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Molecular Phylogeny of Tintinnid Ciliates (Tintinnida, Ciliophora)

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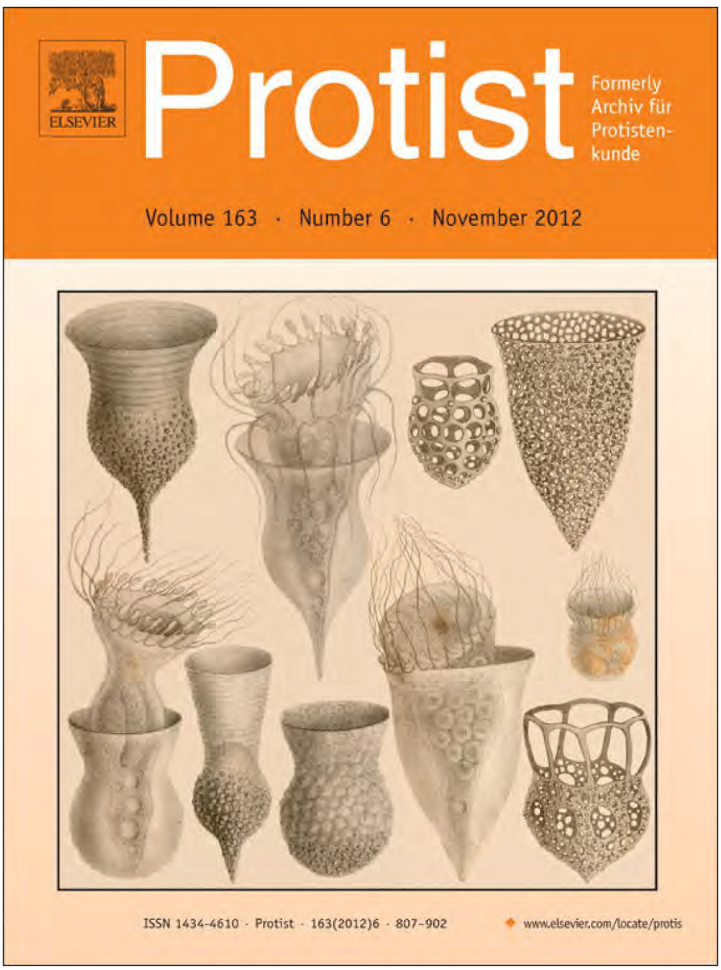

Cover: A composite of drawings taken from the first publication devoted to tintinnid ciliates, that of Ernst Haeckel in 1873, "Ueber einige pelagische Infusorien" published in *Jenaische Zeitschrift für Medizin und Naturwissenschaft* 7: 561–568, Plates 27–28. From Plate 28: the two upper figures on the left are what is now known as *Tintinnopsis campanula*, the lorica (left) and extended cell (right). The lower figures from the left show *Codonella galea*, the cell (left) and the lorica covered with coccoliths (right). The middle lower figure is now known as *Codonellopsis orthoceras*. The five drawings on the right taken from Plate 27 depict members of the family Dictyocystidae that are characterized by their clathrate silica shells. The cover illustration was kindly provided by John R. Dolan (Villefranche-sur-Mer). On page 873–887 of this issue Bachy and colleagues present a molecular phylogeny of tinntinnid ciliates.

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We investigated the phylogeny of tintinnids (Ciliophora, Tintinnida) with 62 new SSU-rDNA sequences from single cells of 32 marine and freshwater species in 20 genera, including the first SSU-rDNA sequences for *Amphorides*, *Climacocylis*, *Codonaria, Cyttarocylis*, *Parundella*, *Petalotricha*, *Undella* and *Xystonella*, and 23 ITS sequences of 17 species in 15 genera. SSU-rDNA phylogenies suggested a basal position for *Eutintinnus*, distant to other Tintinnidae. We propose Eutintinnidae fam. nov. for this divergent genus, keeping the family Tintinnidae for *Amphorellopsis, Amphorides* and *Steenstrupiella*. *Tintinnopsis* species branched in at least two separate groups and, unexpectedly, *Climacocylis* branched among *Tintinnopsis* sensu stricto species. *Tintinnopsis* does not belong to the family Codonellidae, which is restricted to *Codonella*, *Codonaria*, and also *Dictyocysta* (formerly in the family Dictyocystidae). The oceanic genus *Undella* branched close to an undescribed freshwater species. *Metacylis*, *Rhabdonella* and *Cyttarocylis* formed a well supported clade with several *Tintinnopsis* species at a basal position. *Petalotricha ampulla* and *Cyttarocylis cassis* SSU-rDNA and ITS sequences were identical or almost identical. Therefore, we propose *Cyttarocylis ampulla* comb. nov. for them. Intensive use of single-cell isolation and sequencing revealed unexpected complexity in the evolutionary history of these relatively well-studied ciliates. Notably, the diversity of freshwater forms suggests multiple marine-freshwater invasions.

Key words: Alveolates; rDNA phylogeny; loricate ciliates; single-cell PCR.

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Abbreviations: AU, Approximately Unbiased; bp, base pairs; BI, Bayesian Inference; BV, bootstrap value; ITS, Internal Transcribed Spacer; ML, maximum likelihood; PCR, polymerase chain reaction; rDNA, ribosomal deoxyribonucleic acid; s.l., sensu lato; s.s., sensu stricto; SSU, small subunit.

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Introduction

Microzooplankton, composed largely of ciliates and heterotrophic dinoflagellates, plays a major role in the transfer of energy and matter through the pelagic food web by consuming a majority of the primary production (Calbet and Landry 2004). Planktonic ciliates are ubiquitous and much attention has been given to their role as primary consumers of pico- and nanosized autotrophs and heterotrophs, as well as nutrient regenerators and as important food sources for metazoan zooplankton and fish larvae (Dolan et al. 2002; Pierce and Turner 1992). They also represent one of the most morphologically diverse groups in the plankton. Commonly, planktonic ciliates are divided into loricate (tintinnids) and aloricate forms. The identification of aloricate ciliates requires slide mounting, cytological staining (i.e., protargol silver staining) and examination under high magnification (Montagnes and Lynn 1987). By contrast, by examining untreated specimens in plankton settling chambers using an inverted microscope, tintinnids can be easily differentiated from other species based on their secreted shell, the lorica. These loricae range in size from ca. 20 μm up to several hundred micrometers, and can be hyaline or agglutinated with mineral or biological particles attached.

The existing classification of tintinnids is entirely based on the characteristics of the loricae, including differences in the size, general shape, ornamentation, fine-scale surface structures and the presence or absence of agglutinated particles. There are numerous detailed descriptions at the species level, especially with the conspectus of Kofoid and Campbell (1929) in which 627 species were distinguished based on characteristics of the lorica. The first attempt to reconstruct tintinnid phylogeny was also lorica-based and considered the agglutinated lorica as an ancestral character (Kofoid and Campbell 1939). However, reliance on lorica characteristics in tintinnid taxonomy is problematic. In fact, some lorica characteristics, typically its length, have long been known to be quite variable (e.g. Gold and Morales 1975; Laackmann 1908). In natural populations, significant lorica plasticity has been suspected in a variety of species (e.g. Boltovskoy et al. 1990; Davis 1981; Santoferrara and Alder 2009; Wasik and Mikolajczyk 1994). For example, a morphometric study of the genus *Cymatocylis* (Williams et al. 1994) found that 45 lorica morphologies described as separate species could only be reliably sorted into 5 distinct morphotypes. However, the real limitations of lorica-based taxonomy and phylogeny were revealed using cultures with the demonstration that a single species of *Favella* can show lorica characteristic thought to be typical of different genera (Laval-Peuto 1977, 1981, 1983). Unfortunately, for the overwhelming majority of species, there is no information on other morphological characters such as the ciliary pattern or the kinetome. The structure of the kinetome is known for only twenty species, too few for a rigorous analysis and insufficient for the demonstration of clear apomorphies within the Tintinnida (Agatha 2010a; Agatha and Strüder-Kypke 2007; Choi et al. 1992; Foissner and Wilbert 1979).

Ciliates of the subclass Choreotrichia Small & Lynn, 1985 are characterized by an adoral zone of polykinetids, used in locomotion and feeding, which forms a closed membranellar zone (Lynn 2008). Within this subclass, the presence of a lorica distinguishes the order Tintinnida Kofoid & Campbell, 1929 from the order Choreotrichida (strobiliids). In the last decade, advances in molecular phylogeny have allowed evaluation of these diagnostic criteria. Most of the available sequences corresponded to small subunit rRNA gene (SSU-rDNA) sequences. The first studies were based on species available in culture or from pooling cells collected in field samples (Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003). Nevertheless, most species are difficult to culture or to find in abundance in natural samples. Fortunately, techniques such as single-cell PCR and sequencing are especially useful for tintinnids and other ciliates that usually have numerous copies of highly expressed genes in their macronuclear genomes (Prescott 1994).

There are more than 50 accepted genera of extant tintinnids (Lynn 2008). However, publicly available sequences are restricted only to species of 12 genera, with a strong bias towards coastal species of temperate waters (Duff et al. 2008; Gao et al. 2009; Li et al. 2009; Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003, 2008). Nevertheless, tintinnid diversity is highest in tropical or sub-tropical waters (Dolan et al. 2006; Gómez 2007a). Analyses of the relatively small number of sequences have confirmed the monophyly of Tintinnida and supported some higher order groups of families but also have suggested the paraphyly of certain genera (Strüder-Kypke and Lynn 2003, 2008). This suggested that the use of lorica morphology to form natural groups or to reconstruct the evolutionary history of tintinnids is questionable.

Insufficient taxon sampling, namely the lack of molecular information for numerous tintinnid genera, has greatly hindered advances in determining the classification and evolutionary history of

tintinnids. We have addressed this issue by providing a comprehensive molecular phylogeny of Tintinnida and establishing diagnostic characters for a classification supported by new 62 SSU-rDNA sequences of 32 species from marine and freshwater environments. SSU-rDNA sequences of members of the genera *Amphorides* Strand, *Climacocylis* Jörgensen, *Codonaria* Kofoid & Campbell, *Cyttarocylis* Fol, *Parundella* Jörgensen, *Petalotricha* Kent, *Undella* Daday, *Xystonella* Brandt are determined for the first time. We also provide 25 ITS sequences of 17 species in 15 genera in order to compare the phylogeny of Tintinnida using another ribosomal molecular marker.

Results

Specimens Isolated for SSU-rDNA Sequencing

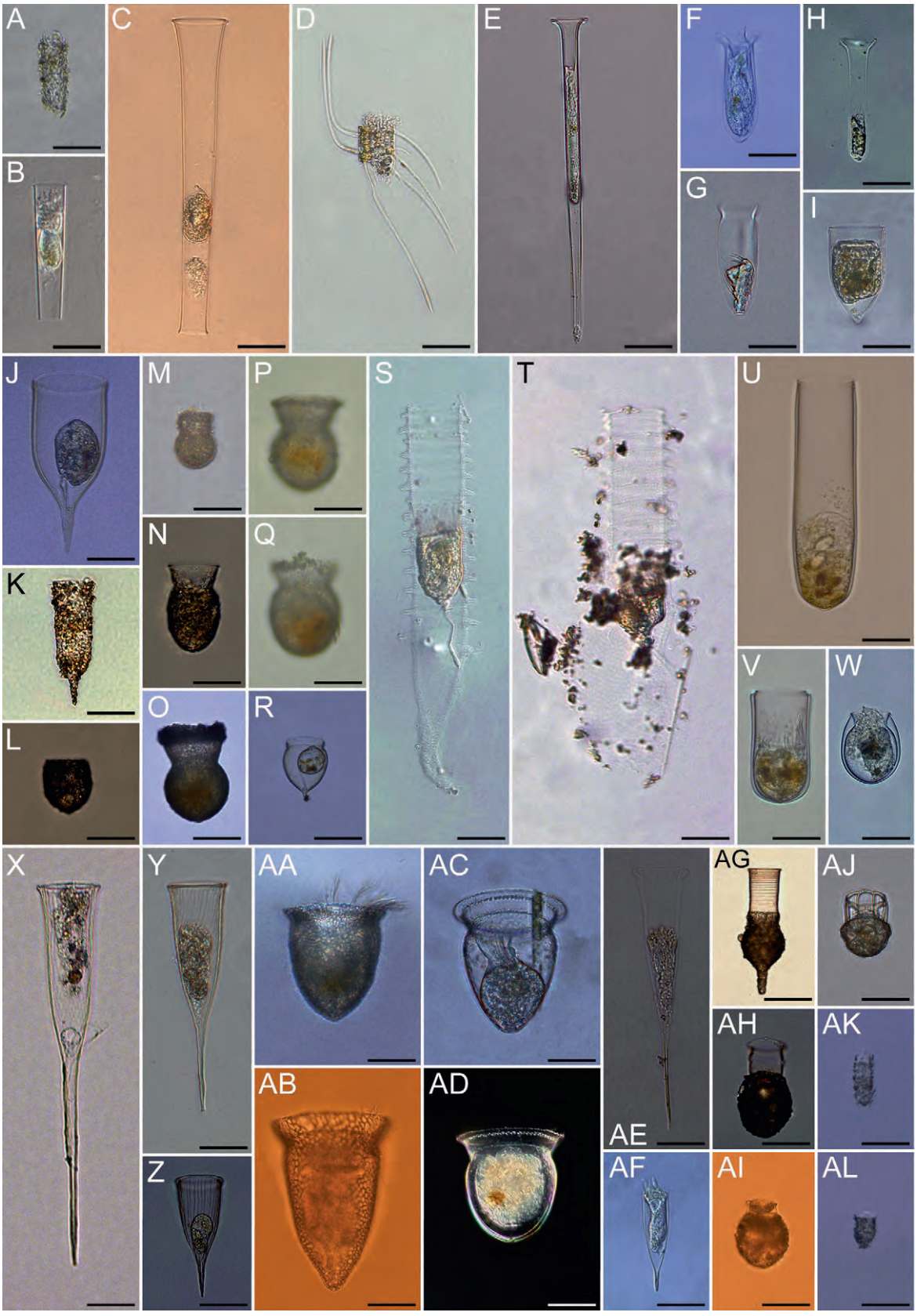
Over the two-year period of our study, a total of 62 SSU-rDNA sequences from 32 morphologically-distinguished species from marine and freshwater environments were determined. All specimens were individually identified, photographed and isolated under the microscope. These species are representative of a wide range of loricae morphologies, lengths, shapes, number of apertures and type of agglomeration (Fig. 1; Supplementary Fig. S1). In some cases, for a single species we included specimens collected in different locations and dates. In addition, ITS and 5.8 rRNA gene sequences were obtained for 17 species. All isolates for which we obtained either the SSU-rDNA sequence or both the SSU-rDNA and ITS sequences are listed in Supplementary Table S1. A detailed description of the isolated specimens classified by family is available in the Supplementary Materials.

SSU-rDNA Phylogeny

Sequence identity between the tintinnid SSU rDNA sequences ranged from 90% to 100%. In particular, the sequences of *Codonella*, *Codonellopsis*, *Stenosemella* and *Dictyocysta* species were very similar (98%-100%). By contrast, the sequences of the congeneric species of *Eutintinnus* exhibited higher dissimilarity (ranging from 95%-97% nucleotide identity). This implies a higher difference between the *Eutintinnus* species than between other species belonging to different genera. We also found possible evidence of intra-specific genetic variation in the SSU-rDNA locus of the species *Amphorides quadrilineata*. The distance between the two isolates FG1141 and FG293 (from two different Mediterranean sites) was almost 3%, while genetic variation was not found in other tintinnid species (e.g. *Tintinnopsis cylindrica* or *Codonellopsis morchella*).

There were no differences among the maximum likelihood (ML) and Bayesian inference (BI) tree topologies for all well-supported nodes in the tintinnid SSU-rDNA phylogeny. Moreover, the use of different sequence alignment and masking methods did not affect these well-supported nodes. Oligotrichs and aloricate choreotrichs branched as sister groups of a large monophyletic group containing all tintinnid sequences with the exception of *Tintinnidium* species (Fig. 2). In fact, in all our analyses, *Tintinnidium* species branched close to the Strombilidiidae, although with weak statistical support (ML bootstrap value -BV- 60%, Bayesian posterior probability -PP- 0.67). To check the robustness of this result, we carried out an Approximately Unbiased (AU) test on the SSU-rDNA data set to assess the monophyly of tintinnid species. The test could not significantly reject the constrained topology where all the tintinnids *sensu stricto* (with a lorica) were forced to be monophyletic ($p = 0.22$) (Supplementary Table S2).

In addition to the divergent clade of *Tintinnidium*, the species of the genus *Eutintinnus* formed a basal branching group within the tintinnid core. This clade is divided into two sister groups, the first composed of *Eutintinnus apertus*, *E. pectinis* and an unidentified species of this genus, and the second of *Eutintinnus fraknoi* and *E. tubulosus* (Fig. 2). The genera *Salpingella*, *Amphorellopsis*, *Amphorides*, and *Steenstrupiella* branched together in a clade with a strong support (BV 100%). In the classical taxonomical schemes, these genera are placed in the family Tintinnidae. Within this clade, our sequence identified as *Salpingella acuminata* branched in a basal position, forming a paraphyletic group with a second sequence retrieved from GenBank under the same name *S. acuminata* (EU399536). The sequences of *Amphorellopsis*, *Amphorides*, and *Steenstrupiella* formed a well supported group (BV 100%) with a long basal branch, indicating an acceleration of evolutionary rate before the diversification of these three genera. Our five sequences of *Amphorides quadrilineata* showed different positions. Three of them formed a group with *Steenstrupiella* whereas, surprisingly, the sequences of our isolates *A. quadrilineata* FG1141 and FG249 formed a group with *Amphorellopsis acuta*. We were unable to clearly find morphological differences between the five *A. quadrilineata* specimens (Fig. 1G and H).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Under the same morphology these specimens clearly formed two cryptic phylotypes.

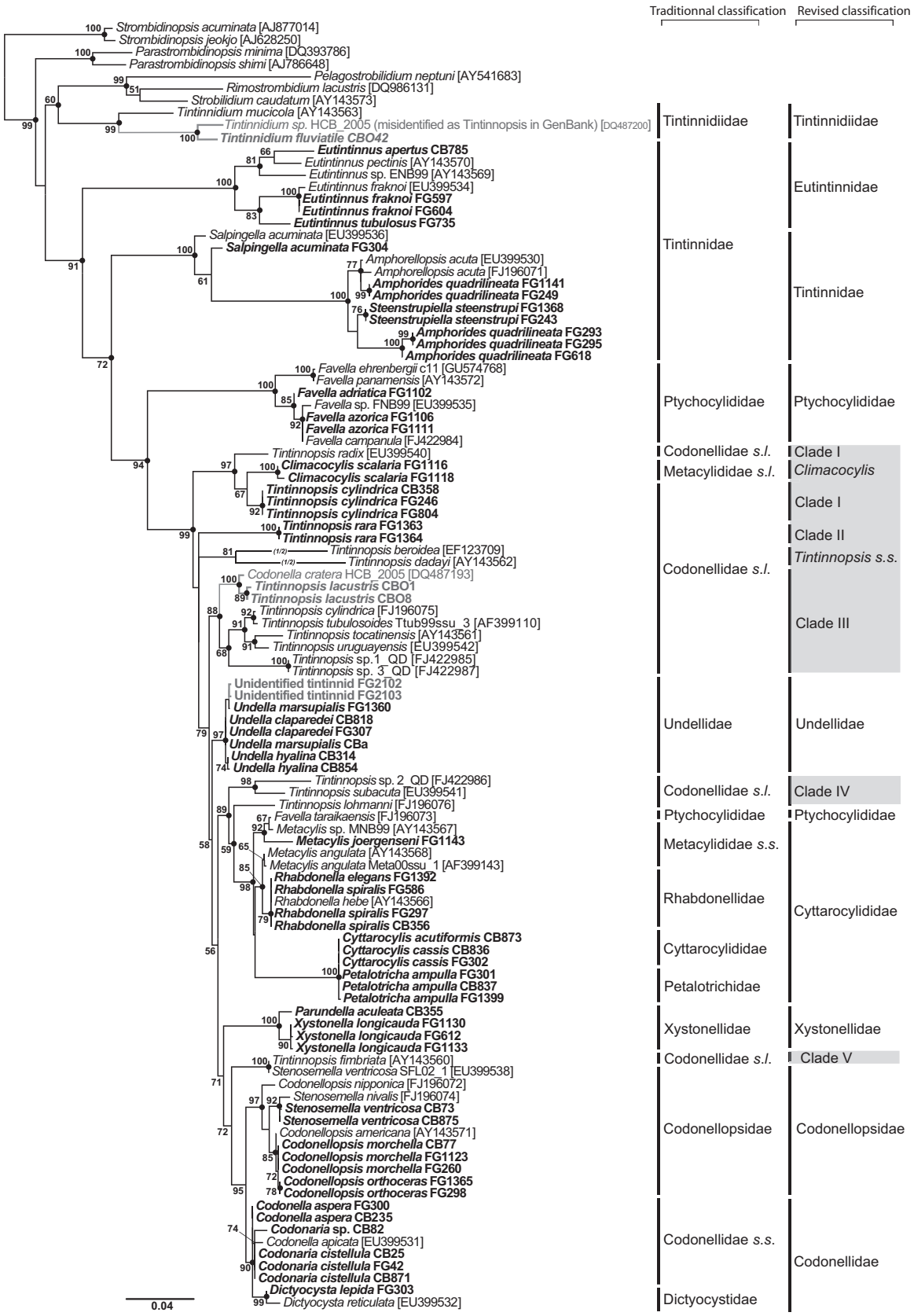
A clade with a strong support (BV 100%) was formed exclusively of species of *Favella*, with the exception of a GenBank sequence named *Favella taraikaensis* (FJ196073), which branched close to *Metacylis* spp. The sequences of our isolates *Favella azorica* FG1106 and *F. azorica* FG1111 were identical between them and to that of *F. campanula* (Schmit) Jörgensen, (GenBank FJ422984), while the isolate *F. adriatica* FG1102 was more distantly related to them. All these specimens were collected in the same location, Bay of Villefranche, and in an interval of one week (Supplementary Table S1).

The rest of species emerged within a strongly supported group (BV 99%, PP 1), within which different sub-clades grouped species with high support but the relationships among them remained unresolved. Notably, the sequences of the genus *Tintinnopsis* were scattered among several subclades. A sequence retrieved from GenBank under the name *Tintinnopsis beroidea* Stein (EF123709), if correctly identified, corresponded to the *Tintinnopsis* type species (Li et al. 2009). Most other *Tintinnopsis* species were grouped in several clades branching in the same region of the tree as the type species. Nevertheless, there were two groups distantly related to the group of the type species. One was composed of *T. subacuta* Jörgensen and *T. lohmanni* Laackmann in addition to sequences of *Favella*, *Metacyclis Rhabdonella*, *Cyttarocyclis* and *Petalotrica* species. The second one was represented only by a sequence named *Tintinnopsis fimbriata* Meunier (Strüder-Kypke and Lynn 2003), which was 99% identical to a *Stenosemella ventricosa* sequence, suggesting the possible misidentification of the sequence submitted to GenBank. Interestingly, the sequences of *Climacocylis* branched in the main group of sequences of *Tintinnopsis* (BV 97%, Fig. 2). This was unexpected because the lorica morphology of *Climacocylis* is radically different from *Tintinnopsis* (see Fig. 1K and S) and no taxonomic scheme has ever proposed a close relationship between these genera.

As all the *Tintinnopsis* species did not cluster together and appeared in different subclades in the tree, AU tests were carried out on the SSU-rDNA data set to assess the polyphyly of *Tintinnopsis* spp. (Supplementary Table S2). The test significantly rejected the constraint that all *Tintinnopsis* species formed a monophyletic clade ($p = 0$), contradicting the lorica-based morphological classification. However, as long as we did not consider *Tintinnopsis fimbriata* AY143560, *Tintinnopsis subacuta* EU399541, *Tintinnopsis* sp. 2 FJ422986 and *Tintinnopsis lohmanni* FJ196076, the AU test could not reject the constrained topology where the 11 other *Tintinnopsis* spp. were forced to be monophyletic ($p = 0.16$). We could not discard the possibility that the fast evolving sequences of the type species *Tintinnopsis beroidea* and of *Tintinnopsis dadayi* (see the long branches in Fig. 2) formed a monophyletic group because of a long-branch attraction artefact (Bergsten 2005).

A well-supported clade was composed of the species of *Undella* (BV 97%). The sequence difference between *Undella marsupialis*, *U. claparadei* and *U. marsupialis* were extremely low (>99.9% identity). Unexpectedly, two sequences of two specimens of an unidentified species branched in this clade. These specimens were collected in a freshwater lake and showed a lorica with

Figure 1. Light micrographs of specimens of *Tintinnida* collected for single-cell PCR analysis **A-AL**. See Table S1 for collection dates, locations and accession numbers. **A.** *Tintinnidium fluviatile* isolate CBO42. **B.** *Eutintinnus tubulosus* isolate FG735. **C.** *E. fraknoi* isolate FG604. **D**. *E. apertus* isolate CB785. **E**. *Salpingella acuminata* isolate FG304. **F**. *Amphorides quadrilineata* isolate FG1141. **G**. *Amphorides quadrilineata* isolate FG293. **H**. *Steenstrupiella steenstrupi* isolate FG1368. **I**. *Favella azorica* isolate FG1111. **J**. F. *adriatica* isolate FG1102. **K**. *Tintinnopsis cylindrica* isolate CB358. **L**. *Tintinnopsis rara* isolate FG1364. **M**. *Tintinnopsis lacustris* isolate CBO1. **N**. *Codonella aspera* isolate FG300. **O**. *Codonaria cistellula* isolate FG42. **P**. *Codonaria cistellula* isolate CB871. **Q**. *Codonaria* sp. isolate CB82. **R**. *Metacylis joergenseni* isolate FG1143. **S**. *Climacocylis scalaria* isolate FG1118. **T**. *Climacocylis scalaria* isolate FG1116. **U**. *Undella hyalina* isolate CB854. **V**. *U. marsupialis* isolate CBa. **W**. *U. claparedei* isolate CB818. **X**. *Rhabdonella spiralis* isolate CB356. **Y**. *R. spiralis* isolate FG586. **Z**. *R. elegans* isolate FG1392. **AA**. *Cyttarocylis cassis* isolate FG302. **AB**. *C. acutiformis* isolate FG873. **AC**. *Petalotricha ampulla* isolate FG301. **AD**. *Petalotricha ampulla* isolate CB837. **AE**. *Xystonella longicauda* isolate FG1133. **AF**. *Parundella aculeata* isolate CB355. **AG**. *Codonellopsis orthoceras* isolate FG298. **AH**. *Codonellopsis morchella* isolate FG260. **AI**. *Stenosemella ventricosa* isolate CB875. **AJ**. *Dictyocysta lepida* isolate FG303. **AK**. Unidentified tintinnid isolate FG2102. **AL**. Unidentified tintinnid isolate FG2103. Scale bar 50 μm. Pictures of additional tintinnid cells from some of these species for which we obtained sequences are available in Supplementary Figure S1.



agglomerated particles (Fig. 1AK and AL). In sharp contrast, the species of *Undella* are typically oceanic and their loricae are smooth (Fig. 1, U-W). The identity of this freshwater taxon needs further research.

As mentioned above, another clade with relatively strong support (BV 89%, PP 0.93) was composed of sequences from the genera *Tintinnopsis* (*T. subacuta* and *T. lohmanii*), *Metacylis*, *Rhabdonella*, *Cyttarocylis* and *Petalotricha*. Beyond *Tintinnopsis,* the other genera have a long history of re-classification as they have been ascribed to several different families. The sequences of *Metacylis joergenseni*, *Metacylis* sp. MNB99 and *Metacylis angulata* branched in two different subclades, whereas the sequences of *Rhabdonella elegans*, *R. spiralis* and *R. hebe* were identical. Similarly, despite the morphological differences between specimens (Fig. 1, AA-AD), the three sequences of *Petalotricha ampulla* were identical or almost identical to the three sequences of *Cyttarocylis cassis* and *C. acutiformis*. Consequently, we can consider that these six sequences corresponded to a single genus, or even a single species, in contrast with classical taxonomical schemes where the genera *Petalotricha* and *Cyttarocylis* belong to the two different families Petalotrichidae and Cyttarocylididae (Lynn 2008).

Another clade (BV 100%) was composed of *Parundella* and *Xystonella*, placed together in the family Xystonellidae. The most distal group was composed of a variety of sequences of the genera *Stenosemella*, *Codonellopsis*, *Codonella, Codonaria* and *Dictyocysta*. The loricae of these genera are totally or partly agglutinated with mineral or biological particles. The highly supported clustering of these genera (BV 95%) provided a strong evidence for the close relationships between the family Codonellopsidae, Codonellidae *sensu stricto* and Dictyocystidae.

ITS+5.8S-rDNA and Concatenated ITS+5.8S-rDNA+SSU-rDNA Analyses

For the specimens analyzed in this study, the ITS and 5.8S rRNA gene sequences were less conserved than the SSU-rDNA and diverged twice as much (Supplementary Fig. S2), with relatively high regularity ($r^2 = 0.66$). ITS+5.8S-rDNA phylogenies were quite congruent with the corresponding SSU-rDNA phylogenies, at least for the strongly supported nodes (Supplementary Fig. S3). The only noteworthy exception was *Tintinnidium fluviatile*, which branched at a basal position with some non-tintinnid sequences in the complete SSU-rDNA phylogeny (Fig. 2) but as sister of a *Salpingella+Amphorides* clade in the ITS+5.8S-rDNA phylogeny (Supplementary Fig. S3). Given this overall congruence between the SSU-rDNA and the ITS+5.8S-rDNA topologies, a concatenated alignment of the SSU-rDNA, ITS and 5.8S-rDNA sequences was compiled in order to increase the phylogenetic signal to evaluate the phylogenetic relationships among Tintinnida (Fig. 3). According to this concatenated analysis, tintinnids were divided into three clades: the first one only contained the two sequences of *Eutintinnus fraknoi* (BV 100, PP 1); the second one (weakly supported: BV 70%, PP 0.75) grouped *Tintinnidium fluviatile, Salpingella acuminata* and *Amphorides quadrilineata*; and the last one (strongly supported: BV 100%, PP 1) corresponded to the large complex of species also observed in the SSU-rDNA phylogeny. This complex was divided into two sister groups. The first sub-clade (BV 67%, PP 0.8) included *Xystonella longicauda*, *Undella claparedei*, *U. hyalina, Tintinnopsis lacustris*, *Rhabdonella elegans, R. spiralis*, *Cyttarocylis cassis* and *Petalotricha ampulla.* The second sub-clade (BV 97%, PP 1) grouped, on the one hand, *Codonella aspera*, *Codonaria cistellula* and *Codonaria* sp. CB82 with, on the other hand, *Codonellopsis morchella* and *Stenosemella ventricosa*. Once again, our sequences formed well-supported lineages, but despite of the supplementary amount of nucleotide positions the internal relationships among those lineages remained poorly resolved.

Discussion

Reconciling Tintinnid Molecular Phylogeny and Morphological Taxonomy

Until recently, the phylogenetic robustness of the lorica-based tintinnid taxonomy was difficult to

Figure 2. Maximum likelihood rooted phylogenetic tree of choreotrich SSU-rDNA sequences, based on 1,240 aligned positions. Names in bold represent sequences obtained in this study. Traditional and revised taxonomic groups are shown on the right; light grey background indicates incertae sedis groups. Numbers at nodes are bootstrap values (values under 50% are omitted). Bayesian posterior probabilities higher than 0.90 are indicated by filled circles. Accession numbers are provided between brackets. Grey lines and names indicate the freshwater lineages. The scale bar represents the number of substitutions for a unit branch length.

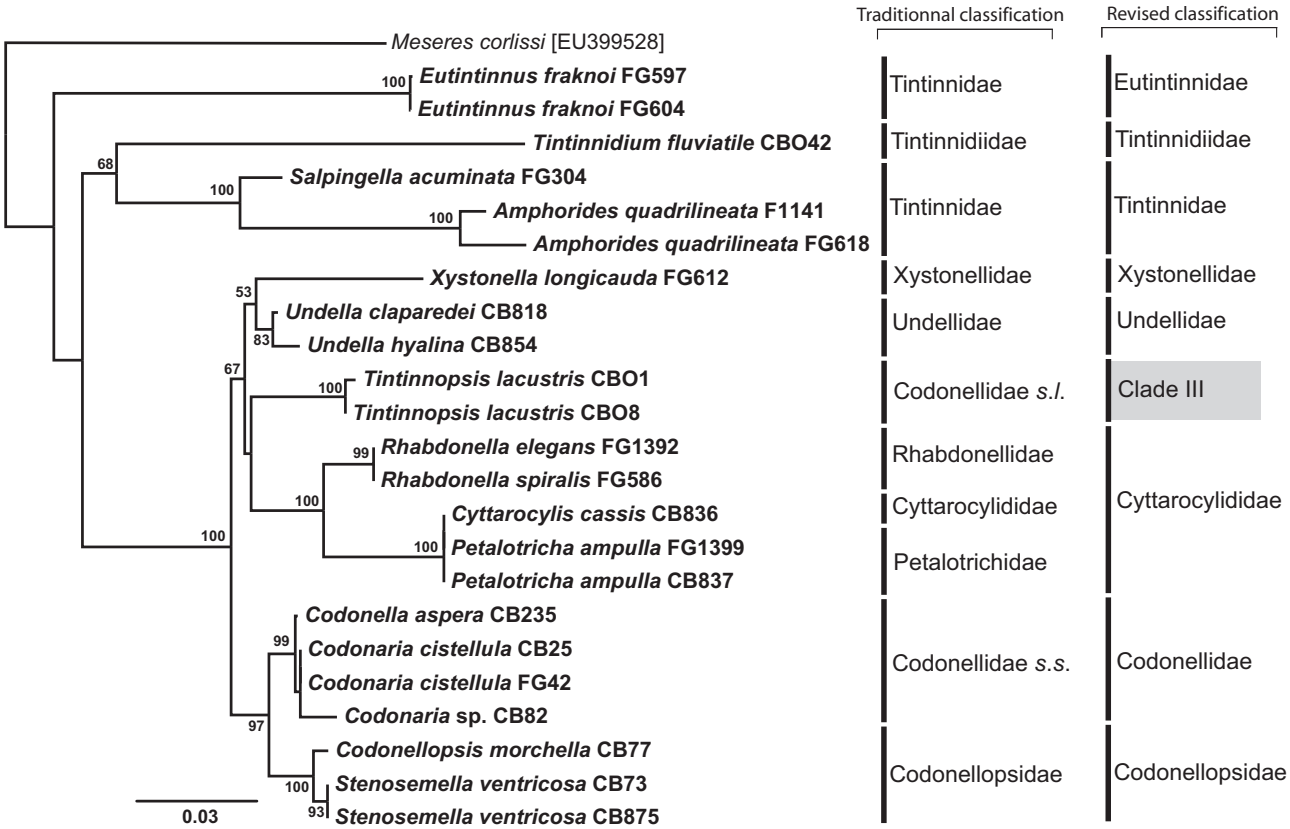


Figure 3. Maximum likelihood rooted phylogenetic tree of tintinnid SSU-rDNA, 5.8S-rDNA and ITS sequences, based on 1,682 aligned positions. Traditional and revised taxonomic groups are shown on the right; light grey background indicates incertae sedis groups. Numbers at nodes are bootstrap values (values under 50% are omitted). Bayesian posterior probabilities higher than 0.90 are indicated by filled circles. Accession numbers are provided between brackets. The scale bar represents the number of substitutions for a unit branch length.

test. This is now possible thanks to the development of molecular phylogeny. With this aim, we assembled a comprehensive data set of tintinnid sequences, including 62 SSU-rDNA and 25 ITS+5.8S-rDNA sequences covering 13 of the 15 families currently defined by morphological classifications (Lynn 2008).

To describe the evolutionary history of tintinnids, one essential issue is to define the position of the root within this ciliate order. Previous studies based on lorica morphology (Kofoid and Campbell 1929, 1939), infraciliature pattern (Laval-Peuto and Brownlee 1986) and SSU-rDNA phylogeny (Duff et al. 2008; Strüder-Kypke and Lynn 2003) suggested that *Tintinnidium* spp. were located at the base of the tintinnid tree. To investigate the most basal lineage of the tintinnid tree, we used a variety of other choreotrichs and oligotrichs as outgroup taxa. However, our results showed little confidence in the exact position of the tintinnid root in both the ML and Bayesian trees (Fig. 2), and also in our concatenated rDNA analysis (Fig. 3). To check whether this uncertainty was due to the use of a too narrow set of outgroup sequences, we carried out phylogenetic analyses including a diversity of additional outgroup sequences from different groups within the large ciliate class Spirotrichea (Supplementary Figs S4 and S5). As in our first analysis, these new phylogenies failed to place *Tintinnidium* spp. robustly. Nevertheless, they showed a different position for the Eutintinnidae clade, which was close to the base of Tintinnida in the initial tree but emerged either as sister of the *Salpingella-Amphorellopsis-Amphorides-Steenstrupiella* group (BV < 50%) or at the next branch after this group (BV 64%). At any rate, statistical support for the basal part of the tintinnid tree remained weak. Therefore, it appeared clear that supplementary sequence information, in particular sequences from other conserved markers, is needed to assess the phylogenetic position of *Tintinnidium* spp. Likewise,

further work will be needed to robustly infer the phylogenetic position of the families Strombilidiidae and Parastrombidinopsidae, which tend to group with the Tintinnidiidae rather than with the Strombidinopsidae.

Previous studies employing sequence data reported the paraphyly of the genus *Tintinnopsis* (Li et al. 2009; McManus and Katz 2009; Snoeyenbos-West et al. 2002). Our results with a richer taxonomic sampling support this view. It is often difficult to observe details of the loricae of *Tintinnopsis* because the agglomerated particles often conceal diagnostic characteristics of the lorica. This likely explains that under the genus *Tintinnopsis* have traditionally been described species that, according to molecular phylogeny analyses, should belong to different genera. Moreover, annotation errors and possible misidentifications may lead to incorrect interpretations. For example, the GenBank sequence DQ487200 annotated as *Tintinnopsis* sp. seems to correspond to a *Tintinnidium* species in the published article (Duff et al. 2008). Similarly, in the case of *Tintinnopsis fimbriata*, an unambiguous identification seems not possible from the picture of the specimen shown (Strüder-Kypke and Lynn 2003). Not surprisingly, our molecular analysis reveals that the phylogeny of the genus *Tintinnopsis* is even more complex than previously thought. For example, we have found a freshwater genus that shares the external appearance of *Tintinnopsis*, with an entirely agglomerated lorica but that clearly branched with the oceanic genus *Undella*, with a smooth lorica. *Climacocylis scalaria*, with a distinctive hyaline lorica, represented a similar case since it branched very close to *T. cylindrica* and *T. radix* within one of the *Tintinnopsis* clades. Moreover a potential monophyletic grouping of the majority of *Tintinnopsis* sequences from GenBank and those added in this study cannot be excluded, as attested by our AU tests (see above). Taking all this into account, it seems clear that the taxonomic value of the "agglomeration of particles" or "agglutinated lorica" as a diagnostic character at the family level is not supported by the molecular data. With no doubt, the current classification of the genus *Tintinnopsis* needs a complete revision in order to establish a taxonomic scheme compatible with molecular phylogeny.

In contrast with the poorly informative *Tintinnopsis* lorica, other species that have distinctive and easily visible lorica morphologies are ideal to investigate the phylogenetic value of the lorica characters. Our results reveal that the species of the genus *Eutintinnus* form a cohesive monophyletic group in a basal position in the tintinnid phylogeny (Fig. 2). Compared to other tintinnids, the lorica of *Eutintinnus* is relatively simple, just a smooth tube lacking ornamentations (horns, annuli, collar, spines...) except for the toothed oral rim of *E. pectinis*. In most *Eutintinnus* species the diameter of the oral and aboral aperture are quite similar while in other tintinnids the aboral end is closed or, if open, has a very small diameter compared to the oral diameter. This morphological character together with the support of the molecular data argues in favor of the erection of a new family for *Eutintinnus* (see Taxonomic Appendix).

An additional problem concerns the consistency of lorica morphology. For example, studies of the life cycle of some *Favella* species have revealed important changes in lorica morphology during cell development, with individuals often exhibiting morphologies previously considered to belong to separate genera, such as *Coxiella* (Laval-Peuto 1981). These observations are also supported by molecular data. In fact, Kim et al. (2010) used cultures and observed lorica polymorphism for specimens of *Favella ehrenbergi* that shared identical SSU-rDNA sequences. Similarly, our Mediterranean specimens of *Favella azorica*, without an aboral pedicel, showed identical SSU-rDNA and, moreover, these sequences were identical to those of *F. campanula*, with an aboral pedicel, isolated from China (Gao et al. 2009). The phylogenetic position of the *F. taraikaensis* Hada sequence obtained by Li et al. (2009), which unexpectedly branched with *Metacylis* sp., is also questionable. Additional sequences of this taxon are required to ascertain the monophyly of the genus *Favella.* At present, *Favella* is the only representative of the Ptychocylididae for which sequences are available so that the monophyletic character of this family also remains to be tested.

The studies of the life cycle of tintinnids are nearly restricted to common coastal species that are maintained in temporary cultures. As a consequence, very little is known about the life cycle and changes in the lorica morphology for rare and oceanic species. Single-cell molecular analyses offer an opportunity to study these species. Thus, our work using this approach has revealed conflicting cases comparable to those cited for *Favella*. For example, in the case of the genera *Petalotricha* and *Cyttarocylis*, our sequences of *P. ampulla* (Fig. 1, AC-AD) branched in the same clade as those of *Cyttarocylis acutiformis* (Fig. 1, AB) and *C. cassis* (Fig. 1, AA). These six *Petalotricha* and *Cyttarocylis* specimens collected at different dates and places showed identical or almost identical SSU-rDNA sequences, and

even the more rapidly evolving ITS and 5.8S-rDNA sequences were identical. Such genetic identity of the two distinct forms, one with the lorica wall finely sculptured with polygonal reticuli (*Cyttarocylis* morphotype) and the other with a smooth lorica with irregular small fenestrae in the subnuchal area (*Petalotricha* morphotype), suggests that they may just represent different stages of lorica development in a single genus or even in a single species. This is unexpected if we consider that in all previous taxonomical schemes, *Petalotricha* and *Cyttarocylis* have been classified into two separate families based on the conspicuous differences in the lorica structure (Fol 1881; Kent 1882; Kofoid and Campbell 1929; Marshall 1969). *Cyttarocylis* Fol, 1881 has the priority over *Petalotricha* Kent, 1882 and, consequently, we propose a new combination to place *Petalotricha ampulla* under the genus *Cyttarocylis* (see Taxonomic Appendix).

For the genus *Undella*, numerous species have been described based on minute variations of the lorica (Daday 1887; Kofoid and Campbell 1929). Our phylogenetic analysis shows a very close relationship between the clearly distinct morphotypes of *Undella hyalina*, *U. marsupialis* and *U. claparedei*. It also supports that the genus *Proplectella* Kofoid & Campbell, 1929, which grouped the small and round-shaped Undellidae with an inner collar (*U. claparedei* morphotype; Fig. 1W), could be considered a junior synonym, as already supposed by Balech (1975).

Simple morphological characters, such as the lorica length, seem to have low taxonomic value in the case of genera such as *Rhabdonella.* Our *Rhabdonella* spp. specimens showed lorica with lengths between 100 μm and 400 μm (Fig. 1, X-Z). Although described as different species, our specimens of *R. spiralis* and *R. elegans* shared identical SSU-rDNA, ITS and 5.8S-rDNA sequences. In the case of *Codonaria cistelulla,* our specimens showed different degrees of collar agglutination (Fig. 1, O-P) but, again, their SSU-rDNA sequences were also identical.

The monograph of Kofoid and Campbell (1929) catalogued 697 species, raising to species status many forms previously described as varieties, often based on slight lorica differences. In this study, we have found cases of little or none genetic difference between species with distinct loricae belonging to the genera *Undella*, *Rhabdonella* or *Cyttarocylis*. This suggests that the number of species have most likely been excessively inflated. On the other hand, *Tintinnopsis* may require a split into several genera, even families. The species *Amphorides quadrilineata* is an exception. Our sequences branched in two distinct clades that appear to hide a cryptic speciation.

Marine-Freshwater Transitions

As observed in many eukaryotic lineages (Logares et al. 2009), there are genera composed of marine and freshwater species. In those cases, the phylogenies do not support a deep ancient divergence between members of marine and freshwater habitats but a number of more or less recent independent colonizations. If our tintinnid phylogenetic tree topology is correct, at least three transitions between marine and freshwater/brackish environments appear to have occurred during the evolutionary history of these ciliates. Both parsimony and maximum likelihood character optimization suggest that the ancestor of tintinnids *sensu stricto* was a marine species, as it is the case for most other choreotrich ciliates, and, consequently, that the three colonizations inferred occurred from marine ancestors to freshwater environments (Supplementary Fig. S6). Until now, few tintinnid species living in freshwaters have been described. Our results suggest that freshwater colonizations are not restricted to families characteristic of coastal waters but also include at least one representative of typical open water forms (Undellidae). This phenomenon appears therefore to be phylogenetically diverse within the Tintinnida.

The generic affiliation of the freshwater species *Codonella cratera* is probably incorrect, as inferred by cytological and ciliature features (Agatha 2010a, b; Agatha and Strüder-Kypke 2007; Laval-Peuto and Brownlee 1986). In our SSU-rDNA tree, a freshwater lineage strongly grouping *Codonella cratera* (Duff et al. 2008) with *Tintinnopsis lacustris* branches as sister clade of marine *Tintinnopsis* spp. This confirms that *C. cratera* should be removed from the genus *Codonella* to become a *Tintinnopsis* species.

Classification into Families

Our results revealed that the genera traditionally ascribed to the family Tintinnidae formed at least three distantly related clades, suggesting that they may define different families. The clearest example is *Eutintinnus* spp. that forms a separate cohesive monophyletic group (BV 100%, PP 1) distant to other members of the Tintinnidae (Fig. 2). From a morphological point of view, *Eutintinnus* differs from the other Tintinnidae in the wide aboral opening and the lack of longitudinal aboral fins. By contrast, the very rarely reported type genus *Tintinnus* is characterized by a homogeneous wall with a closed

aboral end (Kofoid and Campbell 1939). Therefore, both the morphological and phylogenetic molecular data converge to support the exclusion of *Eutintinnus* from the family Tintinnidae. For this reason, we propose in the taxonomical appendix the erection of Eutintinnidae fam. nov. to host the members of the genus *Eutintinnus*. This new family is characterized by a hyaline lorica, cylindrical or nearly so, clearly opened at the both ends. Some species of *Eutintinnus* are characterized by the association with diatoms (*Chaetoceros*, *Hemiaulax*) (Gómez 2007b).

While *Eutintinnus*, with simple lorica morphology, tends to branch in a basal position in the tintinnid phylogeny, the genus *Dictyocysta*, with a peculiar reticulate lorica, branches in the most distal position (Fig. 2). In the SSU-rDNA phylogeny, our sequences of *D. lepida* and that of *D. reticulata* (Strüder-Kypke and Lynn 2008) branch with high support (BV 90%, PP 1) with species of the genera *Codonaria* and *Codonella*, type of family Codonellidae. In the classical taxonomic schemes, *Dictyocysta* has been placed in the family Dictyocystidae. However, the very close phylogenetic relationship between *Dictyocysta* and members of the Codonellidae suggests the placement of *Dictyocysta* in this family. This is supported by cytological characteristics shared by these genera: the presence of a lorica sac and closing apparatus (see review in Agatha 2010b).

Tintinnopsis has been classified in the family Codonellidae. However, the *Tintinnopsis* sequences form, at least, two separate groups and are never related to Codonellidae sensu stricto (Fig. 2). The group of *Tintinnopsis subacuta* and *T. lohmanni* appeared in a clade (BV 89%) that also contained the genera *Metacylis*, *Rhabdonella* and *Cyttarocylis*, which belong to three separate families. This strongly suggests that *T. subacuta* and *T. lohmanni* do not belong to the genus *Tintinnopsis*. Detailed morphological studies are required for these species before proposing the transfer into another existing genus or the erection of a new genus. Concerning the entire clade to which *T. subacuta* and *T. lohmanni* belong, it also groups the families Metacylididae, Rhabdonellidae and Cyttarocylididae. However, their branching pattern does not support any particular relationship among these families, indicating that the three families could be merged into a single one. In so doing, the family Cyttarocylididae would have the priority to name the entire family.

Most *Tintinnopsis* species, including the type species *T. beroidea*, formed a paraphyletic group with several distinct lineages. Unexpectedly, *Climacocylis* (family Metacylididae) appears closely related to several of the *Tintinnopsis* species, far from *Metacylis*, the type of the family. Consequently, *Climacocylis* should be excluded from the Metacylididae. However, we have been unable to find common morphological characters that could support the placement of *Tintinnopsis* and *Climacocylis* under a single family or the tentative transfer of *Tintinnopsis* species such as *T. radix* or *T. cylindrica* into the genus *Climacocylis*. Given the observed complexity, the assignment of *Tintinnopsis* and *Climacocylis* to any existing family requires further research.

Methods

Sampling and cell isolation: Our primary sampling site was the Bay of Villefranche-sur-Mer in the NW Mediterranean Sea. Additional samples were collected from 2 other coastal sites in the NW Mediterranean, Marseille and Banyuls-sur-Mer, and 2 sampling stations in the open Mediterranean, one near the center of the Western basin and another in the Central basin. We also collected samples in freshwater lakes (see below). Sampling in the Bay of Villefranche sur Mer, Ligurian Sea, was performed at a long-term monitoring site called 'Point B' (43°41′10″N, 7°19′00″E; water column depth ~80 m). Sampling in double oblique angle was done with a custom-made conical phytoplankton net (53 μm mesh size, 54 cm diameter and 280 cm length). Additional samples were collected at discrete depths by using 12-L Niskin bottles, and concentrated by screening water through 20 μm Nylon mesh (Nitex Co.). The material was examined in Utermöhl chambers and individual tintinnids were isolated and photographed using an inverted microscope (Olympus IX51) equipped with an Olympus DP71 digital camera. Based on microscopic observations, species designations were made employing characteristics of lorica morphology. The main taxonomic works consulted included Jörgensen (1924), Kofoid and Campbell (1929, 1939) and Balech (1959). We followed the classification into families and genera proposed by Lynn (2008). The identified and photographed specimens were micropipetted individually with a fine capillary into a clean chamber and washed several times in serial drops of 0.2-μm filtered and sterilized seawater. The isolated washed cells were placed in 0.2 ml Eppendorf tubes filled with several drops of absolute ethanol. The tubes were stored at 4 °C or at room temperature and in darkness until the molecular analysis could be performed. Other specimens were collected from the end of the pier (depth 3 m) of the Station Marine d'Endoume, Marseille (43°16′48″N, 5°20′57″E). A strainer with netting of 20 μm mesh-size was used to collect the organisms, with a filtered volume ranging between 10 and 100 liters according to the concentration of particles. The concentrated material was processed in a similar manner as described above, using a Nikon Eclipse TE200 inverted microscope equipped with a Nikon Coolpix E995 camera. Tintinnids were also collected from surface waters of the harbour of Banyuls-sur-Mer (42°28′50″N, 3°08′09″E) following the same procedure. The specimens were observed with an Olympus inverted microscope (Olympus IX51) and photographed with an Olympus DP71 digital camera. Specimens from open waters of the Mediterranean specimens were isolated from samples collected during the BOUM (Biogeochemistry from the

Oligotrophic to the Ultra-oligotrophic Mediterranean) cruise in the Mediterranean Sea in June-July 2008. Material was from surface water sampling at the permanent stations of the Western Basin, 'Station A' (39°6′N, 5°30′E), and the Central Ionian Sea, 'Station B' (34°8′N, 18°45′E) (see Christaki et al. 2011 for details). Ten litres were collected from the surface with a bucket and filtered by using a strainer of 20-μm netting aperture. The retained material was fixed with absolute ethanol to a final concentration of 50% concentrated seawater sample and 50% ethanol. The fixed sample was subsequently examined and processed as described above. Tintinnids were also collected from freshwater lakes in the Chevreuse Valley (Étang du Perray, 48°41′49″N, 1°51′37″E; Étang de Pourras, 48°42′52″N, 1°50′39″E; Étang des Vallées 48°41′20″N, 1°54′59″E), near Paris, France, and from La Albufera (39°21′32″N, 1°38′22″W), a large shallow lake near Valencia, Spain. The specimens were observed, isolated and processed as described above.

PCR amplification of small subunit rRNA genes, internal transcribed spacers ITS1 and ITS2, and 5.8S rRNA genes: The specimens fixed in ethanol were centrifuged gently for 5 min at 3,000 rpm. Ethanol was then evaporated in a vacuum desiccator and single cells were resuspended directly in 25 μl of Ex TaKaRa buffer (TaKaRa, distributed by Lonza Cia., Levallois-Perret, France). PCR reactions were done in a volume of 30 μl reaction mix containing 10-20 pmol of the forward eukaryotic-specific SSU-rDNA primers EK-42F (5′-CTCAARGAYTAAGCCATGCA-3′) and the reverse eukaryotic large subunit rDNA EK-28S564R (5′-TGGTCCGTGTTTCRAGACG-3′) (López-García et al. 2001). The PCR reactions were performed under the following conditions: 2 min denaturation at 94 °C; 10 cycles of 'touch-down' PCR (denaturation at 94 °C for 15 s; a 30 s annealing step at decreasing temperature from 65 down to 55 °C employing a 1 °C decrease with each cycle, extension at 72 °C for 2 min); 20 additional cycles at 55 °C annealing temperature; and a final elongation step of 7 min at 72 °C. Internal primers were designed for this study to specifically target Tintinnida ribosomal genes and to avoid any possible PCR contamination by other eukaryotes (e.g. ingested preys or parasites). A nested PCR reaction was then carried out using 3 μl of the first PCR reaction diluted by 1/10 in a GoTaq (Promega, Lyon, France) polymerase reaction mix containing the tintinnid-specific primers 18S-Tin3F (5′-GCGGTATTTATTAGATAWCAGCC-3′) and 28S-TinR1 (5′-TGGTGCACTAGTATCAAAGT-3′) and similar PCR conditions as described above. At this step, if amplicon bands were not visible on agarose gels, a third, semi-nested PCR was carried out using the eukaryotic-specific SSU-rDNA primer EK-1498R (5′-TAACAATACAGGGCATCCAT-3′) and keeping the forward primer TIN3F. Negative controls without template DNA were used at all amplification steps. Amplicons of the expected size (~2,000 bp after the nested PCR and ~1,500 pb after the semi-nested one) were then entirely sequenced using the primers 18S-Tin3F, EK-1498R and 28S-TinR1 with an automated 96-capillary sequencer ABI PRISM 3730xl (Beckman Coulter Genomics, Takeley, U.K.). Sequences were deposited in GenBank with accession numbers JQ408154-JQ408215 (see also Supplementary Table S1).

Phylogenetic analyses: We carried out a multiple alignment with our sequences and publicly available complete or nearly complete (>1,400 bp) SSU-rDNA sequences of spirotrich ciliates using the profile alignment option of MAFFT (Katoh et al. 2002) and MUSCLE 3.6 (Edgar 2004). We kept in our data set the 37 tintinnid sequences from specimens that were associated with micrographs in the references given in GenBank to minimize the inclusion of mis-identifed species. As outgroup, we used 17 sequences belonging to hypotrich, stichotrich, oligotrich (Strombidiidae) and choreotrich ciliates (Strobilidiidae and Strombidinopsidae). Partial sequences too short to be included in our data set were eliminated (e.g. *Eutintinnus* sp., EU399533, 1309 bp) to keep the maximum of sequence information. Pairwise sequence comparisons (percentage of sequence identity) were calculated with the package ClustalX (Larkin et al. 2007). The resulting alignment was manually inspected using the program ED of the MUST package (Philippe 1993). Ambiguously aligned regions and gaps were excluded from phylogenetic analyses. A second sequence data set was constructed through the same procedure that included the sequences of tintinnids for which we succeeded to amplify the SSU-rDNA, the ITS1 and ITS2 and the 5.8S rRNA genes. Conserved alignment sites were trimmed using GBLOCKS (Castresana 2000) or BMGE (Criscuolo and Gribaldo 2010) with default parameters. Then, we carried out Maximum Likelihood (ML) phylogenetic analyses with the program TREEFINDER (Jobb et al. 2004) applying a GTR + Γ + I model of nucleotide substitution with a Γ-shaped distribution of substitution rates with four rate categories. This model was selected using the model selection tool implemented in TREEFINDER. Bootstrap values were calculated using 1,000 pseudoreplicates with the same substitution model. The Bayesian Inference (BI) analyses were carried out with the program MrBayes (Huelsenbeck and Ronquist 2001), with two independent runs and 1,000,000 generations per run. After checking convergence (maximum difference between all bipartitions <0.01) and eliminating the first 3,500 trees (burn-in), a consensus tree was constructed sampling every 100 trees. Comparison of different tree topologies was carried out by applying the Approximately Unbiased (AU) test (Shimodaira 2002) implemented in TREEFINDER. Finally, the most parsimonious pattern of marine-freshwater transitions was inferred for the set of tintinnid species using MESQUITE 2.75 (Maddison and Maddison 2011).

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Appendix A. Taxonomic appendix

Based on the information obtained from molecular phylogenetic analyses, we propose the following classification of the tintinnid genera for which molecular data are available.

- Subclass Choreotrichia Small & Lynn, 1985.
- Order Tintinnida Kofoid & Campbell, 1929.

Family Tintinnidiidae:
Tintinnidium
Family Eutintinnidae fam. nov. Bachy *et al.*:
Eutintinnus
Family Tintinnidae:
Salpingella, *Amphorellopsis*, *Amphorides*, *Steenstrupiella*

Family Ptychocylididae
Favella
Family Undellidae:
Undella
Family Cyttarocylididae:
Metacylis, *Rhabdonella*, *Cyttarocylis* (=*Petalotricha*)
Family Xystellonellidae:
Xystonella, *Parundella*
Family Codonellopsidae:
Codonellopsis, *Stenosemella*
Family Codonellidae (including Dictyocystidae):
Codonella, *Codonaria*, *Dictyocysta*
Incertae sedis:
Tintinnopsis sensu stricto (type species), *Climacocylis, Tintinnopsis* clades I/II/III/IV/V

New Taxonomical Proposals

Combinatio nova:

Cyttarocylis ampulla (Kent) C. Bachy, J.R. Dolan and P. López-García, comb. nov.

Basionym: *Petalotricha ampulla* Kent 1882. A manual of the infusoria: including a description of all known flagellate, ciliate, and tentaculiferous Protozoa, British and foreign, and an account of the organization and affinities of the sponges. Vol. II. D. Bogue, London. p. 627, figs 1-2.

Synonyms: *Tintinnus ampulla* Fol (1881), *Petalotricha major* Jörgensen (1924), *Petalotricha serrata* Kofoid & Campbell (1929).

Familia nova:

- Subclass Choreotrichia Small & Lynn, 1985.
- Order Tintinnida Kofoid & Campbell, 1929.
- Family Eutintinnidae fam. nov. (=Tintinnidae, pro parte).
- Diagnosis: The family is characterized by a hyaline and smooth lorica, cylindrical or nearly so, clearly opened at the both ends. The family differs from Tintinnidae in the wide aboral opening and the lack of longitudinal aboral fins. Free-living, marine. Some species are associated with an ectosymbiont diatom.
- Type genus: *Eutintinnus* Kofoid & Campbell, 1939.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.protis.2012.01.001.

References

Agatha S (2010a) Redescription of *Tintinnopsis parvula* Jörgensen, 1912 (Ciliophora: Spirotrichea: Tintinnina), including a novel lorica matrix. Acta Protozool **49**:213–234

Agatha S (2010b) A light and scanning electron microscopic Study of the closing apparatus in tintinnid ciliates (Ciliophora, Spirotricha, Tintinnina): A forgotten synapomorphy. J Eukaryot Microbiol **57**:297–307

Agatha S, Strüder-Kypke MC (2007) Phylogeny of the order Choreotrichida (Ciliophora, Spirotricha, Oligotrichea) as inferred from morphology, ultrastructure, ontogenesis, and SSrRNA gene sequences. Eur J Protistol **43**:37–63

Balech E (1959) Tintinnoinea del Mediterraneo. Trabajos del Instituto Español de Oceanografia **28**:1–88

Balech E (1975) La famillia Undellidae (Protozoa, Ciliophora Tintinnina). Physis Sec A **34**:377–398

Bergsten J (2005) A review of long-branch attraction. Cladistics **21**:163–193

Boltovskoy D, Dinofrio EO, Alder VA (1990) Intraspecific variability in Antarctic tintinnids: the *Cymatocylis affinis/convallaria* species group. J Plankton Res **12**:403–413

Calbet A, Landry MR (2004) Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. Limnol Oceanogr **49**:51–57

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol **17**:540–552

Choi JK, Coats DW, Brownlee DC, Small EB (1992) Morphology and infraciliature of three species of *Eutintinnus* (Ciliophora; Tintinnina) with guidelines for interpreting protargol-stained tintinnine ciliates. J Eukaryot Microbiol **39**:80–92

Christaki U, Van Wambeke F, Lefevre D, Lagaria A, Prieur L, Pujo-Pay M, Grattepanche JD, Colombet J, Psarra S, Dolan JR, Sime-Ngando T, Conan P, Weinbauer MG, Moutin T (2011) Microbial food webs and metabolic state across oligotrophic waters of the Mediterranean Sea during summer. Biogeosciences **8**:1839–1852

Criscuolo A, Gribaldo S (2010) BMGE (Block Mapping and Gathering with Entropy): A new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC Evol Biol **10**:210

Daday Ev (1887) Monographie der Familie der Tintinnodeen. Mittheilungen aus der Zool. Station zu Neapel **7**:473–591

Davis CC (1981) Variations of lorica shape in the genus *Ptychocylis* (Protozoa: Tintinnina) in relation to species identification. J Plankton Res **3**:433–443

Dolan JR, Jacquet S, Torréton J-P (2006) Comparing taxonomic and morphological biodiversity of tintinnids (planktonic ciliates) of New Caledonia. Limnol Oceanogr **51**: 950–958

Dolan JR, Claustre H, Carlotti F, Plounevez S, Moutin T (2002) Microzooplankton diversity: relationships of tintinnid ciliates with resources, competitors and predators from the Atlantic Coast of Morocco to the Eastern Mediterranean. Deep-Sea Res part I **49**:1217–1232

Duff RJ, Ball H, Lavrentyev PJ (2008) Application of combined morphological-molecular approaches to the identification of planktonic protists from environmental samples. J Eukaryot Microbiol **55**:306–312

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **19**:113

Foissner W, Wilbert N (1979) Morphologie, Infraciliatur und Ökologie der limnischen Tintinnina: *Tintinnidium fluviatile* Stein, *Tintinnidium pusillum* Entz, *Tintinnopsis cylindrata* Daday und *Codonella cratera* (Leidy) (Ciliophora, Polyhymenophora). J Protozool **26**:90–103

Fol H (1881) Contribution to the knowledge of the family Tintinnodea. Annals Mag Nat Hist **12**:73–88

Gao S, Gong J, Lynn D, Lin X, Song W (2009) An updated phylogeny of oligotrich and choreotrich ciliates (Protozoa, Ciliophora, Spirotrichea) with representative taxa collected from Chinese coastal waters. Syst Biodivers **7**:235–242

Gold K, Morales EA (1975) Seasonal changes in lorica sizes and the species of Tintinnida in the New York Bight. J Eukaryot Microbiol **22**:520–528

Gómez F (2007a) Trends on the distribution of ciliates in the open Pacific Ocean. Acta Oecol **32**:188–202

Gómez F (2007b) On the consortium of the tintinnid *Eutintinnus* and the diatom *Chaetoceros* in the Pacific Ocean. Mar Biol **151**:1899–1906

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics **17**:754–755

Jobb G, von Haeseler A, Strimmer K (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol **4**:18

Jörgensen (1924) Mediterranean Tintinnids. Report on the Danish Oceanographical Expeditions 1908-10 to the Mediterranean and Adjacent Seas, Vol 2 (Biology), J.3., 1–110

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res **30**:3059–3066

Kent WS (1882) A Manual of the Infusoria, Including a Description of All Known Flagellate, Ciliate, and Tentaculiferous Protozoa, British and Foreign and an Account of the Organization and Aaffinities of the Sponges. David Bogue, London

Kim SY, Yang EJ, Gong J, Choi JK (2010) Redescription of *Flavella ehrenbergii* (Claparède and Lachmann, 1858) Jörgensen, 1924 (Ciliophora: Choreotrichia), with phylogenetic analyses based on small subunit rRNA gene sequences. J Eukaryot Microbiol **57**:460–467

Kofoid CA, Campbell AS (1929) A Conspectus of the Marine and Freshwater Ciliata Belonging to the Suborder Tintinnoinea, with Descriptions of New Species Principally from the Agassiz Expedition to the Eastern Tropical Pacific 1904–1905. Univ Calif Publ Zool

Kofoid CA, Campbell AS (1939) The Ciliata: the Tintinnoinea. Bull Mus Comp Zool Harvard

Laackmann H (1908) Ungeschlechtliche und geschlechtliche Fortpflanzung der Tintinnen. Wissensch Meeresunters, Abteilung Kiel **10**:13–38+Plates1–3

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics **23**:2947–2948

Laval-Peuto M (1977) Reconstruction d'une lorica de forme *Coxliella* par le trophonte nu de *Favella ehrenbergii* (Ciliata, Tintinnina). C R Acad Sci **284**:547–550

Laval-Peuto M (1981) Construction of the lorica in Ciliata Tintinnina. In vivo study of *Favella ehrenbergii*: Variability of the phenotypes during the cycle, biology, statistics, biometry. Protistologica **17**:249–272

Laval-Peuto M (1983) Sexual reproduction in *Favella ehrenbergii* (Ciliophora, Tintinnina) taxonomical implications. Protistologica **19**:503–512

Laval-Peuto M, Brownlee DC (1986) Identification and systematics of the Tintinnina (Ciliophora): evaluation and suggestions for improvement. Institut océanographique, Paris, France

Li Z, Yi Z, Yang J, Gong J, Clamp JC, Al-Rasheid KAS, Al-Arifi S, Al-Khedhairy AA, Song W (2009) Phylogenetic investigation on five genera of tintinnid ciliates (Ciliophora, Choreotrichia), based on the small subunit ribosomal RNA gene sequences. Progr Nat Sci **19**:1097–1101

Logares R, Brate J, Bertilsson S, Clasen JL, Shalchian-Tabrizi K, Rengefors K (2009) Infrequent marine-freshwater transitions in the microbial world. Trends Microbiol **17**: 414–422

López-García P, Rodriguez-Valera F, Pedros-Alio C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409**:603–607

Lynn DH (2008) The Ciliated Protozoa. Characterization, Classification, and Guide to the Literature. 3rd ed Springer, Dordrecht

Maddison W, Maddison DR (2011) Mesquite: a modular system for evolutionary analysis. Version 2.75. http://mesquiteproject.org

Marshall SM (1969) Protozoa, order Tintinnia. Conseil International pour l'exploration de la Mer, Fiches d'Identification de Zooplancton:fiches 117–127

McManus GB, Katz LA (2009) Molecular and morphological methods for identifying plankton: what makes a successful marriage? J Plankton Res **31**:1119–1129

Montagnes DJS, Lynn DH (1987) A quantitative Protargol stain (QPS) for ciliates: Method description and test of its quantitative nature. Mar Microb Food Webs **2**:83–93

Philippe H (1993) MUST, a computer package of Management Utilities for Sequences and Trees. Nucleic Acids Res **21**:5264–5272

Pierce RW, Turner E (1992) Ecology of planktonic ciliates in marine food webs. Rev Aquat Sci **6**:139–181

Prescott DM (1994) The DNA of ciliated protozoa. Microbiol Mol Biol Rev **58**:233–267

Santoferrara LF, Alder VA (2009) Morphological variability, spatial distribution and abundance of *Helicostomella* species (Ciliophora: Tintinnina) in relation to environmental factors (Argentine shelf; 40-55 S). Sci Mar **73**:701–716

Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol **51**:492–508

Small EB, Lynn DH (1985) Phylum Ciliophora Doflein, 1901. In Lee JJ, Hutner SH, Bovee EC (eds) An Illustrated Guide to the Protozoa. Society of Protozoologists. Allen Press, Lawrence, Kansas, pp 393–575

Snoeyenbos-West OLO, Salcedo T, McManus GB, Katz LA (2002) Insights into the diversity of choreotrich and oligotrich ciliates (Class: Spirotrichea) based on genealogical analyses of multiple loci. Int J Syst Evol Microbiol **52**: 1901–1913

Strüder-Kypke MC, Lynn DH (2003) Sequence analyses of the small subunit rRNA gene confirm the paraphyly of oligotrich ciliates sensu lato and support the monophyly of the subclasses Oligotrichia and Choreotrichia (Ciliophora, Spirotrichea). J Zool **260**:87–97

Strüder-Kypke MC, Lynn DH (2008) Morphological versus molecular data - Phylogeny of tintinnid ciliates (Ciliophora, Choreotrichia) inferred from small subunit rRNA gene sequences. Denisia **23**:417–424

Wasik A, Mikolajczyk E (1994) Annual cycle of tintinnids in Admiralty Bay with an emphasis on seasonal variability in *Cymatocylis affinis*/*convallaria* lorica morphology. J Plankt Res **16**:1–8

Williams R, McCall H, Pierce RW, Turner JT (1994) Automatic categorisation of five species of *Cymatocylis* (Protozoa, Tintinnida) by artificial neural network. Mar Ecol Prog Ser **107**:263–272