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Discrepancy between conventional morphological systematics and nuclear phylogeny of tintinnids (Ciliophora: Choreotrichia)

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Abstract: Tintinnid ciliates have been traditionally classified according to morphological and morphometric features of the lorica. To examine if the morphological characteristics of loricae reflect the phylogenetic relationships, we collected 23 tintinnid morphospecies (11 genera) and reconstructed phylogenetic trees based on 84 partial sequences of nuclear small subunit rRNA (SSrRNA) gene fragments of 44 morphospecies from a total of eight families (55 sequences from this study, and 29 sequences from the literature). We found that tintinnid ciliates could be classified into six clades consisting of five major clades. While one clade consisted of one family, each of the other five clades consisted of more than two families. Although two families were found only in one clade, the remaining six families were found in more than two clades. In addition, each of six morphospecies was found in more than two major clades, indicating that they are polyphyletic. Principal coordinate analysis showed that morphology of the lorica overlapped substantially between the clades. These results imply that most of the morphological and morphometric traits of marine tintinnid species do not reflect SSrRNA genetic distances even at family levels. It is necessary to identify new characteristics that reflect phylogenetic relationships robustly.

Key words: marine ciliates, morphological variation, nuclear SSrRNA, phylogeny, Tintinnida

Introduction

Planktonic ciliates with vase-shaped shells called loricae are classified as tintinnids (order Tintinnida) and contain about 1,200 species belonging to >100 genera and 15 families (Pierce & Turner 1993, Taniguchi 1997, Lynn 2008). These ciliates are <0.2 mm in cell size and inhabit fresh water, coastal waters and the deep oceans (Beers & Stewart 1967, Heinbokel & Beers 1979, Uye et al. 1996). Because their prey includes suspended bacteria (Gast 1985, Karayanni et al. 2008), tintinnids are recognized as one of the crucial members making up aquatic microbial loops (Legendre & Rassoulzadegan 1995). In addition, tintinnid ciliates graze on phytoplankton (Rassoulzadegan & Etienne 1981) and often ingest more than 20% of the primary production (Capriulo & Carpenter 1983, Verity 1985). There are even species that feed preferentially on dinoflagellate (Stoecker et al. 1981) and terminate algal blooming such as red tides through their grazing pressure (Watras et al. 1985). Thus, tintinnid ciliates are an important microzooplankton component playing multiple roles in channeling and transferring energy and materials in aquatic food webs.

Tintinnid ciliate loricae are made of hyaline pseudochitin excreted from the cells and often ornamented by grains and particles available from the surrounding environment. Because the shape, size and structure of the lorica, as well as the pattern and materials used for agglutination, are impressive and likely species-specific (Kofoid & Campbell 1939), classical taxonomy and systematics of tintinnid ciliates have been based on morphological characteristics of the loricae (Kofoid & Campbell 1939, Pierce & Turner 1993, Taniguchi 1997), although these characteristics are not necessarily related to infraciliary structures of the cells that are generally used in the taxonomy and systematics of other Choreotrichia (Laval-Peuto & Brownlee 1986).

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Apart from these taxonomic studies, recent studies have shown that some morphological characteristics of loricae have important ecological implications. For example, a series of studies done by Dolan and colleagues showed that lorica width at the oral part of tintinnid ciliates restricted the size range of edible food and was highly related to avoidance ability against copepod predation (Dolan et al. 2002, Dolan et al. 2006, Dolan et al. 2009, Dolan 2010). These findings suggest that tintinnid species live in restricted ecological niches imposed by the morphology of the lorica as a phylogenetic constraint. Alternatively, tintinnid species may have evolved distinctive morphological characteristics of the lorica through natural selection to adapt to their local environment, regardless of phylogeny. This possibility implies that some morphological characteristics may diverge between closely related species and converge among distantly related species, and thus that they may not necessarily reflect the phylogenetic relationship among tintinnid species.

Recent developments in molecular-biology-based techniques have enabled us to analyze genetic distances between organisms and reconstruct phylogenetic trees without information on the morphological features (Edwards 2009). DNA sequences of nuclear small subunit rRNA (SSrRNA) gene fragments have been used to construct phylogenetic trees of ciliates, including tintinnids (Snoeyenbos-West et al. 2002, Strüder-Kypke & Lynn 2003, Gao et al. 2009). Strüder-Kypke & Lynn (2008) and Li et al. (2009) reconstructed phylogenies based on the SSrRNA gene sequences of several tintinnid species. However, since the number of species (DNA sequences) that they examined was limited, it is not yet clear to what extent the morphological characteristics of loricae reflect the phylogenetic relationships among tintinnid ciliates. In this study, we analyzed the DNA sequence of the nuclear SSrRNA gene fragments of 23 morphospecies (55 sequences) collected along the northeast coast of Japan. Incorporating sequence data from previous studies, we reconstructed SSrRNA phylogeny using a total of 44 tintinnid morphospecies (84 sequences). We then examined how well the morphological characteristics of the loricae reflect the phylogenetic relationships among these tintinnid ciliates.

Materials and Methods

Collection and identification

Tintinnid ciliates used for genetic analyses were collected at several sites in Sendai Bay and its vicinities during the period from June to October 2009 (Fig. 1). Live tintinnids were collected by buckets or water samplers and concentrated into plastic bottles using 20 μm mesh-size net. These live samples were observed under a light microscope at magnifications of ×100 and ×400 to identify morphospecies based on morphological characteristics of the loricae according to Taniguchi (1997). We also referred to descriptions by several authors: Bakker and Phaff (1976) for *Tintinnopsis minuta* Wailes, Hada (1937) for *T. elongata* Daday, Hada (1938) for *T. karajacensis* Brandt, Roxas (1941) for *T. mortenseni* Schmidt, and Cordeiro & Sassi (1997) for *T. tubulosa* Levander. In this study, specimens that we could not identify to species level due to uncertainties in morphological characteristics were not used. After the identification, typical specimens of each morphospecies were photographed and individually fixed with 99% ethanol and stored for genetic analyses. Of the morphospecies examined, microscopic images of *Steenstrupiella steenstrupii* (Claparède & Lachmann) Kofoid & Campbell and *T. lobiancoi* Daday are not shown in Fig. 2 because we failed to take well-focused photographs capturing, the morphological characteristics of these species.

DNA extraction, PCR amplification, cloning and sequencing

Total genomic DNA was extracted from a single cell using QuickExtract DNA Extraction solution (Epicenter, Madison, WI, USA). Samples were incubated at 65°C for 2 h and at 95°C for 20 min in 20 μL of the QuickExtract solution, and were stored at −20°C. We amplified a fragment of the nuclear small subunit ribosomal RNA (SSrRNA) gene from specimens using the nested PCR protocol described by Shimano et al. (2008) except for *Tintinnopsis mortenseni* and *T. tenuis* Stein. Each 30 μL of the first PCR reaction consisted of 3.0 μL of 10× *Ex Taq* buffer, 2.5 mM of each dNTPs, 0.3 μM of each primer of SR1 and SR12 (Nakayama et al. 1996), 0.75 U of *Ex Taq* DNA polymerase (Takara, Tokyo, Japan), and 22.85 μL of extracted DNA. Each 30 μL of the final PCR reaction consisted of 3.0 μL of 10× *Ex Taq* buffer, 2.5 mM of each dNTPs, 0.3 μM of ciliate-specific primer CS322F (Puitika et al. 2007) and 0.3 μM of SR12 (Nakayama et al. 1996), 0.75 U of *Ex Taq* DNA polymerase (Takara, Tokyo, Japan), and 0.1 μL of the first PCR product. We purified the final PCR products using ExoSAP-IT (USB Cleveland, OH, USA), performed cycle-sequencing reactions with a BigDye Terminator Cycle sequencing kit (Applied Biosystems Inc., Foster City, CA, USA), and obtained sequences in both directions with an ABI Prism 3130 sequencer (Applied Biosystems Inc). Following this protocol, the DNA sequences of *T. mortenseni* and *T. tenuis* were not readable because of overlapping peaks in the sequence data. In these specimens, therefore, the first PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Burlington, ON, Canada). Subsequently, three colonies were chosen from each plate in the kit to re-amplify the SSrRNA gene fragment with the primers CS322F and SR12, and the purified PCR products as above. Then, these products were sequenced as above. Within each of these specimens, difference in the sequence among colonies was less than 1% and did not affect phylogenetic analyses.

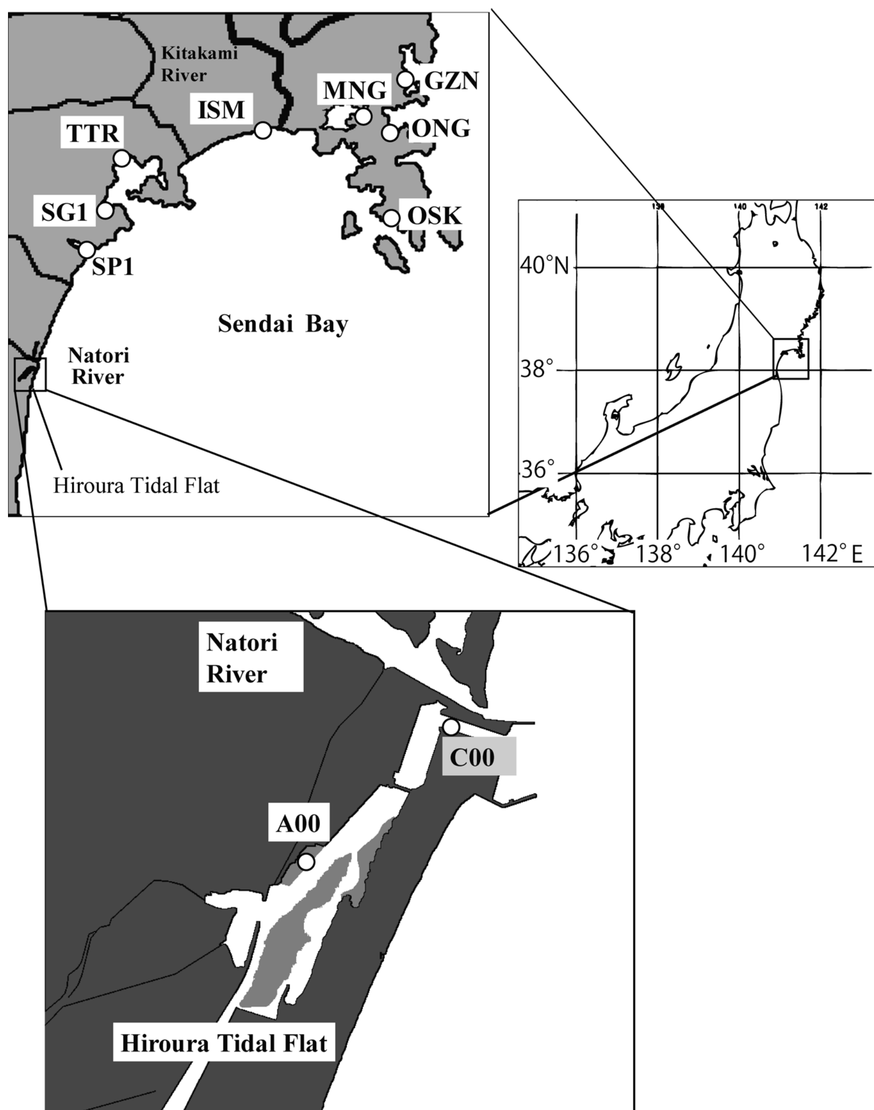

Fig. 1. The location of Sendai Bay and Hiroura tidal flat in Japan and of sites where samples were collected. SP1, Sendai Port; SG1, Shiogama Port; TTR, Tetaru; ISM, Ishinomaki Port; MNG, Mangokuura: OSK, Oshika; ONG, Onagawa; GZN, Gozen; A00, Hiroura tidal flat; C00, river mouth adjacent to Hiroura tidal flat.

Phylogenetic analyses

We obtained 55 sequences of the nuclear SSrRNA gene fragments from 23 morphospecies in Sendai Bay and its vicinities. With sequence data from previous studies (Agatha & Strüder-Kypke 2007, Duff et al. 2008, Gao et al. 2009, Li et al. 2009, Snoeyenbos-West et al. 2002, Strüder-Kypke & Lynn 2003, 2008), we reconstructed SSrRNA phylogeny using a total of 44 tintinnid morphospecies (84 sequences). We aligned the sequences with the CLUSTAL W algorithm (Thompson et al. 1994) and manually adjusted the alignment using BioEdit (Hall 1999) and MEGA 4 (Tamura et al. 2007). For phylogenetic analysis, we used a 1,072 bp fragment of SSrRNA that was common among the sequences examined. We selected a best-fit model of nucleotide substitution with KAKUSAN 4 (Tanabe 2007) and reconstructed the phylogenetic tree applying Maximum-Likelihood (ML), Bayesian Inference (BI) and Neighbor-Joining (NJ) methods. The ML tree was computed with 1,000 bootstrap replicates using TREEFINDER (Jobb 2008). BI analysis was performed using MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck 2003) with 2,000,000 iterations of Markov Chain Monte Carlo (MCMC) sampling. The convergence of MCMC parameters and effective sample size (>100, Kass et al. 1998) were confirmed by Tracer vl.5.0 (Rambaut & Drummond 2009). The NJ tree was reconstructed with 1,000 bootstrap replicates and the Kimura 2-parameter model of nucleotide substitution using MEGA 4. Eight species of Strombidinopsidae, Hypotrichea, Oligotrichia and Stichotrichia (*Strombidinopsis acuminate, S. jeokjo, Laboea strobili, Novistrombidium testaceum, Gastrostyla steinii, Cyrtohymena citrine, Eu-*

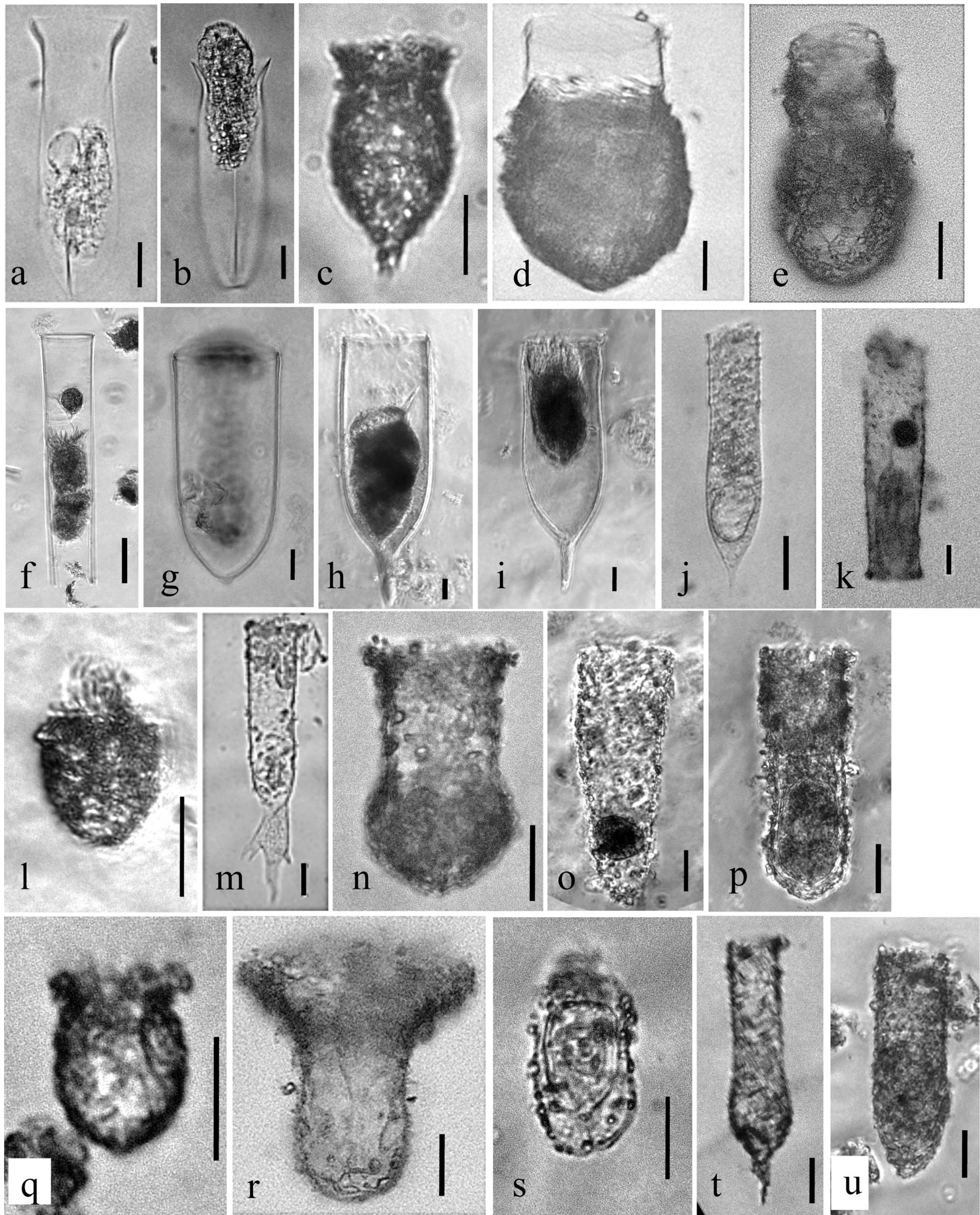

Fig. 2. Light microscope images of tintinnid species photographed under differential interference contrast. a. *Amphorellopsis acuta*, b. *Amphorides amphora*, c. *Codonella amphorella*, d. *Codonellopsis morchella*, e. *Codonellopsis ostenfeldi*, f. *Eutintinnus lususundae*, g. *Favella azorica*, h. *Favella ehrenbergii*, i. *Favella taraikaensis*, j. *Helicostomella fusiformis*, k. *Leprotintinnus pullucidus*, l. *Stenosemella nivalis*, m. *Tintinnopsis corniger*, n. *Tintinnopsis dadayi*, o. *Tintinnopsis elongata*, p. *Tintinnopsis karajacensis*, q. *Tintinnopsis minuta*, r. *Tintinnopsis mortenseni*, s. *Tintinnopsis tenuis*, t. *Tintinnopsis tocantinensis*, u. *Tintinnopsis tubulosa*. Scale bars: 20 μm.

plotidium arenarium, Aspidisca steini) were used as outgroups.

Morphological and morphometric analyses

We selected 35 morphological traits of the lorica (Table 1) that have been incorporated into taxonomic keys for classifying and identifying species, genera and familes of tintinnids in Taniguchi (1997), and used presence/absence of these traits for the analysis (yes-1/no-0): if a specimen had a particular morphological trait, we scored "1" for that specimen regarding that characteristic, otherwise we scored "0". In addition to the scores of specimens collected in this study, we also estimated the scores of morphospecies that were not collected in this study but were used for the genetic analyses. There were determined based on descriptions, illustrations and photographs of the morphospecies from the literature. We obtained a matrix of binary data on the lorica reflecting the morphology of a total of 44 morphospecies, and calculated pairwise similarity values between the morphospecies by simple matching (Sokal & Michener 1958). Then, we performed principal coordinate (PCo) analysis based on the similarity matrix and calculated scores of the first four PCo axes for each morphospecies. These statistical analyses were performed with the aid of the statistical software package R, version 2.10.0. (R Development Core Team 2010).

Using the photographs or illustrations, we measured width and length of the loricae using the software package Image J (Abramoff et al. 2004). Then we calculated the aspect ratio (AR), which is the ratio of the width to length of the lorica, because it has often been reported as one of characters used who discribing tintinnids (Hada 1938, Roxas 1941).

Results

Collected species

We collected and identified a total of 23 morphospecies (11 genera, 6 families) based on lorica morphology (Fig. 2, Appendix Table 1). While we analyzed a nuclear SSrRNA gene fragment from only a single specimen of each morphospecies of *Amphorides amphora* Claparède & Lachmann, *Codonellopsis morchella* (Cleve) Jörgensen, *C. ostenfeldi* (Schmidt) Kofoid & Campbell, *Favella azorica* (Cleve) Jörgensen, *Steenstrupiella steenstrupii*, *Stenosemella nivalis* Jörgensen*, Tintinnopsis elongata* and *T. tubulosa* due to their rare occurrence, we analyzed fragments from more than two specimens each for the other morphospecies. We obtained a total of 55 partial sequences from the 23 morphospecies (Appendix Table 1).

Phylogene tic analyses

The phylogenic tree of nuclear SSrRNA gene fragments (1,072 bp) was reconstructed using 84 unique sequences from 44 morphospecies belonging to a total of eight families with the maximum-likelihood (ML), Bayesian inference (BI), and neighbor-joining (NJ) methods (Fig. 3, Appendix Fig. 1, Appendix Fig. 2). The sequences used in these analyses were 55 sequences from the 23 morphospecies collected for this study, and 29 sequences from 28 morphospecies reported in the literature (eight morphospecies were common to this study). A general time-reversible plus Gamma distributed model (GTR+G) was selected as the best model of nucleotide substitution for both ML and BI analyses according to Bayesian Information Criteria (Tanabe 2007). The BI and NJ trees were mostly congruent with the ML tree. Mean and maximum genetic distances in the ML tree within the order Tintinnida were 4.2% and 39%, respectively. Five major clades (A–E) were supported by higher bootstrap values of ML (>65%) and NJ (>53%) and clade credibility values of BI (>0.98, Fig. 3, Appendix Fig. 1, Appendix Fig. 2). In clade A, a paraphyletic group that was genetically distant from other morphospecies (>8%) was treated as subclade A-1 and the residuals were grouped as subclade A-2. The subclades were also supported by higher bootstrap values of ML (82%) and NJ (80%) and clade credibility values of BI (0.65). However there was a slight difference between these trees. For example, *T. beroidea* EF123709 belonged to subclade A-1 in the ML tree and the NJ tree but was placed under subclade A-2 in the BI tree.

Each clade consisted of several morphospecies belonging to more than two families. The exception was clade B, which contained only morphospecies from the family Ptychocylididae, which is characterized by a hyaline lorica with prongs and visible reticulation. However, morphospecies of Ptychocylididae were also found in subclade A-2. Similarly, although each of the families Rhabdonellidae and Dictyocystidae were found only in one clade, the other families were found spread over at least two clades. Among clades (A–E), subclade A-2 was the most diverse and consisted of morphospecies belonging to eight families. Clade E was the most ancestral and consisted of the families Tintinnidiidae and Codonellidae. The morphospecies of the family Codonellidae were found in all clades except for clade B.

Some morphospecies were found in more than two clades (Fig. 3): *Amphorellopsis acuta* Kofoid & Campbell was found in subclade A-2 and clade D; both *Favella ehrenbergii* Claparéde & Laachmann and *F. taraikaensis* Hada were found in subclade A-2 and clade B; *Helicostomella fusiformis* (Meunier) Jorgensen was found in subclades A-1 and A-2; *Tintinnopsis dadayi* Bonnetto & Ezcurra de Drago was found in subclades A-1 and A-2, and clade C; *T. karajacensis* appeared in subclade A-1, clade E and clade F, and had morphological variation in terms of lorica length. An elongated type of this species occurred in clade D and clade E (AB640659: Fig. 4a) while a short lorica type occurred in subclade A-1 (AB640660: Fig. 4b).

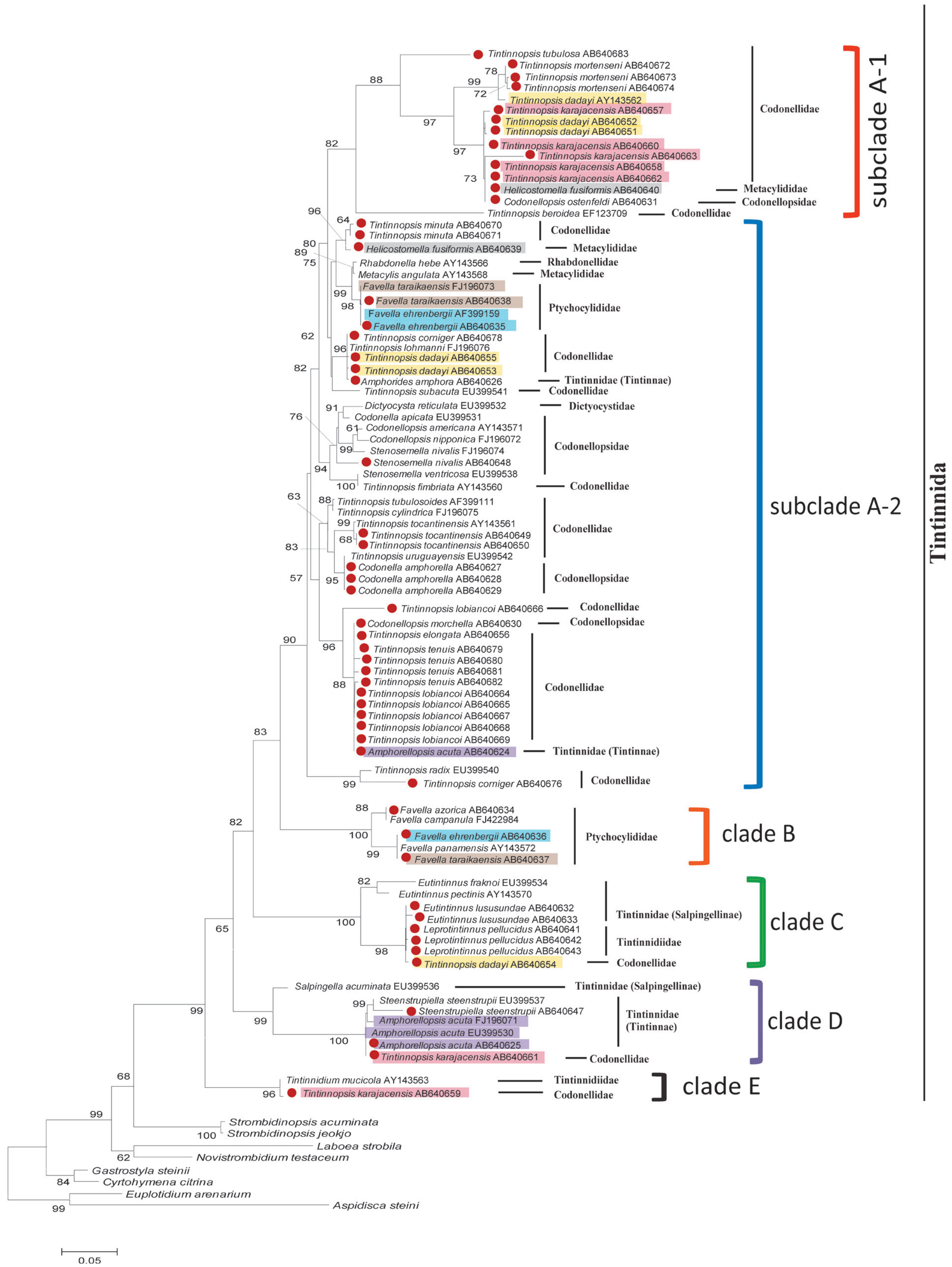

Fig. 3. A phylogenetic tree by the maximum-likelihood (ML) method for tintinnid nuclear SSrRNA sequences. The numbers at nodes represent ML bootstrap support values of more than 50%. Red circles denote morphospecies, which were newly sequenced and analyzed in this study. All of pairwise genetic distances among the five major clades (Clade A to E) and two subclades (A-1, A-2) were more than 8%. Polyphyletic morphospecies have been highlighted with the same color.

Table 1. Morphological characteristics of the lorica that have been used for classifying and identifying species (S), genera (G) and families (F) of tintinnids, and eigen vector values of the characteristics to the first four axes (PCo I to IV) of the principal coordinate analysis. The levels of the top four loadings to each axis are denoted by bold letters.

Trait No.	Lorica portion	Morphology	Classicication level			Eigen vectors			
			F	G	S	PCo I	PCo II	PCo III	PCo IV
1	Surface	Lorica agglutinates substances or grains.	*			**0.619**	−0.024	−0.086	−0.166
2		Lorica has patterns (ex: band or strial) on the surface.	*			−0.141	0.274	0.068	0.176
3		Lorica has fenestrae.	*		*	−0.257	0.057	−0.108	0.001
4		Lorica is annulated with 5–10 spiral turns in anterior part.	*		*	−0.263	−0.003	−0.015	−0.023
5		Lorica is almost hyaline with hardly any visible reticulation.	*			−0.184	0.211	0.064	0.120
6		Lorica is ornamented with vertical striae.	*			−0.275	0.010	−0.054	0.005
7		Lorica is shaped with thick annular bulge.		*		−0.251	0.101	−0.041	0.023
8	Bowl	Lorica is rounded shape in cross-section.		*		**0.948**	0.139	0.044	−0.118
9		Bowl is thickened at central or anterior part.			*	−0.137	−0.093	0.178	**−0.310**
10		Bowl is thickened at posterior part.			*	0.449	−0.228	**−0.470**	0.138
11		Bowl width is longest at posterior part.			*	0.234	0.073	**−0.455**	−0.117
12	Oral	Lorica has oral collar or flare.		*	*	0.293	**−0.491**	**0.329**	0.089
13		Oral part of collar is narrower than bowl diameter.			*	0.129	−0.380	0.177	**0.285**
14		Oral part of collar is wider than bowl diameter.			*	−0.228	−0.067	−0.097	0.003
15		Oral part is cone-shaped.			*	−0.269	0.006	−0.037	0.011
16		Oral part is distinctly funnel-shaped (flaring steeply).			*	0.116	**−0.403**	−0.002	**0.332**
17		Lorica has inner-collar (double oral rims).		*	*	−0.143	−0.004	0.210	−0.172
18		Collar is distinctly partitioned against bowl.			*	−0.054	−0.191	0.260	**−0.226**
19		Collar has no fenestrae.	*		*	−0.235	−0.033	0.037	−0.177
20		Collar has many fenestrae (porous).	*		*	−0.265	−0.017	−0.019	−0.101
21		Inner-collar is longer than one third the length of body.			*	−0.200	−0.049	0.072	−0.216
22		Collar with substances or grains on the surface.	*			0.026	−0.293	−0.107	0.114
23		Collar has minutely dentate oral rim.		*	*	−0.257	0.057	−0.108	0.001
24		Coller is annulated with spiral turns.			*	−0.249	0.009	−0.019	−0.084
25	Aboral	Aboral part is closed-end.	*			**0.924**	0.077	0.032	−0.016
26		Aboral part is abruptly narrowing or rounded-end.			*	**0.862**	0.116	−0.071	−0.119
27		Aboral part is moderately tapering-end.			*	−0.206	−0.047	0.057	0.111
28		Aboral part is closed square shape.			*	−0.279	−0.018	−0.064	−0.006
29		Aboral part is flaring (only opened-end shape).			*	−0.264	−0.001	−0.077	−0.043
30		Aboral part is sharp or has a prong.			*	0.422	0.299	**0.353**	0.085
31	Prong	Lorica has a prong.		*	*	−0.014	**0.442**	0.078	**0.226**
32		Prong is distinctly elongated.			*	−0.014	**0.442**	0.078	**0.226**
33		Prong is ornamented by segments, needle or skirt.			*	−0.265	0.045	−0.058	−0.004
34		Prong is ornamented by ridges.			*	−0.287	−0.008	−0.074	−0.023
35		Ridge part of prong is longer than one third length of body.			*	−0.287	−0.008	−0.074	−0.023

Morphological and morphometric analyses

Relationships among the traits of lorica morphology were summarized well according to several axes of the principal coordinate (PCo) analysis (Table 1). The first four axes (I–IV) explained >75% of variance in the lorica morphology. The first principal coordinate axis (PCo I) was mainly correlated to morphological traits Nos. 1, 8, 25 and 26 in Table 1. Thus, large PCo I values reflected such loricae that are agglutinated and have a rounded shape incross

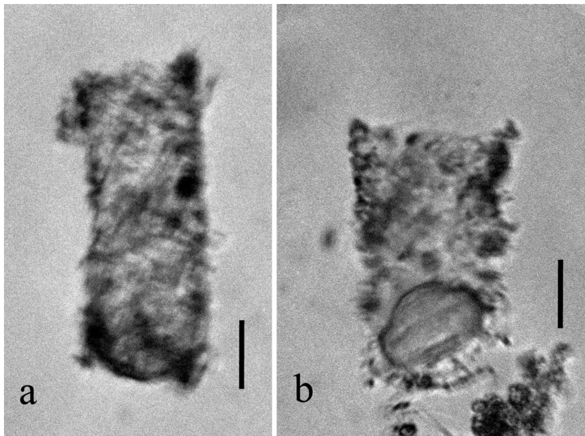

Fig. 4. Morphospecies found in two different clades. a. Tintinnopsis karajacensis (AB640660) in subclade A-1, b. T. karajacensis (AB640661) in clade E in Fig. 3. Scale bars: 20 μm.

section with a closed aboral part that is round or narrows in shape. PCo II was correlated mainly with morphological traits Nos. 12, 14, 31 and 32 and described loricae with slightly flaring oral portions or no collar, and with an elongated prong. The PCo III was correlated mainly with morphological traits Nos. 10, 11, 12 and 30 and described loricae with an oral collar, a sharpened aboral part and a bowl that is not thickened or wide at the posterior part. The PCo IV was correlated mainly with morphological traits Nos. 9, 13, 16 and 18 and described hyaline loricae with a funnel-shaped oral part that was not separated from the bowl, and the bowl is not thickened in any part. We calculated values of the PCos axes of the 44 morphospecies, and plotted these in two-dimensional planes of paired axes according to clades (Fig. 5a, c, d) together with associations of the main morphological traits correlated to these axes (Fig. 5b, d, f). Morphospecies in clades A–D overlapped each other in all the graphs. Among these, morphospecies from subclade A-2 were scattered most widely over the two-dimensional trait space, indicating that this subclade included members with a variety of loricae that covered largely all the morphological variations found in the other clades. Clade B had higher values for PCo II compared with clades C and D (Fig. 5b), because morphospecies of this clade had loricae with a sharpened aboral part or with a prong, and these characteristics were not found in the latter two clades. However, there were no large differences in PCo I, III and IV among these clades (Fig. 5d, f).

Morphometric analyses are shown in Fig. 6. Again, subclade A-2 showed large variations in the morphometrics of the loricae and covered the morphometric variations found in subclade A-1 and the other clades. In general, morphospecies in clade B had longer loricae compared with those in subclade A-1, clade C or clade D (Fig. 6a). In addition, morphospecies in clades C and D tended to have loricae with narrow width relative to the length, as shown by a larger AR (width: length ratio, Fig. 6b), although no significant difference was detected in the AR among the clades (p=0.016, Kruskal-Wallis test).

Discussion

Previous studies on the phylogeny of the Choreotrichia (including the order Tintinnida) were unable to clearly elucidate the phylogeny within the Tintinnida based on nuclear SSrRNA gene fragments (Snoeyenbos-West et al. 2002, Strüder-Kypke & Lynn 2003, 2008, Gao et al. 2009, Li et al. 2009), because they analyzed a limited number of tintinnid species. Here we newly analyzed 55 sequences of the SSrRNA gene fragments of tintinnid species (23 morphospecies, 11 genera and 6 families) collected in Sendai Bay in Japan and its vicinity. Incorporating sequence data from the previous studies, we reconstructed a phylogenetic tree composed of 84 sequences from 44 morphospecies in total of eight families, and enhanced our understanding of tintinnid phylogeny. The results showed that at least six families (Codonellidae, Codonellopsidae, Ptychocylididae, Tintinnidiidae, Tintinnidae and Metacylididae) are polyphyletic.

We analyzed whether or not the morphology and morphometry of the loricae are traits reflecting genealogical relationships among tintinnid ciliates. The results showed that among the six clades that we identified genetically, subclade A-2 was the most diverse in terms of lorica morphology and morphometry. Because subclade A-2 is a large paraphyletic group within clade A, variation in lorica morphologies within subclade A-1 was contained within that of subclade A-2. Although lorica morphology did not overlap between clade B (consisting of Ptychocylididae alone) and clade C (the sole clade containing *Eutintinnus*), or between clade B and clade D (consisting mainly of Tintinnidae), their lorica morphologies overlapped those of clade A to a large degree. Furthermore, even though lorica length and width variation also differed between clade B and clade C, there was significant overlapping with clade A-2. These results suggest that there is no systematic relationship between lorica morphology and nuclear SSrRNA, although some morphological features are useful to taxonomically separate species in clade B from those in clade C and clade D: species in the former clade can be distinguished from those in the latter two clades in that the loricae have prongs, although the same characteristic is also found in species belonging to clade A.

Because agglutinated or hyaline lorica is a very noticeable features, it has been used as a key character separating Codonellidae, Codonellopsidae and Tintinnidiidae from other families of Tintinnida (Kofoid & Campbell 1939). However, morphospecies of Codonellidae, Codonellopsidae and Tintinnidiidae belonged to polyphyletic clades and some clustered with morphospecies belonging to other families lacking substrates on te lorica surface. Moreover, there was no genetic difference between two morphospecies *Codonellopsis ostenfeldi* (Codonellopsidae,

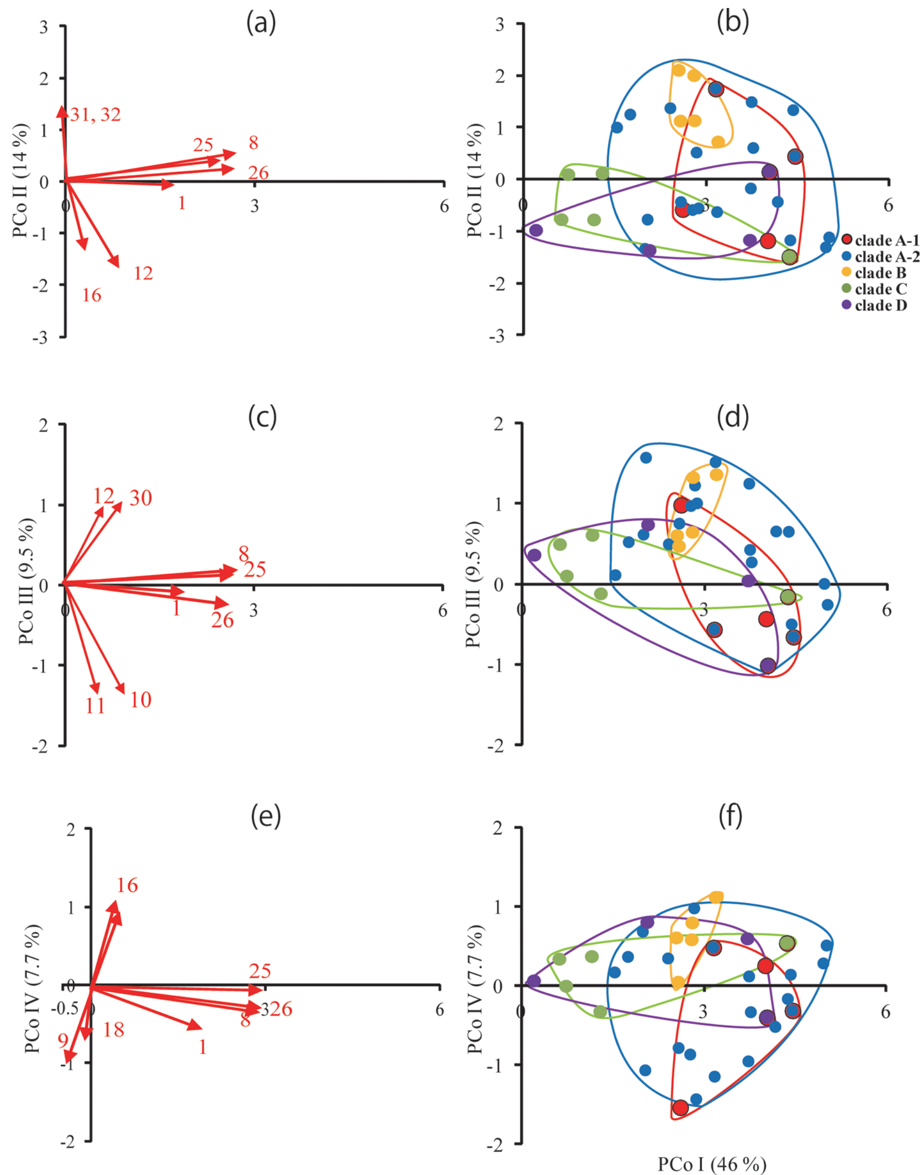

Fig. 5. Results of principal coordination analysis for morphologies of loricae in tintinnids. Loadings of major morphological traits in the first four PCo axies are shown by lines with arrows in two-dimensional ordination spaces determined by the paired axes (a, c and e). Loadings of eigen vectors are multiplied by 3 for comparison and morphological traits are denoted by the numbers in Table 1. Ranges and values of morphospecies in different clades shown in Fig. 3 to these axes are shown by plots and lines with different colors (b, d and f). The ordination spaces are shown as PCo I against PCoII (panel a and b), PCo III (panel c and d) and PCo IV (panel e and f). Values in parentheses are the percentage contribution of PCo axes.

a family with substrates on the lorica surface) and *Helicostomella fusiformis* (Metacylididae, a family without substrates on the lorica surface), or between the two morphospecies *Tintinnopsis dadayi* (Codonellidae, a family with substrates on the lorica surface) and *Amphorides amphora* (Tintinnidae, a family without substrates on the lorica surface). These results indicate that the presence or absence of substrates on the lorica surface may be a trait that has evolved independently in different lineages without being constrained phylogenetically, and thus should not be used as a key trait for tintinnid taxonomy at generic, family or even species levels. It is known that *Favella campanula* Schmidt can change the morphology of the lorica through metamorphosis (Gold 1969). *Tintinnopsis* produce agglutinated loricae with particle or mineral grains in natural settings, but have hyaline loricae when cultured in the laboratory without a supply of substrates for the grains (Gold 1968, 1973). It therefore seems that the type of lorica (hyaline or agglutinated) is not necessarily genetically fixed and could be a plastic trait.

Strüder-Kypke & Lynn (2008) suggested that *T. beroidea* and *T. dadayi* belonged to the “true” *Tintinnopsis*

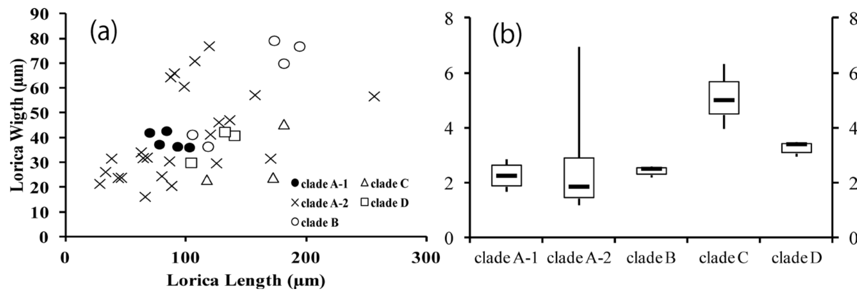

Fig. 6. Relationship between length and width of lorica (a) and box plots (median, quantiles and range) of the aspect ratios (=lorica width/length) in different clades shown in Fig. 3 (b).

genus as a monophyletic group and were phylogenetically distinguishable from other species of *Tintinnopsis*. In this study, however, *T. dadayi* AY143562 was genetically more similar to *Codonellopsis ostenfeldi* and *Helicostomella fusiformis* than to *T. beroidea* EF123709. Thus, the species of "true" *Tintinnopsis* are genealogically unrecognizable. Due to morphological and genetic diversity, the genus *Tintinnopsis* has been thought to be paraphyletic (Laval-Peuto & Brownlee 1986, Agatha & Riedel-Lorje 2006, Strüder-Kypke & Lynn 2008). In this study, morphospecies of *Tintinnopsis* were found not only in clade A-1 but also in clades A-2, C, D and E, which contained species from various families. This result implies that this genus is polyphyletic rather than paraphyletic, and is composed of genetically diverse species. According to classical classification, *Tintinnopsis* spp. have three typical characteristics of the lorica: agglutination of particles or grains, absence of a hyaline collar, and a non-funnelled shape at the oral part. This study suggests that these morphological features are not phylogenically fixed, but are traits that have converged among genealogically different tintinnid groups.

Recently, Kim et al. (2010) showed that specimens of *Favella ehrenbergii* from the coastal waters of Incheon, Korea differed genetically from those collected by Snoeyenbos-West et al. (2002) along the east coast of North America. According to the nuclear SSrRNA phylogeny, specimens of *F. ehrenbergii* from Korea were close to *F. panamensis* Kofoid & Campbell and *F. campanula,* while specimens of *F. ehrenbergii* from eastern North America were close to *F. taraikaensis* (from northern China) and *Metacylis* sp. Since lorica morphology is very similar between *F. ehrenbergii* and *F. taraikaensis*, Kim et al. (2010) suggested that Snoeyenbos-West et al. (2002) might have misidentified their species. However, both specimens of *F. ehrenbergii* and *F. taraikaensis* from Sendai Bay were found not only in clade A but also in clade B, and formed subclades within each clade with other morphospecies. These results imply that *Favella* is not a monophyletic genus and it is hard to identify species of this genus accurately according to the morphology of the lorica.

We also found that morphospecies of *Amphorellopsis acuta, Helicostomella fusiformis*, *Tintinnopsis dadayi* and *T. karajacensis* were polyphyletic and contained diverse lineages. The differences in partial sequence of SSrRNA among specimens within each of these morphospeices cannot be explained due to PCR or sequence reading errors because these differences (>8%) were much larger than those cause by such errors (<1%). This result implies that there are many cryptic species within the Tintinnida, just as with other planktonic taxa such as diatoms (Amato et al. 2007), copepods (Chen & Hare 2008) and aloricate ciliates (Katz et al. 2005). On the one hand, studies with laboratory cultures of tintinnid species have shown that size and morphology of the lorica can vary greatly even within a single species (Gold 1968, 1973, Kim et al. 2010). On the other hand, the present study showed that even if there are no notable differences in the size and shape of the loricae between tintinnids, they do not necessarily share the same descendant and thus the same genealogical position. Therefore, characteristics of the lorica are not necessarily fixed traits and both divergence and convergence in the morphology of loricae has occurred in tintinnids due to evolutionary processes. For these reasons, most morphological features of the lorica are not useful for classifying tintinnid species, even at the family level. To classify species of Tintinnida on a morphological basis without discrepancy with genetic data (Mcmanus & Katz 2009), it may be better to use cytological traits such as inner cell form, cell size and cilia, although only a few studies have as yet conducted cytological analyse, and only for a limited number of tintinnid species (Laval-Peuto & Brownlee 1986, Agatha & Riedel-Lorj 2006, Agatha & Strüder-Kypke 2007).

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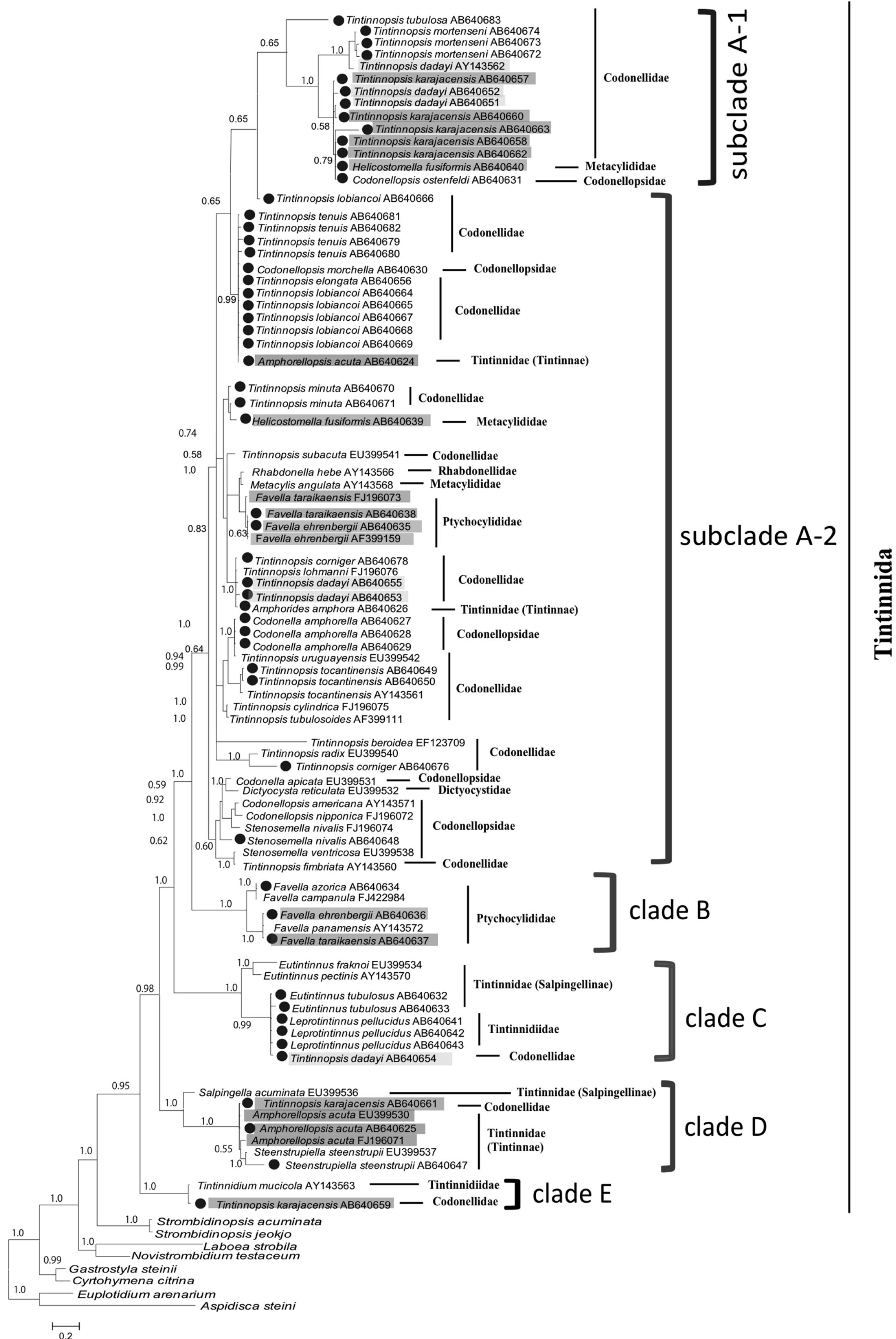
Appendix Table 1. DDBJ accession numbers, sampling sites (see Fig.1), sampling dates, sequence lengths, and GC contents of nuclear SSrRNA sequences from specimens obtained in this study.

	Species	Accession Nos.	Site	Date	Length (bp)	GC content (%)
1	*Amphorellopsis acuta*	AB640624	SP1	13-Aug	1371	46.90
2	*Amphorellopsis acuta*	AB640625	OSK	26-Aug	1288	48.91
3	*Amphorides amphora*	AB640626	C00	26-Aug	1330	45.94
4	*Codonella amphorella*	AB640627	TTR	22-Oct	1366	47.80
5	*Codonella amphorella*	AB640628	TTR	22-Oct	1333	48.16
6	*Codonella amphorella*	AB640629	TTR	22-Oct	1353	48.04
7	*Codonellopsis morchella*	AB640630	SP1	13-Aug	1092	45.42
8	*Codonellopsis ostenfeldi*	AB640631	SP1	3-Sep	1312	47.26
9	*Eutintinnus lususundae*	AB640632	SP1	12-Jul	1319	49.13
10	*Eutintinnus lususundae*	AB640633	SP1	12-Jul	1345	49.52
11	*Favella azorica*	AB640634	C00	28-Jun	1641	47.17
12	*Favella ehrenbergii*	AB640635	SP1	12-Jul	1337	47.94
13	*Favella ehrenbergii*	AB640636	SP1	12-Jul	1353	48.04
14	*Favella taraikaensis*	AB640637	SG1	22-Oct	1354	48.08
15	*Favella taraikaensis*	AB640638	SG1	22-Oct	1334	48.50
16	*Helicostomella fusiformis*	AB640639	SP1	3-Sep	1299	48.65
17	*Helicostomella fusiformis*	AB640640	SP1	3-Sep	1349	46.70
18	*Leprotintinnus pellucidus*	AB640641	SG1	3-Sep	1349	49.59
19	*Leprotintinnus pellucidus*	AB640642	SG1	3-Sep	1320	49.62
20	*Leprotintinnus pellucidus*	AB640643	SG1	3-Sep	1334	49.48
21	*Steenstrupiella steenstrupii*	AB640647	C00	13-Aug	1329	49.36
22	*Stenosemella nivalis*	AB640648	C00	26-Aug	1303	46.97
23	*Tintinnopsis corniger*	AB640676	MNG	26-Aug	1289	48.41
24	*Tintinnopsis corniger*	AB640678	C00	26-Aug	1295	46.80
25	*Tintinnopsis dadayi*	AB640651	TTR	3-Sep	1346	46.66
26	*Tintinnopsis dadayi*	AB640652	TTR	3-Sep	1344	46.73
27	*Tintinnopsis dadayi*	AB640653	SG1	3-Sep	1320	47.35
28	*Tintinnopsis dadayi*	AB640654	SG1	3-Sep	1379	49.17
29	*Tintinnopsis dadayi*	AB640655	SG1	3-Sep	1375	47.27
30	*Tintinnopsis elongata*	AB640656	SG1	3-Sep	1303	47.51
31	*Tintinnopsis karajacensis*	AB640657	C00	13-Aug	1286	46.66
32	*Tintinnopsis karajacensis*	AB640658	ISM	3-Sep	1295	47.03
33	*Tintinnopsis karajacensis*	AB640659	TTR	3-Sep	1323	47.92
34	*Tintinnopsis karajacensis*	AB640660	TTR	3-Sep	1312	47.10
35	*Tintinnopsis karajacensis*	AB640661	SG1	3-Sep	1382	48.12
36	*Tintinnopsis karajacensis*	AB640662	SG1	3-Sep	1363	46.96
37	*Tintinnopsis karajacensis*	AB640663	SP1	3-Sep	1375	46.76
38	*Tintinnopsis lobiancoi*	AB640664	C00	12-Jul	1333	47.56
39	*Tintinnopsis lobiancoi*	AB640665	SP1	12-Jul	1276	46.71
40	*Tintinnopsis lobiancoi*	AB640666	ONG	26-Aug	1277	47.22
41	*Tintinnopsis lobiancoi*	AB640667	GZN	22-Oct	1373	47.41
42	*Tintinnopsis lobiancoi*	AB640668	GZN	22-Oct	1343	47.13
43	*Tintinnopsis lobiancoi*	AB640669	GZN	22-Oct	1370	47.30
44	*Tintinnopsis minuta*	AB640670	ISM	3-Sep	1330	48.42
45	*Tintinnopsis minuta*	AB640671	TTR	3-Sep	1375	48.36
46	*Tintinnopsis mortenseni* clone-1	AB640672	C00	28-Jun	1254	46.81
47	*Tintinnopsis mortenseni* clone-2	AB640673	C00	28-Jun	1237	47.13
48	*Tintinnopsis mortenseni* clone-3	AB640674	C00	28-Jun	1237	47.13
49	*Tintinnopsis tenuis* clone-1	AB640679	C00	28-Jun	1296	47.30
50	*Tintinnopsis tenuis* clone-2	AB640680	C00	28-Jun	1250	46.80
51	*Tintinnopsis tenuis*	AB640681	ONG	26-Aug	1320	46.67
52	*Tintinnopsis tenuis*	AB640682	ONG	26-Aug	1322	47.28
53	*Tintinnopsis tocantinensis*	AB640649	C00	13-Aug	1343	47.51
54	*Tintinnopsis tocantinensis*	AB640650	A00	26-Aug	1364	47.95
55	*Tintinnopsis tubulosa*	AB640683	SP1	3-Sep	1305	48.05

Appendix Fig. 1. Phylogenetic tree by the Bayesian Inference method for tintinnid nuclear SSrRNA sequences. The numbers at nodes represent Bayesian clade credibility values.



Appendix Fig. 2. Phylogenetic tree by Neighbor-Joining (NJ) method for tintinnid nuclear SSrRNA sequences. The numbers at nodes represent NJ bootstrap support values.