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).
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., T. ehrenbergii, Metacylis 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 sequences 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 REDExtract-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 G3’) were used to amplify the ITS1-5.8S-ITS2 region (Miao et al. 2008). The PCR was performed with TAKARA high fidelity Ex Taq polymerase (Takara, Shuzo, Japan), which has an error rate of about 2 × 10⁻⁴ (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 (Swoford 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).

<table>
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Publikacja objęta jest prawem autorskim. Wszelkie prawa zastrzeżone. Kopiowanie i rozpowszechnianie zabronione.
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).

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<th>Species</th>
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<th>Family</th>
<th>Length</th>
<th>ITS1</th>
<th>5.8S</th>
<th>ITS2</th>
<th>ITS2 GC content</th>
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<td>153</td>
<td>192</td>
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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 choreotrichid species 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 choreotrichid 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);
Phylogenetic Analyses on the Tintinnid Ciliates

(2) the ITS2 secondary structures of *Stenosemella nivalis* (Fig. 2D) and *Codonellopsis nipponica* (Fig. 2E), which both belonged to the family Codonellopsidae, are similar; (3) *Tintinnopsis* sp. 1 (Fig. 2J) and *Tintinnopsis* sp. 3 (Fig. 2O) share the same structure since their sequences are identical, and they differ from other *Tintinnopsis* spp. in having a bulge in the position labeled IV; (4) *Favella campanula* (Fig. 2M) differs from *F. taraikaensis* (Fig. 2K) and *F. ehrenbergii* (Fig. 2L) in lacking a bulge in the position labeled II. The estimated thermodynamic energy of putative secondary structures ranges from −51.70 kcal/mol (*T. cylindrica*) to −73.44 kcal/mol (*F. campanula*) (Table 2).

Phylogenetic analyses of tintinnid ciliates based on ITS1-5.8S-ITS2 region and SSU rDNA sequences

Almost identical tree topologies are obtained following phylogenetic analyses based on SSU rDNA and ITS1-5.8S-ITS2 region sequences, although there are some slightly unstable clades (Figs 3, 4). Noteworthy features include the following: (1) the monophyly of choreotrichs is strongly supported in all analyses (Fig. 3); (2) *Strombidinopsis* sp., a member of the order Choreotrichida, is basal to the order Tintinnida; (3) the cluster of *Tintinnopsis lohmanni* and *Favella sp./F. taraikaensis/Metacylis angulata* is supported in trees based on both datasets; (4) *Favella campanula* is separated from the other two *Favella* species.

The discordances between the two trees may be summarized as follows: (1) the relationships among *Tintinnopsis* spp. show poor resolution between clades of *Tintinnopsis* sp. 1/sp. 2/sp. 3 and *Codonellopsis nipponica/Stenosemella nivalis* in ITS1-5.8S-ITS2 trees; (2) *Favella campanula* is basal to *Eutintinnus pectinis* in ITS1-5.8S-ITS2 trees (Fig. 3); (3) the relationship between the clades of *Codonellopsis nipponica/Stenosemella nivalis* and *T. cylindrica/T. tubulosoides* is unstable in the ITS1-5.8S-ITS2 tree.
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 – Metacylis 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 μm.
Strombidinopsis sp. branches basally within the chlo- 
etrich 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 chloetrichs, namely A. acuta and 
Favella campanula, have similar ITS2 secondary struc-
tures 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. Inter-
estingly 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, how-
ever, that the relative positions of these two species dif-
fer 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, Ptychocylididae, 
Metacylididae 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 an-
gulata and Favella spp. (except F. campanula) share 
a similar ITS2 secondary structure and form a high sup-
ported clade in both phylogenetic trees, although there 
is a 3-bp insertion in M. angulata that is absent in Fa-
veilla spp. (Fig. 1, Table 2). Bachy et al. (2012) noted 
that the monophyly of the family Ptychocylididae 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 rela-
tionship between Favella spp. (family Ptychocylididae) 
and M. angulata (family Metacylididae), and the placement 
of F. campanula in a basal position within the chlo-
etrich assemblage, suggest that the family Ptychocylidi-
dae 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 ex-
plain the basal position of this species in the phyloge-
netic trees (Figs 3, 4). Given the distant relationship of 
F. campanula and F. taraikaensis, our data do not sup-
port 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 Co-
donellidae with another 27 extant and 16 fossil genera 
incertae sedis (Lynn 2008). It is noteworthy that Tin-
ninnopsis 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 nucleo-
tide in the ITS1-5.8S-ITS2 region, and (3) are morpho-
logically 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 
most identical to those of T. cylindrica (Fig. 51), sug-
gesting 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. 

A tight relationship between Codonellopsis nip-
ponica and Stenosemella nivalis is inferred based on 
both ITS1-5.8S-ITS2 and SSU rDNA tree, ITS2 sec-
ondary structure and the possession of lorica with ag-
glutinated 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 analy-
ses of the order Tintinnida, based on ITS1-5.8S-ITS2 
region and SSU rDNA sequence data, are nearly con-
gruent 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 morphologi-
cal 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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