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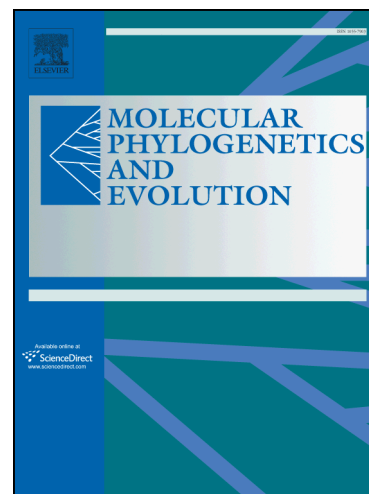
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Running title: multi-gene based phylogenetic analysis on oligotrich ciliates

Multi-gene-based phylogenetic analysis of oligotrich ciliates with emphasis on two dominant groups: cyrtostrombidiids and strombidiids (Protozoa, Ciliophora)

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ABSTRACT

Phylogenetic analyses of ciliated protists are frequently based on single molecular markers, usually the small subunit ribosomal RNA gene (SSU rDNA), despite the well-known limitations of this approach. Here, 78 new sequences of three linked genes (SSU rDNA, ITS1-5.8S rDNA-ITS2, LSU rDNA) were characterized and applied to phylogenetic analyses of oligotrichs (s. str.). It was found that: (1) three taxa, that is tontoniids, pelagostrombidiids and cyrtostrombidiids should be split from the family Strombidiidae (s.l.), which supports Agatha's classification based on morphological characters; (2) the families Tontoniidae and Cyrtostrombidiidae are both monophyletic whereas Strombidiidae is polyphyletic; (3) the positions of the families Cyrtostrombidiidae and Pelagostrombidiidae varied in different trees although with low support values; (4) the close relationship between *Varistrombidium* and *Apostrombidium* is confirmed, which updates the evolutionary hypothesis for oligotrichs based on ciliary patterns; and (5) two relatively stable clades were found in the family Strombidiidae.

Keywords: Oligotrichia; Cyrtostrombidiidae; Pelagostrombidiidae; phylogeny; rDNA

1. Introduction

The oligotrichous ciliates (s. l.) are a group that are often present in great abundance in oceanic waters and play an important role in the microbial loop (Calbet and Saiz, 2005; Edwards and Burkill, 1995; Pierce and Turner, 1992). Nevertheless, the taxonomy and phylogeny of this lineage are insufficiently studied. It is conservatively estimated that there are more than 1000 oligotrich species worldwide, but only about 100 well described morphospecies, representing ca. 15 genera, have been documented (Liu et al., 2015a; Liu et al., 2015b; Song, 2005; Song and Bradbury, 1998; Song and Packroff, 1997; Song et al., 2000; Tsai et al., 2010; Xu and Song, 2006; Xu et al., 2006). Molecular phylogenetic analyses of oligotrichs are even more poorly documented, since only about 30 species from the subclass Oligotrichia have available SSU rDNA sequences.

For much of its history, the subclass Oligotrichia was not subdivided because somatic ciliary patterns did not seem to be restricted to distinct genera (Agatha, 2004b). Agatha divided Oligotrichia into four families (Tontoniidae, Pelagostrombidiidae, Cyrtostrombidiidae, and Strombidiidae) based on their morphological, ontogenetic and ultrastructural characters (Agatha, 2004b). However, in a later classification scheme proposed by Lynn (2008), species of the families Pelagostrombidiidae and Cyrtostrombidiidae were conservatively assigned into Strombidiidae. Until now, these two schemes have not been tested in terms of the placement of Pelagostrombidiidae, since only two very short SSU rDNA sequences (~170bp) are available for the family Pelagostrombidiidae. More sequences, especially from the family Pelagostrombidiidae, are urgently needed to reveal the evolutionary relationships among oligotrich taxa.

Hitherto, most molecular phylogenetic studies focusing on oligotrichs have been based only on SSU rDNA data (Gao et al., 2009; Liu et al., 2015a; Liu et al., 2012; McManus et al., 2010; Song et al., 2013; Tsai et al., 2010; Zhang et al., 2010), and phylogenetic assignments of some taxa are ambiguous due to conflicting results from SSU rDNA trees and morphological characters. For example, in a recent phylogenetic study based on SSU rDNA sequences (Tsai et al., 2015), *Cyrtostrombidium* is sister to the cluster consisting of the family Tontoniidae and the genus *Apostrombidium*. However, in cladistic analyses inferred from morphological data (Agatha, 2004a), *Cyrtostrombidium* is closer to some genera in Strombidiidae than to Tontoniidae. In recent years the ITS1-5.8S rDNA-ITS2 region and the large subunit ribosomal RNA gene (LSU rDNA), which are tandemly linked to the SSU rDNA gene, are increasingly used in phylogenetic analyses of ciliates (Gao et al., 2014; Hewitt et al., 2003; Huang et al., 2014; Liu, 2011; Marande et al., 2009; Yi et al., 2014; Zhao et al., 2015). Previously, phylogenetic trees inferred from just a few ITS1-5.8S rDNA-ITS2 region sequences have been applied in analyses of evolutionary relationships within the Oligotrichia (Li et al., 2013; Snoeyenbos-West et al., 2002). Although the LSU rDNA has not been utilized to analyze Oligotrichia, its utility has been demonstrated in an investigation of a related group, the Tintinnida (Spirotrichea: Choreotrichia) (Santoferrara et al., 2013).

As mentioned above, the phylogeny of oligotrichs is still poorly understood, which is mainly due to: (i) low numbers of available sequences for morphologically described species and (ii) low numbers of gene markers (usually SSU rDNA) applied in phylogenetic analyses. Therefore, in the present investigation, 76 new sequences (17 SSU rDNA, 27 ITS1-5.8S rDNA-ITS2, 32 LSU rDNA) of

oligotrichs were added and multi-gene-based analyses were applied in order to increase our understanding of the phylogeny of this group.

2. Materials and methods

2.1 Sampling and identification

Sampling information for the 36 newly sequenced taxa (Fig. 1) are listed in Table 1. Species identifications were made based on microscopical observation of specimens both *in vivo* and following silver staining (Song et al., 2013). The genomic DNA is used in some taxa which were investigated in previous studies (Gao et al., 2009; Li et al., 2013; Liu et al., 2012; Liu et al., 2011; Zhang et al., 2010). Terminology and systematics follow Agatha (2004a, below order level) and Lynn (2008, subclass level).

2.2 DNA extraction, PCR amplification and sequencing

One or more individuals of each species were isolated for DNA extraction. Total genomic DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) or the DNeasy Blood & Tissue Kit (Qiagen, CA), following the manufacturer's instructions.

The PCR amplification of the SSU rDNA was performed using the eukaryotic universal forward 18SF (5'-AAC CTG GTT GAT CCT GCC AGT-3') and the reverse 18SR (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') primers (Medlin et al., 1988). A fragment of about 500bp covering the ITS1, 5.8S ribosomal gene and ITS2 was amplified using primers ITS-F and ITS-R as described in Gao et al. (2012). Primers 28S-1F and 28S-3R were used to amplify part (about 1800 bp) of the LSU rDNA gene

(Moreira et al., 2007). In some cases, the ITS1-5.8S rDNA-ITS2 region and the part of the LSU rDNA gene were amplified together using primers ITS-F and 28S-3R. PCR conditions were as follows: 5 min initial denaturation at 94°C; 35-40 cycles of 15-30s at 94°C, 60-75s at 56-60°C and 60-90s at 72°C; with a final extension of 7-10 min at 72°C.

The PCR product was purified and inserted into the pMD™ 19-T vector (Takara Biotechnology, Dalian Co., Ltd.). Subsequently, the vector was transferred into competent *E. coli* DH5α cells. Commercial sequencing was carried out on an ABI-PRISM 3730 automatic sequencer (Applied Biosystems, USA). Wherever possible, we selected the same DNA source for the amplification of SSU rDNA, ITS1-5.8S rDNA-ITS2, and LSU rDNA.

2.3 Sequence alignment and phylogenetic analyses

The newly obtained sequences were deposited in the NCBI database (for accession numbers see Table 1 in bold). Other sequences used in the present analyses were downloaded from the NCBI database. Three hypotrich and two halteriid taxa were selected as the outgroup. Sequences of SSU rDNA, ITS1-5.8S rDNA-ITS2, and LSU rDNA were aligned using the online server GUIDANCE (<http://guidance.tau.ac.il/>) with the alignment algorithm MAFFT (Penn et al., 2010). Ambiguous columns in the alignment were removed based on confidence scores calculated by GUIDANCE. The resulting alignment was further manually checked in SeaView v. 4 (Gouy et al., 2010). The final alignments included 1635 sites of SSU rDNA (75 taxa), 400 sites of ITS1-5.8S rDNA-ITS2 (52 taxa), and 1719 sites of LSU rDNA (49 taxa). The individual gene alignments were then concatenated to build

a three-gene, 75-taxon matrix with 3754 characters using the concatenate option in SeaView v. 4. The taxa whose whole sequence data of some genes are unavailable were treated as missing data.

Phylogenetic trees were constructed as described in Gao et al. (2016). Maximum likelihood (ML) analyses with different gene selections (SSU rDNA, ITS1-5.8S rDNA-ITS2, LSU rDNA, and all three concatenated) were performed on the CIPRES Science Gateway (URL: http://www.phylo.org/sub_sections/portal) (Miller et al., 2010), with RAxML-HPC2 on XSEDE using the GTR + I + G model as selected by Modeltest v. 3.4 (Posada and Crandall, 1998). Searches for the best tree were conducted starting from 100 random trees, and 1000 nonparametric bootstrap replicates were done to assess the reliability of the internal branches. Bayesian inference (BI) analyses were also performed on the CIPRES Science Gateway using MrBayes v. 3.1.2 on XSEDE with the GTR + I + G model selected by MrModeltest v. 2.2 (Nylander, 2004). Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains for 4,000,000 generations. Sampling frequency is every 100 generations. The first 25% of sampled trees were discarded as burn-in prior to consensus tree construction. Trees were viewed with MEGA v5 (Chen et al., 2015).

2.4 Topology testing

To test the monophyly of each oligotrich taxon and the robustness of phylogenetic associations of particular interest, the Approximately Unbiased (AU) tests was used (Shimodaira, 2002). Ten constraint ML topologies were generated base on the concatenated data and then compared with the unconstrained ML topology. For all constraints, internal relationships within the constrained groups were unspecified, and relationships among the remaining taxa were likewise unspecified. The site-wise

likelihoods for the resulting constrained topologies and the non-constrained ML topology were calculated using PAUP (Swofford, 2002) and then analyzed in CONSEL (Shimodaira and Hasegawa, 2001) with standard parameters to obtain p -values.

3. Results

3.1 Topology based on SSU rDNA

The topologies generated by BI and ML are generally consistent, therefore only the BI phylogenetic tree is presented with posterior probability values from BI and bootstrap values from ML analysis (Fig. 2). In the SSU rDNA tree, the traditional oligotrichs form two main clades. One clade is composed of the subclass Choreotrichia, the other clade comprises all the members of Oligotrichia (0.99 BI, 67% ML).

In the subclass Oligotrichia, there are 60 species/populations representing 16 genera from four families (Tontoniidae, Cyrtostrombidiidae, Pelagostrombidiidae and Strombidiidae.). For Tontoniidae, three representative genera (*Laboea*, *Pseudotontonia* and *Spirotontonia*) group together with full support. Cyrtostrombidiidae, represented by *Cyrtostrombidium longisomum* and *C. paralongisomum*, clusters with the clade of Tontoniidae in the BI analysis (0.58 BI), but branch basally to Tontoniidae in the ML analysis (22% ML). Pelagostrombidiidae, represented by *Limnostrombidium viride*, clusters with *Strombidium* cf. *capitatum* in the BI analysis (0.72 BI), while groups with *Parallelostrombidium conicum* in the ML analysis (18% ML). The species-rich family Strombidiidae, represented by the genera *Strombidium*, *Sinistrostrombidium*, *Spirostrombidium*, *Varistrostrombidium*, *Apostrombidium*, *Novistrostrombidium*, *Parallelostrombidium*, *Antestrostrombidium*, *Omegastrostrombidium* and *Williophrya*, is

not monophyletic. For example, *Strombidium conicum* always branches before the other oligotrichs, followed by *S. chlorophilum*. The rest of the species in Strombidiidae form a polytomy with the other three families, resulting in numerous unresolved relationships.

In the family Strombidiidae, there are two relatively stable clades. One clade includes four genera, *Novistrombidium*, *Parallelostrombidium* (except *P. conicum*), *Antestrombidium*, and *Omegastrombidium* (0.96 BI, 40% ML). In this clade, *Parallelostrombidium* species, with *P. conicum* excluded, form a fully supported clade which then clusters with *Novistrombidium sinicum* (1.00 BI, 69% ML). *Omegastrombidium*, represented by *O. elegans* and *O. cf. elegans*, forms a fully supported group, and branches successively with *Antestrombidium wilberti* (0.63 BI, 34% ML) and *Novistrombidium orientale* (0.95 BI, 36% ML). The other clade comprises *Strombidium stylifer* and ten other congeners plus *Williophrya* (0.99 BI, 50% ML). In this clade, *Strombidium stylifer* clusters with *S. pseudostylifer*, which then groups with *Strombidium* sp. (1.00 BI, 98% ML). *Strombidium sulcatum* and *S. inclinatum* form a fully supported branch, while the other six *Strombidium* species and *Williophrya* form a well-supported group (0.99 BI, 70% ML). Outside the previous two clades, species generally form polytomies except for some small groups comprising closely related species, e.g. *Strombidium basimorphum* and *S. cf. basimorphum* clustering in a well-supported branch (1.00 BI, 86% ML), two populations of *Strombidium paracalkinsi* branching with each other (1.00 BI, 99% ML), *Varistrombidium kielum* pop2 and pop3 clustering together (1.00 BI, 71% ML), and *Novistrombidium apsheronicum* and *N. testaceum* grouping together with high support (1.00 BI, 83% ML).

3.2 Topology based on ITS1-5.8S rDNA-ITS2 region

For the ITS1-5.8S rDNA-ITS2 dataset with 38 oligotrichs and 7 choreotrichs, the BI tree is presented with support values from both BI and ML algorithms (Fig. 3). The topology of the ITS1-5.8S rDNA-ITS2 region tree has some similarities to that of the SSU rDNA tree, for example: (i) *Lynnella* branches with choreotrichs; (ii) the monophyly of Tontoniidae and the non-monophyly of both Strombidiidae and *Strombidium*; (iii) *Novistrombidium* species scattered into three parts, with *N. apsheronicum* and *N. testaceum* forming a fully supported clade; and (iv) *Parallelostrombidium* forms a highly supported clade with *P. conicum* excluded (1.00 BI, 97% ML). There are, however, also some differences in the topology of the ITS1-5.8S rDNA-ITS2 tree compared to that based on SSU rDNA, for example: (i) Tontoniidae and Cyrtostrombidiidae each clusters with *Strombidium* species respectively, rather than grouping together; and (ii) *Omegastrombidium* forms sister groups with *Williophrya* and *Strombidium* cf. *capitatum*, rather than grouping with *Antestrombidium wilberti* and *Novistrombidium orientale*.

3.3 Topology based on LSU rDNA

Hitherto, only four LSU rDNA sequences of oligotrichs were available prior to this study. In this study, 30 newly characterized oligotrichid LSU rDNA sequences are supplied, and a BI phylogenetic tree based on these data is presented with support values from both BI and ML analyses (Fig. 4). The topology of the LSU rDNA gene tree differs from that of SSU rDNA mainly by: (i) *Lynnella* grouping with oligotrichs (1.00 BI, 78% ML) rather than with choreotrichs; (ii) Tontoniidae, represented by *Laboea*, *Spirotontonia* and *Pseudotontonia*, separated into two parts, one with *Laboea* and *Spirotontonia* clustering with *Limnostrombidium* (Pelagostrombidiidae), the other with *Pseudotontonia*

clustering with *Strombidium basimorphum* (Strombidiidae); (iii) *Strombidium* cf. *capitatum* branching parallel with *Strombidium* and *Williophrya* (0.92 BI, 29% ML) rather than with *Limnostrombidium*; (iii) *Novistrombidium sinicum* clustering with the *Limnostrombidium-Laboea-Spirotontonia* clade (0.54 BI, 18% ML) rather than with the *Parallelostrombidium* clade.

3.4 Topology based on concatenated data

The concatenated data of SSU rDNA, ITS1-5.8S rDNA-ITS2 and LSU rDNA sequences comes from the same 60 oligotrichs and the same 8 choreotrichs that were used for the SSU rDNA analyses. The topology of the concatenated gene tree (Fig. 5) is similar to that of the SSU rDNA tree, the main differences being: (i) *Lynnella* grouping with the oligotrichs in the concatenated gene tree rather than with the choreotrichs; (ii) Tontoniidae clustering with the clade of *Strombidium* cf. *basimorphum* and *S. basimorphum* (0.97 BI, 67% ML) rather than grouping with Cyrtostrombidiidae; (iii) *Novistrombidium sinicum* forming a polytomy with other oligotrichs rather than clustering with the *Parallelostrombidium* clade.

3.5 Topology testing

At the 5% significance level, the hypothesized monophylies of the genera *Strombidium* ($p = 2e-007$), *Varistrombidium* ($p = 0.030$), and *Novistrombidium* ($p = 0.001$) were rejected, while the monophyly of *Parallelostrombidium* ($p = 0.092$) were not rejected (Table 2). In addition, the following hypotheses were rejected, (i) the forced grouping of Cyrtostrombidiidae + Strombidiidae ($p = 0.032$); (ii) the grouping of *Apostrombidium* + *Omegastrombidium* ($p = 0.001$); (iii) the grouping of *Varistrombidium*

+ *Omegastrombidium* ($p = 0.003$); (iv) the grouping of *Novistrombidium* + *Parallelostrombidium* ($p = 0.003$). By contrast, the following hypotheses were not rejected, (i) the forced grouping of *Cyrtostrombidiidae* + *Tontoniidae* ($p = 0.082$); (ii) the grouping of *Varistrombidium* + *Apostrombidium* ($p = 0.084$).

4. Discussion

4.1 Classification of families in the subclass *Oligotrichia*

The classification of the subclass *Oligotrichia* has been disputed for a long time. Suggestions for the subdivision at family level within this group have included: no subdivision at family level (Modeo et al., 2003); the recognition of two families, i.e., *Strombidiidae* and *Tontoniidae* (Lynn, 2008); and the recognition of four families, i.e., *Tontoniidae*, *Cyrtostrombidiidae*, *Pelagostrombidiidae* and *Strombidiidae* (Agatha, 2004b). Only one gene sequence is available for the *Pelagostrombidiidae*, so it is impossible for us to infer whether or not this family is monophyletic. The monophyly of *Cyrtostrombidiidae* is indicated in trees based both on SSU rDNA and on concatenated data, and the monophyly of *Tontoniidae* is supported in all trees except that based on LSU rDNA sequences (Fig. 4). The family *Strombidiidae* is not monophyletic in any of our trees, which is consistent with previous investigations (Li et al., 2013; Song et al., 2015). These findings partly support Agatha's classification based on morphological characters, i.e., that the families *Tontoniidae*, *Pelagostrombidiidae*, and *Cyrtostrombidiidae* should be split from the family *Strombidiidae* (Agatha, 2004b).

The family Pelagostrombidiidae was established by Agatha (2004b) based on their freshwater habitat and the presence of the neoformation organelle, a permanent tube in which the oral primordium develops (Krainer, 1991; Krainer, 1995). Two genera, *Pelagostrombidium* and *Limnostrombidium*, were included in this family. According to cladistic analyses based on morphological data (Agatha and Strüder-Kypke, 2014), Pelagostrombidiidae is closely related to Cyrtostrombidiidae and certain genera in the family Strombidiidae (e.g. *Strombidium*, *Foissneridium*, and *Williophrya*). *Limnostrombidium*, the only representative of Pelagostrombidiidae in our phylogenetic analyses, grouped either with *Strombidium* species in the SSU rDNA and concatenated trees or with *Laboea* and *Spirotontonia* (Tontoniidae) in the LSU rDNA trees, which is partly consistent with the morphological data. Unfortunately, molecular data for the type genus *Pelagostrombidium* is still lacking, therefore the systematic position of Pelagostrombidiidae will remain uncertain pending a re-evaluation following the acquisition of additional data, especially from the type genus and species.

In recent cladistic analyses inferred from morphological data, *Cyrtostrombidium* is more closely related to certain genera in Strombidiidae than to Tontoniidae (Agatha and Strüder-Kypke, 2014). By contrast, phylogenetic analysis based on SSU rDNA sequences indicated that *Cyrtostrombidium* is sister to the group consisting of the family Tontoniidae and the genus *Apostrombidium* (Tsai et al., 2015). In the present multi-gene phylogenetic study, the position of Cyrtostrombidiidae was found to be variable in different trees, either clustering with Tontoniidae or grouping with some species of *Strombidium*, albeit with low support (Figs. 2-5). The grouping of *Cyrtostrombidium* and Tontoniidae was not rejected by the AU test while the grouping of *Cyrtostrombidium* and Strombidiidae was

rejected, which may be due to the non-monophyly of Strombidiidae. Therefore, these two possibilities should be further tested when more information becomes available.

4.2 The eyespot clade

In a recent study of oligotrich systematics based on SSU rDNA sequence data, Liu et al. (2016) recovered a highly supported clade comprising *Strombidium apolatum*, *S. rassoulzadegani*, *S. oculatum*, *S. purpureum* and *Williophrya maedai*, suggesting that the presence of an eyespot might be an important synapomorphy of this group. In the present study, this hypothesis is tested for the first time using multi-gene data.

Among Strombidiidae, species with an eyespot (*Strombidium apolatum*, *S. rassoulzadegani*, *S. cf. parastylifer*, *S. guangdongensei*, *S. oculatum*, *S. purpureum* and *Williophrya maedai*) grouped together in the SSU rDNA, LSU rDNA and concatenated gene trees, all with high support values. Of these species, only *S. purpureum* lacks detailed *in vivo* information, while the rest are all known to possess an eyespot (Liu, 2011; Song et al., 2009; Song et al., 2015). Moreover, the eyespot is the only unique morphological character shared among all members of this clade, which can also explain why *Williophrya* clustered within it. It is noteworthy that tontoniid species possessing an eyespot are excluded from this clade suggesting that the eye-spot evolved more than once within the oligotrichs.

4.3 The close relationship between *Apostrombidium* and *Varistrombidium*: updating the evolutionary hypothesis based on oligotrich ciliary patterns

In this study, three isolates of *Varistrombidium kielum* were included: pop 1 (sampled from Qingdao, northern of China) (Xu et al., 2011), pop 2 (sampled from Zhanjiang, southern of China; Table 1), and pop 3 (sampled from Qingdao, present study). Although they share the same morphological features, the SSU rDNA sequence similarity between pop2 and pop3 is 98.4% while pop1 is even more divergent with a similarity as low as 96.7%. The sequence identities of ITS-5.8S and LSU rDNA cannot currently be calculated due to lack of data. The three populations of *V. kielum* clustered with different lineages in the phylogenetic tree based on SSU rDNA (Fig. 2), pop 1 forming a polytomy with other oligotrichs, and pop 2 and pop 3 clustering with *Apostrombidium parakielum* (0.95 BI, 42% ML). The possibility that the three populations cluster together was also rejected by the AU test. This phenomenon may reveal the existence of cryptic species and high gene diversity within the oligotrichs (Snoeyenbos-West et al., 2002).

In previous phylogenetic studies, *Varistrombidium* and *Omegastrombidium* always cluster together, indicating their close relationship (Agatha, 2011; Gao et al., 2009; Liu et al., 2012; Song et al., 2013; Xu et al., 2011; Zhang et al., 2010). This corresponds well with the evolutionary hypothesis proposed by Agatha that the ciliary pattern of *Varistrombidium* and *Apostrombidium* probably developed independently from a Ω -shaped girdle kinety pattern of an *Omegastrombidium*-like ancestor. (Agatha, 2011; Agatha and Strüder-Kypke, 2014).

In the present study, multi-gene information for *Varistrombidium* and *Apostrombidium* is analyzed for the first time making it possible to conduct a further analysis of the evolutionary relationships among these three genera. In the trees based on SSU rDNA, LSU rDNA and concatenated genes, *A. parakielum* always groups with *V. kielum* pop 2 and pop 3 with high support values. Additionally, the

ITS1-5.8S rDNA-ITS2 sequence similarities between *Omegastrombidium* and *Apostrombidium* are 83.4%-84.6%; and those between *Varistrombidium* and *Omegastrombidium* are 84.8%-85.7%. These values are significantly lower than the ITS1-5.8S rDNA-ITS2 sequence similarity between *Apostrombidium* and *Varistrombidium* (92.0%). Furthermore, the groupings of *Varistrombidium* with *Omegastrombidium* and *Apostrombidium* with *Omegastrombidium* are both also rejected by the AU test. These findings allow us to propose that *Apostrombidium* might have a closer relationship with *Varistrombidium* than with genus *Omegastrombidium*. Morphological and ecological data further support this finding as *Varistrombidium* and *Apostrombidium* share a dorsal split of the girdle kinety, long cilia on the dorsal side and both are psammophilic, all of which are lacking in *Omegastrombidium* (Agatha, 2004b; Song et al., 2013; Xu et al., 2011).

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References

- Agatha, S., 2004a. A cladistic approach for the classification of oligotrichid ciliates (Ciliophora : Spirotricha). *Acta Protozool.* 43, 201-217.
- Agatha, S., 2004b. Evolution of ciliary patterns in the oligotrichida (Ciliophora, Spirotricha) and its taxonomic implications. *Zoology* 107, 153-168.
- Agatha, S., 2011. Updated hypothesis on the evolution of oligotrichid ciliates (Ciliophora, Spirotricha, Oligotrichida) based on somatic ciliary patterns and ontogenetic data. *Eur. J. Protistol.* 47, 51-56.
- Agatha, S., Strüder-Kypke, M.C., 2014. What morphology and molecules tell us about the evolution of Oligotrichea (Alveolata, Ciliophora). *Acta Protozool.* 53, 77-90.
- Calbet, A., Saiz, E., 2005. The ciliate-copepod link in marine ecosystems. *Aquat. Microb. Ecol.* 38, 157-167.
- Chen, X., Zhao, X., Liu, X., Warren, A., Zhao, F., Miao, M., 2015. Phylogenomics of non-model ciliates based on transcriptomic analyses. *Protein Cell* 6, 373-385.
- Edwards, E.S., Burkill, P.H., 1995. Abundance, biomass and distribution of microzooplankton in the Irish Sea. *J. Plankton Res.* 17, 771-782.
- Gao, F., Gao, S., Wang, P., Katz, L.A., Song, W.B., 2014. Phylogenetic analyses of cyclidiids (Protista, Ciliophora, Scuticociliatia) based on multiple genes suggest their close relationship with thigmotrichids. *Mol. Phylogenet. Evol.* 75, 219-226.
- Gao, F., Katz, L.A., Song, W.B., 2012. Insights into the phylogenetic and taxonomy of philasterid ciliates (Protozoa, Ciliophora, Scuticociliatia) based on analyses of multiple molecular markers. *Mol. Phylogenet. Evol.* 64, 308-317.
- Gao, F., Warren, A., Zhang, Q., Gong, J., Miao, M., Sun, P., Xu, D., Huang, J., Yi, Z., Song, W.B., 2016. The all-data-based evolutionary hypothesis of ciliated protists with a revised classification of the phylum Ciliophora (Eukaryota, Alveolata). *Sci. Rep.* 6, 24874.
- Gao, S., Gong, J., Lynn, D., Lin, X., Song, W.B., 2009. An updated phylogeny of oligotrich and choreotrich ciliates (Protozoa, Ciliophora, Spirotrichea) with representative taxa collected from Chinese coastal waters. *Syst. Biodivers.* 7, 235-242.

- Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* 27, 221-224.
- Hewitt, E.A., Muller, K.M., Cannone, J., Hogan, D.J., Gutell, R., Prescott, D.M., 2003. Phylogenetic relationships among 28 spirotrichous ciliates documented by rDNA. *Mol. Phylogenet. Evol.* 29, 258-267.
- Huang, J., Chen, Z., Song, W.B., Berger, H., 2014. Three-gene based phylogeny of the Urostyloidea (Protista, Ciliophora, Hypotricha), with notes on classification of some core taxa. *Mol. Phylogenet. Evol.* 70, 337-347.
- Krainer, K.-H., 1991. Contributions to the morphology, infraciliature and ecology of the planktonic ciliates *Strombidium pelagicum* n. sp., *Pelagostrombidium mirabile* (Penard, 1916) n. g., n. comb., and *Pelagostrombidium fallax* (Zacharias, 1896) n. g., n. comb. (Ciliophora, Oligotrichida). *Eur. J. Protistol.* 27, 60-70.
- Krainer, K.-H., 1995. Taxonomische Untersuchungen an neuen und wenig bekannten planktischen Ciliaten (Protozoa: Ciliophora) aus Baggerseen in Österreich. *Lauterbornia* 21, 39-68.
- Li, J., Liu, W., Gao, S., Warren, A., Song, W.B., 2013. 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. *Eukaryot. Cell* 12, 430-437.
- Liu, W., 2011. Studies on the taxonomy and phylogeny of oligotrich ciliates from the coastal waters off Guangdong. South China Normal University, Guangzhou, pp. 27-31.
- Liu, W., Yi, Z., Lin, X., Li, J., Al-Farraj, S.A., Al-Rasheid, K.A.S., Song, W.B., 2015a. Morphology and molecular phylogeny of three new oligotrich ciliates (Protozoa, Ciliophora) from the South China Sea. *Zool. J. Linn. Soc.* 174, 653-665.
- Liu, W., Yi, Z., Lin, X., Warren, A., Song, W.B., 2012. Phylogeny of three choreotrich genera (Protozoa, Ciliophora, Spirotrichea), with morphological, morphogenetic and molecular investigations on three strobilidiid species. *Zool. Scr.* 41, 417-434.
- Liu, W., Yi, Z., Warren, A., Al-Rasheid, K.A.S., Al-Farraj, S.A., Lin, X., Song, W.B., 2011. Taxonomy, morphology and molecular systematics of a new oligotrich ciliate, *Williophrya maedai* gen. nov., sp.

- nov., with redescrptions of *Strombidium basimorphum* and *Pseudotontonia simplicidens* (Protozoa, Ciliophora, Oligotrichia). Syst. Biodivers. 9, 247-258.
- Liu, W., Yi, Z., Xu, D., Clamp, J.C., Li, J., Lin, X., Song, W.B., 2015b. Two new genera of planktonic ciliates and insights into the evolution of the family Strombidiidae (Protista, Ciliophora, Oligotrichia). PloS one 10, e0131726.
- Lynn, D.H., 2008. The ciliated protozoa: characterization, classification and guide to the literature. Springer Verlag, Dordrecht.
- Marande, W., Lopez-Garcia, P., Moreira, D., 2009. Eukaryotic diversity and phylogeny using small- and large-subunit ribosomal RNA genes from environmental samples. Environ. Microbiol. 11, 3179-3188.
- McManus, G.B., Xu, D., Costas, B.A., Katz, L.A., 2010. Genetic identities of cryptic species in the *Strombidium stylifer/apolatium/oculatum* cluster, including a description of *Strombidium rassoulzadegani* n. sp. J. Eukaryot. Microbiol. 57, 369-378.
- Medlin, L., Elwood, H.J., Stickel, S., Sogin, M.L., 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene 71, 491-499.
- Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, Louisiana, pp. 1-8.
- Modeo, L., Petroni, G., Rosati, G., Montagnes, D.J., 2003. A multidisciplinary approach to describe protists: redescrptions of *Novistrombidium testaceum* Anigstein 1914 and *Strombidium inclinatum* Montagnes, Taylor, and Lynn 1990 (Ciliophora, Oligotrichia). J. Eukaryot. Microbiol. 50, 175-189.
- Moreira, D., von der Heyden, S., Bass, D., Lopez-Garcia, P., Chao, E., Cavalier-Smith, T., 2007. Global eukaryote phylogeny: Combined small- and large-subunit ribosomal DNA trees support monophyly of Rhizaria, Retaria and Excavata. Mol. Phylogenet. Evol. 44, 255-266.
- Nylander, J.A., 2004. MrModeltest v2. Uppsala University.
- Penn, O., Privman, E., Ashkenazy, H., Landan, G., Graur, D., Pupko, T., 2010. GUIDANCE: a web server for assessing alignment confidence scores. Nucleic Acids Res. 38, W23-28.

- Pierce, R.W., Turner, J.T., 1992. Ecology of planktonic ciliates in marine food webs. *Rev. Aquat. Sci.* 6, 139-181.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817-818.
- Santoferrara, L.F., McManus, G.B., Alder, V.A., 2013. Utility of genetic markers and morphology for species discrimination within the order Tintinnida (Ciliophora, Spirotrichea). *Protist* 164, 24-36.
- Shimodaira, H., 2002. An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.* 51, 492-508.
- Shimodaira, H., Hasegawa, M., 2001. Consel: for assessing the confidence of phylogenetic tree selection. *Bioinformatics* 17, 1246-1247.
- Snoeyenbos-West, O.L., Salcedo, T., McManus, G.B., Katz, L.A., 2002. Insights into the diversity of choreotrich and oligotrich ciliates (Class: Spirotrichea) based on genealogical analyses of multiple loci. *Int. J. Syst. Evol. Microbiol.* 52, 1901-1913.
- Song, W.B., 2005. Taxonomic description of two new marine oligotrichous ciliates (Protozoa, Ciliophora). *J. Nat. Hist.* 39, 241-252.
- Song, W.B., Bradbury, P.C., 1998. Studies on some new and rare reported marine planktonic ciliates (Ciliophora: Oligotrichia) from coastal waters in North China. *J. Mar. Biol. Assoc. UK* 78, 767-794.
- Song, W., Li, J., Liu, W., Jiang, J., Al-Rasheid, K.A., Hu, X., 2013. Taxonomy, morphology and molecular systematics of three oligotrich ciliates, including a description of *Apostrombidium parakielum* spec. nov. (Ciliophora, Oligotrichia). *Int. J. Syst. Evol. Micr.* 63, 1179-1191.
- Song, W.B., Packroff, G., 1997. Taxonomy and morphology of marine ciliates from China with description of two new species, *Strombidium globosaneum* nov. spec. and *S. platum* nov. spec. (Protozoa, Ciliophora). *Arch. Protistendk.* 147, 331-360.
- Song, W.B., Wang, M., Warren, A., 2000. Redescriptions of three marine ciliates, *Strombidium elegans*, Florentin, 1901, *Strombidium sulcatum*, Claparède & Lachmann, 1859 and *Heterostrombidium paracalkinsi*, Lei, Xu & Song, 1999 (Ciliophora, Oligotrichida). *Eur. J. Protistol.* 36, 327-342.
- Song, W.B., Warren, A., Hu, X., 2009. Free-living ciliates in the Bohai and Yellow Seas, China. Science Press, Beijing.

- Song, W., Zhao, X., Liu, W., Hu, X., Al-Farraj, S.A., Al-Rasheid, K.A.S., Song, W.B., Warren, A., 2015. Biodiversity of oligotrich ciliates in the South China Sea: description of three new *Strombidium* species (Protozoa, Ciliophora, Oligotrichia) with phylogenetic analyses. *Syst. Biodivers.* 13, 608-623.
- Swofford, D.L., 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4., Sunderland, MA.
- Tsai, S.F., Chen, J.Y., Chiang, K.P., 2010. *Spirotontonia taiwanica* n. sp. (Ciliophora: Oligotrichida) from the coastal waters of northeastern Taiwan: morphology and nuclear small subunit rDNA Sequence. *J. Eukaryot. Microbiol.* 57, 429-434.
- Tsai, S.F., Chen, W.T., Chiang, K.P., 2015. Phylogenetic position of the genus *Cyrtostrombidium*, with a description of *Cyrtostrombidium paralongisomum* nov. spec. and a redescription of *Cyrtostrombidium longisomum* Lynn & Gilron, 1993 (Protozoa, Ciliophora) based on live observation, protargol impregnation, and 18S rDNA sequences. *J. Eukaryot. Microbiol.* 62, 239-248.
- Xu, D., Song, W.B., 2006. Hapantotypification and morphological redescription of the marine planktonic ciliate, *Spirostrombidium cinctum* (Kahl, 1932) Petz, Song et Wilbert, 1995 (Ciliophora : Oligotrichida). *Acta Protozool.* 45, 17-25.
- Xu, D., Song, W.B., Sun, P., Chen, X., 2006. Morphology and infraciliature of the oligotrich ciliate *Strombidium rapulum* (Yagiu, 1933) Kahl, 1934 (Protozoa, Ciliophora, Oligotrichida) from the intestine of sea urchin *Hemicentrotus pulcherrimus* Agassiz. *Zootaxa* 1113, 33-40.
- Xu, D., Sun, P., Clamp, J., Ma, H., Song, W.B., 2011. The establishment of a new oligotrich genus *Varistrombidium* gen. nov. and the morphology and phylogeny of a marine ciliates, *Varistrombidium kielum* (Maeda and Carey, 1985) nov. comb. (Protista, Ciliophora). *Acta. Zool. Sin.* 36, 502-511.
- Yi, Z., Strüder-Kypke, M.C., Hu, X., Lin, X., Song, W.B., 2014. Sampling strategies for improving tree accuracy and phylogenetic analyses: A case study in ciliate protists, with notes on the genus *Paramecium*. *Mol. Phylogenet. Evol.* 71, 142-148.
- Zhang, Q., Yi, Z., Xu, D., Al-Rasheid, K.A.S., Gong, J., Song, W.B., 2010. Molecular phylogeny of oligotrich genera *Omegastrombidium* and *Novistrombidium* (Protozoa, Ciliophora) for the systematical relationships within family Strombidiidae. *Chin. J. Oceanol. Limnol.* 28, 769-777.

Zhao, X., Gao, S., Fan, Y., Strueder-Kypke, M., Huang, J., 2015. Phylogenetic framework of the systematically confused *Anteholosticha-Holosticha* complex (Ciliophora, Hypotrichia) based on multigene analysis. *Mol. Phylogenet. Evol.* 91, 238-247.

ACCEPTED MANUSCRIPT

Fig. 1. Protargol impregnation images of oligotrichous organisms used in current work (original). A, *Apostrombidium parakielum*; B, *Antestrombidium wilberti*; C, *Cyrtostrombidium paralongisomum* pop. 2; D, *Limnostrombidium viride*; E, *Laboea strobila* pop. 2; F, *Lynnella semiglobulosa* pop. 2; G, *Strombidium basimorphum*; H, *Parallelostrombidium ellipticum*; I, *Novistrombidium testaceum*; J, *Novistrombidium sinicum* pop. 3; K, *Novistrombidium sinicum* pop. 2; L, *Parallelostrombidium* cf. *ellipticum*; M, *Omegastrombidium elegans* pop. 2; N, *Novistrombidium orientale*; O, *Strombidium tropicum*; P, *Parallelostrombidium conicum*; Q, *Parallelostrombidium obesum*; R, *Varistrombidium kielum* pop. 2; S, *Strombidium guangdongense*; T, *Sinistrostrombidium cupiformum*; U, *Novistrombidium apsheronicum*; V, *Parallelostrombidium paralatatum* pop. 2; W, *Spirostrombidium apourceolare*; X, *Strombidium stylifer* pop. 2; Y, *Spirostrombidium agatha*; Z, *Strombidium rassoulzadegani* pop. 2; Z1, *Strombidium* sp.; Z2, Z3, *Spirotontonia grandis*; Z4, *Spirotontonia turbinata*; Z5, *Strombidium paracalkinsi* pop. 2; Z6, *Strombidium* cf. *parastylifer*; Z7, *Spirostrombidium subtropicum*; Z8, *Varistrombidium kielum* pop. 3; Z9, *Williophrya maedai*; Z10, *Strombidium* cf. *capitatum*.

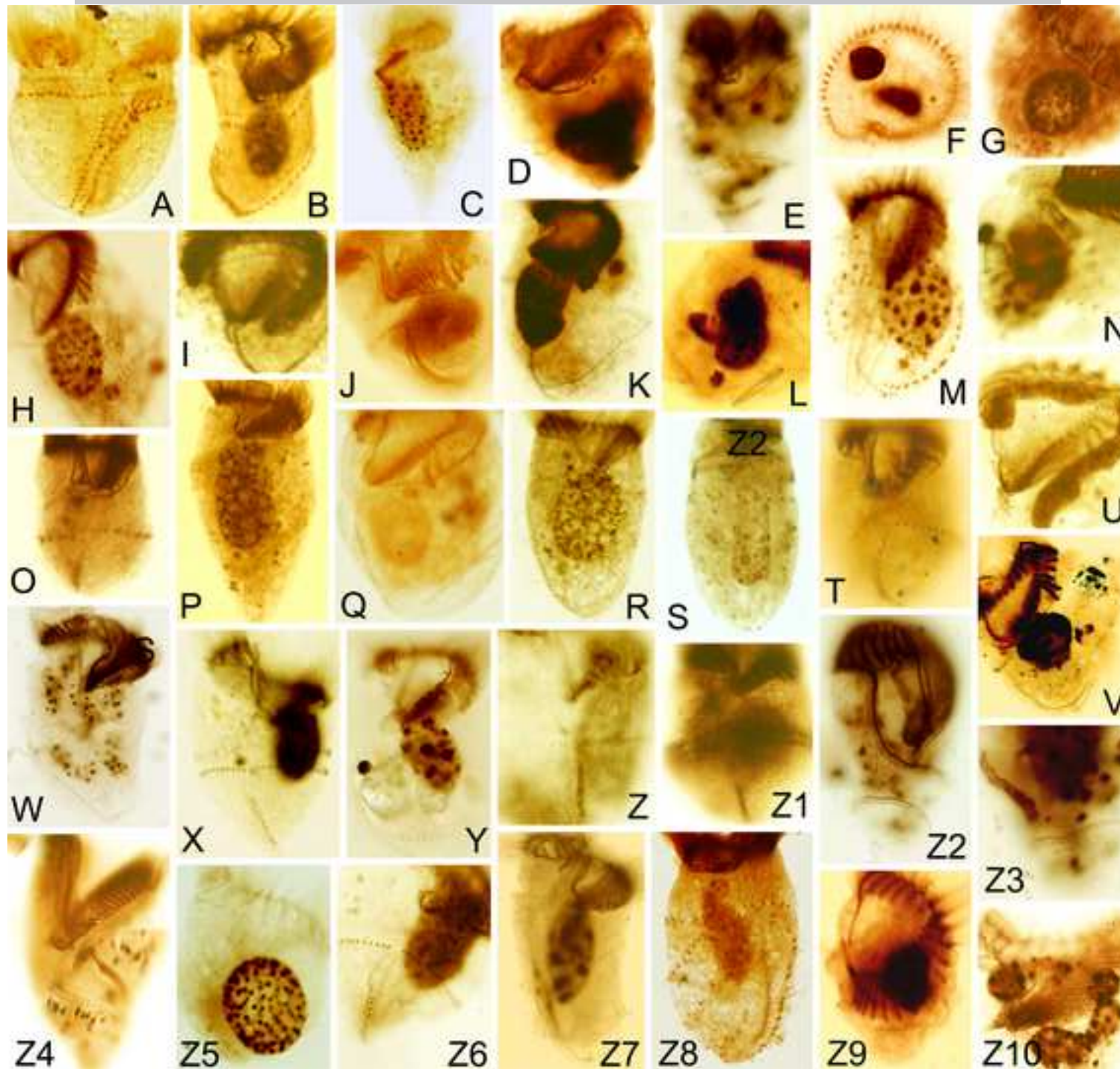
Fig. 2. Bayesian inference (BI) tree of Oligotrichia based on SSU rDNA sequences of 1635 sites from 75 taxa. Numbers at each node are Bayesian posterior probability and ML bootstrap support, respectively. Dashes (-) reflect the disagreement between BI and ML. Dots indicate nodes with full support in both algorithms. Sequences newly obtained are in bold. GenBank accession numbers for each sequence are the codes following the species names. Scale bar represents five substitutions per 100 nucleotides. Systematic classification under subclass Oligotrichia follows Agatha (2004b).

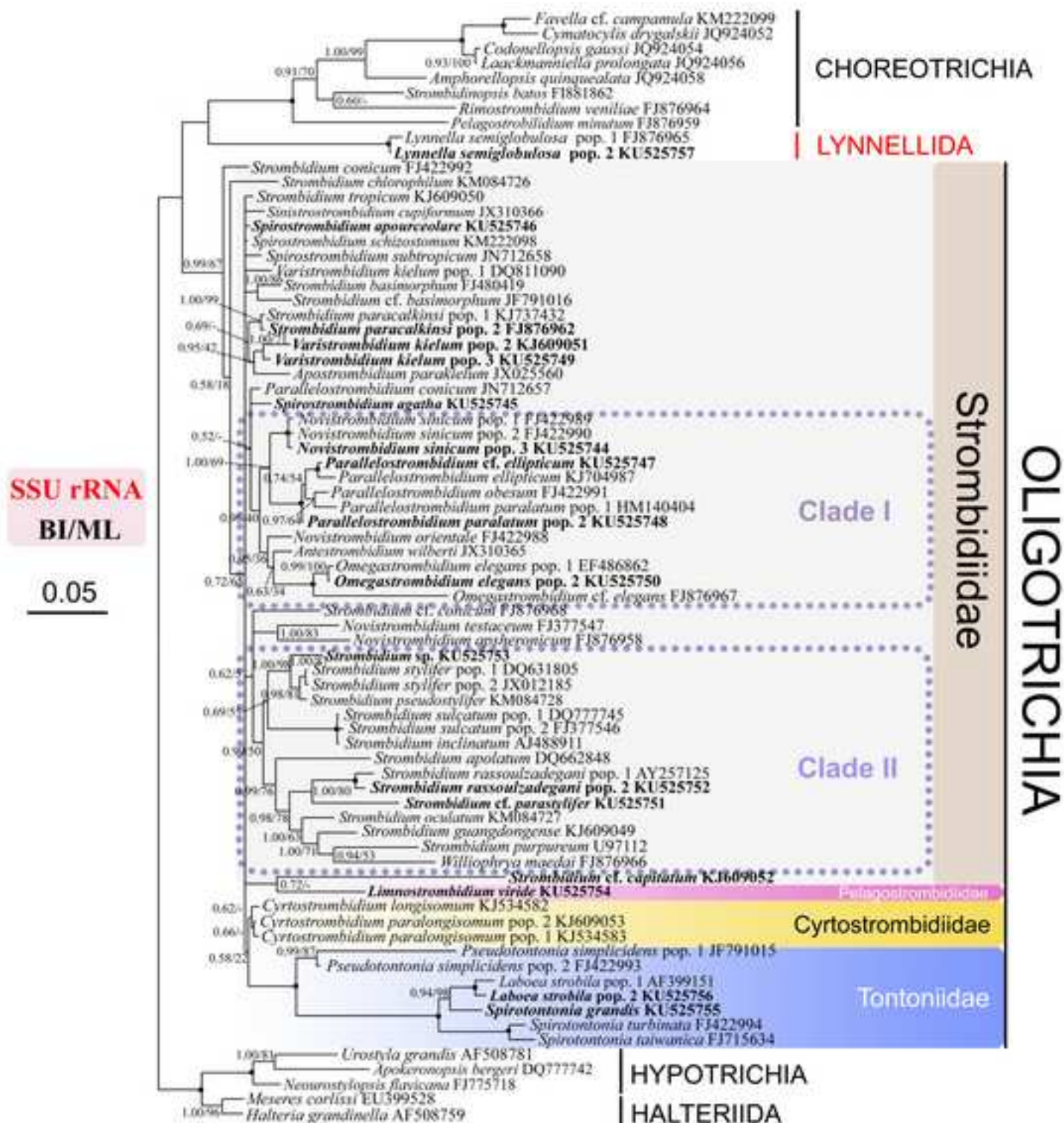
Fig. 3. BI tree of Oligotrichia based on ITS1-5.8S rDNA-ITS2 region sequences of 400 sites from 52 taxa. Numbers at each node are Bayesian posterior probability and ML bootstrap support, respectively. Dashes (-) indicate the disagreement between BI and ML. Sequences newly obtained are in bold. GenBank accession numbers for each sequence are the codes following the species names. Scale bar represents five substitutions per 100 nucleotides.

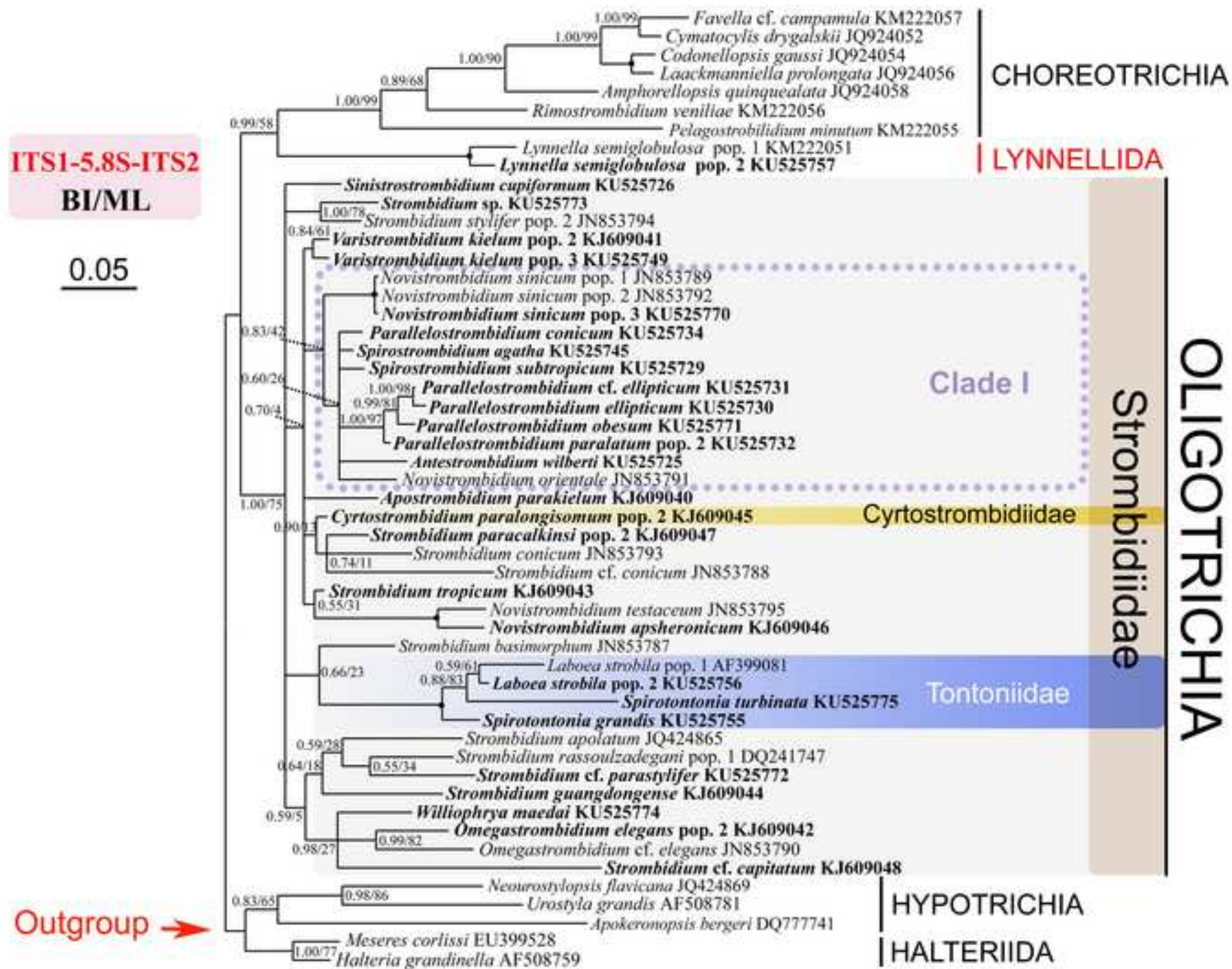
Fig. 4. BI tree of Oligotrichia based on LSU rDNA sequences of 1719 sites from 49 taxa. Numbers at each node are Bayesian posterior probability and ML bootstrap support, respectively. Dashes (-) reflect the disagreement between BI and ML. Dots indicate nodes with full support in both algorithms. Sequences newly obtained are in bold. GenBank accession numbers for each sequence are the codes following the species name. Scale bar represents five substitutions per 100 nucleotides.

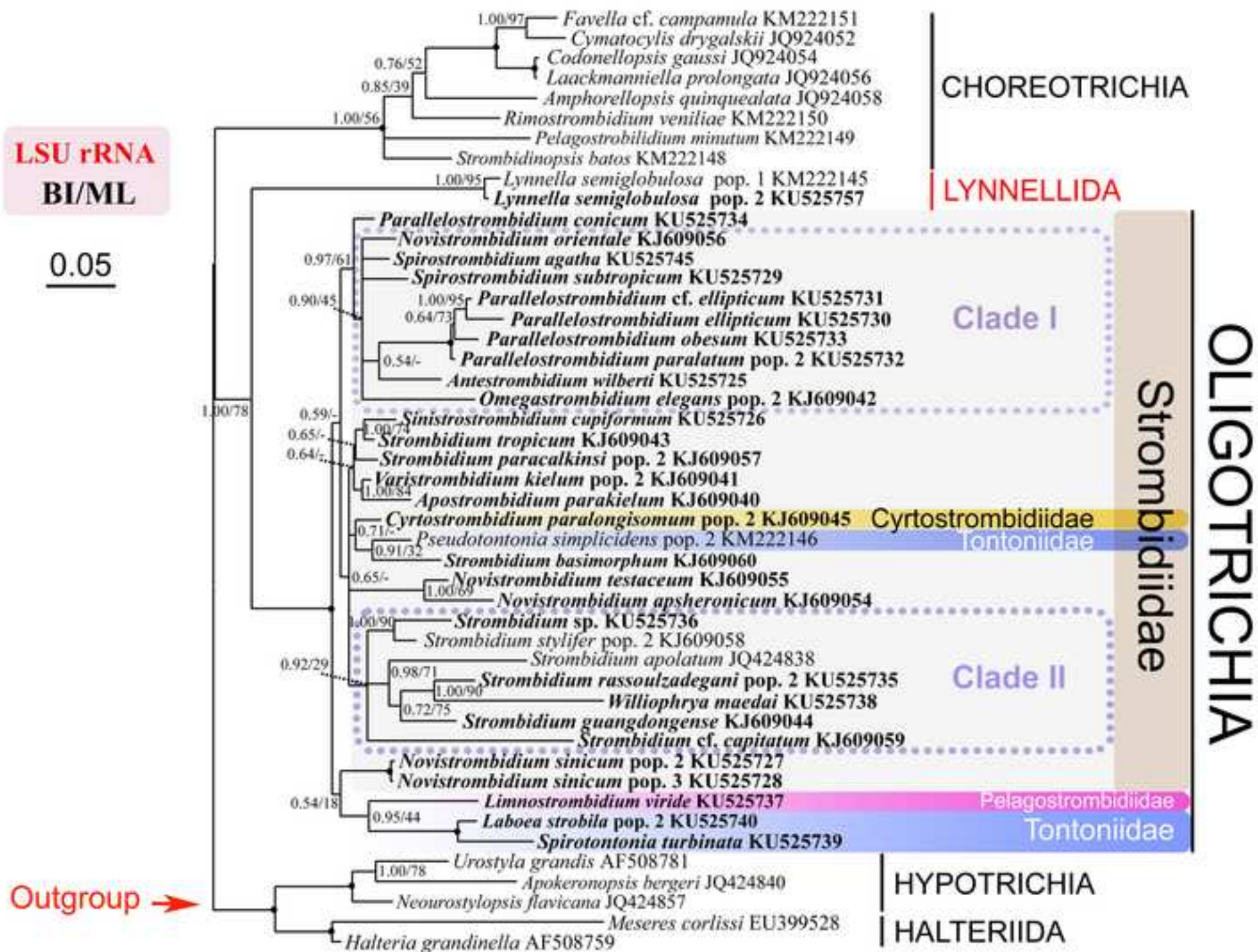
Fig. 5. BI tree of Oligotrichia based on the concatenated data of SSU rDNA, ITS1-5.8S rDNA-ITS2 and LSU rDNA sequences of 3754 sites from 75 taxa. Numbers at each node are Bayesian posterior probability and ML bootstrap support, respectively. Dashes (-) reflect the disagreement between Bayesian and ML. Dots indicate nodes with full support in both algorithms. Scale bar represents five substitutions per 100 nucleotides.

Figure1









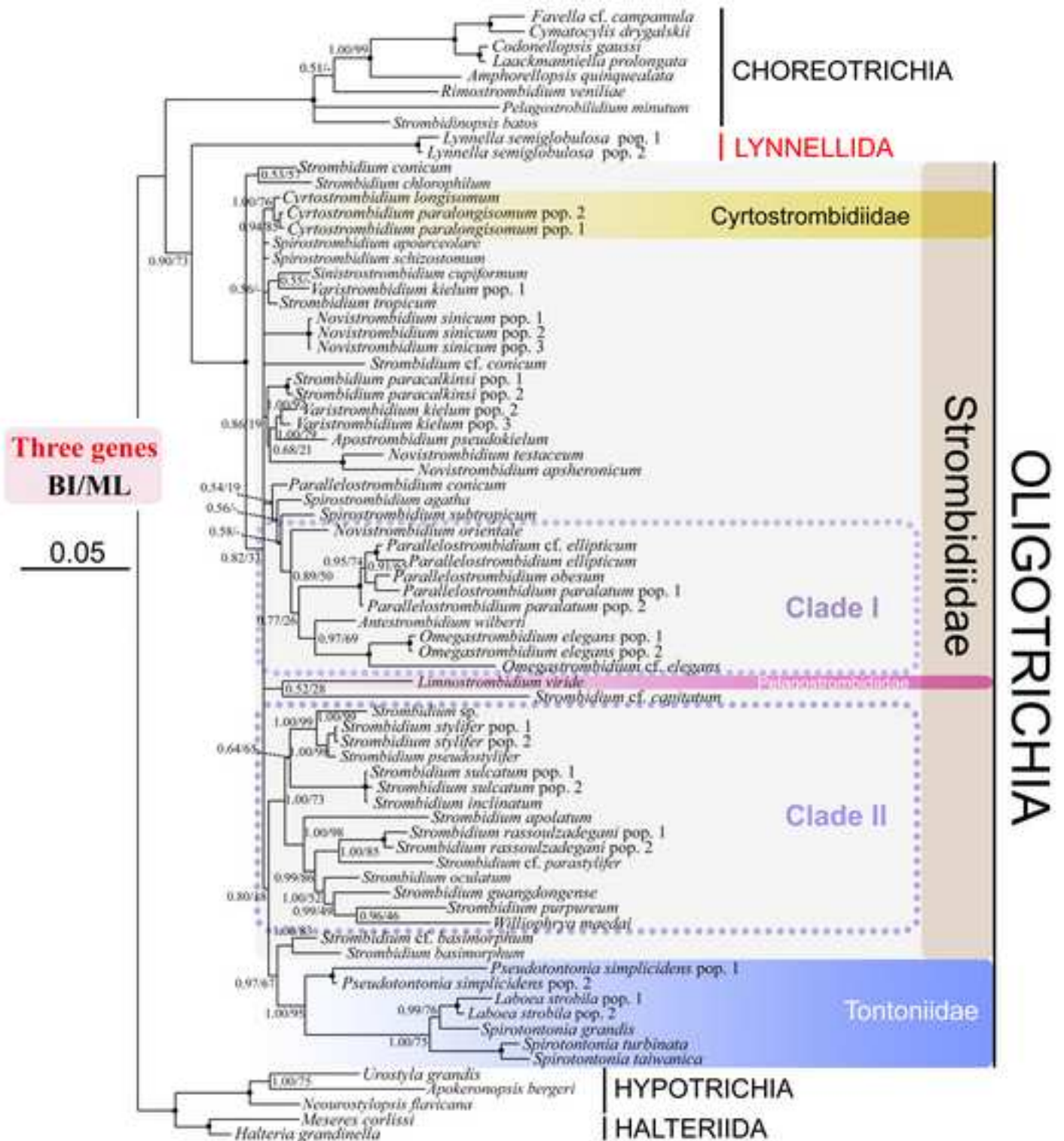


Table 1 Sampling information for newly sequenced oligotrichs in this study (accession numbers in bold are newly sequenced gene fragments in the present study).

Species	Sampled location	Date (M./Y.)	SSU rDNA	ITS1-5.8S rDNA-ITS 2	LSU rDNA
<i>Antestrombidium wilberti</i>	Zhanjiang, Guangdong (21° 38'N, 110° 39'E), China	03/2010	JX310365	KU525725	KU525725
<i>Apostrombidium parakielum</i>	Qingdao, Shandong (39° 10'N, 117° 06'E), China	05/2009	JX025560	KJ609040	KJ609040
<i>Cyrtostrombidium paralongisomum</i> pop. 2	Zhanjiang, Guangdong (21° 16'N, 110° 21'E), China	03/2010	KJ609053	KJ609045	KJ609045
<i>Lynnella semiglobulosa</i> pop. 2	Zhanjiang, Guangdong (21° 16'N, 110° 21'E), China	11/2013	KU525757	KU525757	KU525757
<i>Laboea strobila</i> pop. 2	Zhanjiang, Guangdong (21° 19'N, 110° 42'E), China	11/2013	KU525756	KU525756	KU525740
<i>Limnostrombidium viride</i>	Zhanjiang, Guangdong (23° 10'N, 113° 34'E), China	12/2012	KU525754	—	KU525737
<i>Novistrombidium orientale</i>	Daya Bay, Guangdong (22° 43'N, 114° 32'E), China	03/2008	FJ422988	JN853791	KJ609056
<i>Novistrombidium testaceum</i>	Shenzhen, Guangdong (22° 37'N, 114° 04'E), China	04/2008	FJ377547	JN853795	KJ609055
<i>Novistrombidium apsheronicum</i>	Shenzhen, Guangdong (22° 37'N, 114° 04'E), China	11/2008	FJ876958	KJ609046	KJ609054
<i>Novistrombidium sinicum</i> pop. 2	Zhanjiang, Guangdong (22° 66'N, 114° 59'E), China	04/2008	FJ422990	JN853792	KU525727
<i>Novistrombidium sinicum</i> pop. 3	Zhanjiang, Guangdong (22° 75'N, 113° 62'E), China	11/2011	KU525744	KU525770	KU525728
<i>Omegastrombidium elegans</i> pop. 2	Zhanjiang, Guangdong (21° 19'N, 110° 42'E), China	03/2010	KU525750	KJ609042	KJ609042

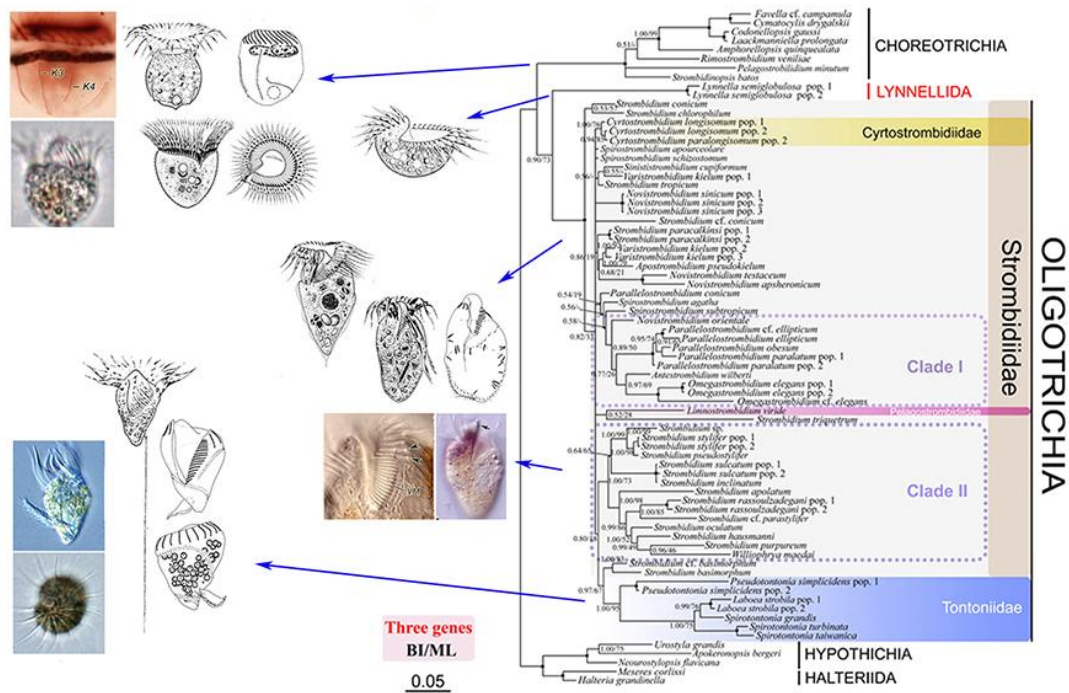
<i>Strombidium</i> sp.	Zhanjiang, Guangdong (21° 19'N, 110° 42'E), China	11/2010	KU525753	KU525773	KU525736
<i>Parallelostrombidium ellipticum</i>	Zhanjiang, Guangdong (21° 38'N, 110° 39'E), China	04/2009	KJ704987	KU525730	KU525730
<i>Parallelostrombidium obesum</i>	Zhanjiang, Guangdong (22° 64'N, 114° 20'E), China	04/2008	FJ422991	KU525771	KU525733
<i>Parallelostrombidium conicum</i>	Zhanjiang, Guangdong (21° 38'N, 110° 39'E), China	03/2010	JN712657	KU525734	KU525734
<i>Parallelostrombidium</i> m cf. <i>ellipticum</i>	Zhanjiang, Guangdong (22° 66'N, 114° 59'E), China	04/2009	KU525747	KU525731	KU525731
<i>Parallelostrombidium paralatum</i> pop. 2	Zhanjiang, Guangdong (21° 08'N, 110° 35'E), China	03/2010	KU525748	KU525732	KU525732
<i>Spirostrombidium apourceolare</i>	Zhanjiang, Guangdong (21° 15'N, 110° 44'E), China	11/2012	KU525746	—	—
<i>Spirostrombidium agatha</i>	Qingdao, Shandong (35° 92'N, 120° 21'E), China	03/2013	KU525745	KU525745	KU525745
<i>Spirostrombidium subtropicum</i>	Zhanjiang, Guangdong (21° 37'N, 110° 43'E), China	03/2010	JN712658	KU525729	KU525729
<i>Sinistrostrombidium cupiformum</i>	Zhanjiang, Guangdong (22° 66'N, 114° 59'E), China	05/2009	JX310366	KU525726	KU525726
<i>Strombidium guangdongense</i>	Zhanjiang, Guangdong (21° 16'N, 110° 21'E), China	12/2009	KJ609049	KJ609044	KJ609044
<i>Strombidium basimorphum</i>	Zhanjiang, Guangdong (21° 13'N, 110° 42'E), China	04/2008	FJ480419	JN853787	KJ609060
<i>Strombidium rassoulzadegani</i> pop.2	Qingdao, Shandong (35° 92'N, 120° 21'E), China	03/2013	KU525752	—	KU525735
<i>Strombidium</i> cf. <i>parastylifer</i>	Zhanjiang, Guangdong (21° 37'N, 110° 43'E), China	10/2012	KU525751	KU525772	—
<i>Strombidium</i> cf.	Zhanjiang, Guangdong	03/2010	KJ609052	KJ609048	KJ609059

<i>capitatum</i>	(21° 16'N, 110° 21'E), China				
<i>Strombidium stylifer</i> pop. 2	Shenzhen, Guangdong (22° 37'N, 114° 04'E), China	03/2008	JX012185	JN853794	KJ609058
<i>Strombidium tropicum</i>	Daya Bay, Guangdong (22° 43'N, 114° 32'E), China	05/2009	KJ609050	KJ609043	KJ609043
<i>Strombidium basimorphum</i> pop. 2	Shenzhen, Guangdong (22° 37'N, 114° 04'E), China	04/2008	FJ480419	JN853787	KJ609060
<i>Strombidium paracalkinsi</i> pop. 2	Daya Bay, Guangdong (22° 43'N, 114° 32'E), China	12/2008	FJ876962	KJ609047	KJ609057
<i>Spirotontonia turbinata</i>	Zhanjiang, Guangdong (22° 66'N, 114° 59'E), China	12/2006	FJ422994	KU525775	KU525739
<i>Spirotontonia grandis</i>	Zhanjiang, Guangdong (21° 19'N, 110° 42'E), China	11/2013	KU525755	KU525755	—
<i>Varistrombidium kielum</i> pop. 2	Zhanjiang, Guangdong (21° 16'N, 110° 21'E), China	12/2009	KJ609051	KJ609041	KJ609041
<i>Varistrombidium kielum</i> pop. 3	Qingdao, Shandong (35° 95'N, 120° 24'E), China	04/2012	KU525749	KU525749	—
<i>Williophrya maedai</i>	Zhanjiang, Guangdong (22° 66'N, 114° 59'E), China	04/2008	FJ876966	KU525774	KU525738

Table 2 Approximately Unbiased test results based on the concatenated data. Rejected monophyly ($p < 0.05$) is highlighted in gray.

Topology constraints	-Ln likelihood	AU value (p)
unconstrained		
<i>Cyrtostrombidium</i> + Tontoniidae	35305.44623486	0.082
<i>Cyrtostrombidium</i> + Strombidiidae	35310.70481386	0.032
<i>Varistrombidium</i>	35311.71614865	0.030
<i>Apostrombidium</i> + <i>Omegastrombidium</i>	35360.23798379	0.001
<i>Apostrombidium</i> + <i>Varistrombidium</i>	35304.61103762	0.084
<i>Omegastrombidium</i> + <i>Varistrombidium</i>	35359.14934567	0.003
<i>Strombidium</i>	35879.02221170	2e-007
<i>Novistrombidium</i>	35356.96363253	0.001
<i>Parallelostrombidium</i>	35313.64954125	0.092
<i>Novistrombidium</i> + <i>Parallelostrombidium</i>	35353.80034956	0.003

Graphical abstract



Highlights

- 1 Positions of pelagostrombidiids and cyrtostrombidiids varied
- 2 Pelagostrombidiids and cyrtostrombidiids should separate from Strombidiidae (s.l.)
- 3 Two relatively stable clades were found in the family Strombidiidae
- 4 The close relationship between *Varistrombidium* and *Apostrombidium* is confirmed