 2013; Pierce and Turner, 1992; Sanders and Wickham, 1993).

Morphologically, these subclasses are characterized by an adoral zone of membranelles that surrounds the apical part of the cell, and a somatic ciliature that is generally reduced. The adoral zone of membranelles forms a closed or slightly opened circle in Choreotrichia, whereas it is C-shaped in Oligotrichia (Lynn, 2008). In Choreotrichia (or choreotrichs), some taxa have an external lorica attached to the cell (order Tintinnida = tintinnids), while the rest (order Choreotrichida = choreotrichids), as well as all of the Oligotrichia (oligotrichs), are aloricate. For most ciliates, taxonomy is based on the cell morphology and ciliary patters, which are studied *in vivo* and by complex staining techniques, especially difficult for the smallest and/or uncultivable species (Agatha, 2011). In contrast, tintinnid taxonomy is based on the lorica, which is relatively easy to sample, preserve and characterize, but it is less reliable for species diagnosis and classification of higher taxa (Agatha and Strüder-Kypke, 2013; Alder, 1999; Laval-Peuto, 1994). As with other organisms, the taxonomic and evolutionary studies of these

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groups have been gradually complemented with DNA sequences over the last 15 years (e.g., Bachy et al., 2012; Liu et al., 2015; Santoferrara et al., 2012; Snoeyenbos-West et al., 2002).

Current limitations in the understanding of choreotrich and oligotrich systematics include: (1) some families still lack data on the ciliary patterns or have never been sequenced reliably, and thus are not represented in cladistic or phylogenetic inferences (Agatha and Strüder-Kypke, 2012, 2014); (2) for some families, data on morphology (cell- and/or lorica-based) and DNA sequences do not agree, and thus increased taxon and character sampling are needed (for example, by using multi-gene approaches that are known to improve phylogenetic accuracy in other ciliate clades; Yi et al., 2014); (3) several families and genera are not monophyletic and require revision, including extremely diverse taxa that are currently difficult to link in taxonomic and ecological studies (e.g., *Tintinnopsis*); and (4) classification systems have not been stable and require constant update, as expected due to increasing knowledge, but in some cases also due to premature conclusions based on incomplete data.

In addition to the known gaps in current systematics, many choreotrich and oligotrich taxa may remain undiscovered. For more than a decade, environmental surveys worldwide allowed the accumulation of ciliate sequences in public repositories (e.g., Stoeck et al., 2003; Doherty et al., 2007; Lie et al., 2014), which provide unique opportunities to reveal uncharacterized diversity. In fact, divergent lineages detected iteratively by environmental sequencing, some of them probably representing families or genera, remain unbounded to morphology (e.g., Forster et al., 2015; Santoferrara et al., 2014). Molecular data also suggest that the number of species currently known for choreotrichs and oligotrichs is inaccurate, for example due to cases of interspecific similarity (crypticity) and intraspecific polymorphism (Katz et al., 2005; Kim et al., 2013; McManus et al., 2010; Santoferrara et al., 2013, 2015). Particularly, tintinnids are suspected of synonymy problems, as many species that were established based on minute lorica differences may actually reflect phenotypic variation due to developmental or environmental factors (Alder, 1999; Dolan, 2016; Laval-Peuto, 1981). As a result, about five times more species have been described for tintinnids than for choreotrichids and oligotrichs combined (>1000 and <200, respectively), also because the aloricate morphospecies remain unexplored in extensive geographical areas (Agatha, 2011).

To help clarify choreotrich and oligotrich taxonomy, evolutionary relationships and global diversity, we focused on the following objectives: (1) to increase the number of families and genera represented in phylogenetic inferences based on three rDNA loci newly studied by single-cell sequencing; (2) to update the classification of these groups based on our novel results and other recent findings; (3) to propose a system for informal classification of ecologically important taxa with uncertain taxonomic position; and (4) to explore the potential for novel diversity within these groups by integrating environmental sequences from multiple studies in a single phylogenetic context. To complete these aims, we retrieved and manually curated all the choreotrich and oligotrich rDNA data in NCBI GenBank, including both morphologically-identified and environmental sequences. There is an increasing need for careful evaluation of DNA sequences available in public repositories, given the well-known issue of inadequate data accumulating along with the useful information (Kozlov et al., 2016). This is true for sequences linked to a named species (e.g., due to misidentifications) and also for environmental sequences (e.g., due to methodological artifacts). Thus, by carefully documenting our curation efforts, we also provide a useful resource for future studies on ciliate phylogenetics and diversity.

## 2. Material and methods

### 2.1. Single cell sequencing

We analyzed isolates of twenty-one species newly collected in summer 2015 and seven species sampled in previous studies, all from Northwest Atlantic waters (3 choreotrichids, 23 tintinnids and 2 oligotrichs; Fig. 1, Supplementary Fig. S1). Of them, twenty-seven species were sequenced for the first time for at least one marker and only one had been sequenced before for the three of them. At least one genus and/or marker were newly sequenced within eight families. Two families (Leegaardiellidae and Ascampbelliellidae), six genera (*Leegaardiella*, *Ascampbelliella*, *Salpingacantha*, *Ptychocylis*, *Parafavella* and *Parundella*) and twelve species (in bold in Supplementary Table S1) had not been sequenced before for any marker. Tintinnid and aloricate taxa were identified based on the lorica or cell morphology, respectively (see detailed information in Supplementary Text 1). Single cells were studied in the microscope, individually subjected to DNA extraction, and sequenced as described before (Santoferrara et al., 2013, 2015). Three primer sets were used for DNA amplification and Sanger sequencing of the small subunit (SSU) rDNA, the 5.8S rDNA combined with the internally transcribed spacer regions 1 and 2 (ITS regions) and the D1-D2 region of the large subunit (LSU) rDNA (Supplementary Table S2). Chromatogram quality was checked individually and sequences in the forward and reverse sense were assembled manually in MEGA v. 5 (Tamura et al., 2011). A total of 60 newly obtained sequences were uploaded in GenBank (accession numbers KY290291 to KY290350). Also, we updated 50 of our previous GenBank records (Supplementary Text 2, Fig. S2A).

### 2.2. Phylogenetic inferences

For phylogenies, we focused on SSU rDNA, ITS regions and LSU rDNA sequences identified to the genus or species level based on morphology. We retrieved and manually curated all the sequences labeled as Choreotrichia or Oligotrichia in NCBI GenBank (1297 and 261, respectively; last updated on February 15, 2017). Records from environmental sequencing as well as low quality and redundant sequences were eliminated; sequences potentially misidentified or lacking published morphological data were retained but flagged (Supplementary Text 3). Our newly obtained sequences were then added, along with four outgroup sequences of the subclass Hypotrichia Stein, 1859. Four final datasets including from 47 to 198 sequences were obtained: SSU rDNA, ITS regions, LSU rDNA, and the three markers concatenated (Supplementary Table S3). For the concatenated dataset, sequences from the same specimen were combined when possible (Supplementary Table S4). Although sequences from the three markers exist for additional species, almost forty of them were excluded due to serious quality concerns (Supplementary Text 3).

Each dataset was aligned with MAFFT v. 7 (Katoh and Standley, 2013). Ambiguous positions were removed with the guidance of Gblocks v. 0.91b under default parameters (Castresana, 2000). Maximum likelihood inferences were done with RAxML v. 8.3.17 (Stamatakis, 2014), with the best-known tree and the node support values inferred out of 200 trees and 10,000 bootstraps, respectively. Bayesian inferences were done with MrBayes v. 3.2.1 (Ronquist et al., 2012). Five million generations were run and trees were sampled each 1000 cycles. The initial 1000 trees were discarded as burn-in, and the remaining 4000 trees were used to estimate the Bayesian posterior probabilities. For each analysis, the GTR model with a $\Gamma$ model of rate heterogeneity and a proportion

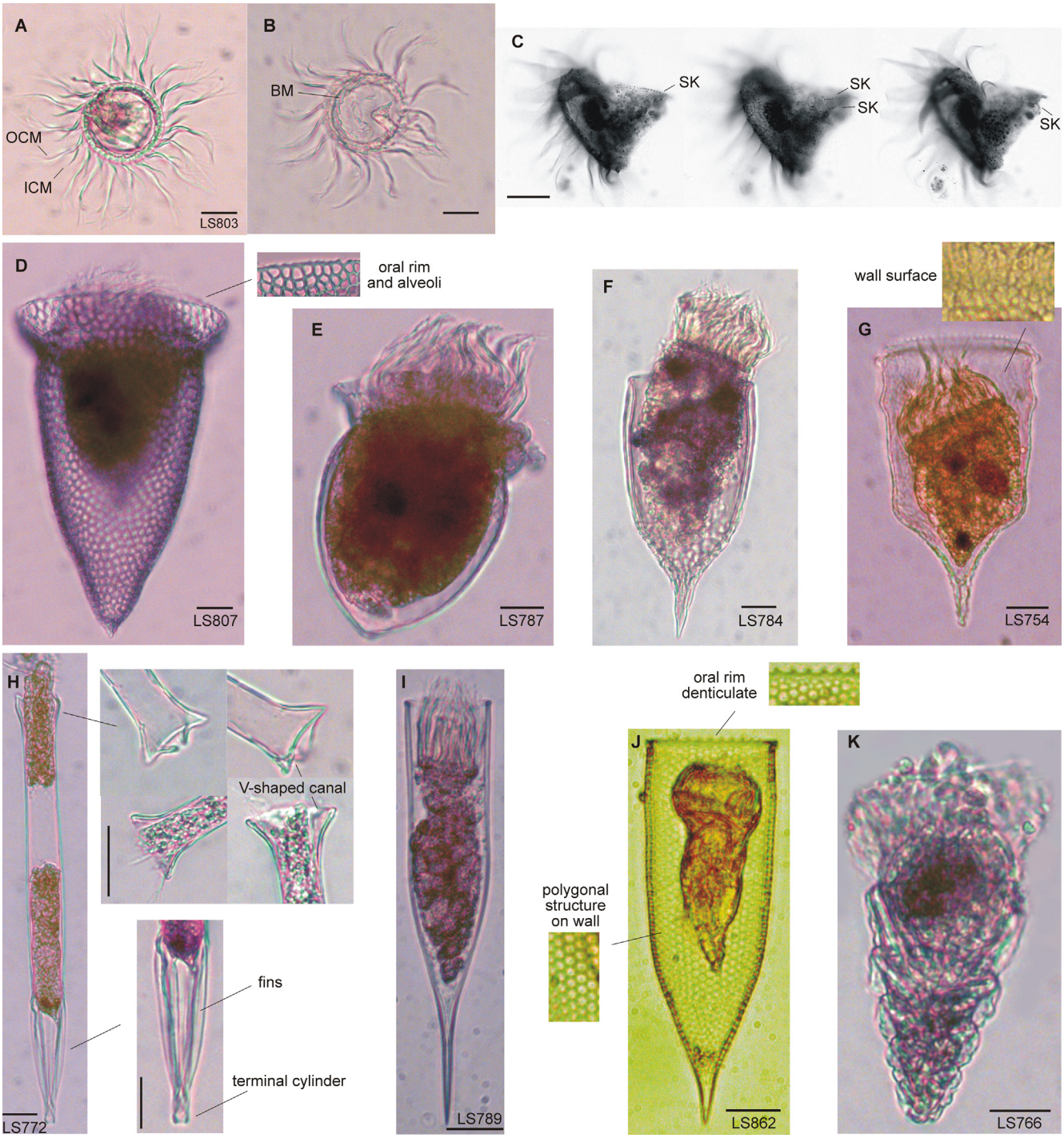


**Fig. 1.** Examples of specimens sequenced in this study. A to C, the choreotrichid *Leegaardiella* sp. As in most Choreotrichia, the adoral zone of membranelles consists of (A) a closed circle of collar membranelles (bipartite in this genus: they consist of an outer and an inner portion with long and short membranelles, OCM and ICM, respectively) and (B) buccal membranelles (BM). The somatic ciliature is reduced, as revealed by protargol impregnation (sequential planes in C); there are four short somatic kineties (SK) consisting of dikinetids in the posterior part of the cell. D to J, the tintinnids *Cyttarocylis acutiformis*, *Petalotricha ampulla*, *Epiplocylis undella*, *Ptychocylis minor*, *Salpingacantha undata*, *Parundella aculeata* and *Parafavella parumdentata*, respectively. Species identification is based on the lorica. K, the oligotrich *Laboea strobila*. Although difficult to see in fixed material, the adoral zone of membranelles is C-shaped; the somatic ciliature includes a spiraled girdle kinety that confers this species a screw-like shape. See additional sequenced specimens and detailed descriptions in the Supplementary Material. Isolate number is shown. Scale = 20 μm. Except for *L. strobila*, we sequenced all species for the first time for at least one marker.

of invariable sites was used, as previously identified with MrModeltest v. 2 (Nylander, 2004) under the Akaike Information Criterion. Based on RAxML bootstrap support and Bayesian posterior probabilities, inference support was considered good (>70%, >0.95), moderate (45–70%, 0.90–0.95) or low (<45%, <0.90).

### 2.3. Exploring the unknown taxa

To explore the proportion of potentially novel taxa in Choreotrichia and Oligotrichia, we considered all the SSU rDNA sequences available in NCBI GenBank. Both morphologically-identified and

environmental sequences from these groups were retrieved and curated in the context of the EukRef initiative (http://eukref.org). A reference dataset of reliable sequences was the seed to iteratively retrieve all the GenBank sequences that are ≥80% similar to the groups of interest, using the BLASTN algorithm (Camacho et al., 2009) against the NCBI non-redundant/nucleotide collection (last updated in July 2015). Sequences shorter than 500 bp (less reliable for phylogenetic analysis; e.g., Dunthorn et al., 2014), chimeras detected with UCHIME (Edgar et al., 2011), and a dataset known to include misidentifications (accession numbers AB640624 to AB640682) were removed. Sequences from the present study were incorporated.

To simplify the bioinformatic steps, the sequences were clustered at 97% similarity with USEARCH (Edgar, 2010). These clusters were subjected to iterative rounds of alignment (MAFFT v. 7; Katoh and Standley, 2013), refinement (trimAl v. 1.2; Capella-Gutiérrez et al., 2009), and maximum likelihood inference (FastTree v. 2; Price et al., 2010) in order to detect and remove any remaining sequence out of the groups of interest or with suspicious quality (e.g., some long branches manually identified as chimeras). The final dataset of 346 clusters (3145 total sequences) was separated into Tintinnida, Choreotrichida and Oligotrichia, re-aligned and analyzed with RAxML as described above (see Section 2.2; the only difference was that 1000 bootstraps were used here). The 3145 final sequences were also clustered at 99% similarity (the cutoff generally accepted as approximation to species in these taxa; Bachy et al., 2013; Santoferrara et al., 2013, 2014), which resulted in 943 clusters. The final datasets will be publicly available as part of EukRef (http://eukref.org).

## 3. Results and discussion

### 3.1. Phylogeny

We expanded the phylogenetic tree of Choreotrichia and Oligotrichia by adding 27 newly sequenced species (Figs. 1 and S1) and by including 18 out of 23 families in concatenated SSU rDNA, ITS regions, and LSU rDNA analyses (Fig. 2). In general, inferences based on concatenated sequences or on each separate marker agreed, although the former had higher support (Figs. 2, 3, S3–S5). All analyses confirmed the monophyly of Choreotrichia and Oligotrichia, but disagreed in which of these subclasses embraces Lynnellidae. This family is basal within Choreotrichia in concatenated and SSU rDNA analyses (Figs. 2 and 3), but affiliated to Oligotrichia or sister to both subclasses in our ITS regions and LSU rDNA trees (Figs. S4 and S5) and previous studies (e.g. Liu et al., 2015, 2016), although usually with moderate or low support. An affiliation of Lynnellidae within Choreotrichia is supported by shared morphological traits (a slightly-open adoral zone of membranelles in *Parastrombidinopsis* and *Parastrombidium*, and the structure of the somatic kinetids in Lohmanniellidae; Agatha and Strüder-Kypke, 2014), even if differences in the position of the oral ciliature weaken this association (Liu et al., 2015).

Regardless of Lynnellidae, Choreotrichida is not monophyletic based on our trees (Figs. 2, 3, S4, and S5) and previous studies of both DNA sequences and morphology (Agatha and Strüder-Kypke, 2014). Within this order, we newly sequenced the family Leegaardiellidae, which forms a long branch between Strombidinopsidae and Strobilidiidae in the concatenated analysis (Fig. 2) and between two known subclades of the paraphyletic Strombidinopsidae (Liu et al., 2016) in the SSU rDNA tree (Figs. 3 and S3A). This contrasts with morphological cladistics, which places Leegaardiellidae as the most basal Choreotrichida due to the singularity of their bipartite collar membranelles (Agatha and Strüder-Kypke, 2012, 2014; Fig. 1). The conflicts in Lynnellidae, Leegaardiellidae, and Strombidinopsidae may be due to the lack of sequences for some key taxa (Lohmanniellidae and *Parastrombidium*). In contrast, Strobilidiidae shows the least problematic position in the order, as it is usually inferred as monophyletic and as the most derived Choreotrichida (e.g., Figs. 2 and 3).

Tintinnida is the best represented group in our trees, and it is confirmed as monophyletic (although with moderate or low support in RAxML analyses; Figs. 2, 3, S4, and S5). The monophyletic Tintinnidiidae, Tintinnidae (including the newly sequenced *Salpingacantha*), Eutintinnidae, and Favellidae (re-established here; see Section 3.2) were sequentially arranged in the trees, in agreement with previous molecular inferences and morphology (mainly the somatic ciliary patterns, lorica ultrastructure and extrusome types; Agatha and Strüder-Kypke, 2012, 2013, 2014). The next taxa in the trees are less clearly resolved. Dictyocystidae and Stenosemellidae appear as sister, monophyletic clades in the concatenated analysis (Fig. 2), but they cluster together in the SSU rDNA tree, where more taxa are included (Fig. S3B). Despite similarities in lorica morphology and extrusome type (Supplementary Text 1), only Dictyocystidae presents a lorica sac, which is considered as an important synapomorphy of this family (Agatha and Strüder-Kypke, 2013, 2014). Xystonellidae, Undellidae (only in the SSU rDNA tree), and a clade with Rhabdonellidae (including *Metacylis*; see Section 3.2), Cyttarocylididae, Ascampbelliellidae (newly sequenced here), Epiplocylididae and Ptychocylididae (excluding *Favella*; see Section 3.2), are all monophyletic, but in some cases are arranged as polytomies (Figs. 2 and 3). Also arranged as polytomies are the most chaotic tintinnids, the paraphyletic *Tintinnopsis* and other *incertae sedis* genera that form up to eleven clades in our trees (see Sections 3.2 and 3.3) and for which at least four kinds of both somatic ciliary patterns and lorica matrix texture are known (Agatha et al., 2013; Agatha and Strüder-Kypke, 2014).

Oligotrichia remains largely under-sampled in our concatenated analyses (Fig. 2). In the SSU rDNA tree (Figs. 3 and S3A), Tontoniidae and Cyrtostrombidiidae are monophyletic, and the only available sequence labeled as Pelagostrombidiidae forms an isolated branch, in agreement with clear morphological differences among these three families (a contractile tail except in *Laboea*, a cyrtos, and a neoformation organelle, respectively; Agatha, 2004). In contrast, Strombidiidae and several of its genera, particularly the species-rich *Strombidium*, are paraphyletic (Figs. S3A, S4, and S5). Probably because several taxa have not been sequenced reliably (not even the type *S. sulcatum*; Supplementary Text 3) or at all, phylogenetic relationships are poorly supported, unstable, and partly inconsistent with evolutionary hypotheses based mainly on the somatic ciliary patterns (Agatha and Strüder-Kypke, 2014; Liu et al., 2015). For now, clades that show molecular and morphological cohesion include (1) *Williophrya* and *Strombidium* species characterized by an eyespot, which may be a major synapomorphy of this group (Liu et al., 2016); and (2) the subgenus *Novistrombidium* (*Novistrombidium*), differentiated by extrusome position, a feature of potential taxonomic value that deserves more study in Strombidiidae (Agatha and Strüder-Kypke, 2014).

### 3.2. Updated classification

We propose an updated classification for Choreotrichia and Oligotrichia (Table 1). This is based on the latest comprehensive classifications for these groups (Agatha, 2011; Agatha and Strüder-Kypke, 2013; Lynn, 2008), the revision of subsequent literature, and our novel findings (Supplementary Table S5). Our intent is to reconcile the existing data in the most conservative way, considering both morphological and molecular support (see Section 3.1). The motivations for this updated classification are three. First, the latest and most widely-used systems disagree in some taxa that are now represented in phylogenetic trees. For

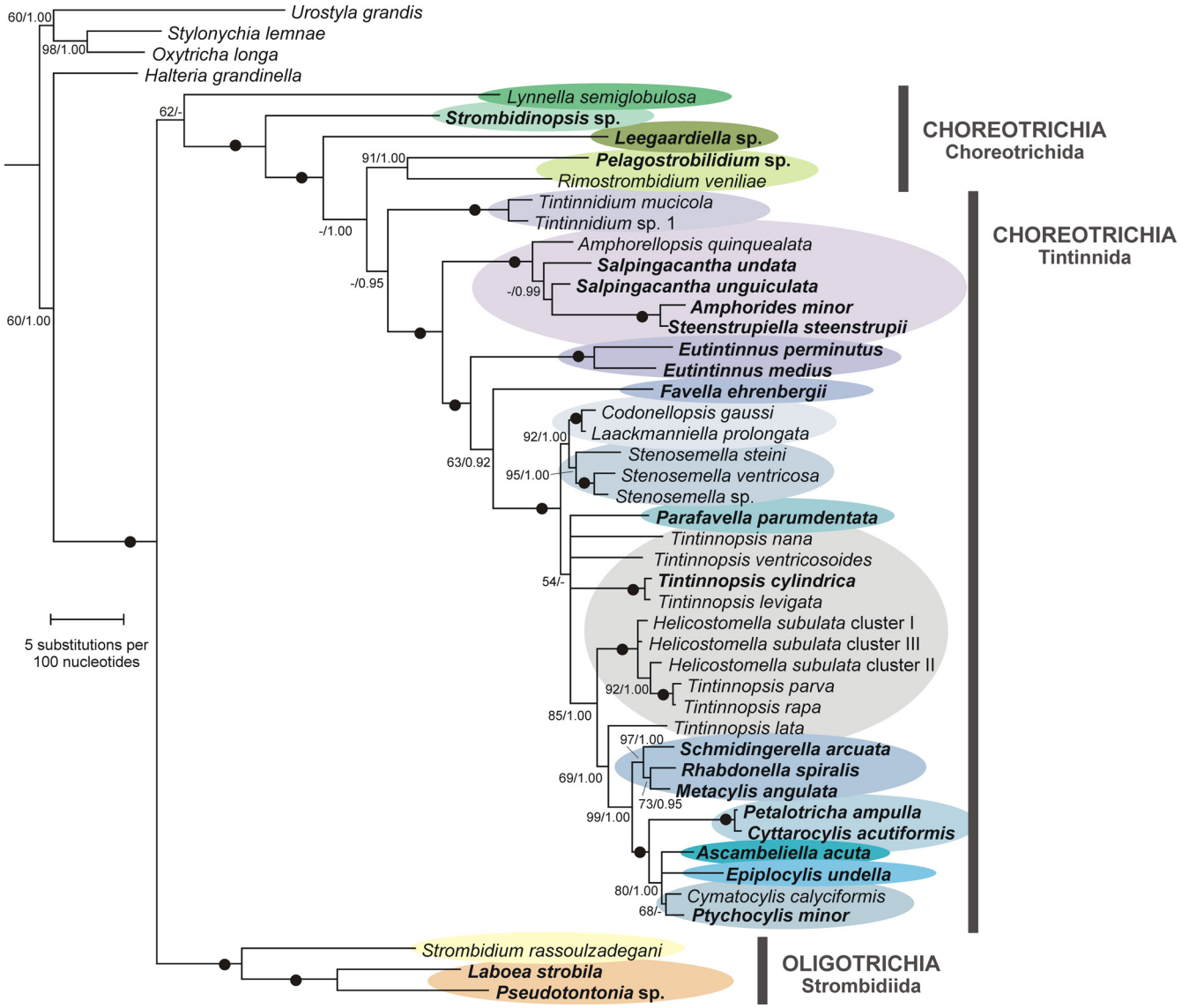


**Fig. 2.** Phylogenetic tree inferred from concatenated SSU rDNA, ITS regions and LSU rDNA sequences. RAxML bootstrap support and MrBayes posterior probability values are shown (only if >45% and >0.90, respectively). A black circle indicates full support in both analyses. Species in bold were sequenced in this study. GenBank accession numbers are shown in Supplementary Table S4. Families (colors) and Tintinnida *incertae sedis* (gray) as in Table 1.

example, *Cyrtostrombidium* has been considered a Strombidiidae (Lynn, 2008), but a separate family is now supported by both its morphology (Agatha, 2004) and DNA sequences (Tsai et al., 2015; Fig. 3). Second, recently-created taxa need to be added in the classification, if justified. For example, the distinctiveness of *Lynnella* has warranted a new family (Liu et al., 2011), but its inclusion in a new order (Liu et al., 2015) seems premature given the morphological similarities to Choreotrichida and unresolved phylogenetic relationships (see Section 3.1). Finally, our new data confirm or reject some rearrangements in tintinnids, as explained below.

We re-establish the family Favellidae Kofoid and Campbell, 1929 and improve its diagnosis (see Section 3.2.1). Campbell (1954) had transfered *Favella* to Ptychocylididae, but this is refuted by the distant position of our novel *Ptychocylis* sequences, which cluster with those of *Cymatocylis* instead (Figs. 2 and S3B). This separation is supported by differences in the ciliary pattern and lorica ultrastructure. *Favella* presents two dorsal kineties in the somatic ciliature, and a lorica wall monolaminar with alveoli and a smooth surface (Agatha and Strüder-Kypke, 2012; Kim et al., 2010). In contrast, *Cymatocylis,* and presumably other Ptychocylididae, have a more developed ciliary pattern with only one dorsal kinety (Kim et al., 2013) and a lorica wall that is also monolaminar with alveoli, but with ridges (also present in *Ptychocylis*; Supplementary Text 1).

*Parundella* and *Dadayiella* are separate genera and both need family reassignment. They have been incorrectly synonymized (Xu et al., 2013), as noticed by Agatha and Strüder-Kypke (2014). Having sequenced them here (Fig. 1) or in previous studies (Santoferrara et al., 2016a), we confirm differences in genes and lorica morphology (Supplementary Text 1). *Parundella* was first established as an *Undella* subgenus given that both taxa show distinct wall laminae (Jörgensen, 1924), but the former was then moved to Xystonellidae without clear reasons (Kofoid and Campbell, 1929). Here, we transfer *Parundella* to Undellidae due to their phylogenetic affinity (Fig. S3B) and similar lorica wall ultrastructure (trilaminar; Agatha and Strüder-Kypke, 2014; Marshall, 1969). *Dadayiella* was affiliated to Tintinnidae, but this placement is not supported by DNA sequences (Fig. S3) or morphology (Kofoid and Campbell, 1929). Thus, we transfer *Dadayiella* as *incertae sedis* in Xystonellidae based on their fully supported

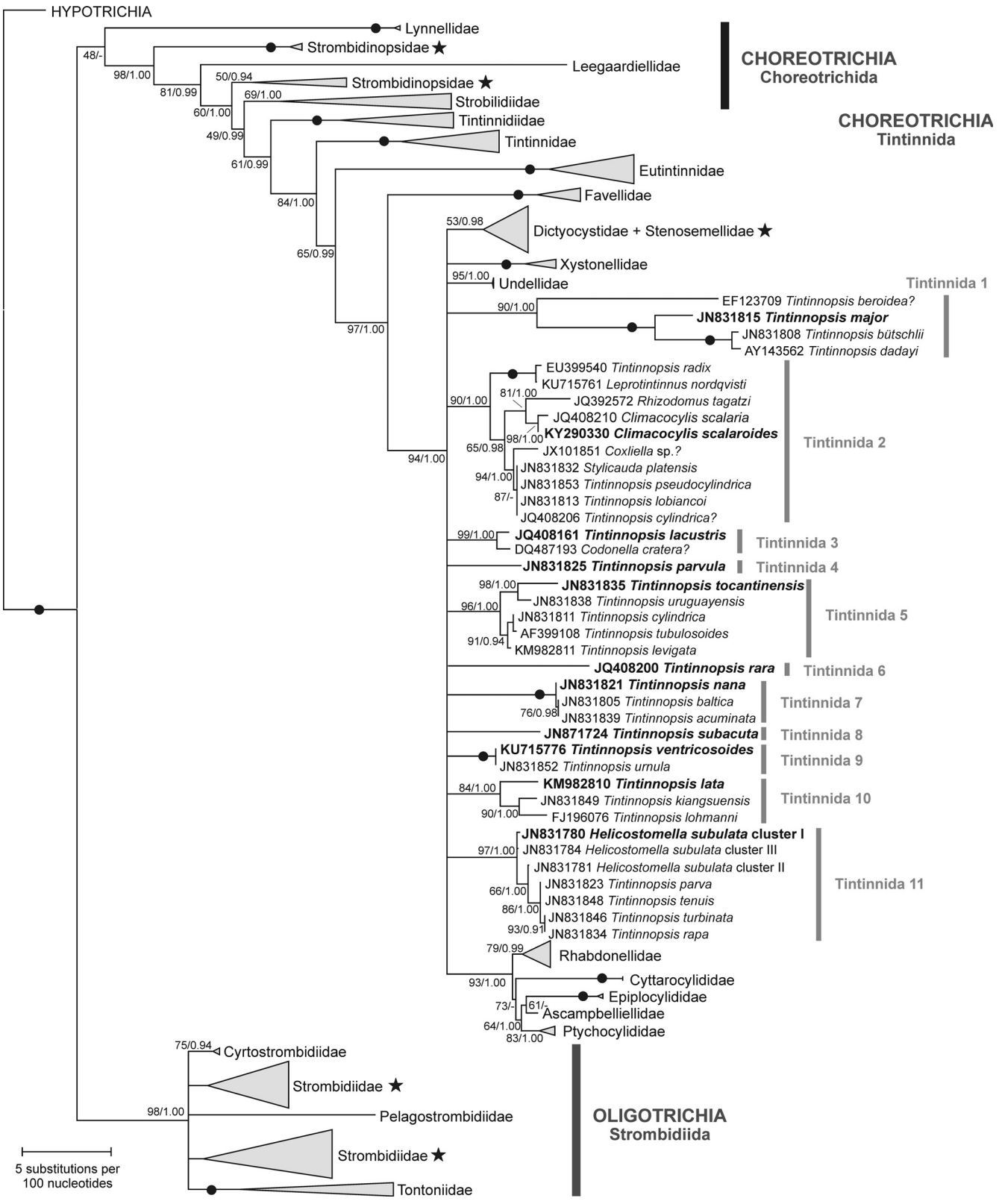


**Fig. 3.** Phylogenetic tree inferred from SSU rDNA sequences. RAxML bootstrap support and MrBayes posterior probability values are shown (only if >45% and >0.90, respectively). A black circle indicates full support in both analyses. A star indicates non-monophyly. Families are collapsed (expanded version in Fig. S3). Tintinnida *incertae sedis* are expanded and enumerated by lineage; for each of them, one sequence (in bold) is selected as representative (the most basal, reliable or distinctive one).

phylogenetic relationship (Fig. S3B), although detailed morphological studies are needed to confirm this affiliation.

*Cyttarocylis* and *Petalotricha* may be separate genera. These genera, their families, and several of their species have been unified based on identical SSU rDNA and ITS regions in specimens from the Mediterranean (Bachy et al., 2012). We found identical sequences for both markers in *C. acutiformis* and *P. ampulla* from the NW Atlantic, but our novel LSU rDNA sequences differ by

**Table 1**
Updated classification of the subclasses Choreotrichia and Oligotrichia.

Choreotrichia Small and Lynn, 1985 (2 orders)
Choreotrichida Small and Lynn, 1985 (5 families)
Leegaardiellidae Lynn and Montagnes, 1988 (1 genus)
*Leegaardiella* Lynn and Montagnes, 1988
Lohmanniellidae Montagnes and Lynn, 1991 (1 genus)
*Lohmanniella* Leegaard, 1915
Lynnellidae Liu et al., 2011 (1 genus)
*Lynnella* Liu et al., 2011
Strobilidiidae Kahl in Doflein and Reichenow, 1929 (3 genera)
*Pelagostrobilidium* Petz, Song and Wilbert, 1995
*Rimostrombidium* Jankowski, 1978
*Strobilidium* Schewiakoff, 1892
Strombidinopsidae Small and Lynn, 1985 (3 genera)
*Parastrombidinopsis* Kim et al., 2005
*Parastrombidium* Fauré-Fremiet, 1924
*Strombidinopsis* Kent, 1881
Tintinnida Kofoid and Campbell, 1929 (14 families)
Ascampbelliellidae Corliss, 1960 (4 genera)
*Acanthostomella* Jörgensen, 1927
*Ascampbelliella* Corliss, 1960
*Incertae sedis: Luxiella* Lecal, 1953
*Incertae sedis: Niemarshallia* Corliss, 1960
Cyttarocylididae Kofoid and Campbell, 1929 (2 genera)
*Cyttarocylis* Fol, 1881
*Petalotricha* Kent, 1881
Dictyocystidae Haeckel, 1873 (6 genera)
*Codonaria* Kofoid and Campbell, 1929
*Codonella* Haeckel, 1873
*Codonellopsis* Jörgensen, 1924
*Dictyocysta* Ehrenberg, 1854
*Incertae sedis: Laackmanniella* Kofoid and Campbell, 1929
*Incertae sedis: Wangiella* Nie, 1934
Epiplocylididae Kofoid and Campbell, 1939 (3 genera)
*Epicancella* Kofoid and Campbell, 1929
*Epiplocylis* Jörgensen, 1924
*Epiplocyloides* Hada, 1938
Eutintinnidae Bachy et al., 2012 (1 genus)
*Eutintinnus* Kofoid and Campbell, 1939
Favellidae Kofoid and Campbell, 1929 (1 genus)
*Favella* Jörgensen, 1924
Nolaclusiliidae Sniezek et al., 1991 (1 genus)
*Nolaclusilis* Snyder and Brownlee, 1991
Ptychocylididae Kofoid and Campbell, 1929 (4 genera)
*Cymatocylis* Laackmann, 1910
*Protocymatocylis* Kofoid and Campbell, 1929
*Ptychocylis* Brandt, 1896
*Wailesia* Kofoid and Campbell, 1939
Rhabdonellidae Kofoid and Campbell, 1929 (7 genera)
*Epirhabdonella* Kofoid and Campbell, 1939
*Metacylis* Jörgensen, 1924
*Pseudometacylis* Balech, 1968
*Protorhabdonella* Jörgensen, 1924
*Rhabdonella* Brandt, 1906
*Rhabdonellopsis* Kofoid and Campbell, 1929
*Schmidingerella* Agatha and Strüder-Kypke, 2012
Stenosemellidae Campbell, 1954 (1 genus)
*Stenosemella* Jörgensen, 1924
Tintinnidae Claparède and Lachmann, 1858 (21 genera)
*Albatrossiella* Kofoid and Campbell, 1929
*Amphorellopsis* Kofoid and Campbell, 1929
*Amphorides* Strand, 1928
*Brandtiella* Kofoid and Campbell, 1929
*Bursaopsis* Kofoid and Campbell, 1929
*Buschiella* Corliss, 1960
*Cantharielle* Kofoid and Campbell, 1929
*Clevea* Balech, 1948
*Daturella* Kofoid and Campbell, 1929
*Epicranella* Kofoid and Campbell, 1929
*Odontophorella* Kofoid and Campbell, 1929
*Ormosella* Kofoid and Campbell, 1929
*Proamphorella* Kofoid and Campbell, 1939
*Prostelidiella* Kofoid and Campbell, 1939
*Rhabdosella* Kofoid and Campbell, 1929
*Salpingacantha* Kofoid and Campbell, 1929
*Salpingella* Jörgensen, 1924
*Salpingelloides* Campbell, 1942
*Steenstrupiella* Kofoid and Campbell, 1929
*Stelidiella* Kofoid and Campbell, 1929
*Tintinnus* Schrank, 1803
Tintinnidiidae Kofoid and Campbell, 1929 (2 genera)
*Membranicola* Foissner, Berger and Schaumburg, 1999
*Tintinnidium* Kent, 1881
Undellidae Kofoid and Campbell, 1929 (7 genera)
*Amplectella* Kofoid and Campbell, 1929
*Amplectellopsis* Kofoid and Campbell, 1929
*Cricundella* Kofoid and Campbell, 1929
*Parundella* Jörgensen, 1924
*Proplectella* Kofoid and Campbell, 1929
*Undella* Daday, 1887
*Undellopsis* Kofoid and Campbell, 1929
Xystonellidae Kofoid and Campbell, 1929 (5 genera)
*Parafavella* Kofoid and Campbell, 1929
*Spiroxystonella* Kofoid and Campbell, 1939
*Xystonella* Brandt, 1906
*Xystonellopsis* Jörgensen, 1924
*Incertae sedis: Dadayiella* Kofoid and Campbell, 1929
*Incertae sedis* in Tintinnida:
*Codonopsis* Kofoid and Campbell, 1939
*Poroecus* Cleve, 1902
*Climacocylis* Jörgensen, 1924
*Helicostomella* Jörgensen, 1924
*Leprotintinnus* Jörgensen, 1900
*Rhizodomus* Strelkow and Wirketis, 1950
*Rotundocylis* Kufferath, 1950
*Stylicauda* Balech, 1951
*Tintinnopsis* Stein, 1867
*Nomen inquirendum: Coxliella* Brandt, 1906

Oligotrichia Bütschli, 1887/1889 (1 order)
Strombidiida Petz and Foissner, 1992 (4 families)
Cyrtostrombidiidae Agatha, 2004 (1 genus)
*Cyrtostrombidium* Lynn and Gilron, 1993
Pelagostrombidiidae Agatha, 2004 (2 genera)
*Limnostrombidium* Krainer, 1995
*Pelagostrombidium* Krainer, 1991
Strombidiidae Fauré-Fremiet, 1970 (12 genera)
*Antestrombidium* Liu et al., 2015
*Apostrombidium* Xu, Warren and Song, 2009
*Foissneridium* Agatha, 2010
*Novistrombidium* Song and Bradbury, 1998
*Omegastrombidium* Agatha, 2004
*Opisthostrombidium* Agatha, 2010
*Parallelostrombidium* Agatha, 2004
*Sinistrostrombidium* Liu et al., 2015
*Spirostrombidium* Jankowski, 1978
*Strombidium* Claparède and Lachmann, 1859
*Varistrombidium* Xu, Warren and Song, 2009
*Williophrya* Liu et al., 2011
Tontoniidae Agatha, 2004 (5 genera)
*Laboea* Lohmann, 1908
*Paratontonia* Jankowski, 1978
*Pseudotontonia* Agatha, 2004
*Spirotontonia* Agatha, 2004
*Tontonia* Fauré-Fremiet, 1914

1.8% between species, in agreement with the marked dissimilarities in lorica morphology (Fig. 1D and E, Supplementary Text 1). This molecular divergence and, especially, the fact that lorica differences are not confirmed as intra-taxon polymorphism (Dolan, 2016) delay potential species and genera synonymizations until more features are studied and unified diagnoses can be provided. Instead, family synonymization is supported phylogenetically (Fig. 2) and by the shared lorica ultrastructure (trilaminar, tubular; Agatha and Strüder-Kypke, 2014). Bachy et al. (2012) included also *Metacylis* and *Rhabdonella* in Cyttarocylididae, but the lack of morphological justification and the increased taxon and character sampling in our inferences (Figs. 2 and S3B) suggest that these transfers are premature. Conservatively, we avoid lumping Cyttarocylididae, Ascampbelliellidae, Rhabdonellidae, Epiplocylididae, and Ptychocylididae, even if they form a highly supported clade in our trees (Figs. 2 and 3) and some of their representatives are

known to share either the lorica texture (the three latter; Agatha and Strüder-Kypke, 2014) or the extrusome type (the first and third; Laval-Peuto and Barría de Cao, 1987).

The family Metacylididae is no longer supported, as noted before (Bachy et al., 2012). *Metacylis* and *Pseudometacylis* are here transferred to Rhabdonellidae, given the phylogenetic position of the former (the second remains unsequenced; Figs. 2 and S3B) and shared lorica texture of all of them (hyaline, monolaminar with alveoli, low surface ridges, and pores; Agatha and Strüder-Kypke, 2012; Balech, 1968; Lackey and Balech, 1966). Other former Metacylididae, *Climacocylis* and *Helicostomella*, share a similar lorica texture (Agatha and Strüder-Kypke, 2014), but they are phylogenetically distant, and instead related to *Tintinnopsis*-like species (Figs. 2 and 3). Also related to *Tintinnopsis* are *Stylicauda*, *Rhizodomus*, and *Leprotintinnus*, the latter no longer supported in Tintinnidiidae due to both phylogenetic distance and unclear morphological affinity (Zhang et al., 2016). The latter six genera are *incertae sedis* in Tintinnida.

#### 3.2.1. Family Favellidae Kofoid and Campbell, 1929

Improved diagnosis: Two loricae types, protolorica, and paralorica. Protolorica frequently with an annulated or spiraled epilorica and a posterior process; paralorica spiraled, usually lacking a posterior process. Lorica wall monolaminar with alveoli and smooth surface. Ciliary pattern characterized by two dorsal kineties, a monokinetidal ventral kinety, and lateral, right, and left ciliary fields. One genus: *Favella*.

### 3.3. Informal classification of incertae sedis: Tintinnopsis and related genera

The taxonomy of *Tintinnopsis* has always been problematic. Because its lorica is densely agglomerated with particles, most diagnostic characters are difficult to study. There is a long history of species splits and unifications (e.g., Bakker and Phaff, 1976), and it has even been considered a "complex" instead of a genus (Alder, 1999). DNA sequencing has revealed that *Tintinnopsis*-like species may actually belong to several genera and families, but a taxonomic revision is currently impossible because most of the about 160 described species still need reexamination with modern methods, including the type *T. beroidea* (Agatha, 2010). The more species are sequenced, the more widespread they are in phylogenetic trees. This has led to attempts to name lineages informally (Agatha and Strüder-Kypke, 2014; Bachy et al., 2012; Zhang et al., 2016). However, these names are inconsistent in the literature and have other limitations in their utility (Supplementary Table S6). For example, such names have not considered that some stable, well-supported clades include not only *Tintinnopsis*-like species, but also other *incertae sedis* taxa with sparsely-agglomerated (*Leprotintinnus, Rhizodomus, Stylicauda*) or particle-free (*Climacocylis*, *Helicostomella*) loricae. For some of these taxa, lorica similarities in particle-free cultures (Fig. S2B) and strong phylogenetic bonds (Santoferrara et al., 2015) suggest that a common affiliation may be reached once data on the lorica matrix and cytology allow for a formal classification.

Taxa such as *Tintinnopsis* and *Helicostomella* are widely distributed and sometimes very abundant in coastal plankton (e.g., Dolan and Pierce, 2013; Santoferrara and Alder, 2009). Thus, finding a stable way to catalog and link them is important not only for phylogenetic studies, but also for ecological surveys, that are increasingly being based on environmental sequencing. Relevant patterns may now remain unrealized just because sequences are difficult to link to distinct lineages. Here we suggest an informal system to name unclassified tintinnid taxa (Fig. 3, Supplementary Table S6). This system has similarities, for example, to recent (but differently aimed) proposals for sequences of foraminifera (Morard et al., 2016) and eukaryotes in general (eukref.org). Eleven lineages including *Tintinnopsis* and related genera are enumerated consecutively with a single Arabic number. As more sequences are added in the tree, potentially split clades that include a representative sequence (GenBank accessions in bold in Fig. 3) should retain their number, while new clades should take the next available number. On the other hand, as clades merge or are formally classified, their numbers should become unavailable.

### 3.4. Unknown lineages in Choreotrichia and Oligotrichia

Choreotrichia and Oligotrichia have a long tradition of morphological description. However, analysis of all the SSU rDNA sequences available in NCBI GenBank (known morphospecies and environmental sequences mostly from clone libraries) suggests a high potential for uncharacterized or novel taxa in these subclasses (Fig. 4). The trends are opposite for loricates and aloricates: most tintinnid sequences represent morphologically-identified taxa, while most choreotrichid and oligotrich sequences derive from environmental surveys (Fig. 4A). Furthermore, choreotrichids and oligotrichs include divergent clades that are entirely formed by environmental sequences, despite low support. Although some of these environmental clades could represent known lineages not sequenced yet, several of them may represent novel families and genera completely unknown from the morphological point of view.

Two conspicuous branching patterns are evident in our trees (Fig. 4A). Lynnellidae and Cyrtostrombidiidae form isolated branches. One possible explanation for this pattern is that primers used in environmental surveys do not capture the real diversity within these taxa; if so, many other novel clades in the same situation may remain undiscovered. Alternatively, these taxa may exemplify heterogeneous levels of SSU rDNA divergence, or dissimilar rates of diversification among families, possibly derived from differences in geographical distributions, ecological niches or other factors (Vamosi et al., 2009; Pyron and Burbrink, 2013). In contrast to these "lonely" taxa, most other clades include a variable number of sequences, with a maximum for Strobilidiidae and the non-monophyletic Strombidiidae, followed by Tontoniidae and Leegaardiellidae (Fig. 4A). Of them, only Strombidiidae is known to be much diversified (12 genera, >90 species; Agatha, 2011; Table 1) and to include cryptic species (Katz et al., 2005; McManus et al., 2010). Our results suggest a strong underestimation of taxonomic diversity and a high degree of crypticity also for Tontoniidae, Strobilidiidae, and Leegaardiellidae.

The proportion of described species versus SSU rDNA sequences supports that there is an underrepresentation of choreotrichids and oligotrichs, as well as an overrepresentation of tintinnids, in global species inventories (Fig. 4B). About 86% of described species correspond to tintinnids, while 14% belong to choreotrichids and oligotrichs combined (Agatha and Strüder-Kypke, 2014). On the other hand, SSU rDNA sequences (this study) suggest that oligotrichs are the most diversified (61%), followed by choreotrichids (25%), and lastly by tintinnids (14%). Although these results support that a high number of synonyms exist among tintinnid morphospecies (Alder, 1999; Dolan, 2016), this situation should not be oversimplified. Examples of either undistinguishable or distinct morphospecies with identical SSU rDNA that consistently differ in more variable, species-level markers (ITS regions and/ or LSU rDNA), and in some cases even ecologically, have been reported (Xu et al., 2012; Santoferrara et al., 2013, 2015; this study). In other words, the conserved nature of SSU rDNA and our incomplete knowledge on intra- and interspecific sequence similarity (or the lack of a universal clustering cutoff equivalent to species) prevent an ultimate estimation of global species richness of Choreotrichia and Oligotrichia using only molecular data. Integration of multigene, morphological, and eco-physiological data is needed to fully

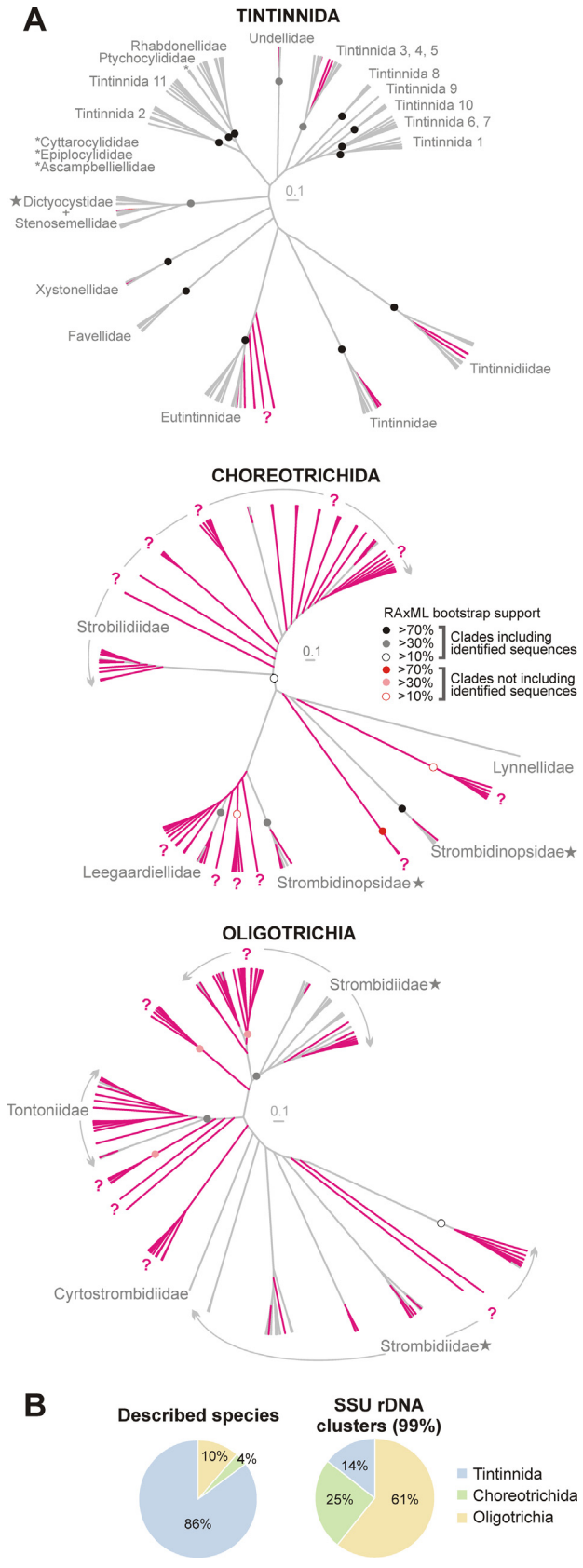


**Fig. 4.** The knowns and unknowns in Choreotrichia and Oligotrichia. A, SSU rDNA clusters (97% similarity) including morphologically-identified (the knowns, in gray) or only environmental (the unknowns, in pink) sequences from NCBI GenBank. Several clades or isolated branches may represent novel taxa (?). A star indicates non-monophyly. B, proportion of SSU rDNA clusters (99% similarity) and described species by order.

characterize ciliate diversity (Agatha, 2011; Santoferrara et al., 2016b).

Because of our curation strategy, we analyzed only sequences longer than 500 bp (see 2.3). However, the current use of environmental high-throughput sequencing (HTS) has produced a massive amount of shorter sequences, which further suggest hidden diversity in ciliates (e.g., Forster et al., 2015; Gimmler et al., 2016). For now, most of this diversity remains morphologically and functionally uncharacterized. Here, single-cell sequencing coupled with morphological identification allows us to link a previously unidentified environmental clade to a known family. We first detected a clade ("cluster X") by HTS and hypothesized that it could correspond to a choreotrichid family not sequenced before (Santoferrara et al., 2014). Although related environmental sequences were found by diverse molecular methods (e.g., Grattepanche et al., 2016; Lie et al., 2014), their taxonomic identity remained a mystery. We now confirm an affiliation to Leegaardiellidae, given the close relationship of these environmental sequences with our novel sequence for this family (Figs. 1A–C and S6).

## 4. Conclusions

We have expanded the phylogenetic inferences based on three rDNA loci for Choreotrichia and Oligotrichia, including two families and six genera never sequenced before. In total, we analyzed 18 families in a multi-gene phylogenetic context, excluding those that lack reliable sequences for at least one locus (Cyrtostrombidiidae, Pelagostrombidiidae and Undellidae) or for the three of them (Lohmanniellidae and Nolaclusiliidae). Based on careful comparison of our molecular results with available information on cytological and ultrastructural characters, we re-established the family Favellidae and updated the classification of these subclasses into 23 total families. Eleven clades that remain *incertae sedis* in Tintinnida, as well as most families in Choreotrichida and Oligotrichia, need additional studies to clarify their taxonomy and evolutionary relationships. Furthermore, entire genera and families remain undescribed among Choreotrichida and Oligotrichia, as suggested by the analysis of all the unidentified, environmental sequences available in GenBank. This analysis provides insights into the environmental diversity of these groups that were not obvious in the individual sequencing efforts. These data also support the fact that aloricates include a high proportion of cryptic species, while loricates include many synonyms.

As more and more environmental sequences are generated, solid references are needed to link these data to the known taxa and to identify hotspots of novel diversity. We used single-cell sequencing to link morphological and molecular data, including in a previously unidentified environmental clade here revealed as Leegaardiellidae. Additionally, we curated almost 4000 sequences from GenBank, which showed problems in both identified sequences (e.g., misidentifications, insufficient or nonexistent published data to confirm identifications, documentation of specimens that cannot be confirmed as the sequenced ones, inconsistent labeling) and environmental sequences (e.g., chimeras and other methodological artifacts). Another alarming issue is the lack of metadata associated with environmental sequences. For example, most choreotrich and oligotrich records lack geographical coordinates, thus limiting studies of spatial distribution. This is particularly important in the current context of climate change that affects, for example, population dynamics and species distribution ranges (Pfenninger et al., 2012; Hofer, 2016). Thus, caution is needed in both the use and contribution to public repositories, given that they are unique resources for evolutionary and diversity studies.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.03.010.

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