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Alpha-Tubulin and Small Subunit rRNA Phylogenies of Peritrichs Are Congruent and Do Not Support the Clustering of Mobilids and Sessilids (Ciliophora, Oligohymenophorea)

YINGCHUN GONG,[a,c] KUIDONG XU,[b] ZIFENG ZHAN,[b] YUHE YU,[a] XUEMEI LI,[a] EDUARDO VILLALOBO[d] and WEISONG FENG[a]

[a]*Key Laboratory of Biodiversity and Conservation of Aquatic Organisms, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China, and*
[b]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China, and*
[c]*State Key Laboratory of Freshwater Ecology and Biotechnology, Wuhan 430072, China, and*
[d]*Departamento de Microbiologia, Facultad de Biologia, Universidad de Sevilla, Sevilla 41012, Spain*

ABSTRACT. Peritrich ciliates have been traditionally subdivided into two orders, Sessilida and Mobilida within the subclass Peritrichia. However, all the existing small subunit (SSU) rRNA phylogenetic trees showed that the sessilids and mobilids did not branch together. To shed some light on this disagreement, we tested whether or not the classic Peritrichia is a monophyletic group by assessing the reliability of the SSU rRNA phylogeny in terms of congruency with α-tubulin phylogeny. For this purpose, we obtained 10 partial α-tubulin sequences from peritrichs and built phylogenetic trees based on α-tubulin nucleotide and amino acid data. A phylogenetic tree from the α-tubulin and SSU rRNA genes in combination was also constructed and compared with that from the SSU rRNA gene using a similar species sampling. Our results show that the mobilids and sessilids are consistently separated in all trees, which reinforces the idea that the peritrichs do not constitute a monophyletic group. However, in all α-tubulin gene trees, the urceolariids and trichodiniids do not group together, suggested mobilids may not be a monophyletic group.

Key Words. α-tubulin, Mobilida, Peritrichia, phylogeny, Sessilida, SSU rRNA.

THE peritrich ciliates, which are assigned to the subclass Peritrichia within the class Oligohymenophorea, are characterized by two prominent bands of cilia that run in counter-clockwise fashion around an expanded oral area, called the peristome (Corliss 1979; Lynn 2008). They are traditionally subdivided into two orders: most representatives of the order Sessilida Kahl (1933) possess a stalk, a scopula, or a lorica to attach to the substrate; the representatives of the order Mobilida Kahl (1933), in contrast, are free-swimming, parasitic (or commensal) and possess an aboral adhesive disc to attach to their hosts.

Systematic studies on the peritrichs have been traditionally based on morphological characters (Lom 1958, 1964; Lom, Corliss, and Noirot-Timothee 1968; Raabe 1963). Recently molecular data have also been used to explore their phylogenetic relationships (Gong et al. 2006; Li et al. 2008; Martín-Cereceda et al. 2007; Miao et al. 2004; Miao, Yu, and Shen 2001; Utz and Eizirik 2007; Williams and Clamp 2007). However, these abundant data, far from being enlightening, have provoked an intense debate about the monophyly of peritrichs. Seemingly, the placement of the peritrichs within the class Oligohymenophorea is the only assumption widely accepted and supported by both morphological characters (Bardele 1981; Lynn 1979, 1981) and molecular phylogenetics (Gong et al. 2006; Li et al. 2008; Miao et al. 2004; Utz and Eizirik 2007). On the other hand, many phylogenetic relationships within the peritrichs have not been well resolved by molecular markers (Clamp and Williams 2006; Itabashi et al. 2002; Miao et al. 2001). Currently, the debate is mainly centered on the relationships between the two recognized orders, Sessilida and Mobilida. The phylogeny based on small subunit (SSU) rRNA showed that the mobilids and sessilids do not cluster together, and so the subclass Peritrichia appears to be paraphyletic (Gong et al. 2006; Zhan et al. 2009).

The disagreement between the molecular and morphological data can be explained in two ways: either the similarity of the sessilids and mobilids in the oral apparatus and the somatic ciliature is homoplastic instead of homologous or the clustering of these two groups is masked in SSU rRNA trees, whose reliability need to be assessed. The simple way is to analyze an additional molecular marker and to check congruency in terms of repeatability of clades (Chen, Bonillo, and Lecointre 2003; Lynn 2008). The α-tubulin gene is a good candidate to analyze the systematic relationships in ciliates as they display a great variety of microtubular arrays made with a reduced genetic repertoire of tubulins highly similar in sequence (Edlind 1998). This gene has proved useful as a molecular marker (Baroin-Tourancheau et al. 1998; Israel et al. 2002; Lee et al. 2008). In ciliates evolution of α-tubulin appears to be severely constrained because of its essential role in structure, making alignments of its sequences less ambiguous and less sensitive to differences in evolutionary rate than alignments of SSU rRNA sequences (Philippe, Germot, and Moreira 2000).

In this paper, we investigated the phylogenetic relationships of the peritrich ciliates by providing the α-tubulin gene information and searched for congruencies with SSU rRNA gene trees.

MATERIALS AND METHODS

Sample collection, isolation, and identification. Six species of mobilids and four species of sessilids were isolated in China and sequenced: *Trichodina heterodentata* Duncan, 1977, *Trichodina nobilis* Chen, 1963, and *Trichodinella myakkae* Mueller, 1937 from different fish hatcheries (30°32′N, 114°23′E) in Wuhan; *Trichodina sinonovaculae* Xu, Song, and Warren, 1999, *Urceolaria korschelti* Zick, 1928, and *Urceolaria urechi* Hirshfield, 1949 from culture beds (36°10′N, 120°56′E for the first two species; 37°47′N, 121°88′E for the last one) off the coast of Shandong; *Carchesium polypinum* Linne, 1785, *Vorticella campanula* Ehrenberg, 1839, *Zoothamnium arbuscula* Ehrenberg, 1839, and *Epistylis* sp. (30°33′N, 114°21′E) from Donghu Lake, Wuhan.

Identification was mainly based on the examination of living and silver nitrate-impregnated specimens. The methods of isolation for mobilids and sessilids followed procedures published elsewhere (Gong et al. 2006; Miao et al. 2001)

Corresponding Author: Y.-H. Yu, Key Laboratory of Biodiversity and Conservation of Aquatic Organisms, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China—Telephone number: +86 27 6878 0773; FAX number: +86 27 6878 0678; e-mail: yhyu@ihb.ac.cn

DNA extraction, polymerase chain reaction (PCR) amplification, cloning, and sequencing. Genomic DNA extraction for each species was done following conventional methods of phenol extraction and ethanol precipitation (Sambrook, Fritsch, and Maniatis 1989). DNA amplifications were done in 25-μl reactions, using peritrich-specific primers designed against α-tubulin (forward: 5′-TGY YTK GAG CAY GGT ATY CAA CC-3′; reverse: 5′-SAG AAY TCT CCT TCY TCC AT-3′), and Taq DNA polymerase (Fermentas, Foster City, CA). Temperature cycling was 30 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 56 °C, and extension for 1 min at 72 °C, followed by 35 cycles in the same manner, but with the annealing temperature increased to 62 °C. Polymerase chain reaction amplifications were performed in a Perkin-Elmer GeneAmp PCR System 9600 (PE Applied Biosystems, Mississauga, ON, Canada).

The PCR products were purified using the Biostar Glassmilk DNA Purification Kit (BioStar International, Toronto, ON, Canada) following the supplier's instructions, and ligated into pGEM-T Easy vector using T4 Ligase (Promega Biotech, Madison, WI). After overnight incubation at room temperature, an aliquot of the ligation reaction was used to transform *Escherichia coli* HB101 competent cells. Clones were selected on blue/white-based screening, subjected to plasmid isolation, and DNA restriction. Positives clones were selected, according to the size of the insert, and submitted to sequencing in an automated DNA sequencer (ABI PRISM 377, Applied Biosystems Inc., Foster City, CA).

Sequence and phylogenetic analyses. The α-tubulin gene sequences of the various taxa were obtained originally in this study or from the GenBank/EMBL databases. All the SSU rRNA gene sequences for the present study were retrieved from the GenBank/EMBL databases. Alignments were performed using CLUSTAL X (Thompson et al. 1997) with default parameters. Chi-square (χ^2) tests of base heterogeneity were implemented in PAUP*4.0b10 to test for compositional biases existing for each codon position of α-tubulin gene sequences across all taxa.

Phylogenetic analyses were based on α-tubulin gene sequences, SSU rRNA gene sequences, and SSU rRNA and α-tubulin genes sequences in combination, respectively. For α-tubulin gene sequences, both the nucleotide sequences, using only the first and second positions of each codon, and the amino acid sequences of a total of 29 partial α-tubulin gene sequences, including the 10 newly sequenced peritrich ciliates, were used to construct the phylogenetic trees. For SSU rRNA gene sequences, the species samples were chosen to be nearly identical to those for α-tubulin, and a total of 28 partial or complete SSU rRNA gene sequences were selected to construct the phylogenetic trees. For SSU rRNA and α-tubulin gene sequences in combination, only 17 species, including 11 peritrich ciliates that have both α-tubulin and SSU rRNA gene sequences information were used for analyses. Here also, the first and second positions of each codon of the α-tubulin gene sequences and the SSU rRNA gene sequences were combined to be used to phylogenetic analysis. In all analyses, *Loxodes striatus* (Karyorelictea) was chosen as outgroup.

Maximum parsimony (MP), neighbor joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) were used to build trees. The MP and NJ analyses were performed with PAUP*4.0b10 (Swofford 2002), ML analysis was conducted with PHYML 2.4.4 (Guindon and Gascuel 2003), and BI was done with MrBayes 3.0b (Huelsenbeck and Ronquist 2001). For MP analysis, characters were not weighted. Tree searches used tree bisection-reconnection branch swapping and 100 simple sequence addition replicates. For NJ, ML, and BI analyses, the tree topologies were inferred using the model 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 resampled 1,000 times, and for Bayesian analysis, the chain length for our analysis was 1,000,000 generations with trees samples every 100 generations, and the first 4,000 generations were discarded as burn-in.

Statistical significance of trees topologies, recovered under different methods of analyses (ML, MP, NJ, and BI), was analyzed using Kishino–Hasegawa (K–H) and Shimodaira–Hasegawa (S–H) tests (Kishino and Hasegawa 1989; Shimodaira and Hasegawa 1999), both tests implemented in PAUP*4.0b10. To test the monophyly of peritrichs and mobilids, we also constructed topologies with the peritrichs and mobilids as monophyletic, respectively, and use the K–H and S–H tests to determine whether the monophyly is rejected. The parameters of tests were 1,000 bootstrap replicates of resampling estimated log-likelihood.

RESULTS

α-Tubulin gene sequences and variations. We obtained 10 new partial α-tubulin gene sequences, whose lengths, GC contents, and accession numbers are shown in Table 1. These sequences show no introns, as they cover amino acids without any detectable intervening sequence.

Chi-square (χ^2) tests showed that nucleotide compositions among all taxa analyzed were homogenous at the first codon positions, a little heterogeneous at the second, but significantly heterogeneous at the third position: first position, $\chi^2 = 82.31$, df = 84, $P = 0.53$; second position, $\chi^2 = 4.73$, df = 84, $P = 1.00$; and third position, $\chi^2 = 2022.48$, df = 84, $P < 0.001$. Considering the significant heterogeneity at the third position, only the first and second positions of each codon were used for the phylogenetic analyses. This means that 716 out of 1,074 positions were used for further analyses, of which 556 were identical (51%), 160 variable (14%), and 108 (10%) informative for parsimony.

Table **1.** Peritrichs α-tubulin gene sequences characteristics.

Species	Orders	Collector	Length (bp)	GC content (%)	Accession number
Trichodina nobilis	Mobilida	Gong	1184	60.8	EF569679
Trichodina heterodentata	Mobilida	Gong	1184	54.4	EF569680
Trichodina sinonovaculae	Mobilida	Zhan and Xu	1047	51.9	FJ890343
Trichodinella myakkae	Mobilida	Gong	1184	60.2	EF569678
Urceolaria korschelti	Mobilida	Zhan and Xu	1047	40.1	GQ285128
Urceolaria urechi	Mobilida	Zhan and Xu	1047	40.5	GQ285127
Carchesium polypinium	Sessilida	Gong and Li	1184	57.7	FJ883553
Epistylis sp.	Sessilida	Gong and Li	1184	39.2	FJ883554
Vorticella campanula	Sessilida	Gong and Li	1184	52	FJ883555
Zoothamnium arbuscula	Sessilida	Gong and Li	1184	57.7	FJ883556

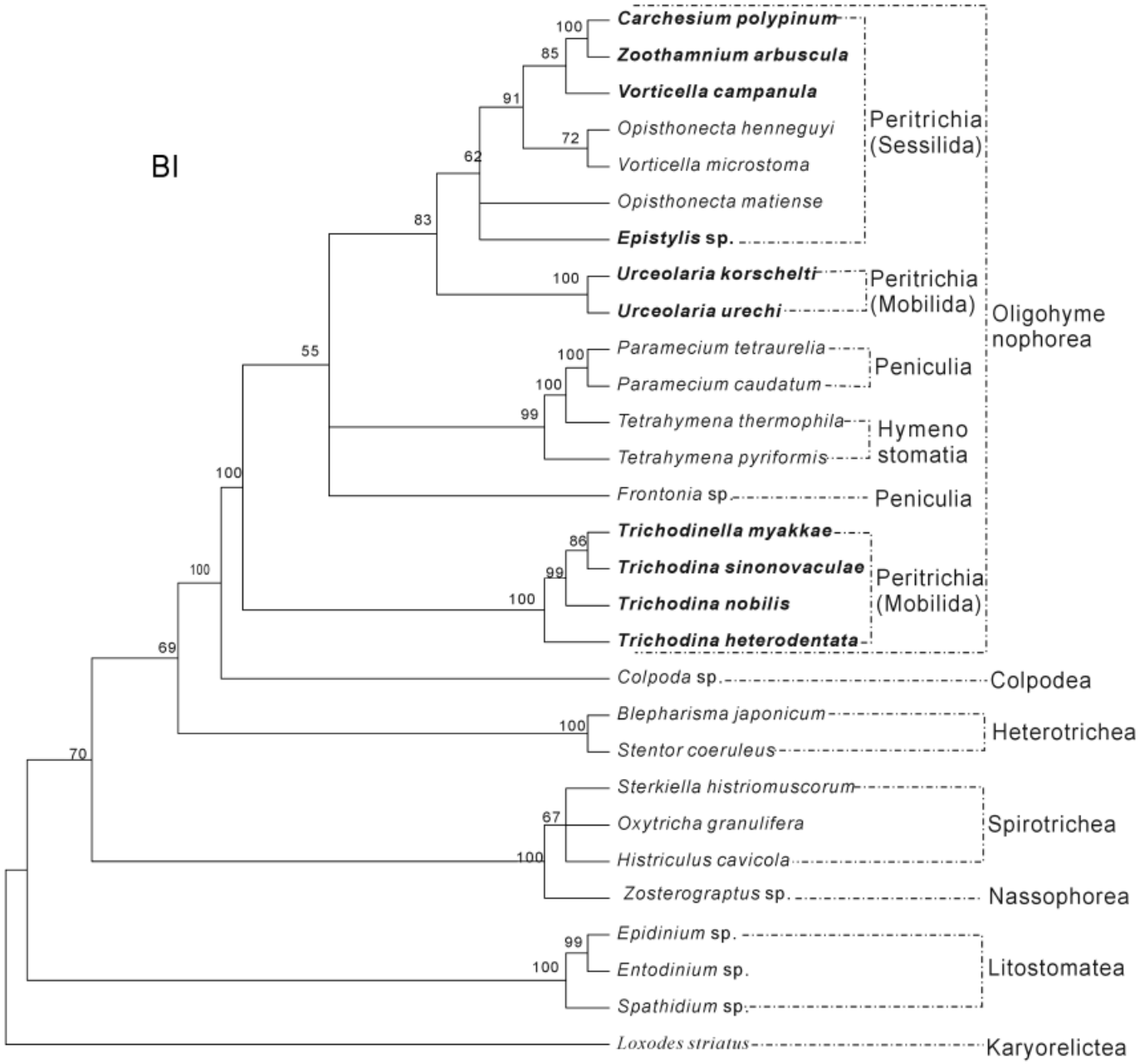

Fig. **1.** The 50% majority rule Bayesian tree inferred from α-tubulin nucleotide sequences of ciliated protozoa with the model of ''GTR+I+G'' selected by AIC in Modeltest 3.7. Only the first and second positions of each codon were used. Numbers on branches indicate the Bayesian posterior probability. New sequences submitted in this study are in bold.

Phylogenetic analyses based on α-tubulin gene sequences. For the phylogenetic trees from nucleotides and amino acids sequences, both the K–H test and S–H test depicted the BI tree as the best, and there were significant differences between the BI tree and MP and NJ trees. Because some bootstraps at the nodes of peritrich ciliates were very low in the ML tree, and there were many parallel branches on the MP and NJ trees, only the BI tree is illustrated (Fig. 1, 2).

In the BI tree based on α-tubulin nucleotides sequences (Fig. 1), the monophyly of the individual ciliate classes was supported. Within the class Oligohymenophorea, the sessilid peritrichs and the mobilids did not cluster together: the sessilids always constituted a terminal clade together with the urceolariid mobilids while the trichodinid mobilids appeared as the basal clade. Besides, both the MP and NJ trees did not support the monophyly of peritrichs and mobilids either.

In the BI tree based on α-tubulin amino acids sequences (Fig. 2), the topology is very similar to the nucleotide tree (cf. Fig. 1): the sessilid peritrichs and mobilid peritrichs still did not cluster together, and the urceolariids and the trichodinids did not group together. Besides, Ml, MP, and NJ trees did not support the monophyly of peritrichs and mobilids either. In this analysis, the urceolariids cluster with Peniculia (Fig. 2) instead of the sessilids (Fig. 1).

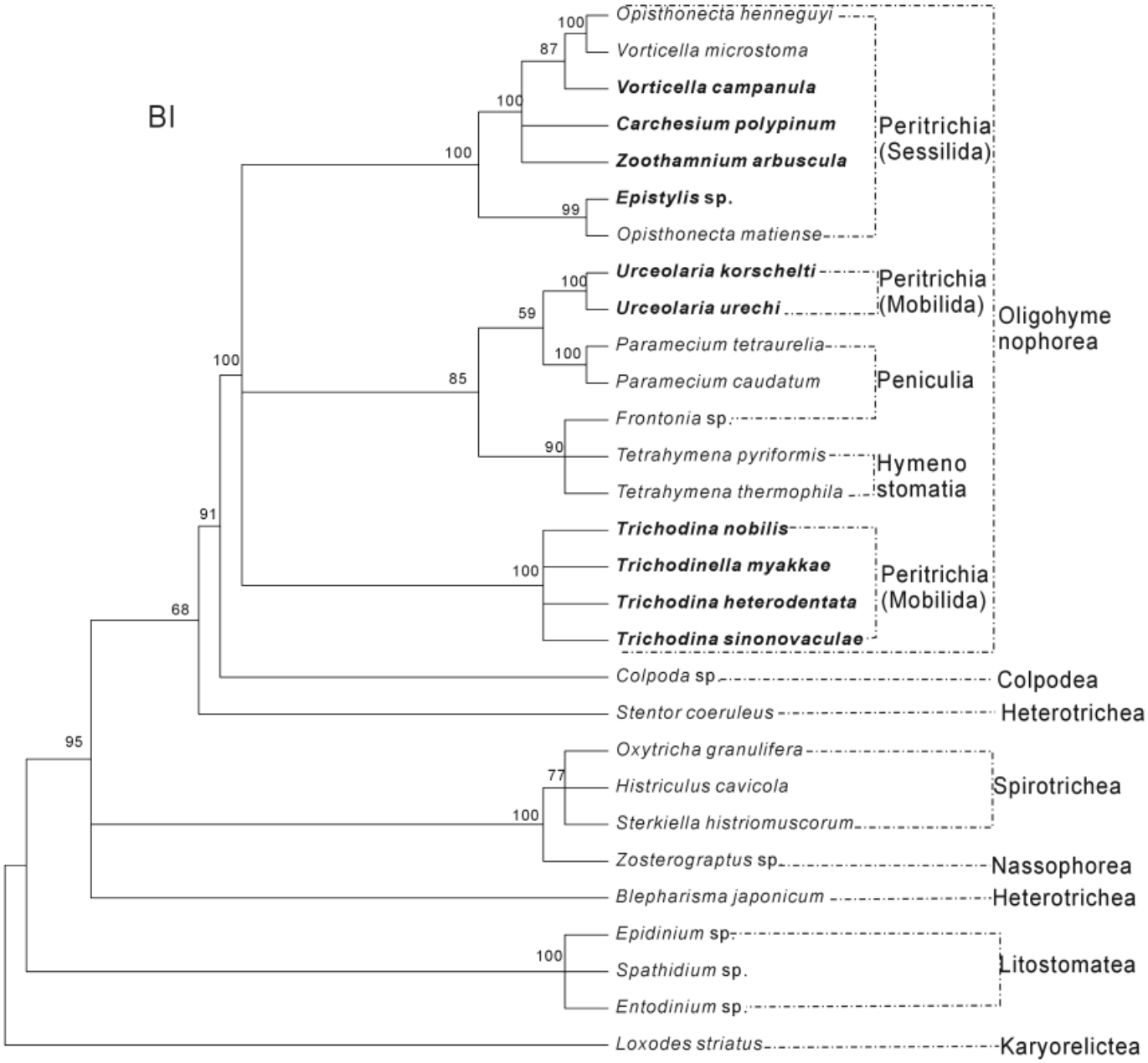

Fig. **2.** The 50% majority rule Bayesian tree inferred from α-tubulin amino acid sequences of ciliated protozoa with the parameters of ''Nst = 6 Rates = gamma.'' Numbers on branches indicate the Bayesian posterior probability. New sequences submitted in this study are in bold.

The K–H and the S–H tests showed that both the monophyly of peritrichs and that of mobilids were not quietly rejected by the analyses based on α-tubulin gene sequences, while the monophyly of Peniculia was rejected.

Phylogenetic analyses based on small subunit rRNA gene sequences. Both the K–H and S–H tests depicted the BI and ML trees (their topologies are quite identical) based on SSU rRNA gene sequences as the best, so the two trees were combined into a consensus tree for the clarity of illustration (Fig. 3). MP and NJ trees that showed a little difference from the best tree were not shown in the paper, and the main difference was that the mobilids and peniculines did not form a sister group in the MP and NJ trees.

In the consensus tree (Fig. 3), the monophyly of all ciliate classes was supported. Within the oligohymenophoreans, the sessilids always constituted a terminal clade associated with the subclass Hymenostomatia, whereas the mobilids were associated with the subclass Peniculia. Within the sessilids, *Vorticella microstoma* and *Opisthonecta henneguyi* always branched together.

Based on SSU rRNA gene sequences, the K–H and the S–H tests showed that the monophyly of peritrichs was not quietly rejected and the monophyly of mobilids and peniculines were supported by the analyses.

Phylogenetic analyses based on small subunit rRNA and α-tubulin gene sequences in combination. The topologies

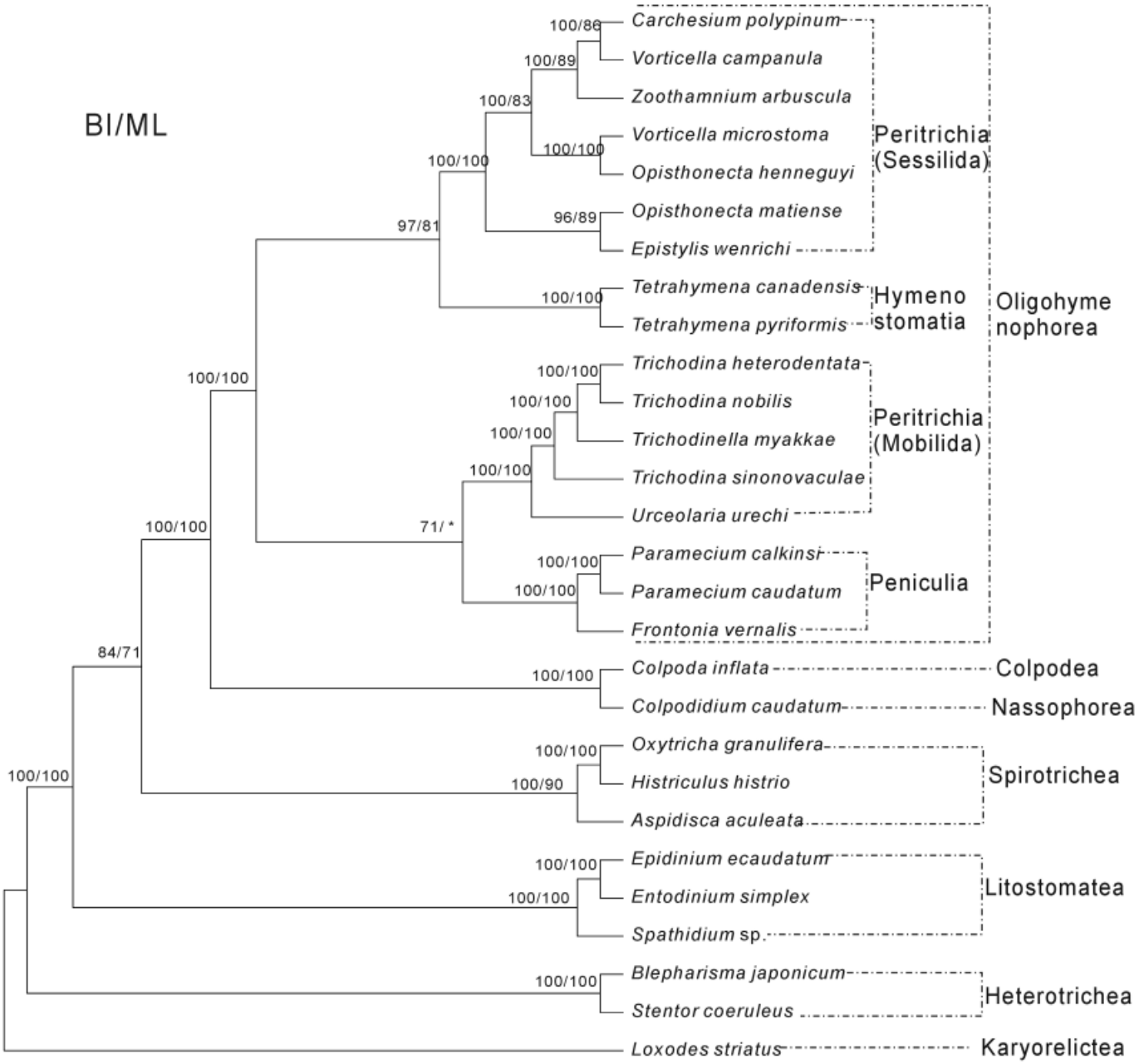

Fig. **3.** The 50% majority rule consensus phylogenetic tree inferred from small subunit rRNA sequences of ciliated protozoa with the model of ''TrN+I+G'' selected by AIC in Modeltest 3.7. Numbers on branches indicate the Bayesian posterior probability followed by the maximum likelihood bootstrap value. Asterisks indicate bootstrap values $<50\%$.

of BI, MP, and ML trees were identical, and were determined as the best by the K–H and S–H tests, so the three trees were combined in a consensus tree (Fig. 4). Within the class Oligohymenophorea, the ciliates were divided into two groups, in which the sessilid peritrichs clustered with Hymenostomatia, while the mobilids grouped with Peniculia. In this analysis, the urceolariids did not cluster with the trichodinids but with Peniculia. As before, *V. microstoma* and *O. henneguyi* invariably branched together.

The K–H and the S–H tests showed that both the monophyly of peritrichs and that of mobilids were not quietly rejected.

DISCUSSION

Phylogenetic relationships between mobilids and sessilids. According to the distinctive oral apparatus and the highly reduced somatic ciliature, the ciliate orders Mobilida and Sessilida have long been assigned to the subclass Peritrichia (Kahl 1933; Lom 1964; Lynn 2008). However, based on SSU rRNA gene sequence data the monophyly of the subclass Peritrichia was questioned (Gong et al. 2006; Li et al. 2008). Recently, Zhan et al. (2009) reinvestigated the phylogenetic relationships of the peritrich ciliates by adding four complete SSU rRNA sequences of the

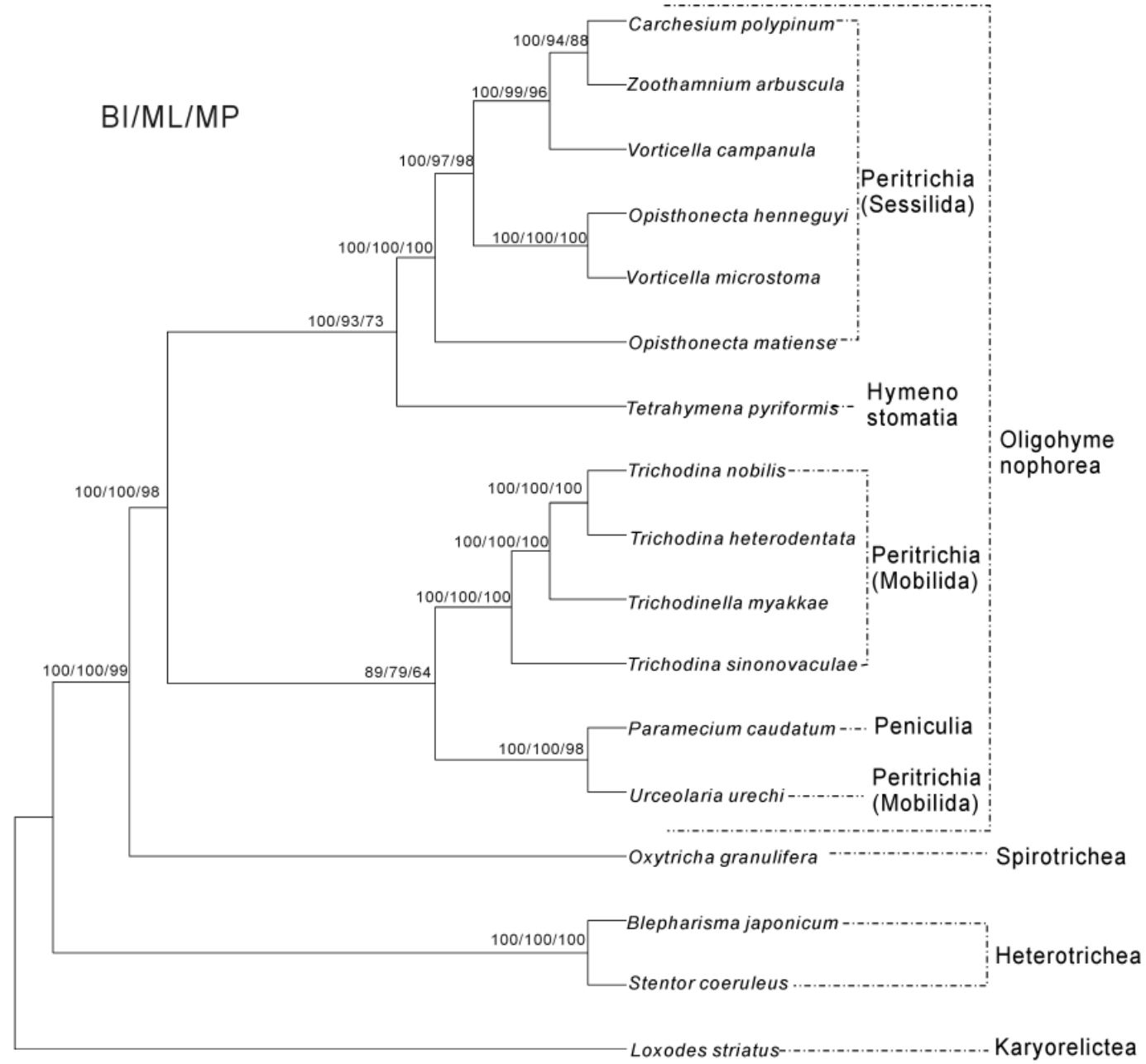

Fig. **4.** The 50% majority rule consensus phylogenetic tree inferred from small subunit rRNA sequences and α-tubulin nucleotide sequences of ciliated protozoa in combination (only the first and second positions of each codon for the tubulin gene were used) with the model of ''TrN+I+G'' selected by AIC in Modeltest 3.7. Numbers on branches indicate the Bayesian posterior probability followed by the maximum likelihood and maximum parsimony bootstrap values.

mobilids, and suggested establishing the new subclass Mobilia Kahl (1933) to contain the order Mobilida Kahl, (1933).

All the phylogenetic trees in the present study, either based on α-tubulin nucleotide sequences, α-tubulin amino acid sequences, SSU rRNA gene sequences, or SSU rRNA and α-tubulin genes sequences in combination, showed that the mobilids and sessilids did not cluster together. This provides further support for the hypothesis that the subclass Peritrichia is not monophyletic. However, the emergence of the orders within the class Oligohymenophorea is somehow different among the α-tubulin and SSU rRNA trees. In the α-tubulin trees, the mobilid family Trichodinidae constitutes a basal clade and the peritrich order Sessilida constitutes a terminal clade, while in the SSU rRNA or combined gene trees, orders/subclasses are paired (i.e. Sessilida/Hymenostomatia and Mobilida/Peniculia), as observed previously (Gong et al. 2006; Li et al. 2008; Utz and Eizirik 2007). On the other hand, the α-tubulin trees supported neither the monophyly of the order Mobilida nor that of the subclass Peniculia, which is certainly contrary to morphological characters of these groups. However, the K–H and S–H tests did not reject the monophyly of the mobilids. It is probable that in a constrained protein like α-tubulin molecular convergence might be responsible for

odd relationships (Edlind 1998), such as that of urceolariids and peniculines.

Nonetheless, our previous claim that the peritrichs do not constitute a monophyletic group is reinforced because the mobilids and sessilids do not cluster together either in the α-tubulin or SSU rRNA tree. Thus, we agree with the arrangement of Zhan et al. (2009), who separated the mobilids from the sessilids (Peritrichia *sensu stricto*) and established a new subclass Mobilia.

Phylogenetic relationships between the trichodinids and urceolariids. Morphologically, the trichodinids and urceolariids have counter-clockwise adoral ciliature encircling a conspicuous peristome and an elaborate denticulate adhesive disc (Basson and Van As 1989; Bradbury 1970). Both groups are considered to constitute the order Mobilida (Corliss 1979; Lynn 2008), which is highly supported by the SSU rRNA gene sequence data (Zhan et al. 2009). However, both the α-tubulin gene and the SSU rRNA and α-tubulin genes in combination do not support the grouping of the urceolariids and trichodinids. This contradiction between the phylogeny based on α-tubulin gene data and that based on the SSU rRNA gene data is puzzling. It raises the question of whether the α-tubulin gene sequences are suitable for phylogenetic analyses of lower levels of ciliate taxa (e.g. at or below the order level). On the other hand, undersampling might obscure the relationships of these groups as there were only two sequences of urceolariid species available for analysis. The only way to make a conclusive determination of this is performing analyses that include a much better sampling of the existing taxa with good representation at the genus and species level.

The comparison between the two small subunit rRNA and α-tubulin genes. With moderate evolutionary rate, the SSU rRNA gene sequences have been widely used to infer the phylogeny of various ciliate groups (Caetano-Anollés 2002). Seen from the SSU rRNA trees in the present study, the topologies constructed by different methods are nearly identical, with usually very high bootstraps. However, it is the case that the SSU rRNA data themselves pose significant phylogenetic problems, suggesting that many genera and families of peritrich ciliates are not monophyletic, such as the genera *Opisthonecta* (Martín-Cereceda et al. 2007), *Vorticella* (Itabashi et al. 2002; Miao et al. 2004), *Epistylis* (Miao et al. 2001), and *Zoothamnium* (Clamp and Williams 2006).

As concerned the α-tubulin gene, it has been used to reconstruct the phylogeny of ciliates at high levels (Baroin-Tourancheau et al. 1998; Israel et al. 2002) because the sequences are quite conserved (Edlind 1998). In correspondence with the SSU rRNA gene data, the α-tubulin gene sequences provide further support for the separation of the mobilid from the sessilid peritrichs. However, like the SSU rRNA gene, the α-tubulin gene may not be suitable for phylogenetic analyses of lower levels of peritrich ciliate taxa because MP and NJ trees based on either the α-tubulin nucleotide or amino acid sequences yielded many parallel branches with often very low bootstraps (data not shown). Thus, it is necessary to find other molecular markers and in particular to make more representative taxon sampling to determine the phylogenetic relationships within sessilid and mobilid ciliates.

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