

## Phylogenetic Analyses on the Tintinnid Ciliates (Protozoa, Ciliophora) Based on Multigene Sequence Data

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**Abstract.** In order to better understand phylogenetic relationships among tintinnid ciliated protozoa, we sequenced and analyzed the SSU rDNA and ITS1-5.8S-ITS2 regions of 10 species belonging to five genera in the order Tintinnida. The secondary structures of the ITS2 region were compared among 8 closely related genera, revealing two stable helices of the palm. In addition, we identified a bulge absence in position II of the ITS2 putative secondary structures of species in basal positions in phylogenetic trees, suggesting the absence bulge might be an ancestral character in the order Tintinnida. Phylogenetic analyses based on SSU rDNA and ITS1-5.8S-ITS2 regions sequence show: 1) divergences within the family Tintinnidae are higher than that among other four families (Codonellidae, Ptychocylididae, Metacylididae and Codonellopsidae), suggesting the subdivision of this family; 2) the family Ptychocylididae is polyphyletic; 3) the subdivision of genus *Tintinnopsis* is suggested, because the *Tintinnopsis* spp. scatter into different clades; 4) species with agglutinated loricae are not clearly separated from that with hyaline ones.

**Key words:** Phylogeny, Ciliophora, choreotrichs, Tintinnida, SSU rDNA, ITS1-5.8S-ITS2 region, ITS2 secondary structure.

### INTRODUCTION

Ciliates are abundant and ubiquitous components of the marine plankton and have a major role in the flux of materials through marine planktonic food webs (Pierce and Turner 1992). Choreotrichs, which include the lorica-bearing tintinnids, are frequently abundant and therefore important components of the marine plankton

(Pierce and Turner 1992, Lynn 2008, Strüder-Kypke and Lynn 2008, Agatha 2011). More than one thousand tintinnid species have been described representing over 100 genera and 15 families (Lynn 2008, Agatha 2010, Kim *et al.* 2010, Jiang *et al.* 2012). Species circumscription and identification are traditionally based on lorica size and shape. These characters, however, are highly variable and this has led to a proliferation of described species, many of which are probably invalid (Laval-Peuto and Brownlee 1986, Lynn 2008, Strüder-Kypke and Lynn 2008). Consequently, molecular data are increasingly used to elucidate the systematics of the tintinnids (Agatha and Strüder-Kypke 2007, Bachy *et al.* 2012).

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To date, more than 200 small subunit rRNA gene (SSU rDNA) sequences of tintinnids have been deposited in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) and several studies have been carried out on their phylogeny based on these data (Snoeyenbos-West *et al.* 2002, Strüder-Kypke and Lynn 2003, Agatha 2004, Agatha and Strüder-Kypke 2007, Bachy *et al.* 2012, Santoferrara *et al.* 2012b). None of these investigations revealed relationships that are consistent with the lorica-based classification. By contrast, there have been few studies on the molecular phylogeny of tintinnid ciliates based on other gene markers (Snoeyenbos-West *et al.* 2002; Santoferrara *et al.* 2012a, b). The internal transcribed spacer 2 (ITS2) of the nuclear ribosomal repeat unit is one of the most commonly applied phylogenetic markers and, because it is a fast-evolving locus, it is appropriate for studies at low taxonomic levels (Coleman 2003, 2005; Keller *et al.* 2009). Among tintinnids, however, ITS1-5.8S-ITS2 region sequences are available for only six species, namely *Tintinnopsis* sp., *T. tubulosoides*, *Favella* sp., *F. ehrenbergii*, *Metacyclis angulata* and *Eutintinnus pectinis*.

In this study we sequenced the ITS1-5.8S-ITS2 region of ten tintinnid species, representing five genera and four families, isolated from coastal waters of northern China (Table 1). These are: *Tintinnopsis* sp. 1, *Tintinnopsis* sp. 2, *Tintinnopsis* sp. 3, *T. lohmanni*, *T. cylindrica*, *Favella campanula*, *F. taraikaensis*, *Amphorellopsis acuta*, *Codonellopsis nipponica* and *Stenosemella nivalis*. Phylogenetic analyses were carried out both on the ITS1-5.8S-ITS2 region and the corresponding SSU rDNA sequenced from isolates of the same species (Table 1). The main aim was to improve our knowledge and understanding of the molecular phylogeny of tintinnids.

## MATERIALS AND METHODS

### Collection and identification of ciliates

Ciliates were collected from coastal waters of the Yellow Sea near the city of Qingdao (36°04'N; 120°23'E), northeastern China (Table 1, Xu and Song 2005, Gao *et al.* 2009, Li *et al.* 2009). Identification and morphological studies were based on lorica characteristics according to previous investigations (Kofoid and Campbell 1929, Agatha 2004, Xu and Song 2005). Terminology and systematics are mainly according to Lynn (2008).

### Extraction of genomic DNA, PCR amplifications, cloning and sequencing

Genomic DNA was extracted using REExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) as described previously and stored at -80°C (Zhang *et al.* 2011). PCR amplifications were performed in a Gene Amp 9700 thermocycler. The forward primer ITS-F (5'-GTA GGT GAA CCT GCG GAA GGA TCA TTA -3') and reverse primer ITS-R (5'-TAC TGA TAT GCT TAA GTT CAG CGG -3') were used to amplify the ITS1-5.8S-ITS2 region (Miao *et al.* 2008). The PCR was performed with TAKARA high fidelity *Ex Taq*<sup>TM</sup> polymerase (Takara, Shuzo, Japan), which has an error rate of about  $2 \times 10^{-6}$  (<http://www.clontech.com/takara>). PCR conditions for the ITS1-5.8S-ITS2 fragments were as follows: 5 min. at 94°C followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min., and a final extension at 72°C for 10 min. The PCR products were purified using the Spin Column PCR Product Purification Kit (Sangon Bio. Co., China) and then inserted into pUCm-T vectors (Sangon Bio. Co., China). Sequencing was performed bi-directionally for 2–3 clones by Sangon Biotech in Shanghai.

### ITS1-5.8S-ITS2 region sequence alignment and ITS2 secondary structure prediction

The 10 newly sequenced ITS1-5.8S-ITS2 regions, adding that of another 5 choreotrichs (obtained from NCBI, see Table 2), were aligned using Geneious 5.6 software (<http://www.geneious.com>, Drummond *et al.* 2012). The ITS2 regions were precisely detected by Hidden Markov Models (HMMs, Keller *et al.* 2009). Putative secondary structures were established by submission of the primary ITS2 sequences to the RNA folding website supporting MFOLD version 3.1 (<http://mfold.rna.albany.edu/?q=mfold/download-mfold>, Zuker 2003) using the default parameters (except T = 25°C) according to Coleman (2005). Finally, the putative secondary structure models were edited for aesthetic purposes with Rna Viz 2.0 (Rijk and Wachter 1997, Rijk *et al.* 2003).

### Phylogenetic analyses

In addition to the 10 newly sequenced species, ITS1-5.8S-ITS2 region sequences of another 36 species, and SSU rDNA sequences of these 46 species (obtained from NCBI database, for accession numbers see Table 1), were used in order to investigate phylogenetic relationships among the tintinnids. All alignments of SSU rDNA and ITS1-5.8S-ITS2 region sequences were imported into BioEdit ver. 7.0.5.4 (Hall 1999), trimmed at the ends, and assembled into contigs. Then we checked for sequencing errors and refined manually. The first dataset included ITS1-5.8S-ITS2 regions of all sequences of tintinnids and other spirotrichs available from the NCBI database; the second dataset included the corresponding SSU rDNA sequences of all taxa of the first dataset.

The ITS1-5.8S-ITS2 region and SSU rDNA sequence data were used to generate phylogenetic trees as follows: (1) Maximum parsimony (MP) trees were calculated by a heuristic search with all characters coded as unordered in PAUP\* 4.0b10 (Swofford 2002); (2) Bayesian inference (BI) analyses were performed with MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) using the GTR+I+G model selected by MrModeltest 2 (Nylander 2004) under the AIC criterion. Two parallel runs were performed

**Table 1.** Species included in the phylogenetic analyses. GenBank accession numbers for ITS1-5.8S-ITS2 region sequences are given in the first line, those for the SSU rDNA sequences are given in the second line. For some species the ITS1-5.8S-ITS2 region sequences and SSU rDNA sequences have a single accession numbers (-); given here as the ITS-5.8S-ITS2 region accession numbers (names of ten newly sequenced tintinnid species are marked bold).

Taxa	GeneBank	Source	Taxa	GeneBank	Source	Taxa	GeneBank	Source	Taxa	GeneBank	Source
<i>Amphorellopsis acuta</i>	JN033241 FJ196071	#China-Qingdao QDA270	<i>Favella tarakaensis</i>	JN033237 FJ196073	#China-Qingdao QDA218	<i>Pseudokeronopsis flava</i>	DQ503579 DQ227798	#China-Qingdao QD-cell bank	<i>Tintinnopsis lohmanni</i>	JN033233 FJ196076	#China-Qingdao QDA251
<i>Apokeronopsis bergeri</i>	DQ777741 DQ777742	#China-Qingdao QD050801	<i>Favella campanula</i>	JN033238 FJ422984	#China-Qingdao QDA254	<i>Pseudokeronopsis rubra</i>	DQ640313 DQ640314	#China-Qingdao QD-cell bank	<i>Tintinnopsis sp. 1</i>	JN033234 FJ422985	#China-Qingdao QDA230
<i>Apokeronopsis crassa</i>	DQ537483 DQ359728	#China-Qingdao QD050821	<i>Gastrostylia steinii</i>	AF508758 AF508758	-	<i>Stenosemella nivalis</i>	JN033240 FJ196074	#China-Qingdao QDA236	<i>Tintinnopsis sp. 2</i>	JN033235 FJ422986	#China-Qingdao QDA269
<i>Codonellopsis nipponica</i>	JN033239 FJ196072	#China-Qingdao QDA235	<i>Holosticha polystylata</i>	AF508760 AF508760	-	<i>Strombidinopsis sp.</i>	AF399067 AF399132	*USA-Groton Sopsis99-1	<i>Tintinnopsis sp. 3</i>	JN033236 FJ422987	#China-Qingdao QDA286
<i>Cyrtohymena citrina</i>	AF508755 AF508755	-	<i>Laboea strobila</i>	AF399081 AF399153	*USA-Groton Lstr00B-3	<i>Strombidium sp. 1</i>	AF399040 AF399116	*USA-Groton Strom00-4	<i>Tintinnopsis tubulosoides</i>	AF399019 AF399110	*USA-Groton clone 99-3
<i>Diophrys apolitothrix</i>	EU189061 EU189068	#China-Qingdao QD06111502	<i>Metacyclis angulata</i>	AF399071 AF399144	*USA-Groton Meta00-2	<i>Strombidium rassoulzadegani</i>	DQ241747 AY257125	*USA-Groton, CT	<i>Uroleptus gallina</i>	AF508779 AF508779	-
<i>Diophrys appendiculata</i>	DQ777740 AY004773	#China-Qingdao QDziguic	<i>Oxytricha longa</i>	AF508763 AF508763	-	<i>Sterkiella histriomuscorum</i>	AF508770 AF508770	-	<i>Uroleptus pisces</i>	AF508780 AF508780	-
<i>Diophrys hystrix</i>	DQ647676 EF486861	#China-Qingdao QD060315	<i>Paraurostyla weissei</i>	AF508767 AF508767	-	<i>Sterkiella nova</i>	AF508771 AF508771	-	<i>Urostyla grandis</i>	AF508781 AF508781	-
<i>Diophrys oligothrix</i>	DQ537485 DQ353850	#China-Qingdao QDliqiuL	<i>Paruroleptus lepisma</i>	AF508765 AF508765	-	<i>Sylonychia lemnae</i>	AF508773 AF508773	-	<i>Uronychia binucleata</i>	EF198666 EF198667	#China-Qingdao QDlifangL1
<i>Diophrys scutum</i>	EU189067 EU189069	#China-Qingdao QDA345	<i>Paradiophrys irmgard</i>	DQ537484 EU189070	#China-Qingdao QD05112201	<i>Sylonychia mytilus</i>	AF508774 AF508774	-	<i>Uronychia setigera</i>	EF198668 EF198669	#China-Qingdao QDlifangL2
<i>Eutintinnus pectinis</i>	AF399105 AF399170	*USA-Groton Epec99-2	<i>Pleurotricha lanceolata</i>	AF508768 AF508768	-	<i>Sylonychia pustulata</i>	AF058775 AF058775	-	-	-	-
<i>Favella ehrenbergii</i>	AF399103 AF399160	*USA-Groton Fehr99-2	<i>Pseudokeronopsis carnea</i>	EF174294 AY881633	#China-Qingdao QD03101101	<i>Tintinnopsis cyindrica</i>	JN033232 FJ196075	China-Qingdao QDA215	-	-	-

**Table 2.** Tintinnid ciliates used in this study. Details of ITS1-5.8S-ITS2 regions and secondary structure of ITS2 are given (The name of newly sequenced species are marked bold).

Species	Order	Family	Length			ITS2 GC content	$\Delta G$ (25°C, kcal/mol)
			ITS1	5.8S	ITS2		
<b><i>Tintinnopsis sp. 1</i></b>	Tintinnida	Codonellidae	99	153	192	42.71%	-69.34
<b><i>Tintinnopsis sp. 2</i></b>			99	153	192	47.40%	-67.71
<b><i>Tintinnopsis sp. 3</i></b>			99	153	192	42.71%	-69.34
<i>Tintinnopsis cylindrica</i>			100	153	192	43.75%	-51.70
<i>Tintinnopsis lohmanni</i>			100	153	192	44.79%	-69.71
<i>Tintinnopsis tubulosoides</i>			100	153	192	43.23%	-69.26
<b><i>Favella campanula</i></b>		Ptychocylididae	114	153	195	47.92%	-73.44
<b><i>Favella taraikaensis</i></b>			99	153	195	46.15%	-70.50
<i>Favella ehrenbergii</i>			99	153	195	46.15%	-70.50
<i>Metacyclis angulata</i>		Metacyclididae	99	153	192	46.35%	-64.49
<b><i>Codonellopsis nipponica</i></b>		Codonellopsidae	101	153	192	45.83%	-73.44
<b><i>Stenosemella nivalis</i></b>			101	153	192	44.79%	-52.10
<i>Eutintinnus pectinis</i>		Tintinnidae	100	153	191	46.60%	-66.54
<b><i>Amphorellopsis acuta</i></b>			97	153	191	45.03%	-70.22
<i>Strombidinopsis sp.</i>	Choreotrichida	Strombidinopsidae	100	153	185	44.86%	-64.25

and the maximum posterior of a consensus tree out of 1,000,000 trees, approximating it with the Markov chain Monte Carlo (MCMC) and sampling every 100<sup>th</sup> generation, was computed. The first 2,500 trees were discarded as burn-in; (3) Maximum likelihood (ML) trees were constructed by PhyML V2.4.4 (Guindon and Gascuel 2003) with GTR+G+I model selected by Modeltest v.3.7 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003); (4) Neighbor joining (NJ) trees were constructed using Phylip version 3.6 (Felsenstein 1989, 2005) with the Kimura-2-parameter model. Additionally, bootstrap analyses with 1,000 replicates were used to examine the robustness of the resulting bifurcations within MP, ML and NJ analyses. Phylogenetic trees were viewed with TreeView v1.6.6 (Page 1996) and edited with MEGA 5 (Tamura *et al.* 2007, 2011).

## RESULTS

### ITS1-5.8S-ITS2 sequence analyses

The ITS1-5.8S-ITS2 region sequences of the 10 tintinnid species have been deposited in the NCBI database (for accession numbers, see Table 1). The lengths of these sequences were similar to each, the longest being that of *Favella campanula* (462 bp) and the shortest *Amphorellopsis acuta* (441 bp) (Fig. 1, Table 2).

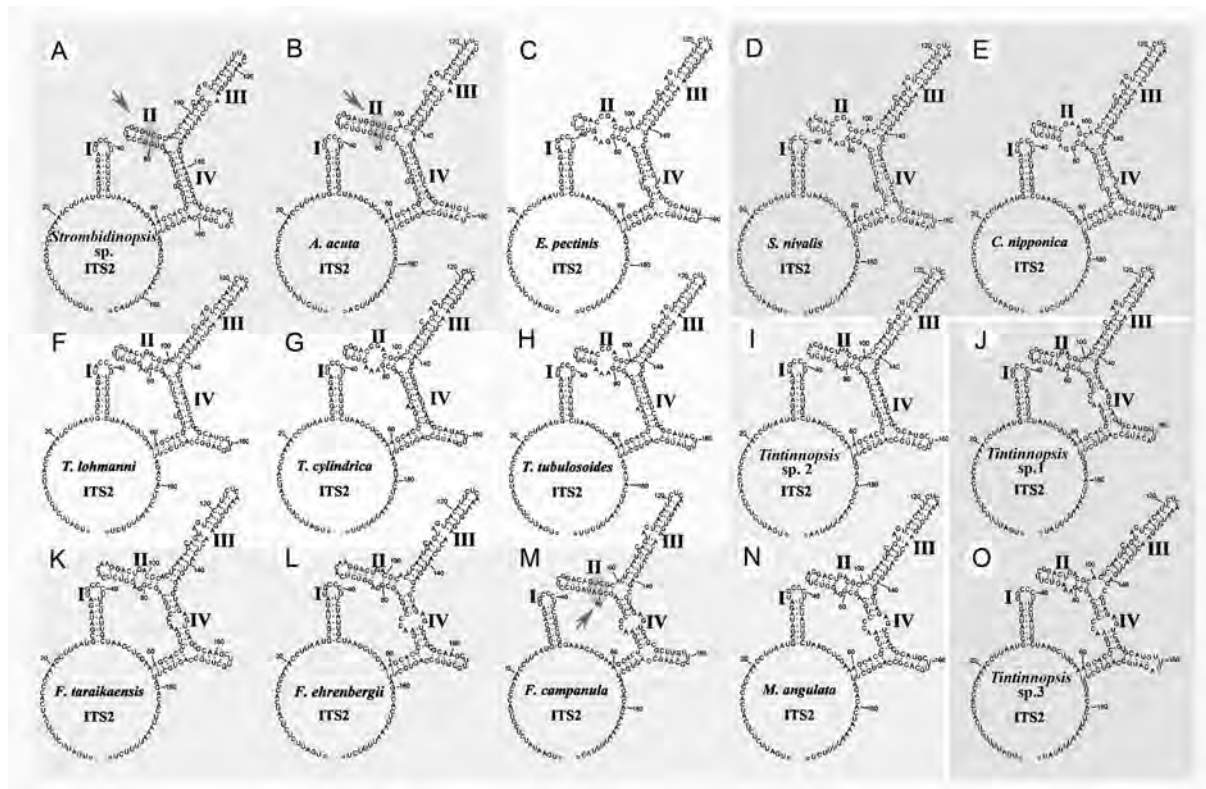
Alignments of the ITS1-5.8S-ITS2 region (Fig. 1) for the 10 newly sequenced species and five other chore-

otrich sequences from the NCBI database reveals four noteworthy features: (1) there are 30 variable sites in the 5.8S gene, and 75 in the ITS1 region and 78 in the ITS2 region; (2) the difference in the ITS1 region between *Favella campanula* and the other 14 choreotrich species is remarkable, the former having a unique 14-bp insertion (arrowed in Fig. 1); (3) the choreotrichid species *Strombidinopsis sp.* has three unique 2-bp and 3-bp insertions and two 3-bp deletions in the ITS1 and ITS2 regions and differs significantly from other tintinnids in the 5.8S gene region (Fig. 1); (4) both *Favella sp.* and *F. taraikaensis*, which differ by only two bases, have a 3-bp insertion in the ITS2 region that is absent in the other 13 species; (5) *Tintinnopsis sp. 1* and *Tintinnopsis sp. 3* differ in just one bp in the 5.8S rDNA and have identical ITS1 and ITS2 region sequences (Fig. 1).

### ITS2 secondary structure and analyses

The ITS2 secondary structures of the 14 tintinnid and one choreotrichid species share the typical model of the Class Spirotrichea, i.e. a palm with two fingers (Coleman 2005). As shown in Fig. 2: (1) *Strombidinopsis sp.* (Fig. 2A) and *Amphorellopsis acuta* (Fig. 2B) have no bulge in the positions labeled II and IV, and thus differs from *Eutintinnus pectinis* (Fig. 2C);





**Fig. 2A–O.** Secondary structure of the internal transcribed spacer 2 (ITS2) RNA transcript of: **A** – *Strombidinopsis* sp.; **B** – *Amphorellopsis acuta*; **C** – *Eutintinnus pectinis*; **D** – *Stenosemella nivalis*; **E** – *Codonellopsis nipponica*; **F** – *Tintinnopsis lohmanni*; **G** – *T. cylindrica*; **H** – *T. tubulosoides*; **I** – *Tintinnopsis* sp. 2; **J** – *Tintinnopsis* sp. 1; **K** – *Favella taraikaensis*; **L** – *F. ehrenbergii*; **M** – *F. campanula*; **N** – *Metacyclis angulata* and **O** – *Tintinnopsis* sp. 3. The diagram illustrates that all these species have a similar ITS2 secondary structure model – one palm with two fingers. *Tintinnopsis* sp. 1 and *Tintinnopsis* sp. 3 have the same ITS2 secondary structure, so are shaded together. Positions labeled II that lack a bulge are marked with arrows. Note that a bulge is present in this position in other species.

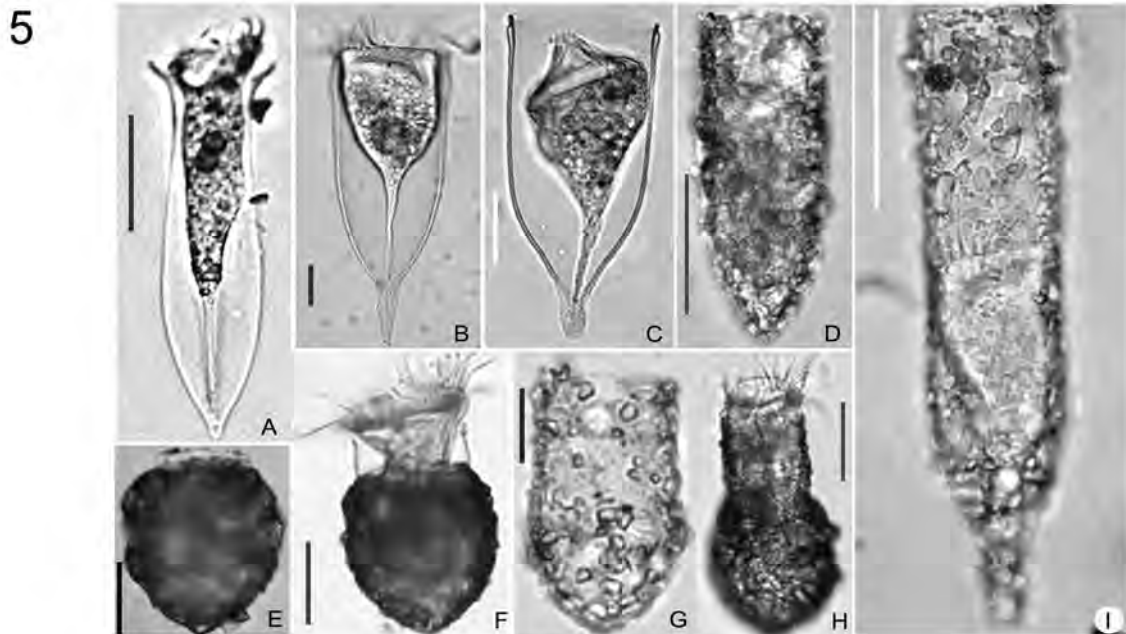
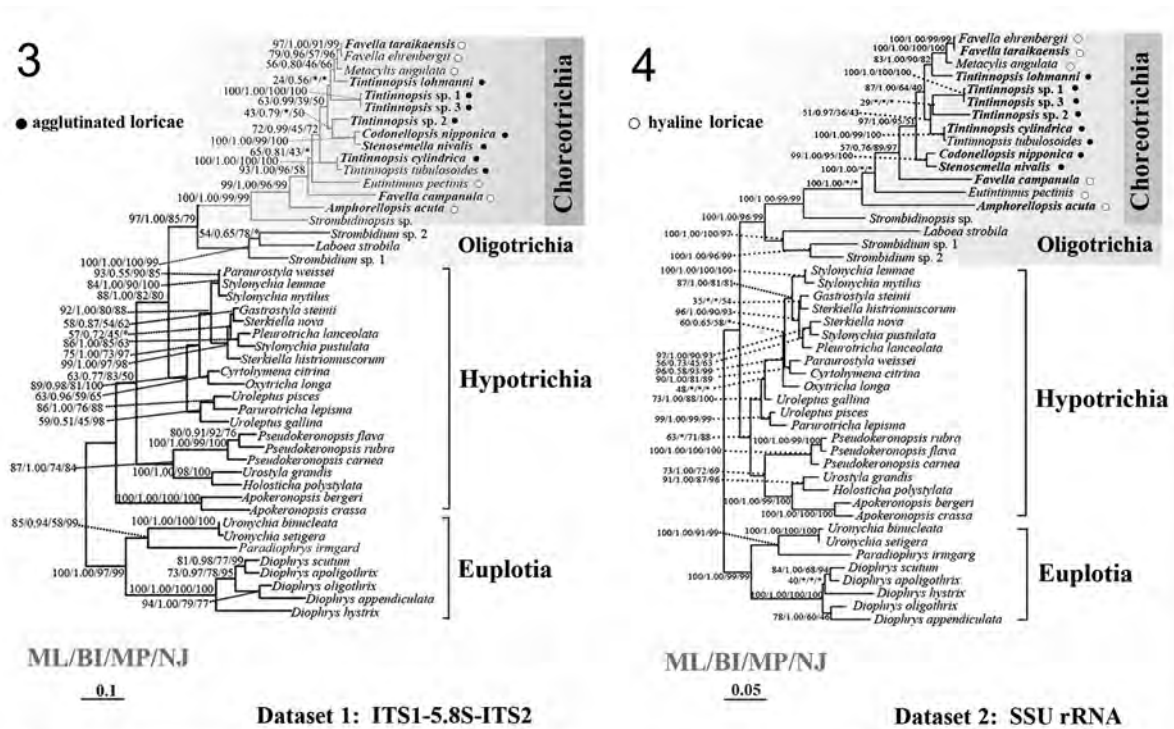
## DISCUSSION

The order Tintinnida contains 15 families and is assigned to the subclass Choreotrichia (Lynn 2008). Traditionally, species identification of tintinnids is mainly based on lorica structure (Kofoid and Campbell 1929, 1939). The relationships among tintinnids are in urgent need of clarification. Several phylogenetic analyses of tintinnids and related taxa have been carried out in the past decade (Snoeyenbos-West *et al.* 2002, Strüder-Kypke and Lynn 2003, Agatha 2004, Agatha and Strüder-Kypke 2007, Strüder-Kypke and Lynn 2008, Li *et al.* 2009, Bachy *et al.* 2012), but each has cast doubt on the primacy of lorica structure in tintinnid systematics. In the present investigation, we have added 10 new ITS1-5.8S-ITS2 region sequences of five genera (*Tintinnopsis*, *Favella*, *Codonellopsis*, *Stenosemella* and *Amphorellopsis*) in four families (Codonellidae,

Ptychocylididae, Codonellopsidae and Tintinnidae) of tintinnids, determined the putative secondary structure of ITS2 of one aloricate choreotrich and 14 tintinnids, and obtained some new findings based on the ITS1-5.8S-ITS2 data.

In all choreotrichs analyzed to date, the ITS2 secondary structures are the same, i.e. a palm with two helices and loops on helices, suggesting that this is a stable character among related species (Coleman 2005). The present study of one aloricate choreotrichid (*Strombidinopsis* sp.) and 14 tintinnids revealed basically the same structure, although there are some variable bulges on one main helix (Fig. 2).

The ITS1-5.8S-ITS2 region of *Strombidinopsis* sp. significantly differed from that of the tintinnids reflecting its placement in the order Choreotrichida rather than Tintinnida, although it has a similar secondary structure to that of *Amphorellopsis acuta* (Figs 1–2).



**Figs 3–5.** Phylogenetic analyses and photomicrographs in this work. **3, 4** – phylogenetic analyses inferred by ML of internal transcribed spacer (ITS) and 5.8S region sequences and small subunit rDNA sequences. Topologies of trees constructed with other methods (BI, MP, or NJ) were essentially identical, lacking only a few nodes indicated by asterisks in the support values. Posterior probability values for branches of the ML tree and bootstrap values for ML, NJ, and MP trees, respectively, are given on nodes. Newly sequenced species are highlighted in bold. Scale bar in 3 corresponds to 10 substitutions per 100 nucleotide positions, scale bar in 4 corresponds to 5 substitutions per 100 nucleotide positions. **5** – photomicrographs of nine of the 10 newly sequenced tintinnid species *in vivo*: **A** – *Amphorellopsis acuta*; **B** – *Favella taraikaensis*; **C** – *F. campanula*; **D** – *Tintinnopsis sp. 2*; **E** – *Stenosemella nivalis*; **F** – *Codonellopsis nipponica*; **G** – *Tintinnopsis sp. 3*; **H** – *T. lohmanni* and **I** – *T. cylindrica*. Scale bars: 25  $\mu$ m.



*Strombidinopsis* sp. branches basally within the choreotrich clade in both the SSU rDNA tree and the ITS1-5.8S-ITS2 region tree (Figs 3, 4). Interestingly, two of the other three species occupying the next most basal positions within the choreotrichs, namely *A. acuta* and *Favella campanula*, have similar ITS2 secondary structures to *Strombidinopsis* sp. in the sense that they lack a bulge in the position marked II (Fig. 2A, B, M). This suggests that the absence of a bulge in position II might be an ancestral character in the order Tintinnida. Interestingly the fourth basal species, *Eutintinnus pectinis*, resembles *A. acuta* in having a hyaline lorica but does not possess a bulge in position II. It is noteworthy, however, that the relative positions of these two species differ in the two trees suggesting that further analyses are needed in order to resolve the phylogeny of the basal tintinnids.

Divergences of ITS1-5.8S-ITS2 region sequences within the family Tintinnidae, represented by *A. acuta* and *Eutintinnus pectinis*, are higher than those among other four families (Codonellidae, Ptychocyliidae, Metacyliidae and Codonellopsidae). These findings support the division of the family Tintinnidae by the erection of the family Eutintinnidae for members of the genus *Eutintinnus* (Bachy *et al.* 2012). *Metacylis angulata* and *Favella* spp. (except *F. campanula*) share a similar ITS2 secondary structure and form a high supported clade in both phylogenetic trees, although there is a 3-bp insertion in *M. angulata* that is absent in *Favella* spp. (Fig. 1, Table 2). Bachy *et al.* (2012) noted that the monophyly of the family Ptychocyliidae could not be confirmed because the only species for which molecular data were available belonged to the genus *Favella*. Based on the present study, the close relationship between *Favella* spp. (family Ptychocyliidae) and *M. angulata* (family Metacyliidae), and the placement of *F. campanula* in a basal position within the choreotrich assemblage, suggest that the family Ptychocyliidae is polyphyletic. Furthermore, we found that the ITS1-5.8S-ITS2 region and SSU rDNA sequences of *F. ehrenbergii* (Snoeyenbos-West *et al.* 2002) are more similar to *F. taraikaensis* (Fig. 5B; Xu and Song, 2005) than to *F. ehrenbergii* (Kim *et al.* 2010). Moreover, the notable 14-bp insertion in the ITS1 region (Fig. 5C) and the absence of a bulge in position II of the putative ITS2 secondary structure (Fig. 2M) in *F. campanula* may explain the basal position of this species in the phylogenetic trees (Figs 3, 4). Given the distant relationship of

*F. campanula* and *F. taraikaensis*, our data do not support the monophyly of the genus *Favella* as suggested by Bachy *et al.* (2012).

The genus *Tintinnopsis*, which is the most diverse tintinnid genus (Strüder-Kypke and Lynn 2003, Bachy *et al.* 2012), is traditionally assigned to the family Codonellidae with another 27 extant and 16 fossil genera *incertae sedis* (Lynn 2008). It is noteworthy that *Tintinnopsis* sp. 1 and *Tintinnopsis* sp. 3: (1) both have a bulge in position IV of the ITS2 secondary structure (Fig. 2J, O) unlike other *Tintinnopsis* spp. (Fig. 2F, G, H, I), (2) differ from one another by only one nucleotide in the ITS1-5.8S-ITS2 region, and (3) are morphologically very similar (Fig. 5G), suggesting that they are two populations of the same species. Likewise, the ITS1-5.8S-ITS2 region and SSU rDNA sequences of *T. tubulosoides* (Snoeyenbos-West *et al.* 2002) are almost identical to those of *T. cylindrica* (Fig. 5I), suggesting that they may be two populations (the former sampled from the USA and the latter sampled from China) of the same species, too. Our findings support the paraphyly of the genus *Tintinnopsis* as suggested in previous studies (Snoeyenbos-West *et al.* 2002; Li *et al.* 2009; Bachy *et al.* 2012; Santoferrara *et al.* 2012a, b).

A tight relationship between *Codonellopsis nipponica* and *Stenosemella nivalis* is inferred based on both ITS1-5.8S-ITS2 and SSU rDNA tree, ITS2 secondary structure and the possession of lorica with agglutinated walls and a hyaline collars (Figs 2D, E, 3, 4, 5D, E). The instability of the codonellopsid clade, represented by *Tintinnopsis* sp. 2, *C. nipponica* and *S. nivalis* (Fig. 3), suggest that additional sequences are required in order to ascertain the monophyly of the family Codonellopsidae.

To summarize, our molecular phylogenetic analyses of the order Tintinnida, based on ITS1-5.8S-ITS2 region and SSU rDNA sequence data, are nearly congruent with previous reports based on SSU rDNA sequence data alone (Snoeyenbos-West *et al.* 2002, Strüder-Kypke and Lynn 2003, Agatha and Strüder-Kypke 2007, Li *et al.* 2009). However, the systematics of its constituent taxa are still confused, especially the genera *Tintinnopsis* and *Favella*. As more morphological and gene sequence data become available, it will be possible to re-evaluate the phylogenetic relationships of those taxa that are discordant with original taxonomy (Kim *et al.* 2010, Bachy *et al.* 2012, Jiang *et al.* 2012, Santoferrara *et al.* 2012a).



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