Reevaluation of the Phylogenetic Relationship between Mobilid and Sessilid Peritrichs (Ciliophora, Oligohymenophorea) Based on Small Subunit rRNA Genes Sequences

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ABSTRACT. Based on morphological characters, peritrich ciliates (Class Olygohymenophorea, Subclass Peritrichia) have been subdivided into the Orders Sessilida and Mobilida. Molecular phylogenetic studies on peritrichs have been restricted to members of the Order Sessilida. In order to shed more light into the evolutionary relationships within peritrichs, the complete small subunit rRNA (SSU rRNA) sequences of four mobilid species, *Trichodina nobilis*, *Trichodina heterodentata*, *Trichodina reticulata*, and *Trichodinella myakkae* were used to construct phylogenetic trees using maximum parsimony, neighbor joining, and Bayesian analyses. Whatever phylogenetic method used, the peritrichs did not constitute a monophyletic group: mobilid and sessilid species did not cluster together. Similarity in morphology but difference in molecular data led us to suggest that the oral structures of peritrichs are the result of evolutionary convergence. In addition, *Trichodina reticulata*, a *Trichodina* species with granules in the center of the adhesive disc, branched separately from its congeners, *Trichodina nobilis* and *Trichodina heterodentata*, trichodinids without such granules. This indicates that granules in the adhesive disc might be a phylogenetic character of high importance within the Family Trichodinidae.

Key Words. Adhesive disc, granule, Mobilida, phylogeny, SSrRNA, *Trichodina*, *Trichodinella*.

PERITRICH ciliates (Ciliophora, Oligohymenophorea), characterized by possession of an expanded oral area, the peristome, and encircled by two prominent bands of cilia that run in counterclockwise fashion, have been long recognized as a higher taxon among ciliates (Corliss 1968). The Subclass Peritrichia is traditionally subdivided into two Orders based on the mode of attachment: the Order Sessilida (Kahl 1933) includes mostly sessile representatives having a scopula as a substrate-attaching organ, while the Order Mobilida (Kahl 1933) includes mostly free-swimming forms, although parasitic form representatives have an aboral adhesive disc as a substrate-attaching organ.

Most species of the Order Mobilida, hereafter referred as mobilids, occur epizootically on various freshwater and marine hosts. More than 280 mobilid species have been reported to infect the skin, gills, and urinary bladders of fishes and amphibians and integument of a few invertebrates (Beers 1964; Van As and Basson 1989; Xu 1999). These peritrichs have been implicated in severe disease and mortalities of fish, causing many economic losses in various parts of the world (Van As and Basson 1987; Van As and Viljoen 1984). Despite their economic and ecological importance, mobilid peritrichs, represented by *Trichodina* and *Urceolaria*, remain less studied than their close-relatives, sessilid peritrichs, represented by *Vorticella* and *Epistylis*. For instance, only sessilid peritrichs with molecular markers have been included in studies of evolutionary relationships within the Class Oligohymenophorea (Greenwood, Sogin, and Lynn 1991b; Miao, Yu, and Shen 2001; Miao et al. 2004). Morphological characters, such as the pattern of ciliary organelles in the oral area and the shape of denticles in the adhesive disc, have been used tentatively to study the systematics of the families Urceolariidae and Trichodinidae of the Order Mobilida (Gong et al. 2005; Raabe 1963; Xu et al. 2000), but these characters have proved inadequate to reconstruct evolutionary history as they are unique features of this order of ciliates and their weighting is difficult to determine. This led us to search for other characters that could be useful for phylogenetic analysis, such as the small subunit (SSU) rRNA gene sequence, to determine the phylogenetic relationship of mobilids within the

Subclass Peritrichia. Four mobilid species, and three different phylogenetic methodologies, namely maximum parsimony (MP), neighbor joining (NJ), and Bayesian analysis were included in this study.

MATERIALS AND METHODS

Samples collection, isolation, and identification. Samples were collected from different fish hatcheries in Wuhan, Hubei Province, China (Table 1). Fishes with symptoms of serious infections were taken and trichodinids present in different organs were isolated using glass micropipettes under a dissecting microscope. Organisms were selected by their dimensions, body shape, characteristic movement, and location on the host. Every specimen was washed three times by double-distilled and autoclave-sterilized water to assure no contaminants were carried over. Specimens were stained by silver nitrate, using a modification of Klein's technique, as described by Lom (1958) and Wellborn (1967). The main structural character used to classify mobilid peritrichs is the precise structure of the adhesive disc (Basson and Van As 1989; Beers 1964). The specimens were identified after visualization in a Zeiss Axioplan microscope (Carl Zeiss, Hallberg-moos, Germany) and comparison with published descriptions (Basson and Van As 1993; Chen 1963; Duncan 1977; Hirschman and Partsch 1955; Mueller 1937).

Sampling is an important factor affecting consistency of phylogentic analyses. We aimed at analyzing as many species as possible, but because culturing trichodinid ciliates is not possible we relied on harvesting individuals to obtain DNA. In the light of these drawbacks, we selected for further work those species that seemed to establish monospecific infections, namely *Trichodina heterodentata* (Duncan 1977), *Trichodina nobilis* (Chen 1963), *Trichodina reticulata* (Hirschman and Partsch 1955), and *Trichodinella myakkae* (Mueller 1937).

DNA extraction. For each species, about 100 individuals were harvested, suspended in lysis buffer (10 mM Tris-HCl, pH 8.0; 1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate [w/v]; 60 μg/ml proteinase K), and incubated at 55 °C for 12–20 h. DNA was extracted using a standard phenol/chloroform method, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; Sambrook, Fritsch, and Maniatis 1989). Polymerase chain reaction (PCR) amplifications were carried out in 25-μl vol. reactions containing 50 ng of

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Table **1.** List of trichodinids used in this paper including name of species and host species, and the place, and year of isolation.

Species	Host species	Origin	Isolator, Year
Trichodinella heterodentata	*Ctenopharynodon idella*	Guanqiao hatchery	F.Y. Zhu, 2001
Trichodinella nobilis	*Hypophthalmichtys molitrix*	Hanyang hatchery	Y.C. Gong, 2004
Trichodinella reticulata	*Ctenopharynodon idella*	Haogou hatchery	Y.C. Gong, 2004
Trichodinella myakkae	*Ctenopharynodon idella*	Hanyang hatchery	F.Y. Zhu, 2001

template DNA, 1 μM of both forward and reverse primers (F: 5′-AACCTGGTTGATCCTGCCAGT-3′; R: 5′-TGATCCTTCTG-CAGGTTCACCTAC-3′) (Medlin et al. 1988), 0.2 mM dNTP, 2 mM MgCl$_2$, and 1 U of *Taq* DNA polymerase (Fermentas, Foster City, CA). Temperature cycling was five cycles of denaturation for 1 min at 94 °C, primer annealing for 2 min at 56 °C, and extension for 2 min at 72 °C, followed by 35 cycles in the same manner, but with the annealing temperature increased to 62 °C. PCR amplifications were performed in a Perkin-Elmer GeneAmp PCR System 9600 (PE Applied Biosystems, Mississauga, ON, Canada).

PCR products were purified using the Biostar Glassmilk DNA Purification Kit (BioStar International, Toronto, ON, Canada) following supplier's instructions. Ligation reactions were performed overnight at 4 °C in 10-μl reaction mixtures containing purified DNA, pGEM-T Easy Vector (Promega Biotech, Madison, WI), and 3 U of T4 DNA ligase (Promega Biotech). Whole ligation reactions were used to transform, following conventional methods (Sambrook et al. 1989), 100 μl of HB101 High Efficient Competent Cells. Positive clones were selected, plasmid DNA isolated and sequenced in both directions with an ABI PRISM 377 automated DNA sequencer (Applied Biosystems Inc., Foster City, CA), using dye terminators and Taq FS with M13 forward and reverse primers.

Sequence database. The nucleotide sequences of 34 ciliates, representing eight classes, are available from the GeneBank databases under the following accession numbers: *Anophryoides haemophila* U51554 (Ragan et al. 1996); *Astylozoon enriquesi* AY049000 (M. C. Strüder-Kypke, unpubl.); *Blepharisma americanum* M97909 (Greenwood et al. 1991a); *Bresslaua vorax* AF060453 (Lynn et al. 1999); *Carchesium polypinum* AF401522 (Miao et al. 2004); *Cyclidium porcatum* Z29517 (Esteban et al. 1993); *Didinium nasutum* U57771 (Wright and Lynn 1997a); *Diplodinium dentatum* U57764 (Wright and Lynn 1997b); *Discophrya collini* L26446 (Leipe et al. 1994); *Epistylis chrysemydis* AF335514 (Miao et al. 2001); *Epistylis urceolata* AF335516 (Miao et al. 2001); *Epistylis wenrichi* AF335515 (Miao et al. 2001); *Euplotes aediculatus* X03949 (Sogin et al. 1986); *Euplotes vannus* AY004772 (Chen and Song 2002); *Frontonia vernalis* U97110 (R P. Hirt et al., unpubl.); *Ichthyophthirius multifiliis* U17354 (Wright and Lynn 1995); *Loxodes striatus* U24248 (Hammerschmidt et al. 1996); *Obertrumia georgiana* X65149 (Bernhard et al. 1995); *Opercularia microdiscum* AF401525 (Miao et al. 2004); *Ophrydium versatile* AF401526 (Miao et al. 2004); *Opisthonecta henneguyi* X56531 (Greenwood et al. 1991b); *Paramecium bursaria* AF100314 (Strüder-Kypke et al. 2000); *Paramecium calkinsi* AF100310 (Strüder-Kypke et al. 2000); *Platyophrya vorax* AF060454 (Lynn et al. 1999); *Pseudocohnilembus marinus* Z22880 (P. L. Dyal, unpubl.); *Pseudomicrothorax dubius* X65151 (Bernhard et al. 1995); *Spirostomum ambiguum* L31518 (Hirt et al. 1995); *Tetrahymena canadensis* M98022 (Sogin et al. 1986); *Tetrahymena pyriformis* M98021 (Sogin et al. 1986); *Trithigmostoma steini* X71134 (Leipe et al. 1994); *Vaginicola crystallina* AF401521 (Miao et al. 2004); *Vorticella campanula* AF335518 (Miao et al. 2001); *Zoothamnium arbuscula* AF401523 (Miao et al. 2004); *Zoothamnopsis sinica* AY319769 (Li and Song, unpubl.).

Phylogenetic analysis. Multiple sequence alignment was performed using ClustalX (Thompson et al. 1997). Considering secondary structural features of the SSrRNA molecule, we further refined the alignment by removing hypervariable regions of helixes E23_1 and E23_2 with the DCSE (Dedicated Comparative Sequence Editor; De Rijk and De Wachter 1993). MP (Farris 1970) and NJ (Saitou and Nei 1987) analyses were performed with PAUP 4.0b10 (Swofford 2002), and Bayesian analysis (Mau 1996; Rannala and Yang 1996) was carried out with MrBayes 3.0b (Huelsenbeck and Ronquist 2001). For MP analysis, characters were not weighted. Tree searches used tree bisection-reconnection (TBR) branch swapping and 1,000 simple sequence addition replicates. For NJ and Bayesian analyses, the tree topologies were inferred using the model ''GTR+I+G,'' which was selected as the best-fit model of nucleotide substitution by AIC in Modeltest 3.7 (Posada and Crandall 1998) and implemented in PAUP 4.0b10. Both parsimony and distance data were bootstrap re-sampled 1,000 times, and for Bayesian analysis, the parameter of Markov chain Monte Carlo (MCMC) was ''number of generations = 1,000,000, sample frequency = 100, number of chains = 4, temperature = 0.5.''

We performed two-tailed Wilcoxon signed-ranks tests (Templeton 1983) and Shimodaira–Hasegawa (S–H) test (Shimodaira and Hasegawa 1999) which were implemented in PAUP 4.0b10 to test whether or not topologies recovered under different methods of analyses (MP, NJ, and Bayesian analysis) were significantly different one from one another. The parameters of S–H test were RELL (resampling estimated log-likelihood), 1,000 bootstrap replicates.

RESULTS

Species and primary structure. Figure 1 shows photomicrographs or diagrammatic drawing from photomicrograph of silver-impregnated cells that were identified as *Trichodina heterodentata* Duncan, 1977 (Fig. 1A), *Trichodina nobilis* Chen, 1963 (Fig. 1B), *Trichodina reticulata* Hirschman and Partsch, 1955 (Fig. 1C), and *Trichodinella myakkae* Mueller, 1937 (Fig 1D). For each species, the SSU rRNA sequence was obtained. The length in basepairs and GeneBank Accession numbers were 1698 and AY788099, 1698 and AY 102172, 1702 and AY741784, 1699 and AY 102176, respectively, for *Trichodina heterodentata*, *Trichodina nobilis*, *Trichodina reticulata*, and *Trichodinella myakkae*.

Phylogenetic analyses. *Loxodes striatus* (Karyorelictea) was chosen as outgroup to test the phylogenetic relationship between mobilid and sessilid peritrichs. Both the Wilcoxon signed-ranks tests and the S–H test (Table 2) show that the topology yielded by MP method was the best among the three topologies. However, the NJ and Bayesian hypotheses were not significantly different from MP hypothesis, as they had very similar log-likelihood scores, and all the *P* values are greater than 0.05. Because the trees topologies were quite similar, we summarized them in one tree (Fig. 2). Tree differences were essentially restricted to the bootstrap value in each node and the emergence of spirotrichs and litostomes. Spirotrichs emerged before the litostomes only when MP and Bayesian methods were used (bootstrap values or
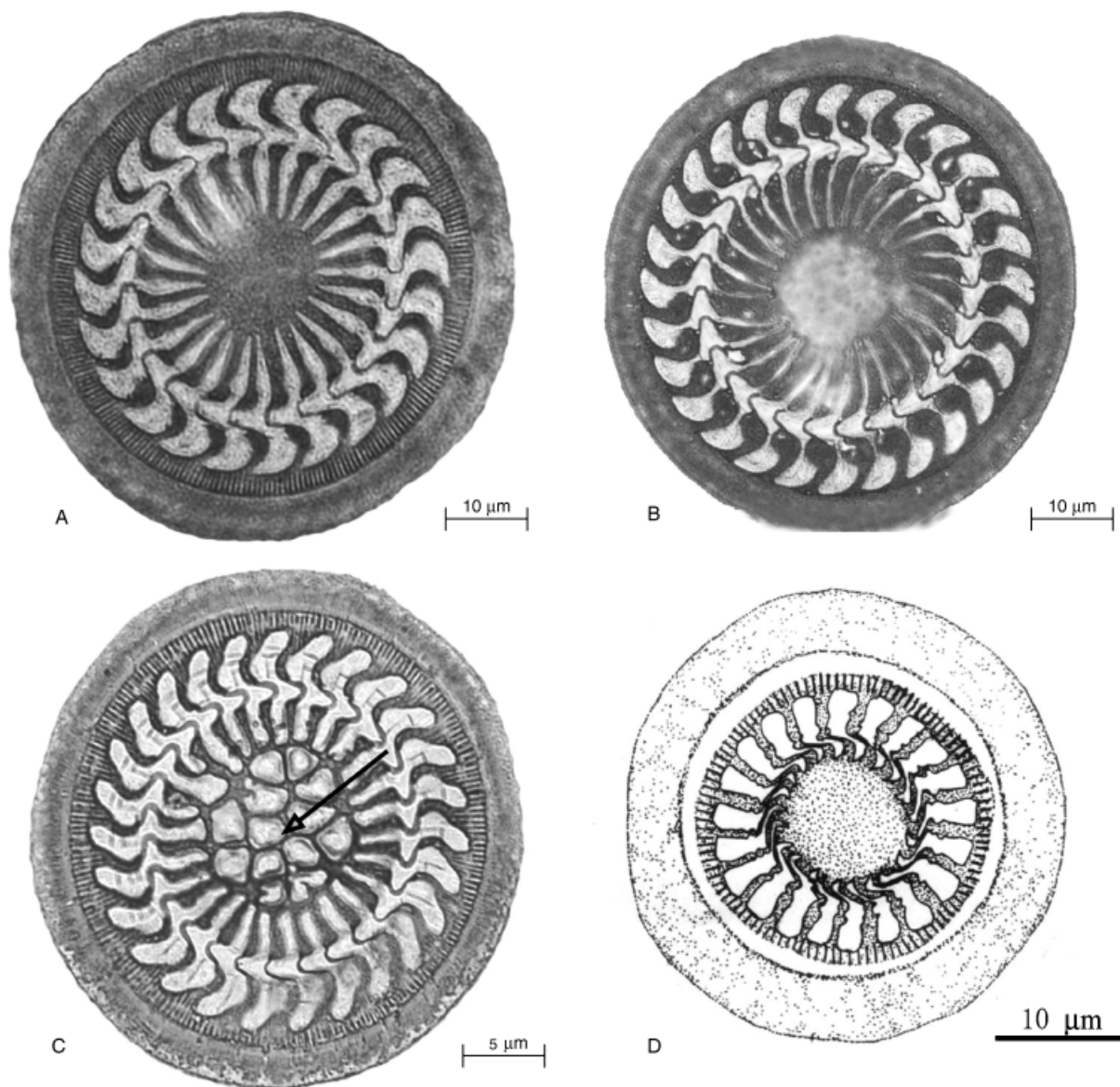
Fig. 1. Detail of the adhesive disc of trichodinids used in this study. Photomicrographs of silver-impregnated cells of: (**A**) *Trichodina heterodentata* Ducan, 1977, (**B**) *Trichodina nobilis* Chen, 1963, and (**C**) *Trichodina reticulata* Hirschman and Partsch, 1955. (**D**). Diagrammatic drawing from photomicrograph of silver-impregnated cells of *Trichodinella myakkae* Mueller, 1937. In panel C, the tip of the arrow point to the granules located at the center of the adhesive disc.

posterior probabilities, 77% [MP], 65% [Bayes]). Concerning the Class Oligohymenophorea, to which peritrichs belong, its monophyly was recovered and supported by bootstrap or posterior probability values (bootstrap values or posterior probabilities, 66% [MP], 92% [Bayes], 92% [NJ]). Within oligohymenophoreans, five different and well-supported clusters were evident and

Table 2. Statistical comparisons of topologies of MP, NJ, Bayesian trees using two-tailed Wilcoxon signed-ranks tests and S–H test.

Topology	Wilcoxon signed-ranks test				S–H test		
	MP				Maximum likelihood		
	Tree length	N	z	P^*	$-\mathrm{Ln}\,L$	$\mathrm{Ln}\,L$ Diff.	P^*
Bayes	4,136	13	-1.3868	0.1655	23,058.57594	1.08637	0.698
NJ	4,143	80	-1.2960	0.1950	23,088.51489	31.02532	0.093
MP	4,131	Best			23,057.48958	Best	

*Significant difference at $P < 0.05$.
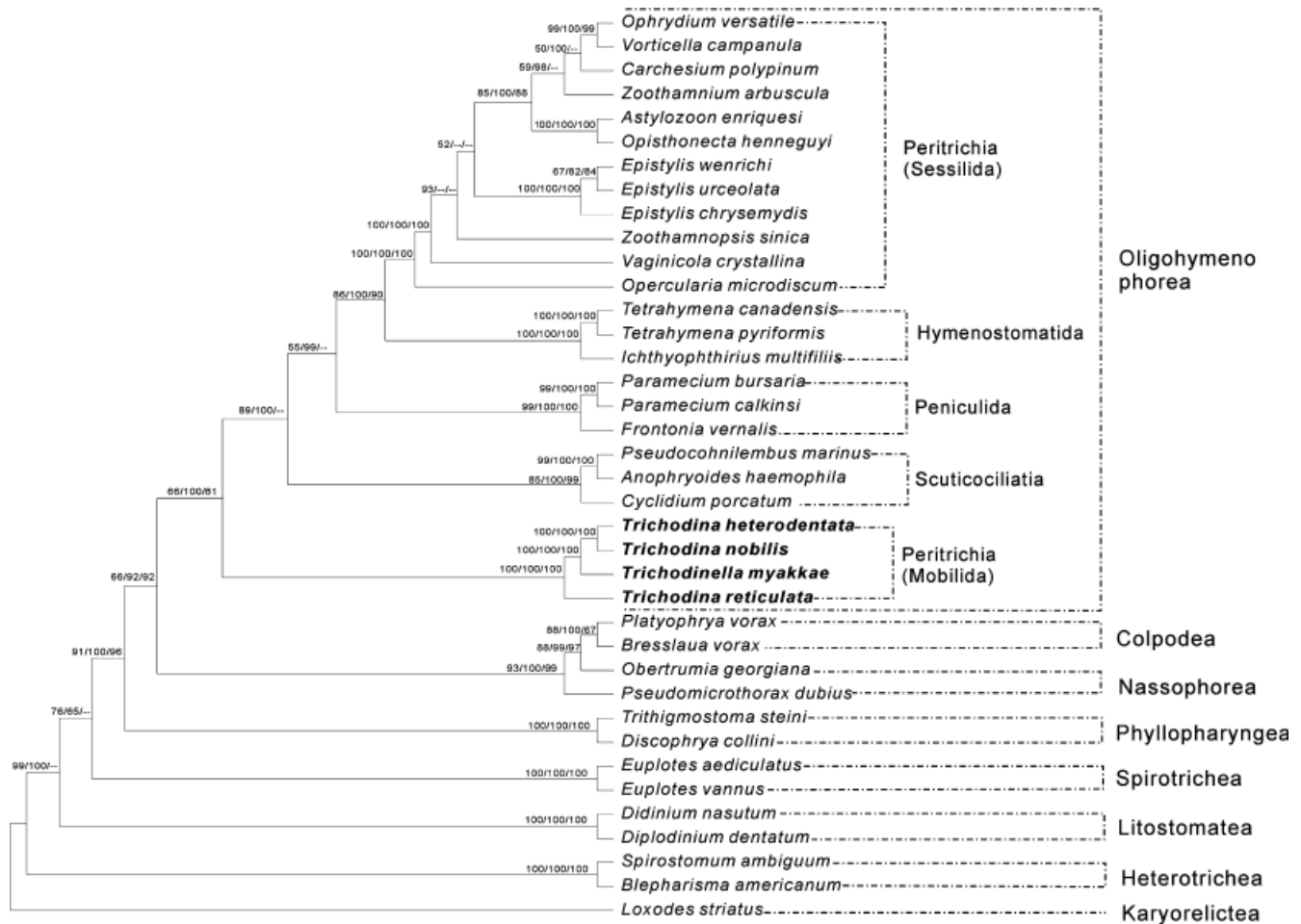MP, maximum parsimony; NJ, neighbor joining; S–H, Shimodaira–Hasegawa.

Fig. 2. The 50% majority rule consensus phylogenetic tree inferred from small subunit rRNA sequences using maximum parsimony (MP), Bayesian analysis and neighbor joining (NJ) methods with the model of ''GTR+I+G'' selected by AIC in Modeltest 3.7 for the last two analyses. The numbers at the nodes represent the bootstrap percentages from 1,000 replicates for MP, the posterior probabilities from 1,000,000 generations for Bayesian analysis, and the bootstrap percentages from 1,000 replicates for NJ, respectively, in order. Dashes represent nodes not existed when applied one of the above-mentioned methods.

corresponded to the hymenostomatids (*Tetrahymena*), peniculines (*Paramecium*), scuticociliates (*Cyclidium*), and the two peritrichs groups, the sessilids and mobilids. Again, differences among trees were essentially restricted to the bootstrap value at each node, and the emergence of *Z. arbuscula* and *Z. sinica*: whatever the tree considered, these two species did not branch together.

One of the most striking features of the tree is that sessilids and mobilids did not branch together. Mobilids constitute a clade emerging early within oligohymenophoreans (bootstrap values or posterior probabilities, 66% [MP], 100% [Bayes], 81% [NJ]), while sessilids constitute a terminal clade consistently associated with hymenostomes (bootstrap values or posterior probabilities, 86% [MP], 100% [Bayes], 90% [NJ]). Another striking feature of these trees is that *Trichodina reticulata* did not branch with its congeners *Trichodina heterodentata/Trichodina nobilis* clade, which were sister taxa to *Trichodinella myakkae*.

DISCUSSION

Evolutionary affinities within peritrichs. It is widely accept-ed that mobilids evolved from telotroch-like stage of sessilids (Lom 1964, 1973; Raabe 1952). Consequently, one would expect mobilids and sessilids forming a monophyletic clade in a phylo-

genetic tree. As stated in the preceding section, our phylogenetic analyses show mobilids and sessilids did not form such a mon-ophyletic group, for mobilids constituted a clade emerging early within oligohymenophoreans, while sessilids constituted a termi-nal clade consistently associated with hymenostomes. Some mobilid and sessilid peritrichs have been found with very uni-form buccal apparatus, which is an important morphological char-acter for peritrichs (Lom 1964). Some authors considered oral areas of mobilids and sessilids analogous instead of homologous structures (Lom, Corliss, and Noirot-Timothee 1968) because of the contradiction of the similarity of morphology and difference in molecular data; perhaps the peritrich type of oral area is simply the result of evolution of the basic Oligohymenophorean oral pat-tern for intensive suspension feeding from two different Oligo-hymenophorean ancestors. Otherwise said, oral structures of per-itrichs are the results of evolutionary convergence, and feeding style could be the evolutionary force that drove such a conver-gence as mobilids and sessilids have a similar food source—both of them feeding on water-dispersed particles, bacteria or algae. Additional data favor this hypothesis that oral structures of per-itrichs are the results of evolutionary convergence. Firstly, the adhesive disc of the mobilids is one of the most highly complex structural elements encountered within the ciliates and perhaps

within eukaryotes, and there is no evidence of homology with the scopula of sessilids (Hausmann and Hausmann 1981b); therefore it seems very improbable that the mobilid adhesive disc might have evolved from the sessilid scopula. Moreover, the aboral locomotor fringe is a peculiarity of mobilids not found in sessilids, though the latter can transiently develop aboral (trochal) bands of cilia for locomotive purposes; there is not, as yet, any TEM information about the internal structural organization of this band (Hausmann and Hausmann 1981a).

However, perhaps this apparent contradiction can be attributable to an artifactual recovery of mobilids in the phylogenetic tree, due to either/both long-branch attraction or undersampling (Felsenstein 1978; Moreira, Le Guyader, and Philippe 1999). Based on the hypothesis of Lom and Raabe (Lom 1964, 1973; Raabe 1952) that the Mobilida evolved from the telotroch-like stage of the Sessilida, it is thinkable that with the adaptation to the new ecological niche we see rapid evolution within this clade, resulting in a complex morphological structure like the adhesive disc and also in highly diverged SSrRNA gene sequences. This can lead to a so-called long-branch attraction that places the Mobilida in a more basal position than they actually are in our analyses; in order to preserve more sequences information, we only removed hypervariable regions of helices E23_1 and E23_2 to avoid long-branch attraction, but perhaps some other hypervariable regions still contributed long-branch attraction. In addition, sampling is considered as an important factor affecting consistency of phylogenetic analyses; perhaps the substantial difference between mobilid sequences and the sessilid sequences is because of our under sampling (only four species in two genera out of a total of over 280 species).

In all, the monophyly of peritrichs was not proved in our analyses based on the SSU rRNA gene sequences, which is inconsistent with currently accepted hypotheses claiming that mobilids originated from the telotroch-like stage of sessilids. However, the results need to be studied further, as we cannot be sure whether long-branch attraction or undersampling affect the topology of mobilids in the phylogenetic tree. In further studies, on the one hand, we will perfect the method to align the SSrRNA gene sequence considering long-branch attraction; on the other hand, we will collect more mobilid species including more genera in the Subclass Mobilida. In addition, more molecular data are also needed to confirm these phylogenies based on the SSrRNA gene. This could be obtained from tubulin, whose trees are often congruent with those based on rDNA (Baroin-Tourancheau et al. 1998; Katz, Lasek-Nesselquist, and Snoeyenbos-West 2003), or actin, which gives trees less congruent with those based on the rDNA genes (Croft et al. 2003). Genetic code determination might also be used as strong evidence, as UAA and UAG codons are translated for glutamine and glutamate in hymenostomes and peritrichs, respectively (Sanchez-Silva et al. 2003).

Phylogenetic relationship among *Trichodina* species. Three *Trichodina* species and one *Trichodinella* species have been studied in the study. The two genera can be differentiated from each other by the denticles in the adhesive disc and the degree of development of the adoral ciliary spiral, characters widely used in generic discrimination of trichodinids (Basson and Van As 1989). While *Trichodina* species have an adoral spiral of 360–540° and well developed denticles that include blades, central parts, and rays, *Trichodinella* species have an adoral spiral of 180–270° and simpler denticles with delicate rays (Lynn and Small 2000). However, it is striking that in our phylogenetic tree one *Trichodina* species, *Trichodina reticulata*, does not branch with its congeners *Trichodina heterodentata*/*Trichodina nobilis* clade, which are sister taxa to *Trichodinella myakkae*. *Trichodina reticulata* can be differentiated from other trichodinids by the constant occurrence of cell-like structures in the center of the adhesive disc, called

granules (Fig. 1), which are the residues of older generations of denticles and evidently add firmness and resiliency to the adhesive disc (Lom and Haldar 1976). Among over 260 species of trichodinids, there are only about 20 *Trichodina* species with such granules (Lom 1970; Xu 1999). We suggest that the granules in the adhesive disc might be an important generic character within the Family Trichodinidae, in addition to the kind of denticles in the adhesive disc and the development of the adoral ciliary spiral. Phylogenetic studies including more species having these structures are essential to test this hypothesis.

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