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http://www.hyxb.org.cn
E-mail: hyxbe@263.net

On four closely related hypotrichous ciliates (Protozoa, Ciliophora, Spirotrichea): molecular characters, interspecific relationships and phylogeny defined with multigene sequence information

GAO Feng[1,2], YI Zhenzhen[1,2*], CHEN Zigui[1], AL-RASHEID Khaled A S[3], SONG Weibo[1,2]

[1] Laboratory of Protozoology, Key Laboratory of Ecology and Environmental Science in Guangdong Higher Education, College of Life Science, South China Normal University, Guangzhou 510631, China

[2] Laboratory of Protozoology, KLM, Ocean University of China, Qingdao 266003, China

[3] Zoology Department, King Saud University, Riyadh 11451, Saudi Arabia

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Abstract
In order to clarify the phylogeny and relationships of the most confused hypotrichous ciliates, *Holosticha*-complex, four closely related holostichids (five populations), *Holosticha bradburyae, H. diademata, Anteholosticha* sp., and *A. manca*, were compared and analyzed using ITS2 secondary structures, ITS1-5.8S-ITS2 region and SSrRNA gene sequences. The ITS1-5.8S-ITS2 region sequences of these four species were first sequenced, and they shared sequence identities ranging from 68.0% to 90.1%, while two populations of *Anteholosticha* sp. differed in three nucleotides (sequence identity 99.8%). There were several minor differences among ITS2 secondary structures of these species, while two populations of *Anteholosticha* sp. had the identical secondary structure. Phylogenetic trees inferred from the ITS1-5.8S-ITS2 region sequences of stichotrichs using multiple algorithms (Neighbor-Joining, Maximum Parsimony and Bayesian) revealed similar topologies. The results show that: (1) *Holosticha bradburyae* and *H. diademata* firmly clustered together with strong bootstrap supports, forming a sister clade with *Anteholosticha* sp., (2) *Anteholosticha* appeared to be a paraphyletic assemblage, in which the morphotype *A. manca* was more closely related to *Diaxonella trimarginata* than to its congener *Anteholosticha* sp. Phylogenetic analyses based on the SSrRNA gene and the combined sequences of SSrRNA gene and ITS1-5.8S-ITS2 region revealed the similar relationships between *Holosticha* and *Anteholosticha*, nevertheless their positions within the subclass Stichotrichia differed from each other inferred from different genes.

Key words: phylogeny, gene sequencing, marine ciliates, SSrRNA, ITS1-5.8S-ITS2

1 Introduction

Ciliates are a major component of the microbial food webs in freshwater and marine systems where they play an important role in mediating the flow of both substances and energy (Corliss, 2002). Furthermore, with their rapid growth and delicate external membranes, ciliates may react more quickly to environmental changes than most other eukaryotic organisms and can thus serve as bioindicators of water pollution (Xu et al., 2009; Coppellotti, 1998). However, ciliates remain insufficiently studied which directly affected their active roles in the basic theoretical research and practical application.

The *Holosticha*-complex, the highly arguable ciliates, are always the most problematic groups among stichotrichs, and have been for a long time supposed as a monophyletic assemblage because all the members share a similar ciliary pattern (Hu et al., 2009; Li et al., 2008; Berger, 2006). However, recent studies indicated that *Holosticha* displays strikingly diverse characters which indicated that members within this genus could be derived from differently originated forms (Hu and Song, 2001). Berger (2006, 2003) redefined the genus *Holosticha*, which he described as "a melting pot for all urostyloids with three distinct frontal cirri, a midventral complex composed of cirral pairs only, transverse cirri, and one left and one right marginal row", and erected the genus *Anteholosticha* based on its morphogenetic features, which separated it from

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*Corresponding author, E-mail: zyi@scnu.edu.cn

the genus *Holosticha*, both belonging to the family *Holostichidae*. However, phylogenetic relationships among *Holosticha*-complex remain confused, despite the fact that numerous morphological and morphogenetic studies of organisms, including molecular phylogeny within these taxa have been performed in recent years (Li, Song et al., 2009; Liu et al., 2009; Shao et al., 2008; Yi et al., 2009; Yi, Song, Shao et al., 2008; Schmidt et al., 2007; Yi et al., 2006). So, more molecular data are necessary to investigate the phylogeny within *Holosticha*-complex.

In this study, the internal transcribed spacer (ITS1-5.8S-ITS2) region for four holostichids species (five populations), *Holosticha bradburyae, H. diademata, Anteholosticha* sp. and *A. manca* were sequenced and analysed. Phylogenetic analyses based on the small subunit ribosomal RNA (SSrRNA) gene as well as combined dataset were also performed. The aims were to provide a new insight on the molecular phylogeny of the holostichids and enhance our understanding of the relationships of four *Holosticha*-complex species.

2 Materials and methods

2.1 *Collection and identification of ciliates*

Samples of five strains were all collected from the coast of the Huanghai Sea (Yellow Sea) in the vicinity of Qingdao (36°04′N; 120°23′E). Identifications and morphological studies were made according to the methods of Li, Zhang et al. (2009) and Berger (2006).

2.2 *Extraction of genomic DNA, PCR amplifications, cloning and sequencing*

Extraction of genomic DNA was carried out as described in the previous study (Yi, Song, Warren et al., 2008). The PCR amplifications were performed using a TaKaRa *ExTaq* DNA Polymerase Kit (TaKaRa Biomedicals, Japan). Primers used for PCR amplification in this work were as follows: 5.8s-like F: 5′-GTA GGT GAA CCT GCG GAA GGA TCA TTA-3′; 5.8s-like R: 5′-TAC TGA TAT GCT TAA GTT CAG CGG-3′. PCR conditions were as follows: 5 min initial denaturation (95 °C), followed by 30 cycles of 0.5 min at 95 °C, 1.0 min at 56 °C; 1.0 min at 72 °C, with a final extension of 10 min (72 °C). Cloning and sequencing were performed according to Yi, Song, Warren et al. (2008).

2.3 *Phylogenetic analyses*

All SSrRNA gene sequences and partial ITS1-5.8S-ITS2 region sequences used in the present investigation were obtained from GenBank. The methods used for phylogenetic analyses were performed as previously described (Yi, Song, Warren et al., 2008).

2.4 *ITS2 secondary structure prediction*

The default settings of the mfold Website (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi) (Zuker, 2003) were used to produce the secondary structure (Miao et al., 2008). The structures were edited for aesthetic purposes with RnaViz 2.0 (Rijk and Wachter, 1997).

3 Results

3.1 *ITS1-5.8S-ITS2 region sequence deposition and comparison*

The ITS1-5.8S-ITS2 region sequences of five strains have been deposited in the GenBank database with the length and the accession numbers as follows: *Holosticha bradburyae*, 488 bp, EU925646; *H. diademata*, 489 bp, EU925647; *Anteholosticha* sp. pop1, 522 bp, EU925644; *Anteholosticha* sp. pop2, 522 bp, EU925645, and *A. manca*, 501 bp, EU925643.

The ITS1-5.8S-ITS2 region sequences of five strains and *Diaxonella trimarginata* were compared (Fig. 1a), and there were five obvious regions of nucleotide insertions (the longest was 13 nucleotides) between *Holosticha* and *Anteholosticha* (noted with arrows). The sequence identity was high (90.5%) between *Holosticha bradburyae* and *H. diademata*. On the contrary, the sequence identity was comparatively low between *A. manca* and two populations of *Anteholosticha* sp. (71.2% and 71.5%, respectively), with *A. manca* having three obvious regions of nucleotide deletion compared with *Anteholosticha* sp., and showing high similarity with *Diaxonella trimarginata* (sequence identity, 93.4%). Only three changes (83, 283 and 323 sites) of ITS1-5.8S-ITS2 region sequences were detected between two populations of *Anteholosticha* sp. (sequence identity 99.8%).

3.2 *ITS2 secondary structures*

As shown in Fig. 2, the secondary structures of four holostichids were predicted based on the ITS2 transcripts. A general model consisting of a multibranch loop and two paired regions (helices A, B) was proposed. The loop was closed, based on the 5.8S-LSU interaction, and its G content was low compared with the paired regions. Compared with the other three species, *Holosticha diademata* had a bigger loop in he-

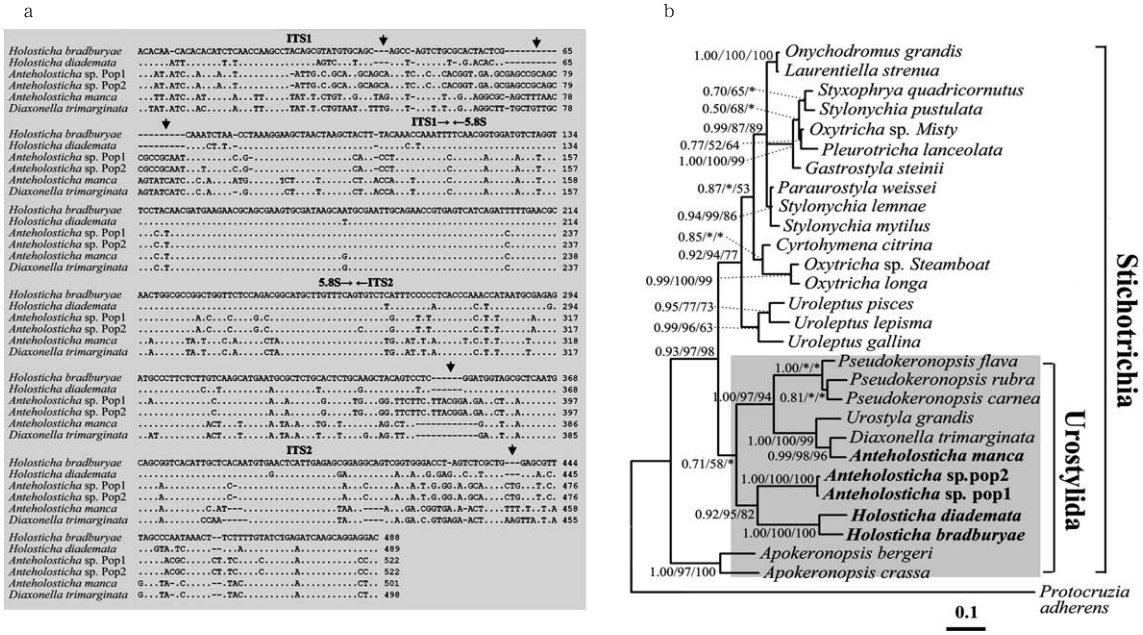

Fig.1. a. ITS1-5.8S-ITS2 sequence alignment of six ciliates. Numbers at the end of the lines indicate the number of nucleotides. Insertions and deletions were compensated by introducing alignment gaps (-). Matched sites are represented in dots (.). b. Bayesian tree inferred from the nucleotide sequences of ITS1-5.8S-ITS2 of stichotrichs. Numbers at nodes represent posterior probability of Bayesian analysis and the bootstrap values of NJ and MP analysis out of 1 000 replicates. Asterisk (*) indicates disagreement between Bayesian and other two methods. The scale bar corresponds to 10 substitutions per 100 nucleotide positions. Sequenced species in this work are highlighted in bold.

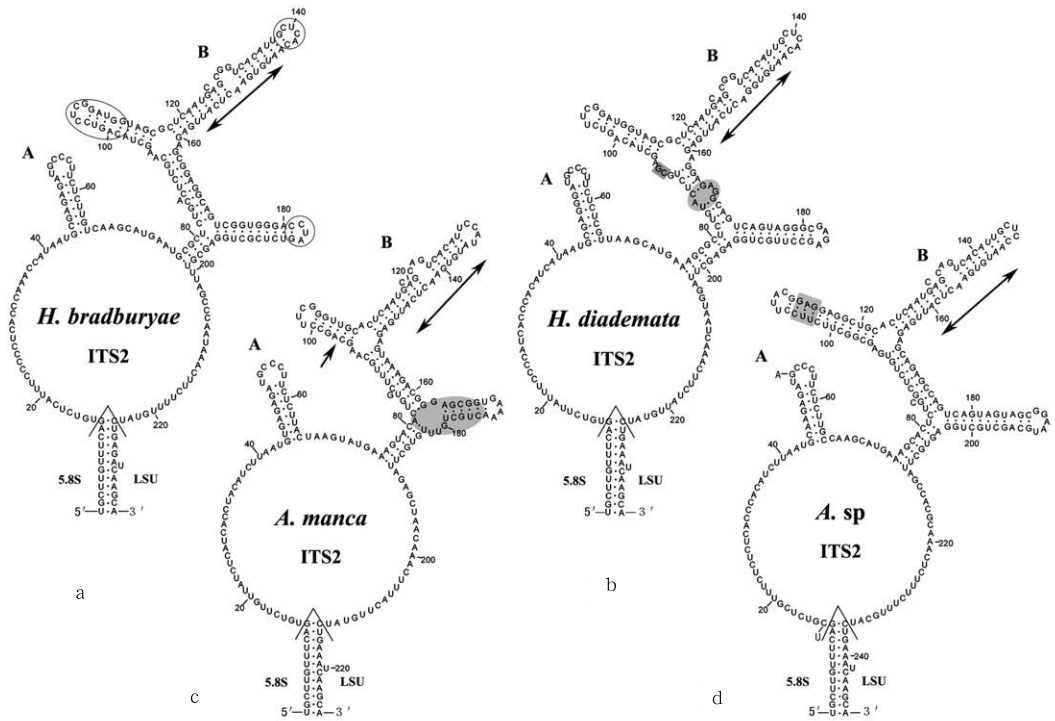

Fig.2. Secondary structures of the internal transcribed spacer 2 (ITS2) RNA transcript of *Holosticha bradburyae* (a), *H. diademata* (b), *Anteholosticha manca* (c) and *Anteholosticha* sp. pop2 (d). The diagrams illustrate the two helices, labeled A and B, present in the Class Spirotrichea (Coleman, 2005). Nucleotide variations between two *Anteholosticha* sp. populations are noted. The region of greatest primary sequence conservation is noted with double arrows. Highly variable termini of subhelices are enclosed in ellipses.

lices B in the denoted region (Fig. 2b, noted in grey squareness), and nearby one more loop was appeared (Fig. 2b, noted in grey ellipse). The two branches in helices B of *Anteholosticha manca* were shorter than those of the other three species, while those of *Anteholosticha* sp. were longer. Two *Anteholosticha* sp. populations shared the same secondary structure, though two nucleotide variations between them were detected (Fig. 2d).

3.3 *Phylogenetic analyses*

Phylogenetic trees inferred from the ITS1-5.8S-ITS2 region sequences using different methods showed essentially the same result (Fig.1b), in which *Holosticha bradburyae* and *H. diademata* always clustered together with highest supports (1.00 BI, 100% NJ, 100% MP), sister to two *Anteholosticha* sp. populations. *Anteholosticha* did not cluster together, but *A. manca* grouped with *Diaxonella trimarginata* and *Urostyla grandis*, forming a well-supported clade with *Pseudokeronopsis* (1.00 BI, 97% NJ, 94% MP).

Phylogenetic trees inferred from the SSrRNA gene sequences (Fig.3) and the combined dataset containing of SSrRNA gene and ITS1-5.8S-ITS2 region sequences (Fig. 4) showed the same result, which indicated that *Anteholosticha manca* was more closely related to *Diaxonella trimarginata*, while *Anteholosticha* sp. formed a separated clade, demonstrating that *Anteholosticha* seems to be paraphyletic. It was different with analysis of ITS1-5.8S-ITS2 region sequences that *Holosticha* branched first from the stichotrichous clade (Fig. 3) instead of *Apokeronopsis* (Fig. 1b), which indicated that the topologies were not stable as inferred from different genes.

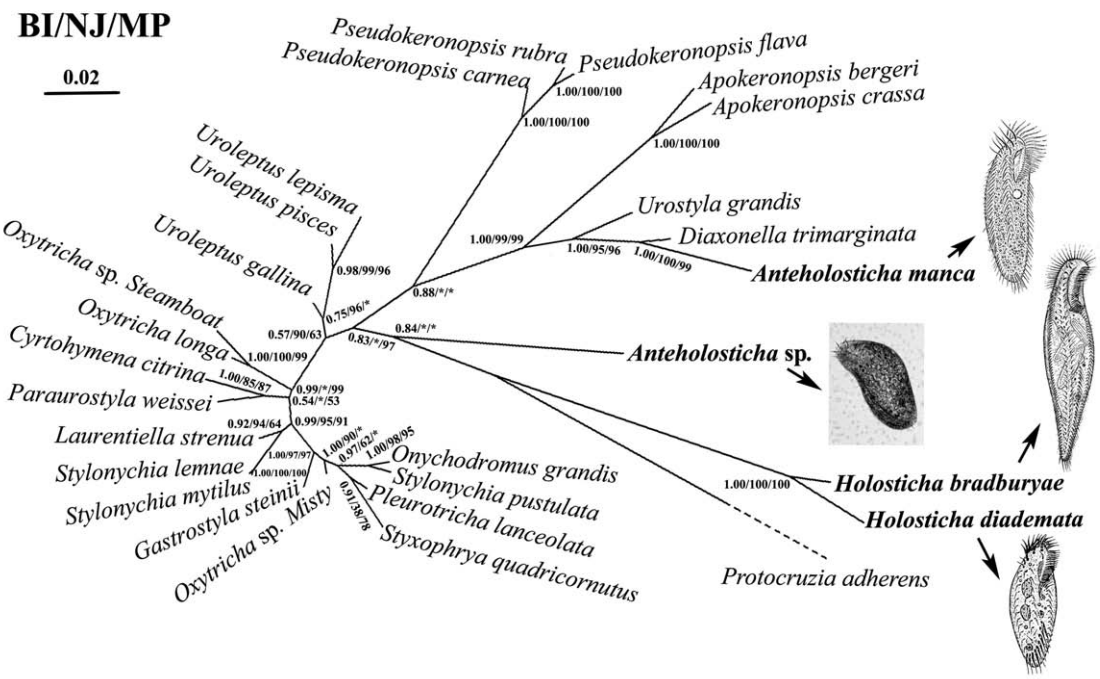

Fig.3. Bayesian tree inferred from the nucleotide sequences of SSrRNA of stichotrichs. Numbers at nodes represent posterior probability of Bayesian analysis and the bootstrap values of NJ and MP analysis out of 1 000 replicates. Asterisk (*) indicates disagreement between Bayesian and other two methods. The scale bar corresponds to 2 substitutions per 100 nucleotide positions. *Holosticha* and *Anteholosticha* are highlighted in bold.

4 Discussion

4.1 *Sequence comparison*

The previous studies indicated that the SSrRNA gene was highly conserved and suitable for interspecies separation but not for biodiversity analysis at intra-species level (Yi, Chen et al., 2008). As compared with SSrRNA, ITS1-5.8S-ITS2 region showed relatively high divergence. Snoeyenbos-West et al. (2002) found that SSrRNA gene sequence diversities among stichotrichs, oligotrichs and choreotrichs were on average 2.97%, 8.17% and 7.48%, respectively,

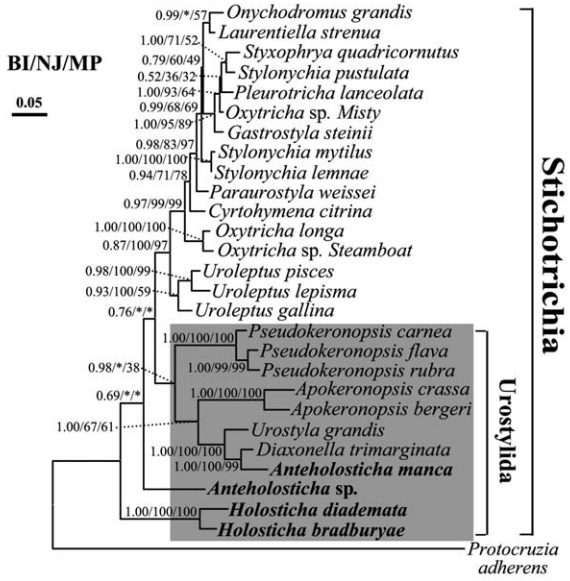

Fig.4. Bayesian tree inferred from the combined sequences of SSrRNA gene and ITS1-5.8S-ITS2 region of stichotrichs. Numbers at nodes represent posterior probability of Bayesian analysis and the bootstrap values of NJ and MP analysis out of 1 000 replicates. Asterisk (*) indicates disagreement between Bayesian and other two methods. The scale bar corresponds to five substitutions per 100 nucleotide positions. *Holosticha* and *Anteholosticha* are highlighted in bold.

while the sequence diversities of ITS1-5.8S-ITS2 region were on average 6.74%, 9.26% and 7.37%, respectively. In our study, the sequence diversities of the ITS1-5.8S-ITS2 region and SSrRNA gene sequence among four holostichids were higher (9.0%–22.6%, 0.4%–10.7% respectively), which supported the opinion that they could be derived from differently originated forms rather than a monophylogenetic group (Hu and Song, 2001).

4.2 *ITS2 secondary structures comparison*

The predicted secondary structures of these four *Holosticha*-complex species (Fig.3) were similar to those of the class Spirotrichea (Yi, Chen et al., 2008; Coleman, 2005). As suggested by Coleman (2005), the helix that we labeled as "B" contained the fifteen nucleotide sequence essentially identical in all Choreotrichia and Stichotrichia (Fig. 2, noted with bidirectional arrows) and three highly variable termini of sub-helices (Fig.2a, noted in the ellipses), and the helix labeled "A" showed a pyrimidine-pyrimidine bulge near the base in almost all cases. Meanwhile, the typical eukaryote association of the 3' region of the 5.8S rRNA gene with the 5' region of the ribosomal LS rRNA gene was also seen in Fig. 2.

Two populations of *Anteholosticha* sp. shared the same secondary structure because all variations of nucleotides in paired regions of the secondary structure preserved the pairing potential with a compensatory base change (CBC) or hemi-CBC (compensatory change on only one side of a helix pairing) (Coleman, 2003). Besides, there were some differences among the ITS2 secondary structures of these four holostichids (e.g., different loop configurations), for which there was no CBC "proof" (Coleman, 2005, 2003). Anyway, the four morphospecies we investigated could be separated using ITS2 secondary structures.

4.3 *Phylogenetic relationships among four holostichids*

Considering the fact that most hypotrichs at generic level possess the same or very similar pattern of morphogenesis (Li et al., 2008; Berger, 2006), *Holosticha* displays strikingly diverse characters which indicate likely that members within this genus could be derived from differently originated forms rather than a monophylogenetic group as accepted for a long time (Hu and Song, 2001). Berger (2003) redefined the genus *Holosticha*, splitting it into four genera (*Holosticha, Anteholosticha, Caudiholosticha* and *Biholosticha*), and two genera (*Holosticha* and *Anteholosticha*) were involved in this study. Owing to the limited number of *Holosticha* and *Anteholosticha* species, and the lack of molecular data on *Caudiholosticha* and *Biholosticha* genera, the precise relationships among the four genera still remain unresolved. However, some useful conclusion could be made.

Our phylogenetic analyses based on different genes, SSrRNA gene (Fig. 3) and ITS1-5.8S-ITS2 region (Fig.1b), and the combined dataset (Fig.4) using different methods consistently reveal that: (1) both *Holosticha* and *Anteholosticha* fell into Urostylida, showing a close relationship to each other; (2) *Holosticha* was monophyletic, while *Anteholosticha* clustered separately from the *Holosticha* and might not be monophyletic, which was also found in the previous investigations (Li, Song et al., 2009; Yi, Song, Shao et al., 2008; Schmidt et al., 2007). Furthermore, Berger (2003) also discussed that *Anteholosticha* was still heterogeneous due to the lack of good apomorphies and

only a combination of plesiomorphies.

Interestingly, the results of sequence comparison, ITS2 secondary structures comparison and phylogenetic trees consistently showed that *Anteholosticha manca* did not cluster with *Anteholosticha* sp. but with *Diaxonella trimarginata*. Considering most of the morphological features (e.g., continuous adoral zone; three enlarged frontal cirri; nuclear apparatus left of midline or scattered; caudal cirri lacking.), *A. manca* was more similar to *Anteholosticha* sp. and the great differences between *D. trimarginata* and *Anteholosticha* were the number of left marginal rows (3-4 vs. 1) and the number of buccal cirri (5-8 vs. 1) (Li, Hu et al., 2008; Shao et al., 2007). However, as to morphogenetic process of frontoventral-terminal anlagen, both *A. manca* and *D. trimarginata* were primary primordial (Li et al., 2008; Shao et al., 2007), while *Anteholosticha* sp. was secondary primordial (Shao, personal communication), and this was consistent with the result of our molecular data.

Our study also indicates that the positions of the morphospecies investigated within the subclass Stichotrichia differed from each other inferred from different genes. The reasons might be: (1) single gene contained limited information; (2) some effects, like different out-groups, different combinations of internal groups, different lengths of gene sequences and different tree-construction algorithms etc., could change the tree topologies (Yi et al., 2007). Therefore, multigene and large numbers of DNA sequences, combined with morphological and morphogenetical characters, should be analyzed to minimize the difference between the phylogenetic and the real trees.

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