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Phylogeny of the Order Tintinnida (Ciliophora, Spirotrichea) Inferred from Small- and Large-Subunit rRNA Genes

LUCIANA F. SANTOFERRARA,[a,b] GEORGE B. McMANUS[a] and VIVIANA A. ALDER[b,c,d]

[a]*Department of Marine Sciences, University of Connecticut, Groton, Connecticut, 06340, USA, and*
[b]*Departamento de Ecología, Genética y Evolución, FCEN, Universidad de Buenos Aires, Buenos Aires, Argentina, and*
[c]*CONICET, Buenos Aires, Argentina and*
[d]*Instituto Antártico Argentino, Buenos Aires, Argentina*

ABSTRACT. Concatenated sequences of small- and large-subunit rRNA genes were used to infer the phylogeny of 29 species in six genera of Tintinnida. We confirmed previous results on the positions of major clusters and the grouping of various genera, including *Stenosemella*, the paraphyletic *Tintinnopsis*, the newly investigated *Helicostomella*, and some species of the polyphyletic *Favella*. *Tintinnidium* and *Eutintinnus* were found to be monophyletic. This study contributes to tintinnid phylogenetic reconstruction by increasing both the number of species and the range of genetic markers analyzed.

Key Words. Ciliate, concatenated phylogeny, LSU rDNA, SSU rDNA, tintinnid.

TINTINNID ciliates play a key role as trophic link in planktonic food webs of estuarine and marine environments (Lynn 2008). They are characterized by the presence of a lorica, which has been the basis for taxonomy (Alder 1999; Kofoid and Campbell 1929). Even if the diagnostic value of the lorica for species identification has been long questioned due to its plasticity, this structure is commonly used in ecological surveys and has provided a powerful tool to analyze patterns of diversity and biogeography (e.g. Dolan et al. 2006; Thompson and Alder 2005). Recently, Santoferrara et al. (2012) have shown that the ability to delimit species is comparable for lorica morphology and genetic data (e.g. the small- [SSU] and large-subunit [LSU] rRNA gene sequences), although the degree of agreement between both criteria depends, among other factors, on the genetic markers and cut-off values used for phylotype discrimination.

Molecular studies on tintinnid evolution have focused on SSU rRNA sequences, which are currently available for fewer than 60 of ~ 1,200 named species. Phylogenetic analyses have shown that lorica morphology and genetic sequences disagree above the species level and that the molecular data currently available are unable to resolve some ambiguous relationships (e.g. Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003, 2008). This emphasizes the necessity for revising current taxonomic schemes by integrating cytological, ultrastructural, and genetic information (Agatha and Strüder-Kypke 2007). In addition, more effort is needed to improve resolution of genealogical inferences, for example, by increasing taxonomic sampling and concatenating SSU and LSU rRNA sequences, as has been achieved for other spirotrich ciliates (Hewitt et al. 2003) and protists in general (Moreira et al. 2007). The aim of our study was to contribute to the tintinnid phylogenetic reconstruction by newly analyzing SSU rRNA sequences from 21 species, and concatenating SSU and LSU rRNA sequences for 29 species.

MATERIALS AND METHODS

DNA sequences. GenBank Accession Numbers for all sequences analyzed in this study are included in Fig. 1. Newly analyzed tintinnid sequences of SSU rRNA and the 5′-end of the LSU rRNA were obtained in a previous study, which includes illustrations and criteria for species identification (Santoferrara et al. 2012). *Strombidinopsis* sp. and *Strombidium rassoulzadegani* were isolated from Long Island Sound (USA; 41°16′N, 72°36′W), cultured as described by McManus et al. (2010), and then subjected to DNA extraction as reported by Santoferrara et al. (2012). For PCR amplification of SSU and LSU rDNA, the universal primers 18Scom-F1 and 18Scom-R1 (Zhang et al. 2005) and 28S-F1a and 28S-R1a (Ortman 2008) were used, respectively. Electrophoresis-isolated products were purified, and then sequenced using Big Dye Terminator v3.1 and a capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis. Sequences of SSU and LSU rRNA were edited using MEGA5 (Tamura et al. 2011) and aligned with CLUSTALW (Thompson et al. 1994). Alignments were refined manually and then combined. Separate phylogenetic analyses were run for SSU rRNA with 1,691 nucleotide positions and SSU + LSU rRNA with 2,387 nucleotide positions. Maximum likelihood (ML) analyses were done using RAxML (Stamatakis et al. 2007), setting 10,000 bootstrap replicates, the GTR model of nucleotide substitution with the Γ model of rate heterogeneity, and a random starting tree. The ML bootstrap support (MLS) was estimated for each node, and additional searches for the Best-Known Likelihood Tree were carried out (200 inferences). Bayesian Inference (BI) analyses were done using MrBayes (Ronquist and Huelsenbeck 2003). Five million generations were run and trees were sampled each 1,000 cycles. The initial 1,000 trees were discarded as burn-in, and the remaining 4,000 trees were used to estimate the Bayesian Posterior Probabilities (BPP). The suitability of the selected burn-in and the achievement of stationarity were checked using Tracer (Rambaut and Drummond 2007). The GTR model with a Γ model of rate heterogeneity and a proportion of invariable sites was used for BI, as previously identified with MrModeltest under the AIC criterion (Nylander 2004).

RESULTS AND DISCUSSION

The analyses based on SSU rRNA and SSU + LSU rRNA provided similar topologies for both ML and BI (Fig. 1). In general, our results agreed with previous reports based on either SSU rRNA (Gao et al. 2009; Kim et al. 2010; Li et al. 2009; Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003, 2008) or both SSU rRNA and cytology (Agatha and Strüder-Kypke 2007). The subclasses Stichotrichia, Oligotrichia, and Choreotrichia were found to be monophyletic, although this cannot be confirmed for Oligotrichia in the SSU + LSU rRNA analysis, as it was represented only by *S. rassoulzade-*

Corresponding Author: L. Santoferrara, Department of Marine Sciences, University of Connecticut, Groton, Connecticut 06340, USA—Telephone number: 1 860 405 9232; FAX number: 1 860 405 9153; e-mail: luciana.santoferrara@uconn.edu

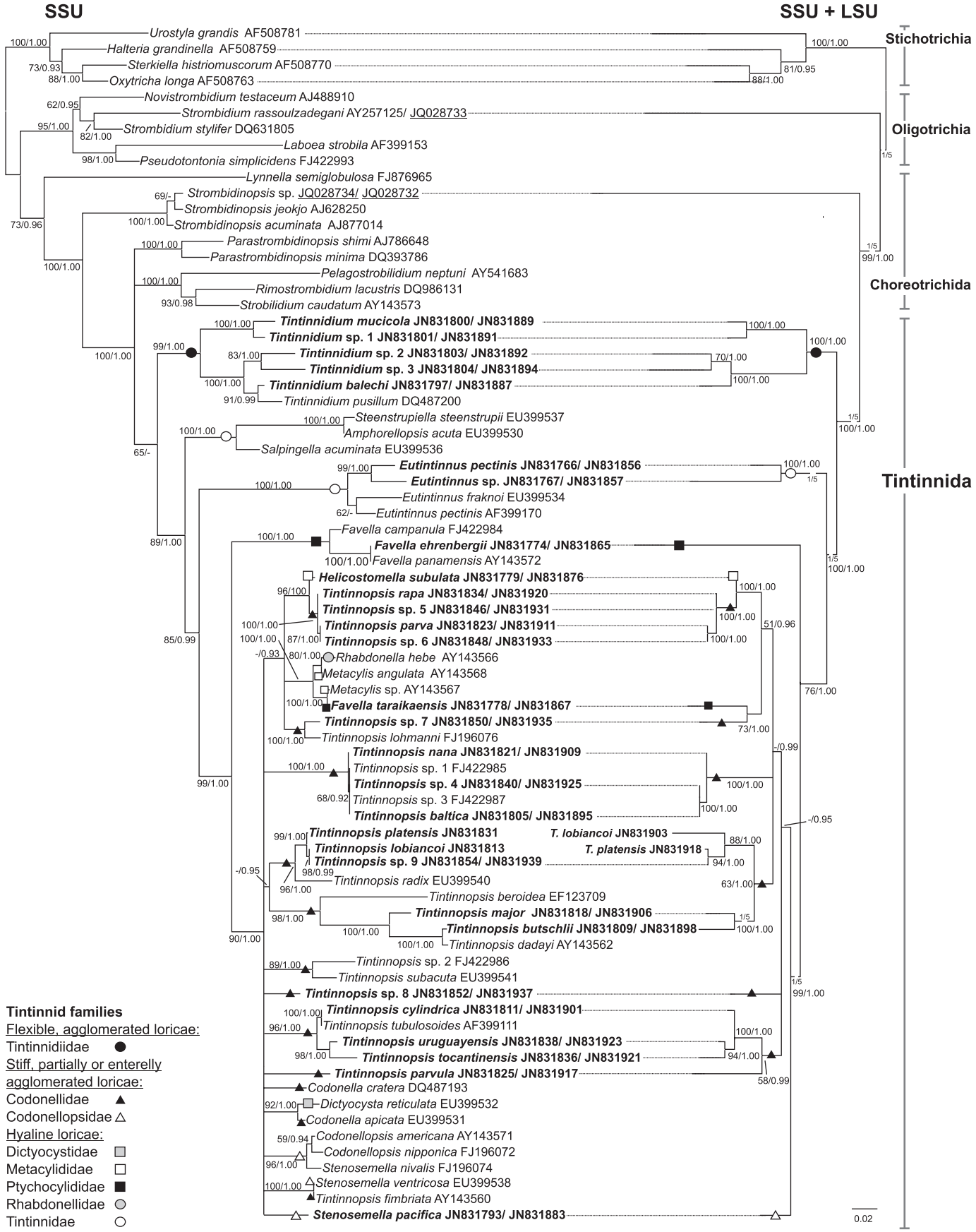
SSU
SSU + LSU
Stichotrichia
Oligotrichia
Choreotrichida
Tintinnida
Urostyla grandis AF508781
Halteria grandinella AF508759
Sterkiella histriomuscorum AF508770
Oxytricha longa AF508763
Novistrombidium testaceum AJ488910
Strombidium rassoulzadegani AY257125/ JQ028733
Strombidium stylifer DQ631805
Laboea strobila AF399153
Pseudotontonia simplicidens FJ422993
Lynnella semiglobulosa FJ876965
Strombidinopsis sp. JQ028734/ JQ028732
Strombidinopsis jeokjo AJ628250
Strombidinopsis acuminata AJ877014
Parastrombidinopsis shimi AJ786648
Parastrombidinopsis minima DQ393786
Pelagostrobilidium neptuni AY541683
Rimostrombidium lacustris DQ986131
Strobilidium caudatum AY143573
Tintinnidium mucicola JN831800/ JN831889
Tintinnidium sp. 1 JN831801/ JN831891
Tintinnidium sp. 2 JN831803/ JN831892
Tintinnidium sp. 3 JN831804/ JN831894
Tintinnidium balechi JN831797/ JN831887
Tintinnidium pusillum DQ487200
Steenstrupiella steenstrupii EU399537
Amphorellopsis acuta EU399530
Salpingella acuminata EU399536
Eutintinnus pectinis JN831766/ JN831856
Eutintinnus sp. JN831767/ JN831857
Eutintinnus fraknoi EU399534
Eutintinnus pectinis AF399170
Favella campanula FJ422984
Favella ehrenbergii JN831774/ JN831865
Favella panamensis AY143572
Helicostomella subulata JN831779/ JN831876
Tintinnopsis rapa JN831834/ JN831920
Tintinnopsis sp. 5 JN831846/ JN831931
Tintinnopsis parva JN831823/ JN831911
Tintinnopsis sp. 6 JN831848/ JN831933
Rhabdonella hebe AY143566
Metacylis angulata AY143568
Metacylis sp. AY143567
Favella taraikaensis JN831778/ JN831867
Tintinnopsis sp. 7 JN831850/ JN831935
Tintinnopsis lohmanni FJ196076
Tintinnopsis nana JN831821/ JN831909
Tintinnopsis sp. 1 FJ422985
Tintinnopsis sp. 4 JN831840/ JN831925
Tintinnopsis sp. 3 FJ422987
Tintinnopsis baltica JN831805/ JN831895
Tintinnopsis platensis JN831831
Tintinnopsis lobiancoi JN831813
Tintinnopsis sp. 9 JN831854/ JN831939
T. lobiancoi JN831903
T. platensis JN831918
Tintinnopsis radix EU399540
Tintinnopsis beroidea EF123709
Tintinnopsis major JN831818/ JN831906
Tintinnopsis butschlii JN831809/ JN831898
Tintinnopsis dadayi AY143562
Tintinnopsis sp. 2 FJ422986
Tintinnopsis subacuta EU399541
Tintinnopsis sp. 8 JN831852/ JN831937
Tintinnopsis cylindrica JN831811/ JN831901
Tintinnopsis tubulosoides AF399111
Tintinnopsis uruguayensis JN831838/ JN831923
Tintinnopsis tocantinensis JN831836/ JN831921
Tintinnopsis parvula JN831825/ JN831917
Codonella cratera DQ487193
Dictyocysta reticulata EU399532
Codonella apicata EU399531
Codonellopsis americana AY143571
Codonellopsis nipponica FJ196072
Stenosemella nivalis FJ196074
Stenosemella ventricosa EU399538
Tintinnopsis fimbriata AY143560
Stenosemella pacifica JN831793/ JN831883
0.02
Tintinnid families
Flexible, agglomerated loricae:
Tintinnidiidae
Stiff, partially or enterelly agglomerated loricae:
Codonellidae
Codonellopsidae
Hyaline loricae:
Dictyocystidae
Metacylididae
Ptychocylididae
Rhabdonellidae
Tintinnidae

Fig. **1.** Phylogenetic analyses of the order Tintinnida based on small-subunit (SSU) rRNA (left) and concatenated SSU + large-subunit (LSU) rRNA (right) sequences. Species from the subclasses Choreotrichia (order Choreotrichida), Oligotrichia, and Stichotrichia were used as outgroups. Topology corresponds to the maximum likelihood (ML) analysis. Numbers on each node are ML bootstrap support (MLS) and Bayesian posterior probability (BPP), respectively. Only nodes with MLS $\geq$ 50% and BPP $\geq$ 0.90 are shown. Complete lines represent genetic distances; broken lines are used to link the same species in the two trees. For convenience of illustration, some long branches in the SSU + LSU rRNA tree were shortened to one-fifth their actual length (labeled as 1/5). The scale bar represents two substitutions per 100 nucleotides. Symbols indicate tintinnid families. For GenBank accession numbers, those in bold correspond to newly analyzed SSU and LSU rRNA sequences, whereas those underlined correspond to newly obtained sequences; those in regular font are from previous studies.

gani. The clustering of *Halteria grandinella* within the Stichotrichia is confirmed in the concatenated analysis, contrary to some cytological and morphogenetic features that suggest an affiliation with the Oligotrichia (Agatha and Foissner 2009). The monophyly of the order Tintinnida was poorly supported in the SSU rRNA-based phylogeny (MLS = 65%; BPP $\leq$ 0.90). Although there was strong support for this node in the SSU + LSU rRNA analysis, this may have been caused by undersampling of the Choreotrichida, as LSU rRNA has been sequenced for only our isolate of *Strombidinopsis* sp.

Within the Tintinnida, the genus *Tintinnidium* formed a basal cluster with high support (MLS = 99–100%; BPP = 1.00). *Tintinnidium* was found to be monophyletic, in contrast with previous molecular phylogenies (e.g. Kim et al. 2010), as a sequence formerly considered *Tintinnopsis* sp. actually corresponds to *Tintinnidium pusillum* (Duff et al. 2008). Our results contrast also with cytological data, which have shown a close relationship between *Tintinnidium* spp. and *Tintinnopsis cylindrata*, although the generic affiliation of the latter species needs confirmation (Agatha and Strüder-Kypke 2007). However, the fact that *Tintinnidium* spp. were separated into two fully supported clades agrees with cytological studies, which indicate that *Tintinnidium* includes more than one supraspecific taxon (Agatha and Strüder-Kypke 2007).

In addition to the *Tintinnidium* clade, three clusters with full support were found: a cluster only represented in the SSU rRNA phylogeny and including *Amphorellopsis acuta*, *Steenstrupiella steenstrupii*, and *Salpingella acuminata;* a monophyletic cluster of *Eutintinnus*; and a cluster comprising *Favella ehrenbergii*, *Favella panamensis*, and *Favella campanula* with LSU rRNA not available for the two latter species. All the other genera and species formed a dense cluster with high support in ML analyses (i.e. 90% for SSU rRNA and 99% for SSU + LSU rRNA) and full support in BI analyses (Fig. 1). For SSU rRNA, this cluster included 28 species within *Tintinnopsis*, and 13 species within *Codonella*, *Codonellopsis*, *Dictyocysta*, *Favella*, *Helicostomella*, *Metacylis*, *Rhabdonella*, and *Stenosemella,* which formed 15 branches or subclusters moderately to highly supported in both ML and BI analyses (MLS = 89–100; BPP = 1.00). In the SSU + LSU rRNA tree, four subclusters with moderate to high support in both ML and BI analyses were found: a subcluster that grouped *Helicostomella subulata, Favella taraikaensis*, and five *Tintinnopsis* species (MLS = 51%, BPP = 0.96), and three subclusters that included only *Tintinnopsis* species (MLS = 58–100%, BPP = 0.99–1.00). *Tintinnopsis* sp. 8 formed an isolated branch. Three of these subclusters grouped together in a larger cluster, and *Stenosemella pacifica* branched basally to all of them, but these relationships had low support in the ML analysis (MLS < 50%, BPP = 0.95–0.99). Altogether, these results confirm the polyphyly of *Favella* and the paraphyly of *Tintinnopsis*.

Of the 15 families accepted for tintinnids based on lorica morphology (Lynn 2008), eight were represented in this study. The molecular data currently available indicate that Tintinnidiidae is monophyletic, Codonellidae, Codonellopsidae, and Metacylididae are paraphyletic, and Ptychocylididae and Tintinnidae are polyphyletic. Dictyocystidae and Rhabdonellidae are underrepresented, thus preventing conclusions to be drawn about them. The relationship between families reflected the paraphyly of tintinnid taxa with hyaline or agglomerated loricae (see Fig. 1). This observation was confirmed also by the newly established position of *H. subulata,* as this hyaline species clustered with four species of the agglomerated genus *Tintinnopsis* (MLS = 96–100%, BPP = 1.00). These results agree with previous findings in that the presence/absence of particles in the lorica is not a useful character for phylogeny (e.g. Snoeyenbos-West et al. 2002; Strüder-Kypke and Lynn 2003, 2008). On the basis of these results, we speculate that the sequence of lorica evolution may have gone from flexible and agglomerated loricae (e.g. *Tintinnidium*) to hyaline loricae (e.g. *Eutintinnus*), and then to stiff, partially or entirely agglomerated loricae (e.g. *Codonellopsis*, *Tintinnopsis*), in agreement with Agatha and Strüder-Kypke (2007).

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