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Multigene-Based Analyses of the Phylogenetic Evolution of Oligotrich Ciliates, with Consideration of the Internal Transcribed Spacer 2 Secondary Structure of Three Systematically Ambiguous Genera

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Oligotrichs are ciliates of great abundance, but their molecular systematics are rarely studied. In this study, nine species representing three genera (*Strombidium*, *Novistrombidium*, and *Omegastrombidium*) of marine oligotrich ciliates were collected from coastal waters of China. The small subunit (SSU) rRNA gene of two species and the internal transcribed spacers and 5.8S region (ITS1-5.8S-ITS2) for all nine species were sequenced for the first time. Phylogenetic trees using both the SSU rRNA gene and ITS1-5.8S-ITS2 region sequences were generated. In addition, the secondary structures of ITS2 RNA transcripts of 11 taxa representing four genera (*Novistrombidium*, *Strombidium*, *Omegastrombidium*, and *Laboea*) were investigated. The phylogenetic analyses show that (i) the family Strombidiidae is polyphyletic, (ii) the genus *Novistrombidium* is probably paraphyletic, containing at least two subclades, which is consistent with recent cladistic analyses based on morphological data, and (iii) the tailless genus *Laboea* is separate from other genera of Strombidiidae, clustering instead with the tontoniids. Comparisons of the secondary structure of ITS2 regions also show that *Laboea* is clearly different from other strombidiids. These findings cast doubt on the monophyly of the family Strombidiidae.

Most members of the ciliate subclass Oligotrichia are thought to be cosmopolitan (1). Because of their high abundance and growth rate, oligotrichs are an important component of marine planktonic ciliate communities and play a critical role in the trophic flux and nutrient cycling of the pelagial realm (2). To date only c. 60% of oligotrichs, or about 120 species, have been described or redescribed using modern methods to reveal details of the infraciliature and other morphological features of taxonomic importance (1, 3–6). Consequently, many issues concerning the systematics of members within the group remain unresolved.

In recent years, molecular methods have been applied to investigate phylogenetic relationships among oligotrichs, mostly based on small subunit (SSU) rRNA gene sequence data (5–9). However, it is increasingly recognized that any single gene has limitations for elucidating evolutionary relationships among ciliates (10). Furthermore, there appears to be more genetic variation among oligotrich and choreotrich genera than among other spirotrich ciliate groups, casting doubt on the likelihood that SSU rRNA gene sequence data alone can reflect this variation (11). Additional molecular markers that are increasingly used for investigating phylogenetic relationships among ciliates include the internal transcribed spacer 2 (ITS2) secondary structure and ITS1-5.8S-ITS2 region sequence data (12, 13). In the present study, we combine data from all three sources (i.e., SSU rRNA gene sequences, ITS1-5.8S-ITS2 region sequences, and ITS2 secondary structure) in order to infer evolutionary relationships within the Oligotrichia. Furthermore, in order to better resolve the phylogeny of the group, we increase the number of sampled taxa by adding molecular data for nine populations, representing seven species and three genera of oligotrichs.

MATERIALS AND METHODS

Ciliate collection and identification. Samples were collected using 20-μm mesh plankton nets. Collection data for each species are given in Table 1. Culturing and morphological examination of these species were performed using the methods of previous studies (3, 14). Species identification was based on the literature (4, 15, 16). Terminology and systematics follow Lynn (17).

DNA extraction, PCR amplification, cloning, and sequencing. Genomic DNA was extracted as described by Liu et al. (16). In brief, 10 to 15 cells were starved overnight in sterile seawater at room temperature to minimize the contents of food vacuoles and eliminate contaminants (13). DNA was extracted using a RdExtract-N-Amp tissue PCR kit (Sigma, St. Louis, MO) according to the manufacturer's protocol, with the slight modification that only 1/10 of the volume suggested for each reagent solution was used.

Primers used for amplifying ITS1-5.8S-ITS2 region sequences were 5.8SF, 5′-GTA GGT GAA CCT GCG GAA GGA TCA TTA-3′, and 5.8SR, 5′-TAC TGA TAT GCT TAA GTT CAG CGG-3′. PCR conditions followed Yi and Song (13). Additionally, the universal eukaryotic forward primer Euk A (5′-AACCTGGTTGATCCTGCCAGT-3′) and reverse primer Euk B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) were used to amplify the SSU rRNA gene (18), using the PCR protocol described by Liu et al. (19).

The purified PCR product was inserted into the pUCm-T vector (Shanghai Sangon Biological Engineering & Technical Service Company, Shanghai, China) and transformed into *E. coli* DH5α cells. Sequencing was carried out on an ABI 3730 sequencer (Applied Biosystems).

Phylogenetic analyses. Other sequences used in this study were obtained from the NCBI GenBank database (accession numbers are given in

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TABLE 1 Oligotrichs sampled for this study

Species	Sample location	Date (mo/yr)
Novistrombidium sinicum pop1	Mangrove wetland, Shenzhen (22°32′N, 114°01′E; southern China)	01/2008
Omegastrombidium cf. *elegans*	Daya Bay, Guangdong (22°42′N, 114°32′E), southern China	01/2008
Strombidium stylifer	Mangrove wetland, Shenzhen	04/2008
Strombidium basimorphum	Mangrove wetland, Shenzhen	04/2008
Novistrombidium testaceum	Mangrove wetland, Shenzhen	04/2008
Novistrombidium orientale	Daya Bay, Guangdong	03/2008
Strombidium conicum	Daya Bay, Guangdong	04/2008
Novistrombidium sinicum pop2	Daya Bay, Guangdong	04/2008
Strombidium cf. *conicum*	Daya Bay, Guangdong	12/2008

Fig. 1 and 2A). Sequences were aligned using Clustal W implemented in BioEdit 7.0.0 (20), and the unique regions for oligotrichs were exported using Seaview 4 (21). Ends were trimmed, and the ambiguously aligned sites were refined, yielding an alignment of 1,532 (versus 2,005 in the original alignment) sites for the SSU rRNA gene, 563 (versus 766 in the original alignment) sites for the ITS1-5.8S-ITS2 region, and 2,190 (versus 2,615 in the original alignment) sites for the concatenated SSU rRNA and ITS-5.8S gene sequences, respectively. For the phylogenetic analyses, the

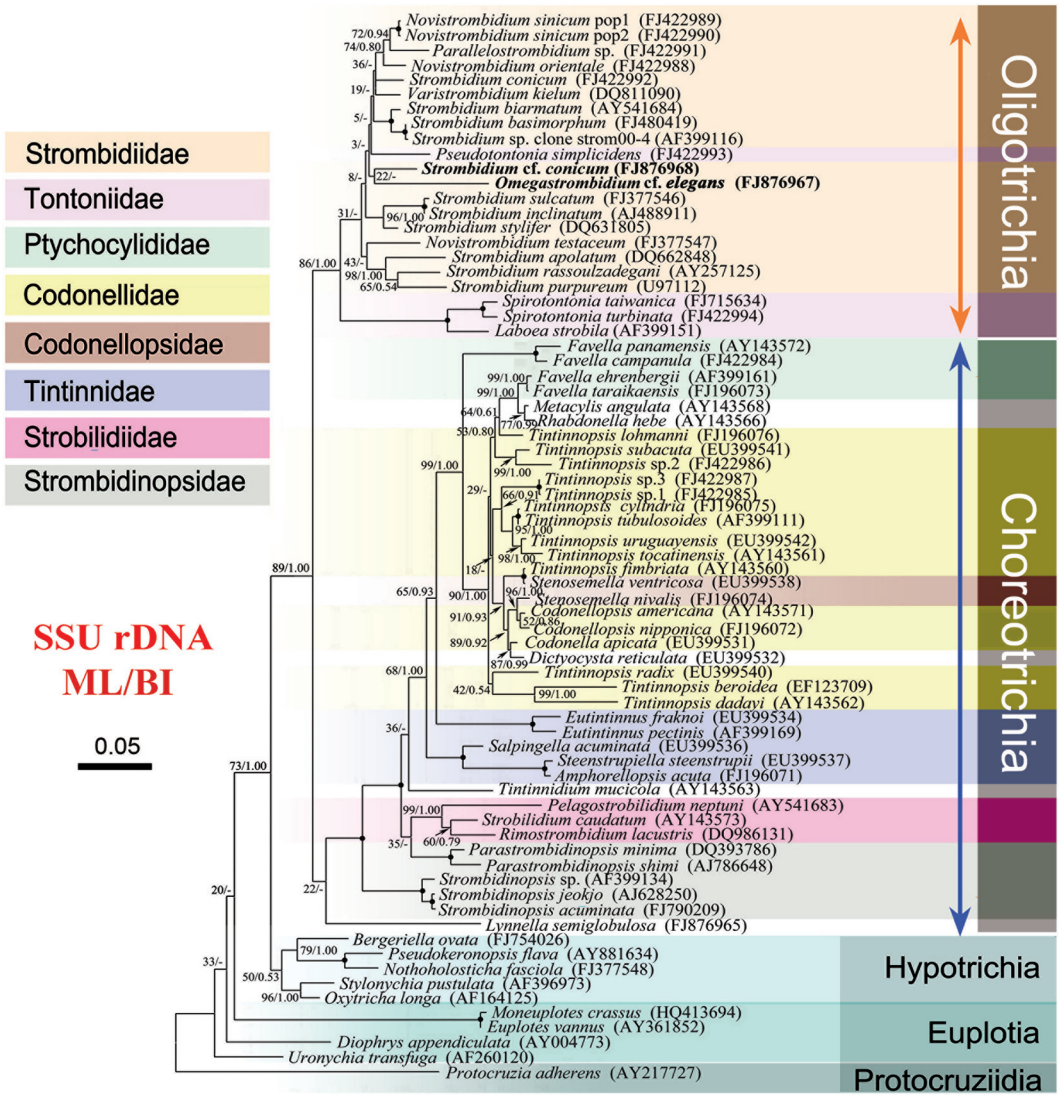

FIG 1 Phylogenetic tree based on 72 SSU rDNA sequences inferred by maximum-likelihood (ML) and Bayesian inference (BI) analyses. Numbers near the nodes of branches represent the bootstrap value for the ML analysis and the posterior probability value of the BI analysis, respectively. Dashes (-) reflect disagreement between the two topologies. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. Newly deposited sequences are in boldface. The codes in parentheses following the species name are the GenBank sequence accession numbers.

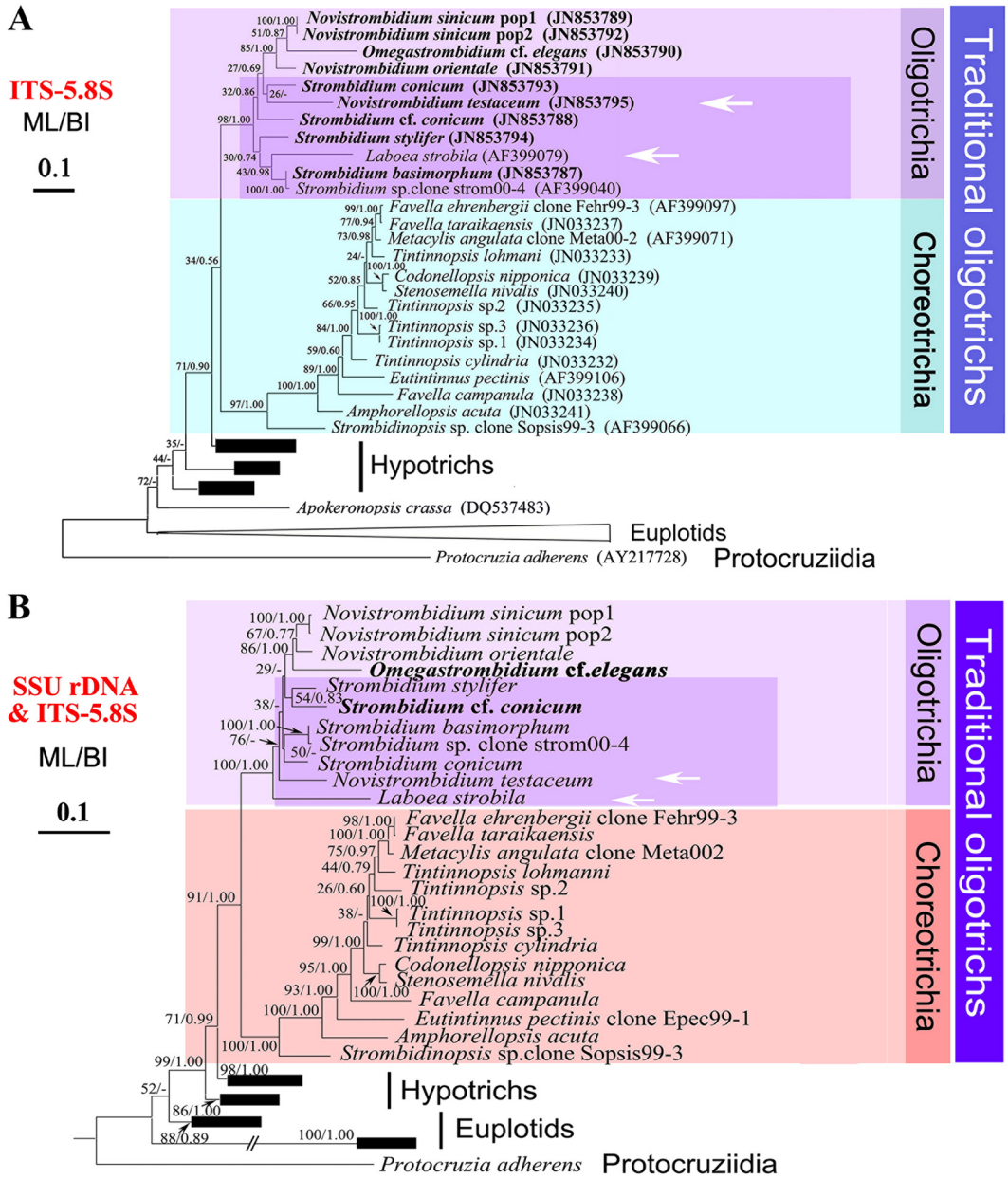

FIG 2 Phylogenetic trees based on ITS1-5.8S-ITS2 region sequences (A) and SSU rRNA gene and ITS-5.8S region sequences (B) inferred by maximum likelihood (ML) and Bayesian inference (BI) analyses. Species newly sequenced in this study are in boldface. The codes following the names are the GenBank sequence accession numbers. Numbers on branches represent the bootstrap values from ML analysis and posterior probability of Bayesian analysis, respectively. Dashes (-) indicate disagreement between the ML and BI methods. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

program Modeltest (22) selected GTR plus I (0.4092) plus G (0.4771) for the SSU rRNA gene, GTR plus I (0.0673) plus G (0.5883) for the ITS1-5.8S-ITS2 region, and GTR plus I (0.3208) plus G (0.5055) for the concatenated sequences as the best models under the AIC criterion, which were then used to construct maximum likelihood (ML) trees. Similarly, MrModeltest, version 2 (23), chose the same best-fitting model with the identical values for Bayesian inference (BI) analyses. ML trees were constructed with the PhyML program, version 2.4.4 (24). The reliability of internal branches was assessed using a nonparametric bootstrap method with 1,000 replicates. The BI analysis was performed with MrBayes 3.1.2 (25). Four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 (2,000,000 for concatenated sequences) generations with a sample frequency of 100. The first 2,500 (5,000 for concatenated sequences) generations were discarded as burn-in. Posterior probabilities were calculated by applying the majority rule consensus. *Protocruzia adherens* of the subclass Protocruziidia was selected as the outgroup taxon in the analyses.

ITS2 secondary structure prediction. The ITS2 sequences were submitted to the mfold website (http://mfold.rna.albany.edu/?q=mfold) for secondary structure prediction with default settings (26). Structures were edited for esthetic purposes with RnaViz 2.0 (27) under the model for ciliates (28).

Topology testing. In order to test the monophyly of the family Strombidiidae and of the genera *Novistrombidium* and *Strombidium*, the approximately unbiased (AU) test was used (29). Steps were as described in reference 12. Eight constrained ML analyses were carried out on the SSU

TABLE 2 List of species for which both the ITS1-5.8S-ITS2 region and SSU-rDNA gene were newly sequenced[a] or obtained from GenBank

Species	ITS1-5.8S-ITS2			SSU-rRNA gene		
	GenBank accession no.	Sequence length (bp)	GC content (%)	GenBank accession no.	Sequence length (bp)	GC content (%)
Strombidium conicum	**JN853793**	546	48.53	FJ422992	1775	47.61
S. cf. *conicum*	**JN853788**	542	46.68	**FJ876968**	1776	47.30
Omegastrombidium cf. *elegans*	**JN853790**	542	44.10	**FJ876967**	1772	43.74
S. stylifer	**JN853794**	541	49.15	DQ631805	1774	48.03
S. basimorphum	**JN853787**	543	47.88	FJ480419	1774	48.08
Novistrombidium orientale	**JN853791**	536	48.51	FJ422988	1772	47.35
N. sinicum pop1	**JN853789**	534	50.37	FJ422989	1773	48.34
N. sinicum pop2	**JN853792**	534	50.56	FJ422990	1773	48.11
N. testaceum	**JN853795**	538	47.21	FJ377547	1770	48.36

[a] Accession numbers for new sequences are in boldface.

rRNA alignment, as follows: (i) 18 strombidiids, (ii) 18 strombidiids and *Pseudotontonia simplicidens*, (iii) 18 strombidiids and *Laboea strobila*, (iv) 18 strombidiids, *P. simplicidens*, and *L. strobila*, (v) four species of *Novistrombidium*, (vi) *Laboea* and *Spirotontonia*, (vii) *Pseudotontonia*, *Laboea*, and *Spirotontonia*, and (viii) 11 species of *Strombidium*. The resulting constrained topologies were then compared to the unconstrained ML topologies. The internal relationships within each constrained group and the relationships among the remaining taxa were unspecified.

Nucleotide sequence accession numbers. The SSU rRNA gene of two species (*Strombidium* cf. *conicum* and *Omegastrombidium* cf. *elegans*) and the ITS1-5.8S-ITS2 region for all nine species were sequenced for the first time. The sequences were deposited in the NCBI GenBank database with accession numbers, lengths, and GC contents as listed in Table 2.

RESULTS

There is only a 1-bp difference in the ITS1-5.8S-ITS2 region sequences between the two populations of *N. sinicum*. The SSU rRNA gene and the ITS1-5.8S-ITS2 region sequences of the 11 strombidiids used in the phylogenetic analyses share similarities of 68.4% to 99.6% (86.0% to 99.6% without *Strombidium* sp.) and 73.2% to 99.8%, respectively.

Secondary structures of the order Strombidiida. Within the order Strombidiida, ITS1-5.8S-ITS2 region sequence data were available for 11 lineages representing four genera (*Novistrombidium*, *Omegastrombidium*, *Strombidium*, and *Laboea*). The predicted secondary structure of the ITS2 region generally consists of a large loop separated by two helices. Considering the primary sequences, as well as the secondary structures, of their ITS2 region, we found that (i) although four genera were represented, the 11 structures were divided into three modes (*Novistrombidium sinicum* population 1 [pop1] and pop2, *Novistrombidium orientale*, *Omegastrombidium* cf. *elegans*, and *Strombidium stylifer* [Fig. 3A to E]; *Novistrombidium testaceum*, *Strombidium* sp., *Strombidium basimorphum*, *Strombidium* cf. *conicum*, and *Strombidium conicum* [Fig. 3F to J]; and *Laboea strobila* [Fig. 3K]), (ii) the only substituted nucleotide between two isolates of *N. sinicum* was located at the 136th site of the ITS2 region (see Fig. S1 in the supplemental material), and (iii) the unique nucleotide contributing to the classification of these three modes, inferred from diagrams in Figure 3, was the 146th base (see Fig. S1).

Phylogenetic trees. Trees were constructed using different algorithms and with different gene sequences. The topologies were identical at higher taxonomic levels, whereas the relationships among certain strombidiid genera were inconsistent.

Trees based on SSU rRNA gene sequences with more taxa selected showed the monophyly of both subclasses, Oligotrichia and Choreotrichia (Fig. 1). *Laboea strobila* clustered with two species of the tontoniid genus *Spirotontonia* with high support values (100% ML and 1.00 BI) and was the sister clade to the strombidiid assemblage.

Trees constructed using the ITS1-5.8S-ITS2 region (Fig. 2A) showed that the subclass Oligotrichia was monophyletic with strong support (98% ML and 1.00 BI) and was sister to the subclass Choreotrichia, which was also monophyletic with strong support values (97% ML and 1.00 BI). There were two clades within the Oligotrichia, (i) *Laboea-Strombidium* (except *S. conicum*) (30% ML and 0.74 BI) and (ii) *Novistrombidium-Omegastrombidium-Strombidium conicum* (27% ML and 0.69 BI). Within the second clade, there were two groups; one comprised the two populations of *N. sinicum*, *O.* cf. *elegans* and *N. orientale*, and the other comprised *N. testaceum* and *S. conicum*.

In order to mitigate the effect of base mutation among isolates, SSU ribosomal DNA (rDNA) sequences were, as far as possible, obtained from the same clones as those used to construct the ITS1-5.8S-ITS2 region tree and then combined with their corresponding ITS-5.8S region sequences. The resulting concatenated tree (SSU rDNA and ITS-5.8S region) shared a similar topology to that of the ITS1-5.8S-ITS2 tree (Fig. 2B). For example, in both trees, (i) both Oligotrichia and Choreotrichia were monophyletic with high support and (ii) both *Strombidium* and *Novistrombidium* were paraphyletic. However, the distribution patterns of the component species were different. In the ITS1-5.8S-ITS2 tree, three of four *Novistrombidium* sequences formed a paraphyletic clade nesting with *Omegastrombidium* species (85% ML and 1.00 BI), while *N. testaceum* clustered with *S. conicum*. In the concatenated tree, *N. testaceum* appeared as the second deepest branch of Oligotrichia and the other three *Novistrombidium* sequences formed a monophyletic clade. The inconsistency also applied to *Strombidium*. In the ITS1-5.8S-ITS2 tree, five species of *Strombidium* scattered into 4 clades, while they clustered into two groups in the concatenated tree. The most obvious difference is the position of *Laboea*. In the concatenated tree (Fig. 2B), *L. strobila* occupied a basal position within the oligotrichs (100% ML and 1.00 BI), whereas in the ITS1-5.8S-ITS2 tree (Fig. 2A), *L. strobila* grouped with two *Strombidium* species (43% ML and 0.98 BI).

Topology testing. The AU test rejected the possibility that *Laboea* belongs to the family Strombidiidae (Table 3, constraint

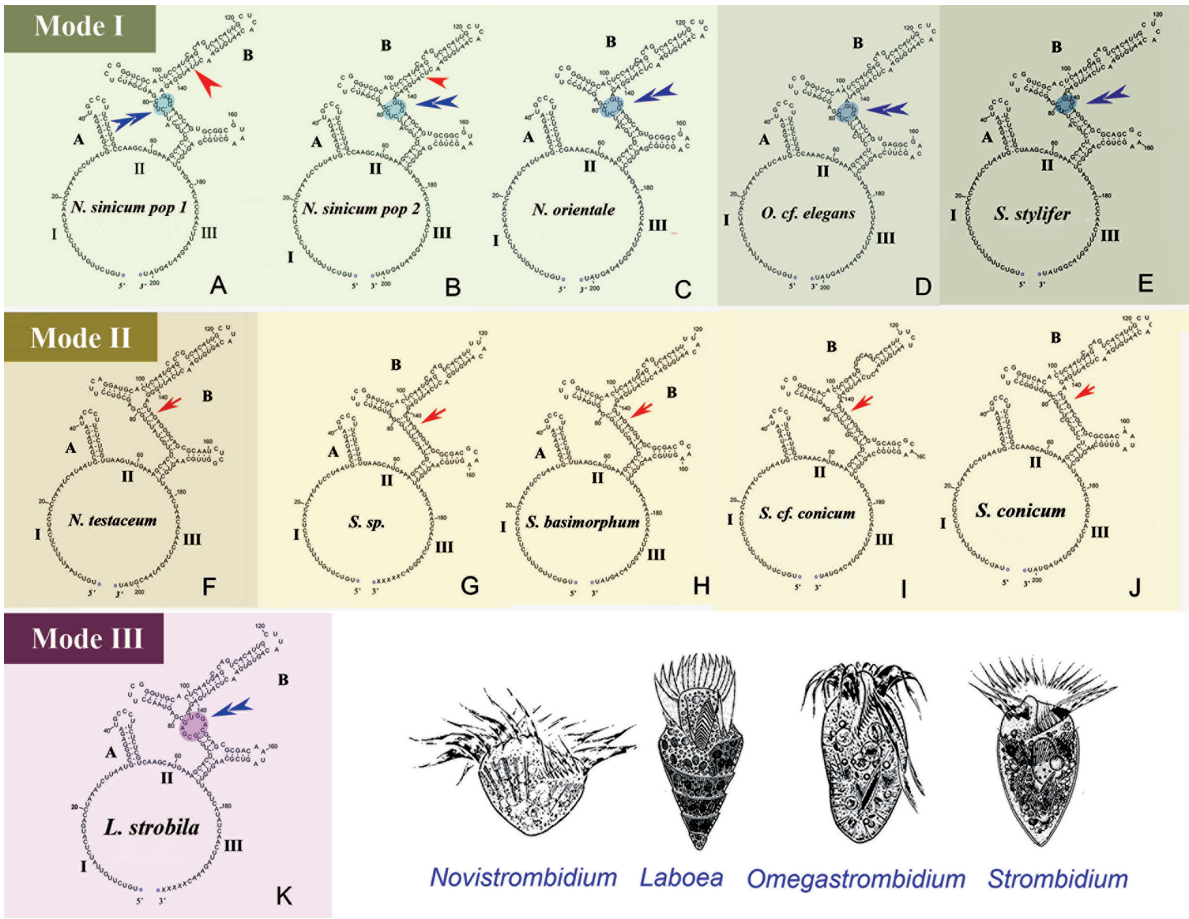

FIG 3 Secondary structures of the internal transcribed spacer 2 (ITS2) RNA transcript of representative species of four oligotrich genera (*Novistrombidium*, *Omegastrombidium*, *Strombidium*, and *Laboea*). The diagrams illustrate the two helices, labeled A and B, present in the class Spirotrichea (28). The three parts of the largest loop are labeled I, II, and III, respectively. The red arrowheads (in panels A and B) mark the only nucleotide variation between two populations of *N. sinicum*. Red arrows or blue double arrowheads indicate the different structures in helix B (elliptical shadowed region) among these three categories.

group 8, $P = 0.002$), but the possibility that all species of Strombidiidae except *Laboea* form a monophyletic group was not refuted (constraint group 3, $P = 0.970$). Moreover, even though species of *Strombidium* and *Novistrombidium* did not form monophyletic clades (Fig. 1), the possibility of monophyly of the genera *Strombidium* and *Novistrombidium* was not rejected (Table 3, constraint group 4, $P = 0.759$, and constraint group 5, $P = 0.404$). Furthermore, the topology was robust for the tontoniid *Spirotontonia* and *Pseudotontonia* together with *Laboea* (Table 3, constraint group 1, $P = 0.970$, and constraint group 2, $P = 0.970$).

TABLE 3 Approximately unbiased test results

Constraint group	Topology constraint	−ln likelihood	AU value (*P*)[a]
1	*Laboea* + *Spirotontonia*	15,809.24,178	0.970
2	*Pseudotontonia* + *Laboea* + *Spirotontonia*	15,809.24,178	0.970
3	18 strombidiids	15,809.24,178	0.970
4	*Strombidium* monophyly	15,813.87,249	0.759
5	*Novistrombidium* monophyly	15,820.11,228	0.404
6	18 strombidiids + *Pseudotontonia*	15,832.74,004	**0.039**
7	18 strombidiids + *Pseudotontonia* + *Laboea*	15,940.07,464	**4e−004**
8	18 strombidiids + *Laboea*	20,311.37,195	**0.002**

[a] AU values (*P*) below 0.05 are in boldface.

DISCUSSION

The genus *Laboea*. In recently published SSU rRNA gene trees of the Oligotrichia, the tail-less genus *Laboea* is more closely related to the tailed tontoniid genera *Spirotontonia* and *Pseudotontonia* than to the tail-less taxa of the family Strombidiidae (6, 8). This relationship was also recovered with maximum support (100% ML and 1.00 BI) in the present analyses based on SSU rRNA gene sequence data (Fig. 1). Moreover, the AU test rejected all constrained trees which supposed the monophyly of Strombidiidae with the inclusion of the genus *Laboea* ($P < 0.01$), and the constrained tree which supposed *Laboea* with *Spirotontonia* was not rejected ($P = 0.970$). Besides the tree topology, there are another three pieces of evidence supporting the closer relationship between *Laboea* and tontoniids, as follows. (i) The sequence identities of *Laboea* with three tontoniids (92.3% to 94.8%) were higher than those of *Laboea* with strombidiids (88.3% to 90.4%). (ii)

Laboea has more common unique characters with tontoniids than with strombidiids both in molecular information (see Fig. S2 in the supplemental material) and in morphological features. The morphology of *Laboea* is very similar to that of the tontoniid *Spirotontonia*, e.g., the irregular cone-shaped body with sinistrally spiraled girdle kinety, giving it a screw-like appearance, and the multiple macronuclear nodules (3) (see Fig. S3 in the supplemental material). (iii) The secondary structure of the ITS2 region shows that *Laboea* clearly differs from other strombidiids, such as *Novistrombidium* and *Strombidium*, by the presence of a loop in helix B composed of 10 nucleotides (versus 8 in *Novistrombidium sinicum* and *N. orientale* and no loop in *Strombidium*) (Fig. 3). These findings were examples of a disagreement between molecular information and morphology. The "tail" may be not a good family-level diagnostic feature for the separation of Tontoniidae from other oligotrich families (21), especially with the consideration of *Laboea*.

Is the family Strombidiidae paraphyletic? As a species-rich group of oligotrichs, the family Strombidiidae is characterized by having a bipartite oral ciliature with anterior and ventral membranelles, a strongly reduced somatic ciliature, and a unique stomatogenetic process which takes place within a transient tube (3, 17). Within the family, the somatic ciliature, which consists of only one to several kineties, exhibits high diversity of arrangement and has been always regarded as an important generic character. Ten genera are currently assigned in the family Strombidiidae, i.e., *Strombidium*, *Spirostrombidium*, *Parallelostrombidium*, *Novistrombidium*, *Omegastrombidium*, *Apostrombidium*, *Varistrombidium*, *Opisthostrombidium*, *Foissneridium*, and *Williophrya* (3, 4, 30) (see Fig. S3 in the supplemental material). Recently, Agatha (3) separated strombidiids with a contractile tail from other family members and placed them in a new family, Tontoniidae, containing four genera: *Tontonia*, *Paratontonia*, *Spirotontonia*, and *Pseudotontonia*.

As mentioned above, members of the family Strombidiidae differ from the Tontoniidae in lacking a conspicuous, elongate, contractile tail (17). Previous studies have reported the monophyly of the family Strombidiidae (9, 11, 31). However, recent studies based on SSU rRNA gene sequence data revealed that the tail-less genus *Laboea* clusters with the Tontoniidae rather the Strombidiidae (8, 32), which renders the family Strombidiidae paraphyletic.

In the present study, the ITS1-5.8S-ITS2 region tree and the concatenated tree were constrained by the limited number of taxa. With the absence of tontoniids, it is hard to test whether the family Strombidiidae is monophyletic. Therefore, we constructed an expanded SSU rDNA tree with 11 additional oligotrichid and 26 additional choreotrichid sequences (Fig. 1). Although the bootstrap values are too low to define relationships among genera in Strombidiidae (Fig. 1), it is clear that the family Strombidiidae is not monophyletic if the genus *Laboea* is considered (Fig. 2 and Table 3).

Besides the intricate assignment of *Laboea*, it is noteworthy that the diversity of strombidiids is probably underestimated, with three new strombidiid genera, i.e., *Williophrya* Liu, 2011, *Foissneridium* Agatha, 2011, and *Opisthostrombidium* Agatha, 2011, being recently established (4, 30). Furthermore, there is a lack of molecular data, particularly multigene information, for many strombidiid species (especially the ITS of *Spirotontonia* and *Pseudotontonia*) and some genera are not represented in any form of molecular information. Consequently, more data are needed to determine the monophyly of the family Strombidiidae.

The known taxa in *Strombidium* belong to a nonmonophyletic assemblage. The species-rich genus *Strombidium* was the basic and earliest-established taxon in the family Strombidiidae. To date, about 65 species have been assigned in this genus (1), while only about 10 nominal and 9 undetermined species have their molecular information in the NCBI database. However, even from this limited information, species of *Strombidium* have exhibited enormous diversities in both sequence identity and tree topology. The identities of the SSU rRNA gene sequences among *Strombidium* species ranged from 69.9% to 96.3% (89.5% to 96.3% without *Strombidium* sp. and *S. biarmatum*). The sequences of *Strombidium* sp. and *S. biarmatum* shared similarity of 95.5%, but they are both notably different from other *Strombidium* species (similarities with others ranged from 69.9% to 75.9%). In SSU rRNA gene trees (Fig. 1), the *Strombidium* species were separated into four clades, as follows: (i) *S. conicum*; (ii) *S. biarmatum*, *S. basimorphum*, and *Strombidium* sp.; (iii) *S. apolatum*, *S. rassoulzadegani*, and *S. purpureum*; and (iv) *S. sulcatum*, *S. inclinatum*, and *S. stylifer*. Intriguingly, the separation of congeners was supported by morphological differences. For example, *S. conicum* revealed itself in a separate branch by possessing a unique hemitheca with longitudinal lines rather than polygonal platelets (33). In addition, *S. biarmatum* and *S. basimorphum* justified a distinct clade by being the only two congeners with extra extrusomes attached to the anterior portion of cells (4, 31). Our findings, together with previous molecularly based results (4), further support that *Strombidium* should be split into several morphologically and ontogenetically defined genera (31). Although the possibility of the monophyly of *Strombidium* was not rejected (Table 3, constraint group 4, $P = 0.759$), it might still be possible that the intrageneric morphological differences are not genuinely reflected in SSU rRNA genes.

The genus *Novistrombidium* is paraphyletic. Previous reports based on SSU rRNA gene sequence data have suggested that the genus *Novistrombidium* is paraphyletic when *N. sinicum* and *N. orientale* are included (4, 6, 8, 15, 16). The results of the present multigene analyses are consistent with this finding (Fig. 1 and 2). Data inferred from the secondary structure of the ITS2 region corroborate the paraphyly of *Novistrombidium*, with *N. sinicum* and *N. orientale* having a mode I structure (with seven loops in helix B) similar to that of *S. stylifer* and *Omegastrombidium*, whereas *N. testaceum* has a mode II structure (with six loops in helix B) similar to the majority of *Strombidium* species (Fig. 3).

It should be noted in the SSU rDNA tree that *N. sinicum* and *N. orientale* reside in the same clade as *Parallelostrombidium*. This topology could be explained by their shared characteristics with *Parallelostrombidium*, including (i) the broadly ellipsoidal cell shape, (ii) extrusomes equidistantly arranged along the girdle kinety, (iii) the presence of thigmotactic membranelles, (iv) an ovoid macronucleus, and (v) the localization of the anterior end of the ventral kinety below the right portion of the girdle kinety (15). Furthermore, the respective sequence identities of *Parallelostrombidium* with *N. sinicum* and *N. testaceum* are 97% and 96.2%, slightly higher than those between *N. testaceum* and two other congeners (95.1% and 95.7%). In contrast, *N. testaceum*, the type species of *Novistrombidium*, has a sausage-shaped macronucleus, extrusomes grouped in bundles, and localization of the ventral

kinety extending longitudinally through the gap in the girdle kinety, which resulted in its separation from the other two *Novistrombidium* species (34). Nevertheless, the monophyly of the genus *Novistrombidium* could not be rejected by the AU test (Table 3, constraint group 5, $P = 0.404$), and this discrepancy between phylogenetic topology and AU test result may stem from undersampling.

Conclusions from the multigene phylogenetic analysis. When the ITS1-5.8S-ITS2 region was first applied to the study of oligotrichs and choreotrichs, it was suggested that the ITS and 5.8S regions could provide adequate polymorphism data to assess genetic variation at the genus/population level within these groups (11). In the meantime, SSU rDNA sequences have been widely used to infer evolutionary relationships among spirotrichs, and phylogenetic trees based on such data are generally concordant with many morphological hypotheses, albeit with some discrepancies (17, 35). The value of using a single gene marker in order to elucidate evolutionary relationships among ciliates has been questioned (10). Therefore, we have used multigenes, i.e., SSU rRNA gene and ITS-5.8S region sequences, to increase the robustness of our analyses of phylogenetic relationships among oligotrichs. In the present study, the overall mean distances in oligotrichs are 0.104 in the SSU rRNA gene and 0.159 in the ITS-5.8S region, while in choreotrichs, they are 0.067 in the SSU rRNA gene and 0.090 in the ITS-5.8S region, confirming that oligotrichs are more genetically variable than choreotrichs in both the SSU rRNA gene and the ITS-5.8S region (11). Our findings support the removal of *Laboea* from the Strombidiidae to the Tontoniidae, thus rendering the family Strombidiidae monophyletic. Furthermore, the monophyly of *Novistrombidium* was doubted in the topologies of trees and morphological features. However, we are currently unable to resolve a number of phylogenetic relationships due to (i) differences between the two genes in length and variation rate, (ii) the lack of available ITS-5.8S region sequence data for oligotrichs, and (iii) undersampling of certain key taxa, such as *Varistrombidium*, *Parallelostrombidium*, and *Omegastrombidium*. Therefore, further studies are required to increase the resolution of the oligotrich systematics.

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