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ORIGINAL ARTICLE

Three rDNA Loci-Based Phylogenies of Tintinnid Ciliates (Ciliophora, Spirotrichea, Choreotrichida)

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Barcoding; genetic diversity; marine protists; microzooplankton; multiple-locus phylogeny.

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ABSTRACT

To improve understanding of diversity, phylogeny and evolution in tintinnid ciliates, it is essential to link multiple molecular markers with properly identified and documented morphospecies. Accordingly, 54 tintinnid morphospecies/isolates mainly from the Yellow and East China Seas were collected and analysed. Using single-cell approaches, sequences were obtained for three rDNA loci (18S, ITS1-5.8S-ITS2, D1-D5 region of 28S). Twenty-six tintinnid morphospecies (29 isolates) are documented by micrographs, measurements, morphologically described, and compared with the original species description. Three rDNA loci-based phylogenetic analyses were then performed for these identified isolates. Sequences from 25 unidentified species/isolates were also included in the comparison of the three rDNA loci. Ribosomal DNA genes of the genus *Leprotintinnus* were analysed for the first time, showing that *Leprotintinnus* was closely related to *Tintinnopsis radix* and branched distinctly apart from the family Tintinnidiidae. Four novel clades (VI to IX) of the *Tintinnopsis* complex emerged in the 18S genealogies. Analyses of the relative variability in the ITS and 28S regions vs. the 18S rDNA showed that the ITS1-5.8S-ITS2 and ITS2 regions well co-varied with the 18S rDNA when the variations of the latter were less than 3%, whereas at difference of less than 1%, no correlation was found between the compared loci. These findings highlight the difficulties in using variable locus-based cut-off divergences in circumscribing tintinnid morphospecies.

TINTINNID ciliates belong to the choreotrichid spirotrichs and represent an ecologically important group of marine planktonic ciliates with a high morphological diversity (Dolan et al. 2013, 2014; Kim et al. 2013). About 1,000 tintinnid species have been described and classified into 14 families and 75 genera (Agatha and Strüder-Kypke 2014), almost exclusively based on lorica features (e.g. size, shape, ornamentation and structure; Kofoid and Campbell 1929, 1939). Although ciliary patterns are generally used as the main criterion in identifying ciliates, only a few tintinnid species (29) have been investigated in this respect (Agatha and Strüder-Kypke 2014). Consequently, studies of tintinnid biogeography and ecology still completely rely on lorica features, which might, however, show a high phenotypic plasticity and considerable interspecific resemblance. Furthermore, the lorica-based tintinnid classification emerged to be artificial in molecular phylogenies and cladistic analyses of the preliminary cell features (Agatha and Strüder-Kypke 2007, 2012, 2014).

In the last decade, efforts had been made to assess the phylogenetic relationships among tintinnids, using mainly a single locus, viz., the 18S rRNA gene (small subunit ribosomal RNA genes or 18S rDNA; Agatha and Strüder-Kypke 2012; Gao et al. 2009; Li et al. 2009; Strüder-Kypke and Lynn 2003, 2008). However, due to the relatively conserved nature of the 18S rDNA, it is not completely efficient in resolving evolutionary relationships at genus and species level (Agatha and Strüder-Kypke 2012). Recently, multiple-locus approaches have been increasingly used in addressing phylogenetic issues of ciliates (Gao et al. 2016;

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Zhang et al. 2012a, 2014) and tintinnid ciliates. In the latter, more variable loci were analysed in combination with the 18S rDNA to refine the phylogenies, for instance, the ITS1-5.8S-ITS2 (internal transcribed spacer sequences plus 5.8.S rDNA, thereafter referred to as the ITS region; Bachy et al. 2012; Li et al. 2013; Snoeyenbos-West et al. 2002; Zhao et al. 2012) and the 28S rRNA genes (large subunit ribosomal RNA genes or 28S rDNA; Santoferrara et al. 2012). Actually, it had been demonstrated that a combination of two rDNA markers might provide a higher resolution of the phylogenetic relationships in tintinnids than a single marker does (Bachy et al. 2012; Santoferrara et al. 2012). However, only a small fraction of the huge tintinnid diversity is currently covered by the molecular phylogenetic assessments, namely, only 31 of the 75 genera; the number is even smaller concerning sequences of multiple loci (15 genera).

Barcoding and thus molecular phylogeny and environmental sequencing depend on trustworthy reference sequences from reliably identified species (Santoferrara et al. 2016; Xu et al. 2012, 2013). Beyond the well-known pitfalls of an exclusively lorica-based tintinnid taxonomy and classification, species determination is also flawed by the application of unsuitable identification literature. Since species circumscriptions in revisionary taxonomic works usually deviate from the original descriptions owing to subjective lumping and splitting of species, the use of the original descriptions or authoritative redescriptions (the redescription that best fits an insufficient original description and provides enough characters for a reliable determination) is recommended (Santoferrara et al. 2016). In the future, the combination of genetic and cytological data will indicate which morphospecies should be considered synonyms.

Ribosomal DNA loci have been commonly applied in molecular ecological studies of protists including tintinnids, in which knowledge of species-level rDNA variations is important for understanding the correlation between genetic diversity and morphospecies diversity (e.g. Bachy et al. 2013). Short and variable rDNA markers, such as the ITS regions and the D1-D2 region of the 28S rDNA, have been evaluated in ciliates (Miao et al. 2008; Stoeck et al. 2014; Sun et al. 2014). For tintinnids, variations of the 18S gene, the ITS region, and the D1-D2 regions of the 28S rDNA within a set of identified morphospecies were found to be up to 1%, 1.5% and 0.6%, respectively (Bachy et al. 2012, 2013, 2014; Santoferrara et al. 2013, 2015). However, it remains to be tested (i) whether the observed discontinuities in the variability can be generally applied for distinguishing tintinnid species and (ii) how these discontinuities differ between the rDNA markers. Using rDNA sequences of tintinnids from single cells or long clone sequences from rDNA libraries of environmental samples, it seems possible to assess the relative variability among the loci, which may be helpful in addressing the issues mentioned above.

In the present study, we collected 54 morphospecies/isolates of tintinnid ciliates mainly from off-shore sites in the northern China seas and tried to obtain sequences of the three rDNA loci from the same individuals or isolates; for 29 isolates, we provide morphological data (measurements and illustrations) and discuss their identifications. These data are important for vetting the determinations of the analysed specimens. Sequence information of seven taxa is provided for the first time in this work (Table 1). For another 25 unidentified isolates, we provide ITS-18S rDNA, 28S-18S rDNA sequence pairs from the same tintinnid individual. In addition, we compiled sequence data from an individual or a clone from rDNA libraries and evaluated the variability of the ITS (including ITS1 and ITS2) and 28S regions relative to that of the 18S rDNA.

MATERIALS AND METHODS

Sampling and morphological identification

The samples were collected from the Berre Lagoon near the city of Marseille, France, and 13 coastal sites in the Yellow Sea (including the Jiaozhou Bay) and East China Sea either on cruises or on individual sampling occasions during the period from May to November of 2011 (Table 1 and Fig. S1). At each site, five litres of surface water were collected with a plastic bucket and filtered through a 5-μm or 10-μm-meshed plankton net. The concentrated material was investigated in vivo (individual sampling occasions) or fixed with nonacidic Lugol's solution to a final concentration of 1% (cruises samples). For species identification, tintinnid cells were isolated by a micropipette under a stereoscope. The observations and measurements were performed at 400–1,000X magnification, using bright field and differential interference contrast microscopy of an inverted microscope (Olympus IX51) equipped with a digital camera (Olympus DP21). Photomicrographs were taken at 200X or 400X magnification. Identification of tintinnids was performed, using the micrographs and measurements as well as the original descriptions or authoritative redescriptions of the species (see Table 1 for references and Fig. 1–3, S2–S4 in the Supporting Information). The classification used follows Agatha and Strüder-Kypke (2013), except for the family Eutintinnidae, which had been established by Bachy et al. (2012).

DNA extraction, PCR, cloning and sequencing

Eighteen isolates were individually picked for photographing and were subsequently subjected to DNA extraction; the remaining 11 isolates (marked by asterisks) for DNA extraction were not from the individuals illustrated in Fig. 1–3, but from morphologically identical specimens in the same sample (Table 1). Another 25 individuals were directly subjected to DNA extraction, i.e. without photographing. DNA extraction was performed according to Gong et al. (2007). The PCR amplifications were carried out with a Fermentas Dreamtaq™ DNA Polymerase Kit (Thermo Fisher Scientific Inc., Waltham, MA). The PCR primers for amplifying nearly full-length 18S rDNA genes (1.7 kb), the ITS regions (0.55 kb) and the partial 28S rDNA genes (1.5 kb) are listed in Table S1. Briefly, the 18S rDNA sequences were amplified, using the primers

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Table 1. A summary of identified isolates, rDNA sequences, sampling sites, literature used for identification (see Supporting Information for discussion), the analyst and the associated micrographs

Morphospecies	Isolate ID[a]	18S[b]	ITS[b]	28S[b]	Date	Location	Lat (N)	Long (E)	Tem (°C)	Sal	Ref.[c]	Analyst[d]	Figure
Amphorellopsis sp.	9203	KU715756			2011-09-20	YUBB	37.46	121.45	23	31	[1]	JD	1C
Amphorellopsis sp.	10191	KU715757			2011-10-19	YUBB	37.46	121.45	18	31	[1]	JD	1D
Amphorellopsis sp.	12	KU715758	KU715794		2011-10-31	Q2BB	36.05	120.35	21	35	[1]	JD	1E
Dictyocysta sp.	J34		**KU715795**	**KU715777**	2011-05-19	ECS, site D9	26.97	126.12	25	35	[1]	WZ	3B
Eutintinnus cf. *apertus*	E	KU715759	KU715796	KU715778	2011-05	France, Berre Lagoon	43.3	5.37	20	30	[2]	WZ	1F
Eutintinnus macilentus	J2		**KU715797**	**KU715779**	2011-06-05	ECS, site BY	21.66	116.63	NA	NA	[3]	WZ	1G
Favella panamensis	7-20	KU715760	**KU715798**		2011-07-11	QJZB, site A3	36.16	120.25	23	30	[1]	WZ	2E
Laboea strobila	85*		KU715799	**KU715780**	2011-06-13	QJZB, site D3	36.04	120.23	18	32	[4]	WZ	3A
Leprotintinnus nordqvisti	7-6	**KU715761**	**KU715800**		2011-07-11	QJZB, site A3	36.16	120.25	23	30	[5, 6]	WZ	1H
Leprotintinnus simplex	7-23*		**KU715801**	**KU715781**	2011-07-11	QJZB, site A5	36.16	120.33	23	31	[7]	WZ	1I
Rhabdonella valdestriata	J33		**KU715802**	**KU715782**	2011-05-19	ECS, site D9	26.97	126.12	25	35	[8]	WZ	1J
Rhizodomus tagatzi	9201	KU715762		**KU715783**	2011-09-20	YUBB	37.46	121.45	23	31	[5, 6]	JD	2F
Stenosemella sp.	4	KU715763	KU715803	KU715784	2011-10-31	Q2BB	36.05	120.35	21	35	[1]	WZ	3D
Stenosemella ventricosa	J19	KU715764	KU715804	**KU715785**	2011-05-27	ECS, site N2	32.04	122.5	17	32	[9]	WZ	3C
Schmidingerella quequenensis	J30	**KU715765**	**KU715805**	**KU715786**	2011-05-22	ECS, site C2	30.6	122.83	18	32	[10]	WZ	2D
Tintinnidium cf. *primitivum*	101*	KU715766	KU715806		2011-06-14	QJZB, site B2	36.13	120.19	18	32	[11]	WZ	1A
Tintinnidium mucicola	27	KU715767	**KU715807**	KU715787	2011-10-31	Q2BB	36.05	120.35	21	35	[5, 6]	WZ	1B
Tintinnopsis baltica	29*		**KU715808**	KU715788	2011-05-10	QJZB, site D5	36.03	120.29	13	31	[12]	WZ	3H
Tintinnopsis brasiliensis	21*	**KU715768**	**KU715809**	**KU715789**	2011-10-31	Q2BB	36.05	120.35	21	35	[1]	WZ	3F
Tintinnopsis cylindrica	7102*	KU715769	**KU715810**		2011-07-10	YUBB	37.46	121.45	23	31	[13]	JD	2G
	31*		KU715811	KU715790	2011-05-10	QJZB, site D5	36.03	120.29	13	31	[13]	WZ	2H
Tintinnopsis fistularis	10*	**KU715770**	**KU715812**		2011-10-31	Q2BB	36.05	120.35	21	35	[14]	WZ	2J
Tintinnopsis hemispiralis	7*		**KU715813**		2011-10-31	Q2BB	36.05	120.35	21	35	[15]	WZ	2I
Tintinnopsis parvula	30*	KU715771	**KU715814**	KU715791	2011-05-10	QJZB, site D5	36.03	120.29	13	31	[16]	WZ	3I
Tintinnopsis radix	J13	KU715772	**KU715815**		2011-05-18	ECS, site E1	28.21	121.8	18	21	[17]	WZ	2A
	J8	KU715773	KU715816		2011-06-01	ECS, site B1	31.13	122.43	19	25	[17]	WZ	2B
	101913	KU715774			2011-10-19	YUBB	37.46	121.45	18	31	[17]	JD	2C
Tintinnopsis sp.	60*	KU715775	KU715817	KU715792	2011-05-11	QJZB, site B2	36.13	120.19	14	30	[1]	WZ	3G
Tintinnopsis cf. *ventricosoides*	58	**KU715776**	**KU715818**	**KU715793**	2011-05-11	QJZB, site B2	36.13	120.19	14	30	[18]	WZ	3E

[a] "*" indicates isolates for which DNA was not extracted from the individuals measured and shown on the micrographs, but from morphologically identical morphotypes in the same sample.

[b] Accession numbers for sequences obtained in the present study. Loci of a given species that have sequenced for the first time in this study are marked in bold.

[c] [1] Kofoid and Campbell (1929); [2] Kofoid and Campbell (1939); [3] Jörgensen (1924); [4] Agatha et al. (2004); [5, 6] Brandt (1906, 1907); [7] Schmidt (1901); [8] Strelkow and Wirketis (1950); [9] Fol (1884); [10] Balech (1945); [11] Busch (1925); [12] Brandt (1896); [13] Agatha and Riedel-Lorjé (2006); [14] Meunier (1919); [15] Yin (1956); [16] Agatha (2010); [17] Jiang et al. (2012); [18] Meunier (1910).

[d] Analyst: JD, Jun Dong; WZ, Wuchang Zhan.

ECS, East China Sea; Q2BB, The 2nd bathing beach in Qingdao; QJZB, Jiaozhou Bay of Qingdao; YUBB, Yantai University bathing beach; Lat, latitude; Long, longitude; Ref, reference of original description or authoritative redescription; Sal, salinity; Tem, temperature.

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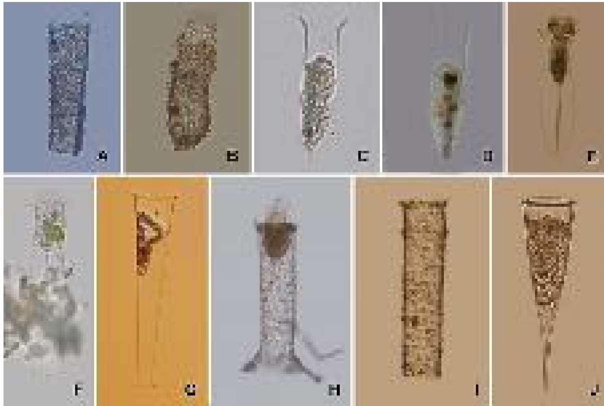

Figure 1 Light micrographs of tintinnids collected for gene sequencing. See Table 1 for sampling sites, isolate numbers and accession numbers. The lorica length (*L*) and the opening diameter (OD) are given in brackets. (**A**) *Tintinnidium* cf. *primitivum* isolate 101 (*L* = 130 μm, OD = 36 μm). (**B**) *Tintinnidium mucicola* isolate 27 (*L* = 120 μm, OD = 35 μm). (**C**) *Amphorellopsis* sp. isolate 9203 (*L* = 166 μm, OD = 54 μm). (**D**) *Amphorellopsis* sp. isolate 10191 (*L* = 183 μm, OD = 53 μm). (**E**) *Amphorellopsis* sp. isolate 12 (*L* = 145 μm, OD = 43 μm). (**F**) *Eutintinnus* cf. *apertus* isolate E (*L* = 198 μm, OD = 37 μm). (**G**) *Eutintinnus macilentus* isolate J2 (*L* = 224 μm, OD = 46 μm). (**H**) *Leprotintinnus nordqvisti* isolate 7-6 (*L* = 156 μm, OD = 45 μm). (**I**) *Leprotintinnus simplex* isolate 7-23 (*L* = 175 μm, OD = 42 μm). (**J**) *Rhabdonella valdestriata* isolate J33 (*L* = 220 μm, OD = 60 μm).

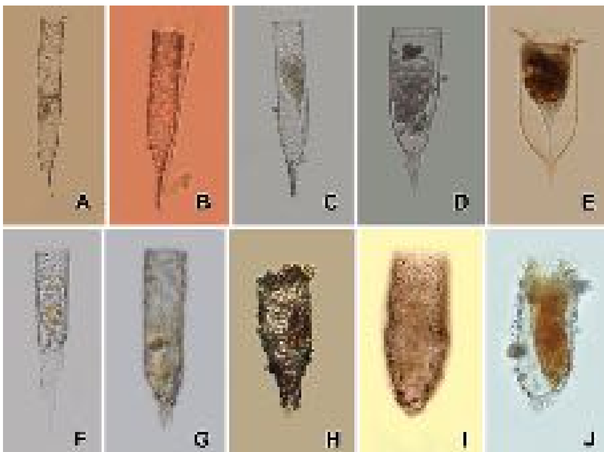

Figure 2 Light micrographs of tintinnids collected for gene sequencing. See Table 1 for sampling sites, isolate numbers and accession numbers. The lorica length (*L*) and the opening diameter (OD) are given in brackets. (**A–C**) *Tintinnopsis radix* isolates J13 (*L* = 340 μm, OD = 45 μm), J8 (*L* = 364 μm, OD = 65 μm), 101913 (*L* = 284 μm, OD = 47 μm). (**D**) *Schmidingerella quequenensis* isolate J30 (*L* = 235 μm, OD = 67 μm). (**E**) *Favella panamensis* isolate 7-20 (*L* = 228 μm, OD = 79 μm). (**F**) *Rhizodomus tagatzi* isolate 9201 (*L* = 174 μm, OD = 33 μm). (**G, H**) *Tintinnopsis cylindrica* isolate 7102 (*L* = 152 μm, OD = 34 μm), isolate 31 (*L* = 107 μm, OD = 38 μm). (**I**) *Tintinnopsis hemispiralis* isolate 7 (*L* = 114 μm, OD = 34 μm). (**J**) *Tintinnopsis fistularis* isolate 10 (*L* = 50 μm, OD = 24 μm).

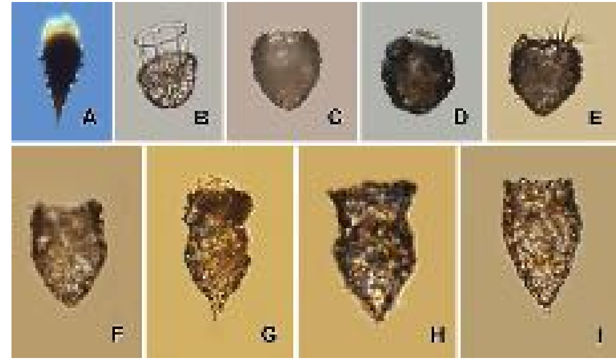

Figure 3 Light micrographs of an oligotrichid (**A**) and tintinnid choreotrichids (**B–I**) collected for gene sequencing. See Table 1 for sampling sites, isolate numbers and accession numbers. The lorica or cell length (*L*) and the opening diameter (OD) or cell width (W) are given in brackets. (**A**) *Laboea strobila* isolate 85 (*L* = 99 μm, W = 47 μm). (**B**) *Dictyocysta* sp. isolate J34 (*L* = 54 μm, OD = 36 μm). (**C**) *Stenosemella ventricosa* isolate J19 (*L* = 81 μm, OD = 43 μm). (**D**) *Stenosemella* sp. isolate 4 (*L* = 72 μm, OD = 32 μm). (**E**) *Tintinnopsis ventricosoides* isolate 58 (*L* = 63 μm, OD = 40 μm). (**F**) *Tintinnopsis brasiliensis* isolate 21 (*L* = 77 μm, OD = 46 μm). (**G**) *Tintinnopsis* sp. isolate 60 (*L* = 65 μm, OD = 29 μm). (**H**) *Tintinnopsis baltica* isolate 29 (*L* = 64 μm, OD = 41 μm). (**I**) *Tintinnopsis parvula* isolate 30 (*L* = 44 μm, OD = 23 μm).

18S-F and 18S-R (Gong et al. 2007). To obtain the ITS and 28S rDNA regions, two pairs of primers (ITS-F/-R or ITS1/4; Cili28s/Cili28s-1000 or 5.8S+/LR7) were applied alternatively for each region (Gardes and Bruns 1993; Gong et al. 2007; Vilgalys and Hester 1990; White et al. 1990; Yi et al. 2009). The PCR products were purified, using the Gel Extraction Kit (Qiagen Inc., Beijing, China), and inserted into a pUCm-T cloning vector (Sangon Biotechnoloyg Inc., Shanghai, China). The clones with the target amplicons were screened by PCR amplifications and sequenced in both directions, employing an ABI 3700 sequencer (Invitrogen Biotechnology Co., Ltd, Shanghai, China), following Liu and Gong (2012).

Phylogenetic analyses

The published rDNA sequences of tintinnids, oligotrichids and outgroup taxa (e.g. hypotrichs and euplotids) were retrieved from GenBank. Sequences of each locus were aligned separately, using the online aligner MAFFT (Katoh and Standley 2013), and manually refined, employing SeaView (Gouy et al. 2010). The highly variable regions and poorly aligned sites were manually excluded prior to analyses. The final alignments comprised 163 sequences with 1,701 positions of the 18S rDNA, 75 sequences with 480 positions of the ITS region, and 49 sequences with 1,227 positions of the 28S rDNA. Maximum likelihood (ML) trees were constructed using RAxML (Stamatakis et al. 2008) with a GTR + I + Γ model for each of the three rDNA loci. The reliability of the internal branches was assessed using a nonparametric bootstrap method with 1,000 replicates for all loci. Bayesian inference (BI) was executed with MrBayes

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3.1.2 (Ronquist and Huelsenbeck 2003) under a GTR + I + Γ model. Markov chain Monte Carlo simulations (MCMC) with four chains (default temperature parameter, sampling frequency 0.01) were run for three million generations at which the mean standard deviation of split frequencies was < 0.01. The first 25% of the generations were abandoned as burn-in. For each locus, monophyletic *Tintinnopsis* groups with bootstrap supports higher than 50% or with posterior probabilities higher than 0.9 were referred to as *Tintinnopsis* clades I-IX (Bachy et al. 2012; thereafter shortened as TIPS I-IX).

Relative variability among 18S, ITS and 28S rDNA sequences

The whole ITS region, ITS1, ITS2 and the D1-D2 region of the 28S rDNA were each compared with the 18S rDNA concerning their sequence variations. We considered two rDNA loci originally obtained from an identical specimen or a clone from rDNA libraries as a locus pair, which was extracted from the published datasets (Bachy et al. 2012, 2013, 2014; Kim et al. 2013; Li et al. 2013; Santoferrara et al. 2012, 2013; Snoeyenbos-West et al. 2002; Zhao et al. 2012) and the present study. A total of 377 pairs of ITS-18S and 117 pairs of 28S-18S sequences of tintinnids were compiled and aligned, using MAFFT. Among these, 33 pairs of ITS-18S and 27 pairs of 28S-18S were contributed by the present work. Pairwise distances were estimated by MEGA (Tamura et al. 2007) with a pairwise-deletion and the P-distance model. Regression and calculation of coefficients (R^2) of locus pairs were performed with Microsoft Excel.

RESULTS

A total of 54 choreotrichid isolates were collected, among which 26 ciliate morphospecies (corresponding to 29 isolates) were identified to species level and 25 isolates were identified to genus or higher taxonomic level and treated as unidentified tintinnids. The identified morphospecies included 25 tintinnid species belonging to 11 genera and the tontoniid oligotrichid species *Laboea strobila* (Table 1 and Fig. 1–3). Cloning and sequencing yielded 21 sequences of 18S rDNA, 25 sequences of ITS regions, and 17 sequences of 28S rDNA, of which 5, 17 and 10 sequences, respectively, were linked for the first time with a species or genus name (Table 1). From the 25 unidentified tintinnids, 25 sequences of the 18S rDNA, 18 sequences of the ITS regions and 21 sequences of the 28S rDNA were obtained (Tables S2, S3) and added to the comparison of 18S rDNA, ITS and 28S rDNA loci. All new sequences have been deposited in GenBank under the accession numbers KU524761 to KU524878 and KX158679 to KX158738 (Tables 1, S2, S3).

Phylogeny inferred from the 18S rRNA genes

The ML tree based on the 18S gene sequences indicates a monophyly of the subclass Choreotrichida with a bootstrap support of 56%, but without BI support (Fig. 4), while the monophyly of the tintinnids is moderately supported (49% ML, 0.92 BI). The aloricate choreotrichids are paraphyletic, comprising three lineages: the Lynnellidae, Strobilidiidae and the nonmonophyletic Strombidinopsidae. The family Tintinnidiidae represented by the genus *Tintinnidium* is sister group to the remaining tintinnid taxa, which form a highly supported clade (92% ML, 1.00 BI).

The new sequences of *Tintinnidium*, *Amphorellopsis, Eutintinnus*, *Favella*, *Stenosemella*, *Schmidingerella, Tintinnopsis* and *Rhizodomus* species generally cluster with their congeners in the families Tintinnidiidae, Tintinnidae, Eutintinnidae, Ptychocylididae, Stenosemellidae and Rhabdonellidae, respectively (Fig. 4, 5); please, note that the genera *Rhizodomus*, *Stylicauda* and *Tintinnopsis* are *incertae sedis* according to Agatha and Strüder-Kypke (2013).

The 18S sequence of the *Favella panamensis* isolate 7-20 differs from those of previously reported *F. panamensis* and *F. ehrenbergii* isolates in only one and two nucleotides, respectively (Fig. 4). The genus *Stenosemella* is not monophyletic, viz., the new *Stenosemella* isolate 4 groups with *Stenosemella nivalis* and the *S. ventricosa* isolate CB875 (JQ408170), while the new *S. ventricosa* isolate J19 is also far apart from the *S. ventricosa* isolate SFL02-1 (EU399538) and *S. pacifica* (Fig. 5). The families Dictyocystidae, Metacylididae, Ptychocylididae, Rhabdonellidae, Stenosemellidae and Tintinnidiidae as well as the genus *Tintinnopsis* are not monophyletic in the 18S rDNA tree (Fig. 4, 5).

The five previously known major clades of the genus *Tintinnopsis*, namely *Tintinnopsis* clade I to V (TIPS I–V), are generally well recovered; yet, TIPS I is expanded and split into two highly supported subclades, i.e. TIPS I-1 and TIPS I-2 (Fig. 5). Likewise, TIPS III is subdivided into three clades with full ML and BI supports for TIPS III-1 and nearly full support (96% ML, 1.00 BI) for TIPS III-3; TIPS III-2 is represented by merely a single species, viz., *Tintinnopsis parvula* (JN831825). Interestingly, the *T. parvula* isolate 30 does not group with the US isolate JN83125. Nine further 18S rDNA sequences of seven *Tintinnopsis* species were added, and four new *Tintinnopsis* clades (TIPS VI-IX; Fig. 5) were established in the present study. Interestingly, the first SSU rDNA sequence of a *Leprotintinnus* species (*L. nordqvisti*) clusters with *Tintinnopsis radix* with strong support (100% ML, 1.00 BI).

Phylogeny inferred from ITS regions

For the first time, ITS sequences of the genera *Dictyocysta* and *Leprotintinnus* are available. The topology of the ITS genealogy matches that of the 18S rDNA tree at family and higher ranks (Fig. 6). Briefly, the monophyly of the Tintinnina is highly supported (91% ML, 1.00 BI), among which the Tintinnidiidae, Tintinnidae, Eutintinnidae and Ptychocylididae successively represent adelphotaxa to the remaining tintinnids. The relationships of the other tintinnids are similar to that in the 18S rDNA genealogy. Two clusters are recognized among the nonmonophyletic TIPSs clades: one is formed by the families Stenosemellidae and Dictyocystidae (76% ML, 1.00 BI), while the

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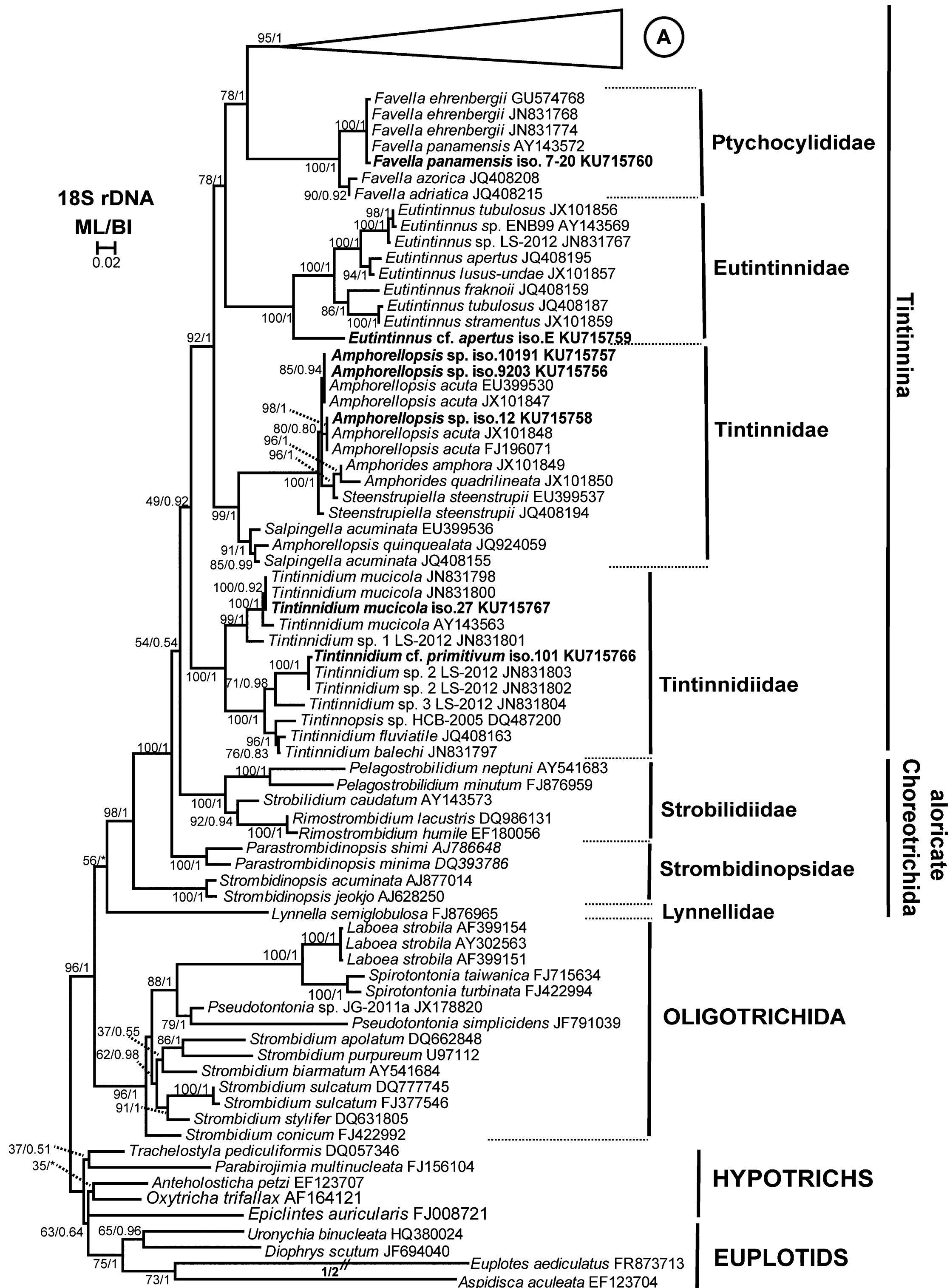

Figure 4 The maximum likelihood (ML) phylogenetic tree of oligotrichid and choreotrichid 18S rDNA sequences, with hypotrichs and euplotids as outgroups. Names in bold represent sequences obtained in this study; accession numbers follow the species names. Numbers at nodes show ML bootstrap values and Bayesian Inference (BI) posterior probabilities; instances in which node was not recovered in the estimated BI tree are designated by an asterisk (*) on the node; bootstrap values less than 30% are not shown. The scale bar corresponds to 0.02 substitutions per nucleotide position. Clade A comprising most *Tintinnopsis* subclades is depicted as a triangle and shown in detail in Fig. 5.

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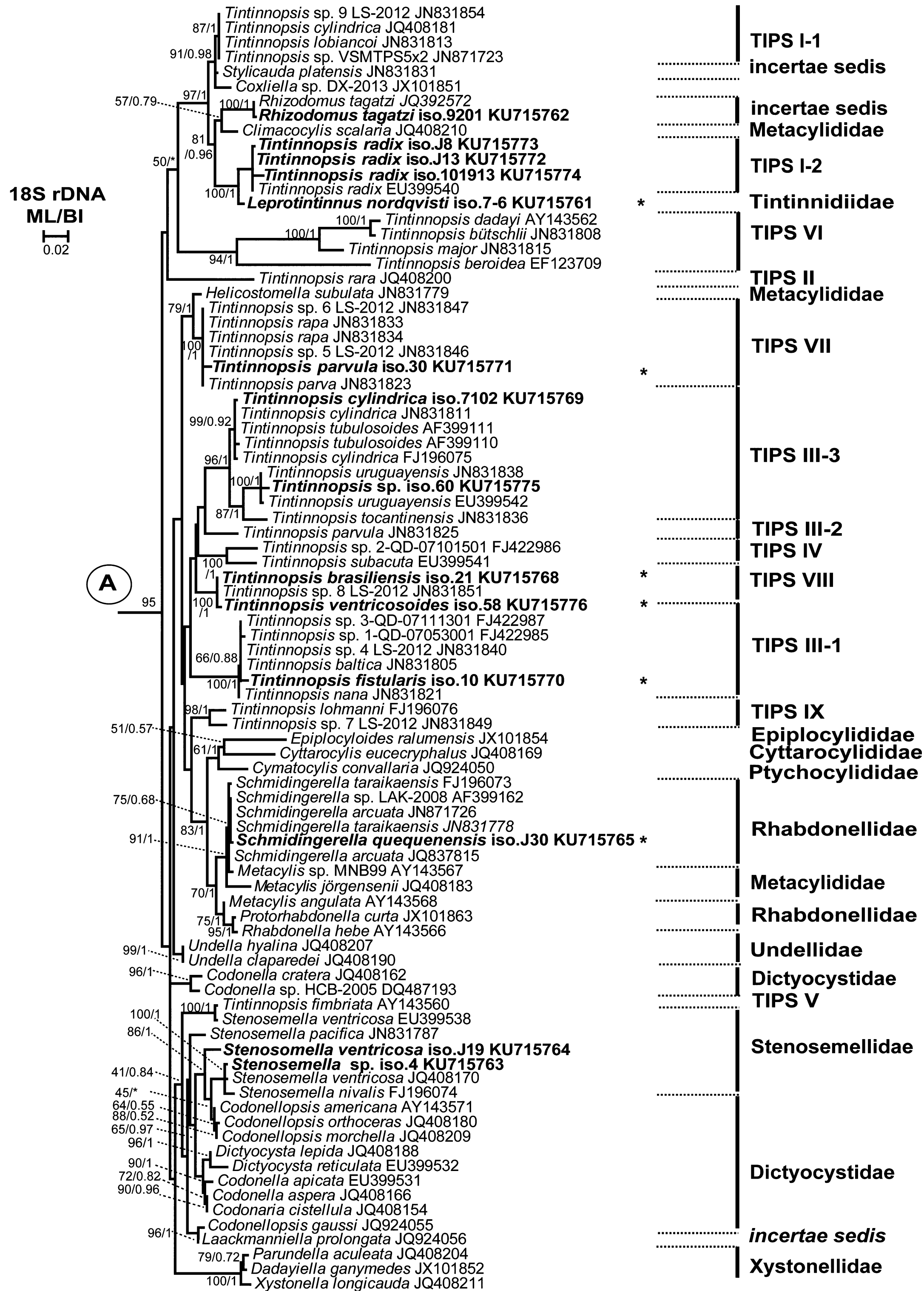

Figure 5 Clade A of the maximum likelihood (ML) tree of 18S rDNA displayed in Fig. 4, showing the nine subclades (TIPS I–IX) of the nonmonophyletic genus *Tintinnopsis*. Names in bold represent sequences obtained in this study; accession numbers follow the species names. Numbers at nodes show ML bootstrap values and BI posterior probabilities; instances in which node was not recovered in the estimated BI tree are designated by an asterisk (*) on the node; bootstrap values less than 30% are not shown. The 18S rRNA genes firstly obtained for morphospecies and genera are labelled with an asterisk "*". The scale bar corresponds to 0.02 substitutions per nucleotide position.

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other assembles the Cyttarocylididae, Rhabdonellidae and Metacylididae. The latter cluster is not as well supported as in the 18S (46% ML, 0.98 BI vs. 83% ML, 1.00 BI).

The ITS dataset is extended by obtaining new sequences of eleven isolates belonging to nine *Tintinnopsis* species. Sequence information of the morphospecies *Tintinnopsis hemispiralis*, *T. fistularis*, *T. brasiliensis* and *T.* cf. *ventricosoides* is first provided in this work. The clades/subclades recognized in the 18S tree (e.g. TIPS I-2, III-1, III-3 and VIII) are well recovered. As in the 18S genealogy, the genus *Leprotintinnus* (represented by *L. nordqvisti* and *L. simplex* differing in 16 sites) is closely related to *Tintinnopsis radix* (100% ML, 1.00 BI). They group with TIPS III-1 represented by *Tintinnopsis baltica* isolate 29, the previously sequenced *Tintinnopsis* sp. AF399014 and *T. fistularis* isolate 10 (Fig. 6), albeit with no support from the ML genealogy (34%ML, 0.97BI). *Tintinnopsis ventricosoides* and *T. brasiliensis* form the new clade TIPS VIII. The newly sequenced species, *T. hemispiralis* and the *T. parvula* isolate 30 do not fall into any TIPSs clade, but form separate branches with variable placements (Fig. 6).

Phylogeny inferred from 28S rRNA gene

In the 28S rDNA tree, *Laboea strobila*, *Strombidium rassoulzadegani*, and one undetermined ciliate represent the Oligotrichida, which form a sister group to the Choreotrichida (Fig. 7). For the first time, 28S sequences of the genera *Dictyocysta*, *Leprotintinnus*, *Rhizodomus*, and *Rhabdonella* are available. The tree topology roughly matches those based on ITS and 18S rDNA analyses (Fig. 4–6).

Two new 28S rDNA sequences of *Tintinnopsis* species were included into the phylogenetic analyses plus one sequence of an unidentified species and three of the already sequenced *T. baltica*, *T. cylindrica* and *T. parvula*. The 28S rDNA sequence of the *T. cylindrica* Qingdao isolate is identical to the former sequence of the US isolate JN831901, whereas the *T. baltica* Qingdao isolate differs by 5.5% from the US isolate JN831895, corresponding to 35 nucleotides. The clades/subclades recognized in the 18S tree (e.g. TIPS I-1, III-1, III-3, VI, VII, VIII, and IX) are again well recovered in the 28S tree.

Relative variability in ITS and 28S regions vs. 18S rDNA

Based on our compiled datasets for tintinnids, we examined the variabilities of four loci, i.e. the ITS, ITS1, ITS2 and D1-D2 regions of the 28S gene and compared them with the variability in the partial 18S rDNA sequences (ca. 1,300-bp fragments covering the V4–V9 regions) in three scenarios (Fig. 8). The distance of each of the four loci covaries well with the 18S differences ranging from 0% to 12% ($R^2 > 0.6$; Fig. 8A–D). When the 18S fragment pairs differ by less than 3%, the differences in the ITS ($R^2 = 0.645$) and ITS2 ($R^2 = 0.565$) are moderately predicted, ranging from 0% to 6.6% and from 0% to 8.2%, respectively (Fig. 8E, G); nevertheless, the variations of the other two loci and the 18S are weakly correlated ($0.35 < R^2 < 0.4$; Fig. 8F, H). Within a 1% difference of the 18S, the corresponding variations of the four loci could not be predicted ($R^2 < 0.17$); Fig. 8I–L.

DISCUSSION

Species identification

All tintinnid isolates sequenced in the present study were identified to the lowest possible taxonomic rank based on the measurements of the lorica length and the opening diameter and the photomicrographs showing the loricae in lateral views under the light microscope. Further characters, such as the lorica shape, the presence of agglutinated particles on the whole lorica or exclusively on its posterior portion, the presence of a posterior opening, and the bowl width were inferred from the micrographs.

Since the species circumscriptions usually deviate in revisionary taxonomic works from the original descriptions owing to subjective lumping and splitting of species, the monographs of Kofoid and Campbell (1929, 1939) and Zhang et al. (2012b) were merely used as first steps of identification; the final determinations were exclusively based on the original descriptions or authoritative redescriptions (in the case the original description is insufficient). Original illustrations (Fig. S2–S4) and detailed discussions of the individual determinations are provided allowing vetting the identifications (Supporting Information).

The determinations of *Eutintinnus macilentus* (Fig. 1G), *F. panamensis* (Fig. 2E), *Rhabdonella valdestriata* (Fig. 1J) and *Schmidingerella quequenensis* (Fig. 2D), all of which have hyaline loricae, as well as of *Rhizodomus tagatzi* (Fig. 2F), *Tintinnidium mucicola* (Fig. 1B), *Stenosemella ventricosa* (Fig. 3C) and *Tintinnopsis radix* (Fig. 2A–C), which have agglutinated loricae, are reliable. However, identification is somewhat more uncertain when the micrographs do not show the sequenced specimens (*Laboea strobila*, *Tintinnopsis baltica*, *T. brasiliensis*, *T. cylindrica*, *T. fistularis*, *T. hemispiralis* and *T. parvula*; Fig. 2G–J, 3A, F, H, I), or it was even impossible when the taxonomically relevant details of the lorica for species discrimination were not recognizable in the micrographs (e.g. *Amphorellopsis* sp.; Table 1 and Fig. 1–3). Species marked by the addition "cf." (confer; to be compared with) somehow deviate from the most similar original description or authoritative redescription (e.g. *Eutintinnus* cf. *apertus* isolate E).

The reports of *Tintinnidium mucicola* from a wide range of salinities indicate a euryhaline species or the presence of a species complex. Isolate 101 (Fig. 1A) is similar in its lorica shape (tube-like with both ends open) and sparse agglutination to *Tintinnidium primitivum*, which, however, has a smaller lorica (length: 39–84 μm vs. 130 μm; opening diameter 21–25 μm vs. 36 μm; Busch 1923, 1925); accordingly, conspecificity is unlikely. In isolate 7-6 (Fig. 1H), the identification required a combination of

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morphological and genetic clues. The lorica shape and size of the isolate match two species, *Leprotintinnus nordqvisti* and *Tintinnopsis brandti*, differing in a posterior lorica opening (present vs. absent), which was not recognizable in the micrograph. Only based on the ITS phylogeny, which revealed a rather close relationship of a typical *Leprotintinnus* species (viz., *L. simplex*; Fig. 1L) and isolate 7-6, its determination as *L. nordqvisti* was feasible. *Leprotintinnus simplex* matches the original description, except for a shorter lorica (175 μm vs. 204 μm). The identification of isolate 58 demonstrates common shortcomings of original literature: Meunier (1910) did not provide lorica measurements, but merely mentioned the diameter of the microscopical field of view in his line drawings. Therefore, it was rather difficult to estimate the original lorica size, and our specimen is thus only tentatively determined as *Tintinnopsis ventricosoides* (see below; Fig. 3E).

Remarks on our newly added species

Our work provides the first sequence information for *Tintinnopsis fistularis* and *T. hemispiralis*. Both, the18S and ITS phylogenies indicate an affiliation of *T. fistularis* with clade TIPS III-1(Fig. 5, 6). *Tintinnopsis hemispiralis* was originally described from Chinese waters (Yin 1956) but had rarely been reported since that time. The analysis of our Chinese isolate suggests that it may represent a potentially distinct subclade in the *Tintinnopsis* complex (Fig. 6).

For *Tintinnopsis brasiliensis*, the tentatively identified *T.* cf. *ventricosoides*, and *Schmidingerella quequenensis*, the present study provides the first 18S rDNA and ITS sequences. According to the original descriptions, *Tintinnopsis brasiliensis* and *T. ventricosoides* are very similar in their lorica shapes and lengths but they apparently differ in their lorica opening diameters [53 μm vs. 26–36 μm as inferred from illustrations in Brandt (1906) and Meunier (1910)]. Yet, *T. brasiliensis* isolate 21 and *Tintinnopsis* cf. *ventricosoides* isolate 58 in this paper have similar oral diameters (40 μm vs. 46 μm), identical 18S and ITS rDNA sequences, and very similar 28S rDNA sequences (differ by two sites). Since the sequence of *Tintinnopsis* cf. *ventricosoides* is not from the specimen whose lorica had been identified, we refrain from any synonymization. The 18S and ITS sequences of *Schmidingerella quequenensis* deviate only by two and four nucleotides, respectively, from those of *S. taraikaensis*, which has a similar-shaped but larger lorica (260–314 × 80–86 μm vs. 219–270 × 72–74 μm). Owing to the absence of cut-off divergences in the marker genes and knowledge about cell features and the intraspecific lorica variability, conspecificity is uncertain in both cases.

Our *Tintinnopsis parvula* isolate 30 does not group with the US isolate JN83125 in the ITS genealogy, and the 28S sequence of the Chinese *Tintinnopsis baltica* isolate 29 differs by about 35 nucleotides from the US isolate JN831895, indicating that in both cases one of the isolates had been misidentified or a homoplasious lorica shape developed in distantly related members of the *Tintinnopsis* complex. Since the micrographs of the US isolates (Santoferrara et al. 2013) and those in this study only provide information on the lorica outlines and sizes, a detailed comparison is impossible and we refrain from any taxonomic decisions. More specimens of these two species need to be inspected simultaneously using both morphological observations and rDNA sequencing to the same individual in the future.

New insights into phylogenetic relationships among tintinnids

The discrepancies between the molecular phylogenies and cladograms based on morphological characters (especially of the cell) on the one hand and the lorica-based classification on the other hand are well known for some years (Agatha and Strüder-Kypke 2007, 2012, 2013, 2014; Bachy et al. 2012; Gao et al. 2009; Li et al. 2009; Santoferrara et al. 2012; Strüder-Kypke and Lynn 2008). Hence, we did not expect that the addition of tintinnid sequences will cause far-reaching changes in the general topology of the gene trees, e.g. resulting in a monophyly of the genus *Tintinnopsis*. However, with additional sequences the analyses of not only the 18S rDNA, but also of the ITS regions and the 28S rDNA should provide a better resolution among congeners. The inclusion of sequences from additional genera (especially *Leprotintinnus*) gave very interesting insights into the phylogenetic relationships of tintinnids.

Firstly, the analyses of *Leprotintinnus* sequences indicate a close relationship with *Tintinnopsis radix* (Fig. 5, 6) in the 18S rDNA and ITS trees and with *Stylicauda platensis* in the 28S rDNA genealogy (Fig. 7). This clearly contradicts the current lorica-based classification, in which the genus is placed together with *Membranicola* and *Tintinnidium* in the Tintinnidiidae based on their (supposedly) soft and often sparsely agglutinated loricae; such loricae were assumed to represent the plesiomorphic character state typical for most freshwater tintinnids. However, the original description by Imhof (1886) and the redescriptions (Jiang et al. 2012; Laackmann 1913) of *Tintinnopsis radix* as well as the original description of *Stylicauda platensis* by da Cunha and da Fonseca (1917; reported as *Tintinnopsis platensis*) also mention a sparse agglutination of particles, but not a softness of the loricae; and actually, the loricae of the sequenced *Leprotintinnus* specimens were not investigated for their rigidity.

The placement in the molecular phylogeny suggests that *Leprotintinnus* has a rather complex somatic ciliary pattern with a ventral kinety instead of the comparatively simple pattern with two ventral organelles as found in the Tintinnidiidae and *Tintinnopsis cylindrata* [for the discussion of its generic assignment, see Agatha and Riedel-Lorjé (2006), Agatha and Strüder-Kypke (2007, 2012, 2013, 2014), and Petz and Foissner (1993)]. This is supported by an anecdotal observation by SA on one poorly impregnated specimen of *Leprotintinnus bottnicus* from the North Sea showing a complex somatic ciliary pattern

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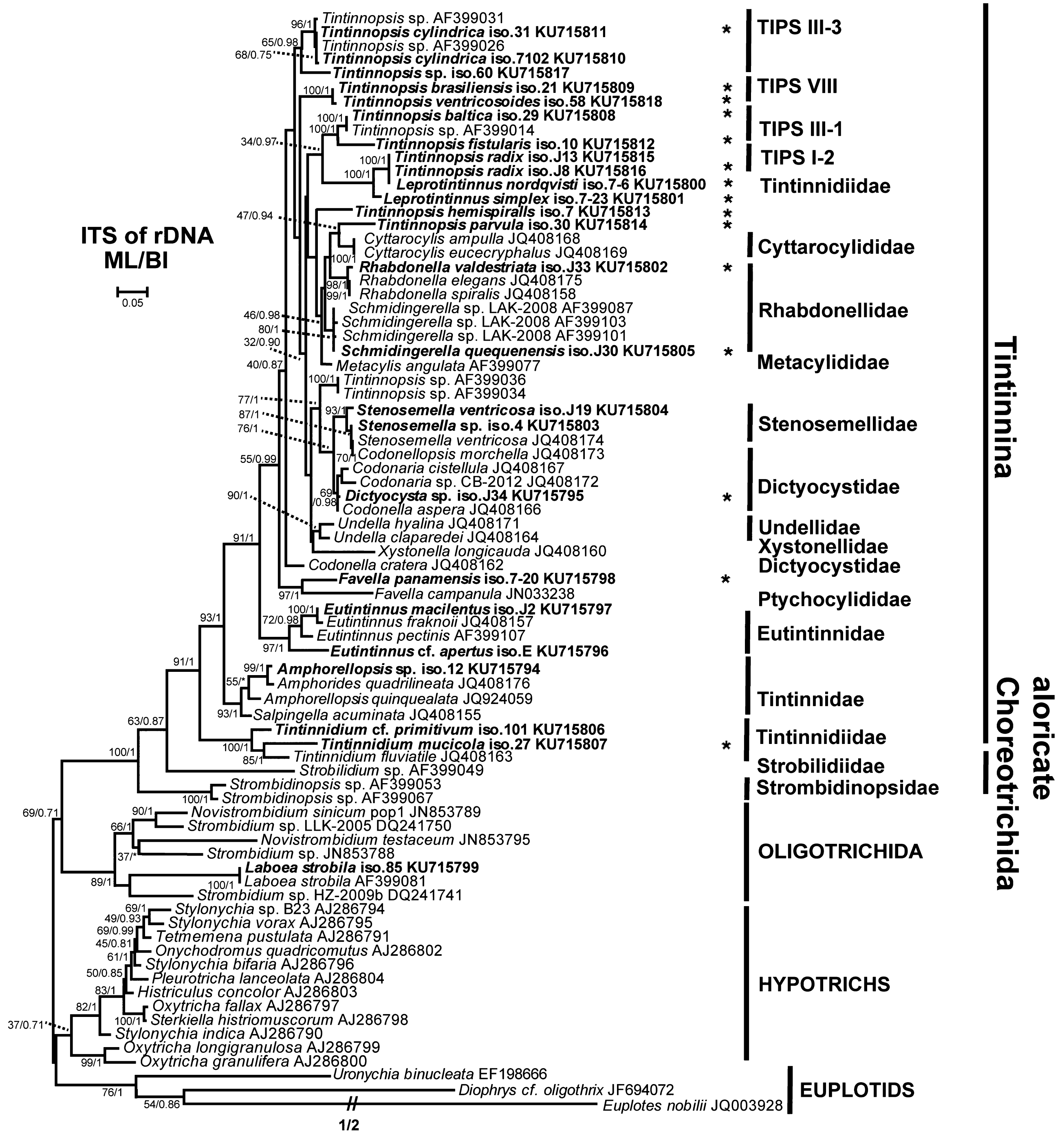

Figure 6 The maximum likelihood (ML) tree based on the ITS1-5.8S-ITS2 (ITS) sequences, with hypotrichs and euplotids as outgroups. Names in bold represent sequences obtained in this study. Numbers at nodes show ML bootstrap values and BI posterior probabilities; instances in which node was not recovered in the estimated BI tree are designated by an asterisk (*) on the node; bootstrap values less than 30% are not shown. ITS regions firstly obtained for morphospecies and genera are labelled with an asterisk "*". The scale bar corresponds to 0.05 substitutions per nucleotide position.

(S. Agatha, data unpublished). Accordingly, the sparse agglutination probably represents a homoplasy. Considering that the lorica matrix material extruded by the ciliate might vary in its stickiness and several genera combine an agglutinated bowl with a hyaline collar (Agatha et al. 2013), such a homoplasy is not surprising. We refrain here from transferring the genus *Leprotintinnus* to another tintinnid family as this necessitates (i) redescriptions of the type species of the genera *Leprotintinnus* (*L. pellucidus*) and *Tintinnopsis* (*T. beroidea*) based on

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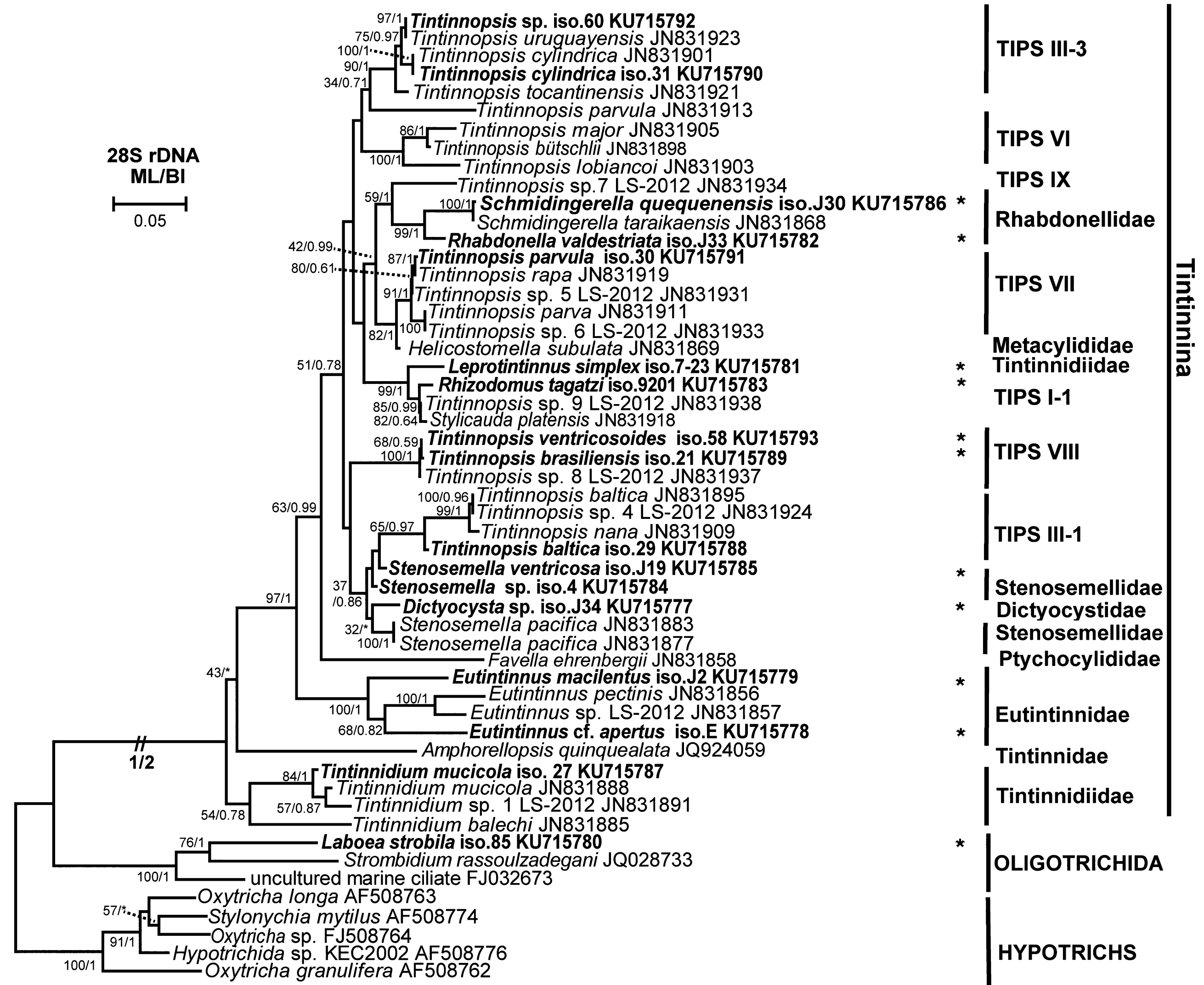

Figure 7 The maximum likelihood (ML) tree for the 28S rDNA dataset of 1,227 nucleotide sites, with hypotrichs as outgroup. Names in bold represent sequences obtained in this study and accession numbers follow the species names. Numbers at nodes show ML bootstrap values and BI posterior probabilities; instances in which node was not recovered in the estimated BI tree are designated by an asterisk (*) on the node; bootstrap values less than 30% are not shown. The 28S rRNA genes firstly obtained for morphospecies and genera are labelled with an asterisk "*". The scale bar corresponds to 0.05 substitutions per nucleotide position.

silver-stained material and (ii) verification that the *T. beroidea* isolate in the gene tree was reliably identified.

Secondly, it is known for a decade that genus *Tintinnopsis* is not monophyletic in both cladistic analyses of the morphological characters and genetic phylogenies (Agatha and Strüder-Kypke 2007, 2012, 2013, 2014; Bachy et al. 2012; Gao et al. 2009; Li et al. 2009; Snoeyenbos-West et al. 2002). It has become obvious that the possession of an entirely agglutinated lorica does not indicate a close relationship, but might merely represent homoplasious character states; actually, the preliminary data suggest that different somatic ciliary patterns might characterize the clades emerging in the genealogies. Accordingly, some genera grouping with the *Tintinnopsis* clades TIPS 1-1 and 2 have totally different lorica structures, e.g. *Climacocylis* with its distinctly spiralled lorica ribs (Fig. 5). Agatha and Strüder-Kypke (2013) kept genera that resemble *Tintinnopsis* in an entirely agglutinated lorica, viz., *Stylicauda* and *Rhizodomus* (both *incertae sedis* like *Tintinnopsis*), as they might be useful in the future to give home to some of the previous *Tintinnopsis* clades.

Although the sequences of the genus *Tintinnopsis* are scattered all over the 18S tree (present and previous analyses), five stable monophyletic clades established by Bachy et al. (2012) are recovered by the present study. We follow their numbering system, but define arbitrarily further monophyletic clades (VI–IX) with a stricter criterion (see "Materials and Methods"). Among these, TIPS I, III-1, III-3, VI, VII, VIII and IX are supported by at least two loci, suggesting stability of these lineages. The

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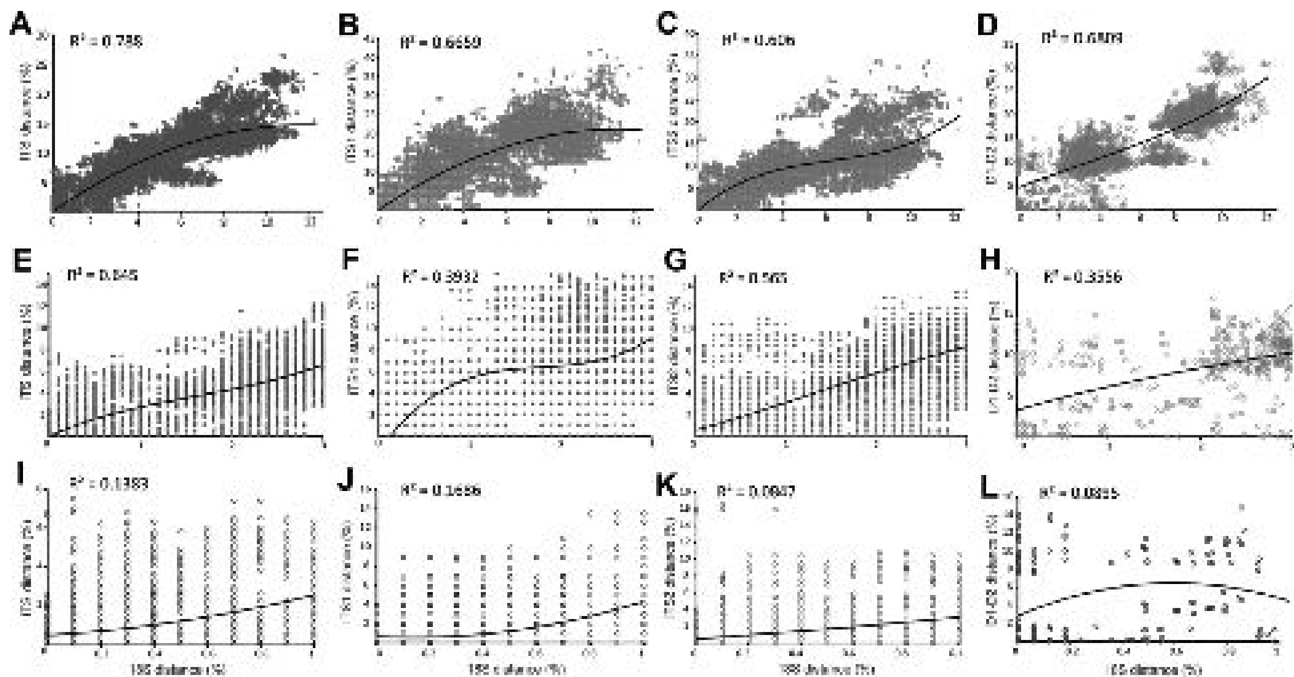

Figure 8 Comparisons of variability in the whole ITS (ITS1-5.8S-ITS2) region (**A, E, I**), two separate regions namely ITS1 (**B, F, J**) and ITS2 (**C, G, K**), and the D1-D2 region of 28S (**D, H, L**) relative to that of the nearly full-length 18S rDNA in tintinnids. Solid regression lines show the best fitted curves describing the relationship between variations of two markers. Relationships between all compiled locus pairs (A–D), those with 18S difference no more than 3% (E–H) and 1% (I–L) are shown separately.

introduction of *Tintinnopsis* subclades facilitates their recognition and the comparison of topologies in genealogies based on different taxa and marker genes.

Thirdly, our 18S rDNA, ITS and 28S rDNA phylogenies do not deviate significantly in their general topology from each other and previous genealogies as anticipated (cp. Fig. 4–7): the moderately supported relationship of the families Stenosemellidae and Dictyocystidae as well as the clustering of the families Cyttarocylididae and Rhabdonellidae in the 18S trees (Fig. 4, 5) were both recovered by the other two rDNA loci. This indicates a well-correlated evolution rate for the three rDNA loci, which corresponds to our comparison of their genetic distances (Fig. 8). In contrast with the ITS and 18S genealogies, the branches of the oligotrichids and hypotrichs are much shorter than that of the choreotrichids in the 28S rDNA phylogeny (Fig. 7), suggesting a higher variability in the choreotrichid 28S rDNA operon; the same had been reported for the choreotrichid α-tubulin genes in a previous study (Snoeyenbos-West et al. 2002).

Towards defining rDNA cut-off divergences for tintinnid morphospecies in ecological studies

In rDNA-based ecological studies of protistan diversity, different cut-off values for rDNA divergences are usually suggested to reflect OTU (operational taxonomic unit) diversity in the communities (Bachy et al. 2013, 2014). Several studies have tried to define cut-off divergences of the 18S, ITS and 28S for delimiting certain tintinnid morphospecies (Bachy et al. 2012, 2013, 2014; Santoferrara et al. 2013, 2015). In the present study, we tested whether the observed discontinuities in the variability in the gene sequences and regions can be generally applied for separating a broad range of tintinnid species and examined the congruence between different cut-off values of rDNA markers in circumscribing morphospecies.

The rDNA loci sequenced in the previous and present study have allowed assessing and comparing the relative variability in each locus in tintinnid ciliates. The variation in the ITS region highly co-varies with difference in the 18S gene of up to 12% (Fig. 8A), which is consistent with the study of Bachy et al. (2013). However, differences in the 18S gene of up to 12% are apparently caused by including not only sequence variations among species, but also of higher taxonomic levels (e.g. genera and families). Inspecting a narrow range of 18S difference may allow us to approach a relationship between the 18S gene and the ITS region at species level. Actually, by constraining the 18S distance within a 3% variability, changes of the whole ITS region and the ITS2 were predictable, suggesting these two loci can be used alternative markers under some conditions (e.g. assessing genetic diversity of tintinnids with a metabarcoding approach). Our analyses based on a broader sampling indicate that the previously estimated intraspecific variations of the rDNA loci in tintinnids are not congruent, especially when the 18S distances range of 0% to 1% was considered for a morphospecies (Bachy et al. 2012, 2013, 2014; Santoferrara et al. 2013, 2015).

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AUTHOR CONTRIBUTIONS

S.A. identified the species; W.Z., J.D. and Y.Y. provided the micrographs and measurements; J.D. and Y.Y. performed the molecular cloning and sequencing; Q.Z. analysed the molecular data; Q.Z., W.Z., N.J. and J.G. provided materials and consumables; Q.Z., S.A. and J.G. wrote the manuscript; and J.G. conceived the research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Map showing the sampling sites of the present study.
Figure S2. Line drawings of loricae from the original descriptions or authoritative redescriptions (A, from Claparède and Lachmann 1858; B–E, from Brandt 1906; F, from Jörgensen 1924; G, H, from Nordqvist 1890; I–L, from Brandt 1906; M, from Schmidt 1901; N–U, from Brandt 1906).
Figure S3. Line drawings of loricae from the original descriptions or authoritative redescriptions (A–C, from Laackmann 1913; D, E, from Jiang et al. 2012; F–I, from Balech 1945; J, from Kofoid and Campbell 1929; K–R, from Strelkow and Wirketis 1950; S, from Daday 1887; T, from Agatha and Riedel-Lorjé 2006; U–X, from Yin 1956; Y, from Meunier 1919).
Figure S4. Line drawings of cell (A, B) or loricae (C–T) from the original descriptions or authoritative redescriptions (A, from Lohmann 1909; B, from Agatha et al. 2004; C, from Claparède and Lachmann 1858; D, from Fol 1884; E, from Marshall 1934; F–M, from Meunier 1910; N, from Kofoid and Campbell 1929; O, from Möbius 1887; P–S, from Brandt 1906; T, from Agatha 2010).
Table S1. Primers used for PCR amplification and sequencing of the small subunit rDNA (SSU, 18S), the large subunit rDNA (LSU, 28S), and the ITS1-5.8S-ITS2 rDNA regions.
Table S2. Tintinnid species used for gene comparison in this study, sequenced in the present study or retrieved from the GenBank: GenBank accession numbers for 18S and ITS-5.8S rDNA.
Table S3. Tintinnid species used for gene comparison in this study, sequenced in the present study or retrieved from the GenBank: GenBank accession numbers for 18S and 28S rDNA.

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