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Reconsideration of the Phylogenetic Position of *Frontonia*-related Peniculia (Ciliophora, Protozoa) Inferred from the Small Subunit Ribosomal RNA Gene Sequences

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Summary. For a long time, the class Oligohymenophorea de Puytorac *et al*., 1974 has been morphologically characterized as a distinct and monophyletic assemblage. However, its relationship of some taxa, especially the *Frontonia*-related Peniculia have not been much concerned yet using molecular techniques. In this report, the SS rRNA genes for three congeners of *Frontonia*, namely *F. lynni*, *F. tchibisovae* and *F. didieri*, were sequenced and characterized. Phylogenetic trees were constructed by means of Bayesian, neighbor-joining (NJ), least-squares (LS) and maximum parsimony (MP) to assess the inter- and intra-generic relationships of the class Oligohymenophorea. All trees show similar topologies with stable bootstrap support and indicate that: (1) four well known groups, i.e., Peritrichia, Hymenostomatia, Scuticociliatia and Peniculia are distinctly outlined within the class Oligohymenophorea; (2) members of *Frontonia* are likely more divergent, that is, morphotypes assigned into this genus based on traditional methods might belong to different assemblages, which hence conflicts with the separation of *Apofrontonia* from the family Frontoniidae, in which the genus *Frontonia* locates; (3) *Apofrontonia* always clusters with *Paramecium* and then groups with the closely related genus *Frontonia*. In contrast to investigations based on morphology and morphogenesis, the SS rRNA gene sequence determines that the well-outlined genus *Frontonia* appears to be a paraphyletic assemblage, in which the morphotype *Frontonia didieri* is more closely related to *Apofrontonia* than to its congeners.

Key words: SS rRNA, *Frontonia lynni*, *F. tchibisovae*, *F. didieri*, Oligohymenophorea, ciliate, phylogeny.

INTRODUCTION

The prominent peniculine ciliates, represented by well-known taxa such as *Paramecium* and *Frontonia* were considered for a long time as a clearly-outlined group within the traditional species-rich class Oligohymenophorea de Puytorac *et al*., 1974. These specimens were characterized by the bucco-kinetal stomatogenesis, similar and well-developed buccal apparatus (i.e., 3 membranelles on the left side of the buccal cavity with a single paroral on the right margin), and basically uniform (hence "primary") somatic ciliature (Corliss 1979, Lynn and Small 1988, Foissner 1996). The systematic relationship among this assemblage was mostly

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deduced from the evidences of somatic kinetid patterns, stomatogenesis and ultrastructure, which usually differ among the higher taxa (Corliss 1979; Small and Lynn 1981, 1985; de Puytorac *et al*. 1984, 1987; de Puytorac 1994).

Compared with other oligohymenophorean groups, however, only a little of work has been reported on the relationship of peniculine ciliates based on molecular information (Lynn and Small 1997, Strüder-Kypke *et al*. 2000). Recently, Fokin *et al*. (2006) discussed the phylogenetic relationship of peniculines with only few taxa inferred from SS rRNA sequences. However, its system conflicts with that by Lynn and Small (1997) in some details. It may be the reason that only a few peniculines have been characterized using suitable molecular markers for phylogenetic analyses at the intra-subclass level (Fokin *et al*. 2006). Meantime, species sampling among Peniculia is quite unbalanced, with relatively few representatives for each genus. Hence, the addition of species from other oligohymenophorean subclasses is needed to resolve their relationships (Strüder-Kypke *et al*. 2000).

For a new survey on evolutionary studies of ciliated protozoa, the SS rRNA gene for three congeners, *Frontonia lynni*, *F. tchibisovae* and *F. didieri*, was sequenced. Inferred from sequence information combining with morphological and morphogenetic characters, topological trees were constructed and analyzed to achieve a better interpretation of the phylogenetic relationships of peniculine ciliates within the class Oligohymenophorea.

MATERIALS AND METHODS

Ciliate collection and identification

Ciliates (Fig. 1) were collected either from the coast of Qingdao (Tsingtao, 36°08′N; 120°43′E) or at the Laizhou Bay (37°24′N; 119°56′E), north China. Culture, morphological studies and identification of ciliates concerned in the present work are according to the methods as used by Long *et al*. (2005).

DNA Extraction and PCR amplification

DNA was isolated according to the protocol in the previous report (Chen and Song 2002). In brief, cells were starved in sterilized seawater at room temperature overnight to minimize the food and contaminants, and then were harvested by centrifugation at 1,500 rpm for 10 mins and treated with the lysis buffer at 56°C for 2~3 hours (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.6% Tween 20, 0.6% Nonidet P40, 60 μg/ml Proteinase K), followed by the phenol-chloroform purification (Shang *et al*. 2003). DNA samples were stored at –20°C. The universal oligonucleotide primers (forward 5'-AACCTGGTTGATCCTGCCAGT-3'; reverse 5'-TGATCCTTCTGCAGGTTCACCTAC-3') were used to amplify the SS rRNA genes of *Frontonia lynni*, *F. tchibisovae* and *F. didieri* (Medlin *et al*. 1988). The SS rRNA gene was amplified using the polymerase chain reaction (PCR) according to Chen and Song (2002). Cycling parameters for PCR amplification were as follows: 1 cycle (94°C 5 mins); 5 cycles (94°C 1 min; 56°C 2 mins; 72°C 2 mins); 35 cycles (94°C 1 min; 60°C 2 mins; 72°C 2 mins); 1 cycle (72°C 10 mins). In order to minimize sequence errors, the high-fidelity TaKaRa ExTaq (TaKaRa, Otsu, Japan) was used for PCR amplification.

Cloning and sequencing of SS rRNA gene

Each PCR product was purified using the TIANgel Midi Purification Kit (TIANGEN Bio. Co., China) after confirmation of appropriate size, and were inserted into a pUCm-T vector (Sangon, Bio. Co., Canada). Plasmids were harvested using the plasmid mini-prep spin column kit (Sangon Bio. Co., Canada) and were sequenced by the INVITROGEN sequencing facility in Shanghai, China. Subsequent sequencing was performed in both directions using primer walking.

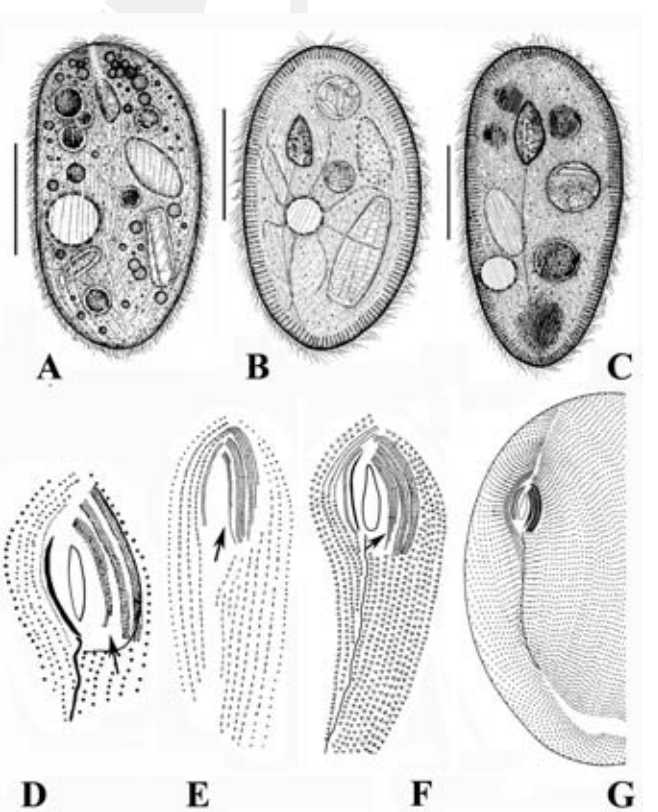

Fig. 1. Morphology of three *Frontonia in vivo* (**A**, **B**, **C**) and after silver impregnations (**D**, **E**, **F**, **G**). **A**, **D** – *Frontonia lynni*. **B**, **E** – *F. didieri*. **C**, **F**, **G** – *F. tchibisovae*. Arrows in D, E, F mark the patterns of the third diagnostic membranelle, different in the number and structure of kinety rows to each other. Scale bars: 50 μm.

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Sequence availability

The SS rRNA gene sequences for other ciliates were available from the GenBank/EMBL databases under the following accession numbers: *Apofrontonia dohrni* AM072621, *Campanella umbellaria* AF401524, *Carchesium polypinum* AF401522, *Cohnilembus verminus* Z22878, *Colpidium campylum* X56532, *Cyclidium porcatum* Z29517, *Dexiotrichides pangi* AY212805, *Epistylis chrysemydis* AF335514, *Frontonia leucas* AM072622, *Frontonia vernalis* U97110, *Glaucoma scintillans* AJ511861, *Ichthyophthirius multifiliis* U17354, *Lembadion bullinum* AF255358, *Mesanophrys carcini* AY103189, *Ophryoglena catenula* U17355, *Paramecium nephridiatum* AF100317, *Paramecium primaurelia* AF100315, *Paramecium tetraurelia* X03772, *Paramecium woodruffi* AF255362, *Paranophrys magna* AY103191, *Parauronema longum* AY212807, *Pseudovorticella punctata* DQ190466, *Tetrahymena australis* M98015, *Tetrahymena hegewischi* M98019, *Urocentrum turbo* AF255357, *Uronema elegans* AY103190, *Vorticella fusca* DQ190468, *Zoothamnium arbuscula* AF401523 and *Zoothamnopsis sinica* DQ190469. A karyorelictid ciliate, *Loxodes striatus* U24248, was selected as the outgroup species.

Phylogenetic analyses

The SS rRNA gene sequences were aligned using a computer assisted procedure, Clustal W, v1.83 (Thompson *et al*. 1994) and refined to be 1,675 bp in length by removing gaps of both termini of the alignment. The computer program, MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) was used for the Markov chain Monte Carlo (MCMC) algorithm to construct a Bayesian tree (BI) under the GTR model of substitution (Lanave *et al*. 1984, Tavare 1986, Rodriguez *et al*. 1990), considering a gamma-shaped distribution of the rates of substitution among sites. This "best" model was evaluated using the program MrModeltest (Nylander 2004). Markov chain Monte Carlo (MCMC) simulations were then run with two sets of four chains using the default settings: chain length 10,000,000 generations, with trees sampled every 100 generations. The first 100,000 generations were discarded as burn-in. The remaining trees were used to generate a consensus tree and calculate the posterior probabilities (PP) of all branches using a majority-rule consensus approach. PHYLIP v3.57 (Felsenstein 1995) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura two-parameter model (Kimura 1980). Distance-matrix trees were then constructed using the Fitch and Margoliash (1967) least-squares (LS) method and the neighbor-joining (NJ) method (Saitou and Nei 1987). Heuristic searches with a parsimony ratchet were used to generate trees for the maximum-parsimony (MP) analysis in PAUP (v4.0b10) (Swofford 2002). For the MP analysis, sequence data were reduced to 590 phylogenetically informative sites. Bootstrap values were generated in PAUP (v4.0b10). For LS, NJ and MP analyses, data were bootstrap resampled 1,000 times.

RESULTS

Sequences and comparisons

The SS rRNA gene sequences of three *Frontonia* were submitted to the NCBI/GenBank database with following accession numbers (with stain names): *Frontonia tchibisovae* QD-pop2 (1748bp, DQ883820), *F. lynni* QD-pop1 (1747bp, DQ196403) and *F. didieri* QD-pop1 (1744bp, DQ885986) (Fig. 2). The GC contents (*F. tchibisovae* 45.25%; *F. lynni* 45.05%; *F. didieri* 45.13%) are in the same range as that of other related ciliates (Li *et al*. 2006). Additional sequencing of clones of each gene revealed identical sequences.

The SS rRNA gene sequence of *Frontonia lynni* differs in 30 nucleotide sites from that of *F. tchibisovae* (sequence identity 98.3%), while 146 from that of *F. didieri* (sequence identity 91.6%). 127 variable sites are observed between that of *F. tchibisovae* and *F. didieri* (sequence identity 92.7%). *F. didieri* and *A. dohrni* are dissimilar to each other in 87 sites of the SS rRNA gene sequence (sequence identity 94.7%) (Table 1).

Phylogenetic analyses based on the SS rRNA gene sequence

To determine the systematic relationships within the class Oligohymenophorea, phylogenetic trees were constructed inferred from the SS rRNA gene sequence. The tree topologies using different algorithms were similar and strongly supported four monophyletic subclasses of Oligohymenophorea available in this study: Peritrichia (BI 0.98, LS 100%, NJ 100%, MP 100%), Hymenostomatia (BI 0.97, LS 100%, NJ 100%, MP 100%), Pe-

Table 1. SS rRNA gene sequence identities (%) of *Frontonia* spp. and *Apofrontonia dohrni*.

	F. tchibisovae	*F. lynni*	*F. didieri*	*F. vernalis*	*F. leucas*	*A. dohrni*
F. tchibisovae	–					
F. lynni	98.30	–				
F. didieri	92.70	91.62	–			
F. vernalis	93.50	91.92	89.94	–		
F. leucas	93.68	92.11	90.22	96.75	–	
A. dohrni	91.92	90.45	94.74	89.76	89.81	–

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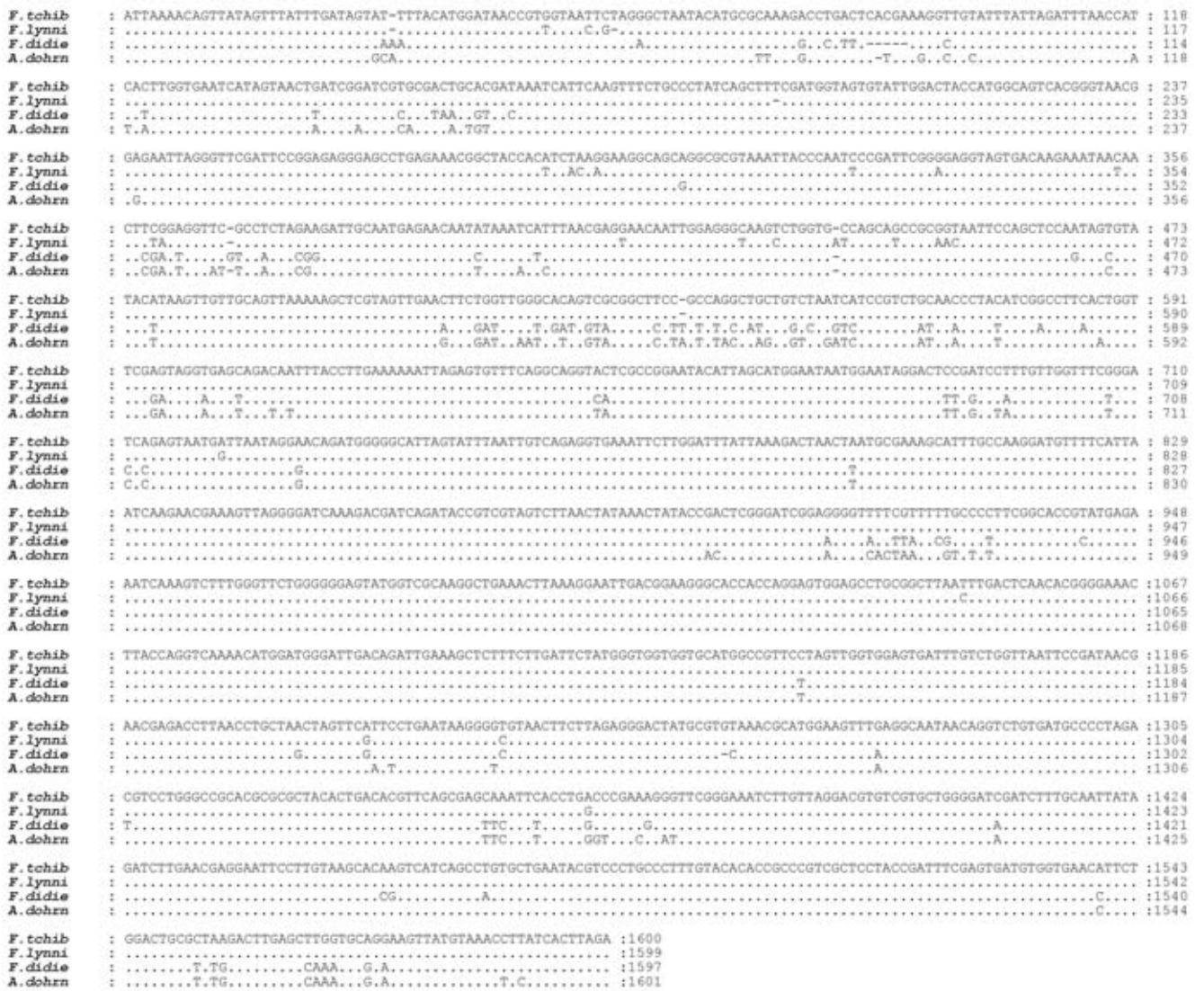

Fig. 2. Small subunit rRNA (SS rRNA) gene sequence alignment of *Frontonia tchibisovae* (*F. tchib*), *F. lynni*, *F. didieri* (*F. didie*) and *Apofrontonia dohrni* (*A. dohrn*). Numbers at the end of lines indicate the number of nucleotides. The differences in sequence length were compensated for by introducing alignment gaps (–) in the sequences. Matched sites are indicated by dots, while unmatched are illustrated by respective nucleotide bases.

niculia (BI 0.97, LS 100%, NJ 100%, MP 81%), and Scuticociliatia (BI 0.81, LS 61%, NJ 84%, MP 78%) although the later was weakly or moderately supported (Figs 3–5).The clade of peniculines branched at a very deep level within the oligohymenophorean assemblage and formed a sister group to the Peritrichia-Hymenostomatia and Scuticociliatia clade.

Except for *Frontonia didieri*, all *Frontonia* species clustered together and formed a sister clade with the branch consisting of *Paramecium* and *Apofrontonia* (BI 0.98, LS 97%, NJ 97%, MP 96%) within the subclass Peniculia, of which *Lembadion* occupied the basal position. Interestingly, *F. didieri* is more closely related to *Apofrontonia dohrni* than to its *Frontonia* congeners. It reveals that the genus *Frontonia* appears to be a paraphyletic clade.

The major aspects of the distance trees (Fig. 4) were generally similar to those of the Bayesian tree (Fig. 3) except that *Apofrontonia dohrni* formed a sister clade with *Frontonia didieri* (LS 99%, NJ 98%), which was also revealed by the maximum parsimony analysis (MP 88%) (Fig. 5).

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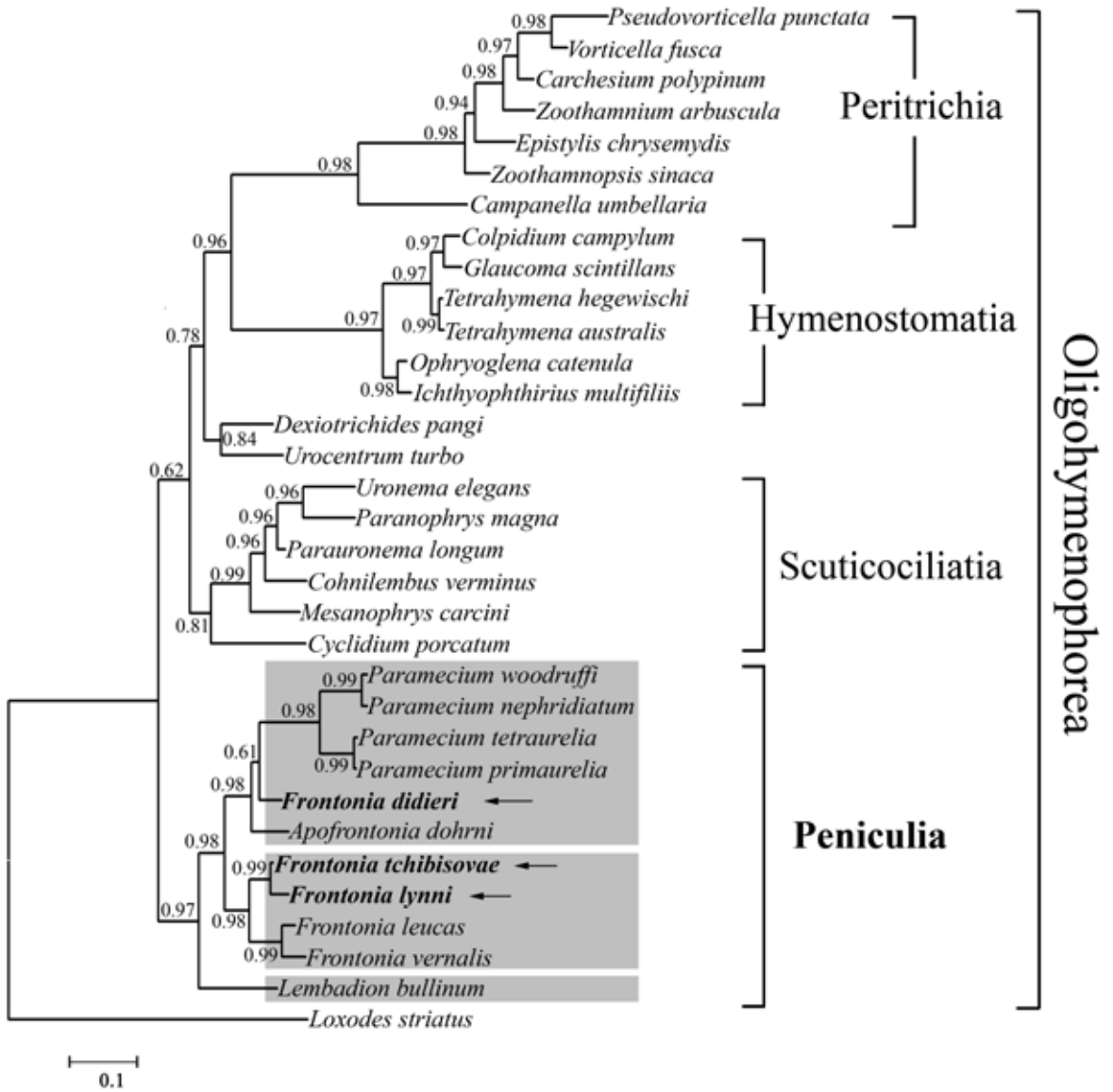

Fig. 3. Bayesian tree inferred from SS rRNA gene sequences of oligohymenophorean ciliates. Species sequenced in this work are highlighted *in Bold* and denoted by arrows. Asterisks indicate bootstrap values less than 0.5. Numbers near branches are posterior probability values. The scale bar corresponds to 1 substitutions per 100 nucleotide positions.

DISCUSSION

In this work, phylogenetic relationships of taxa within the class Oligohymenophorea were determined based on the SS rRNA gene sequence. Results reveal four monophyletic subclasses: Scuticociliatia, Peniculia, Peritrichia and Hymenostomatia, consisting with previous reports based on molecular and morphological data, although the branch orders of some taxa were variable (de Puytorac *et al*. 1987, de Puytorac 1994, Lynn and Small 1997, Strüder-Kypke *et al*. 2000, Fokin *et al*. 2006, Li *et al*. 2006). It could be explained by the difference of characters used for tree construction, such as weighted morphological and morphogenetic charac-

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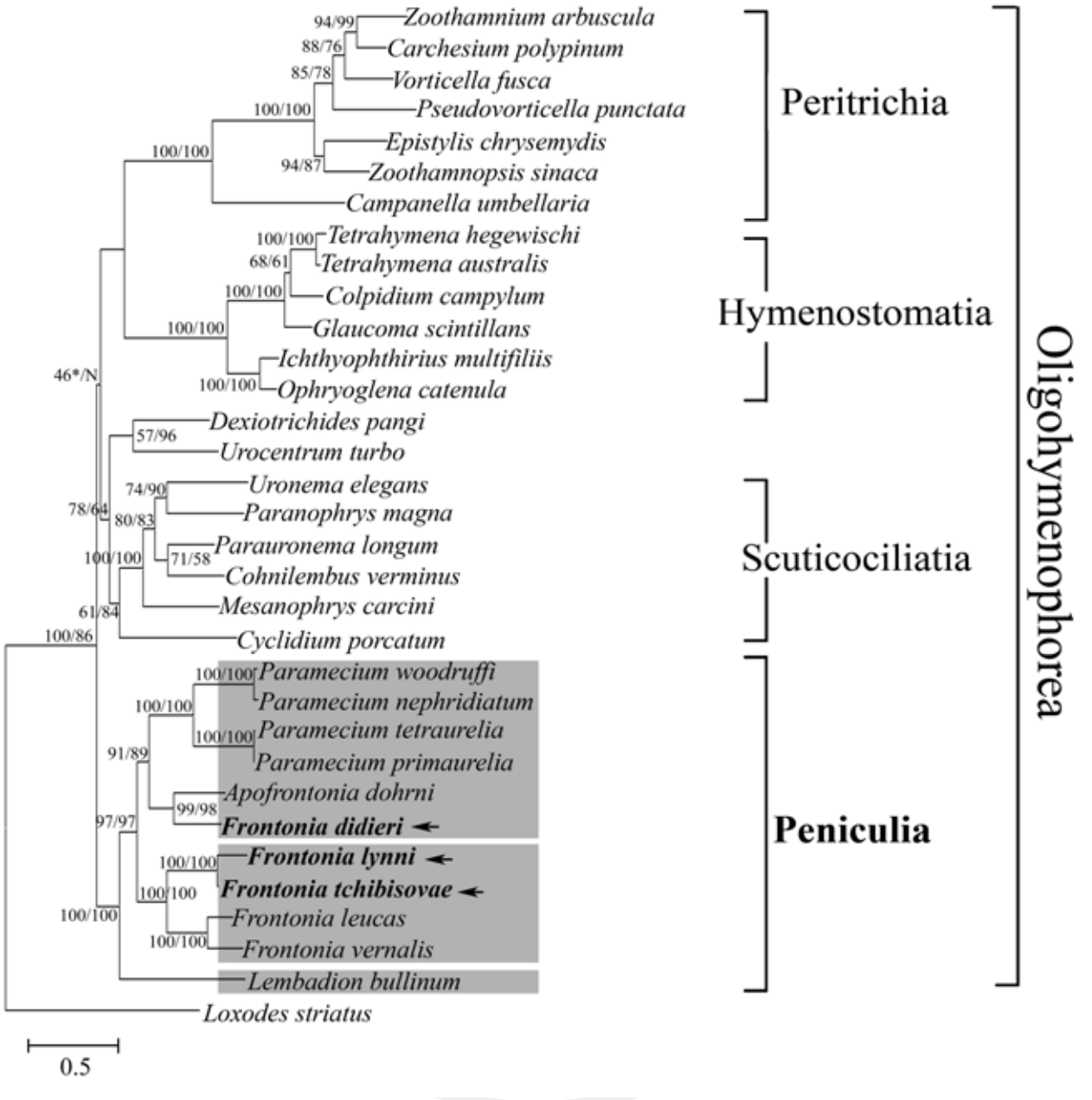

Fig. 4. Distance matrix trees inferred from the SS rRNA gene sequences of oligohymenophorean ciliates with least-squares (LS) and neighbor joining (NJ) methods. Species sequenced in this work are highlighted *in Bold* and denoted by arrows. Numbers on branches indicate support indices of LS and NJ methods from 1,000 bootstrap estimations using the Phylip package. Asterisks indicate bootstrap values less than 50%. "N" reflects disagreement between LS and NJ methods at a given node. Numbers at nodes represent bootstrap values (%) out of 1,000 replicates. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

ters, molecular markers and sequence lengths, and algorithms and taxon combinations.

The genus *Apofrontonia* was newly established by Foissner and Song (2002) and distinguished from its closely-related genus *Frontonia* due to the following features: (1) many long vestibular kineties covering but not extending beyond right buccal wall, (2) buccal cavity bowl-shaped exposing completely three similarly-

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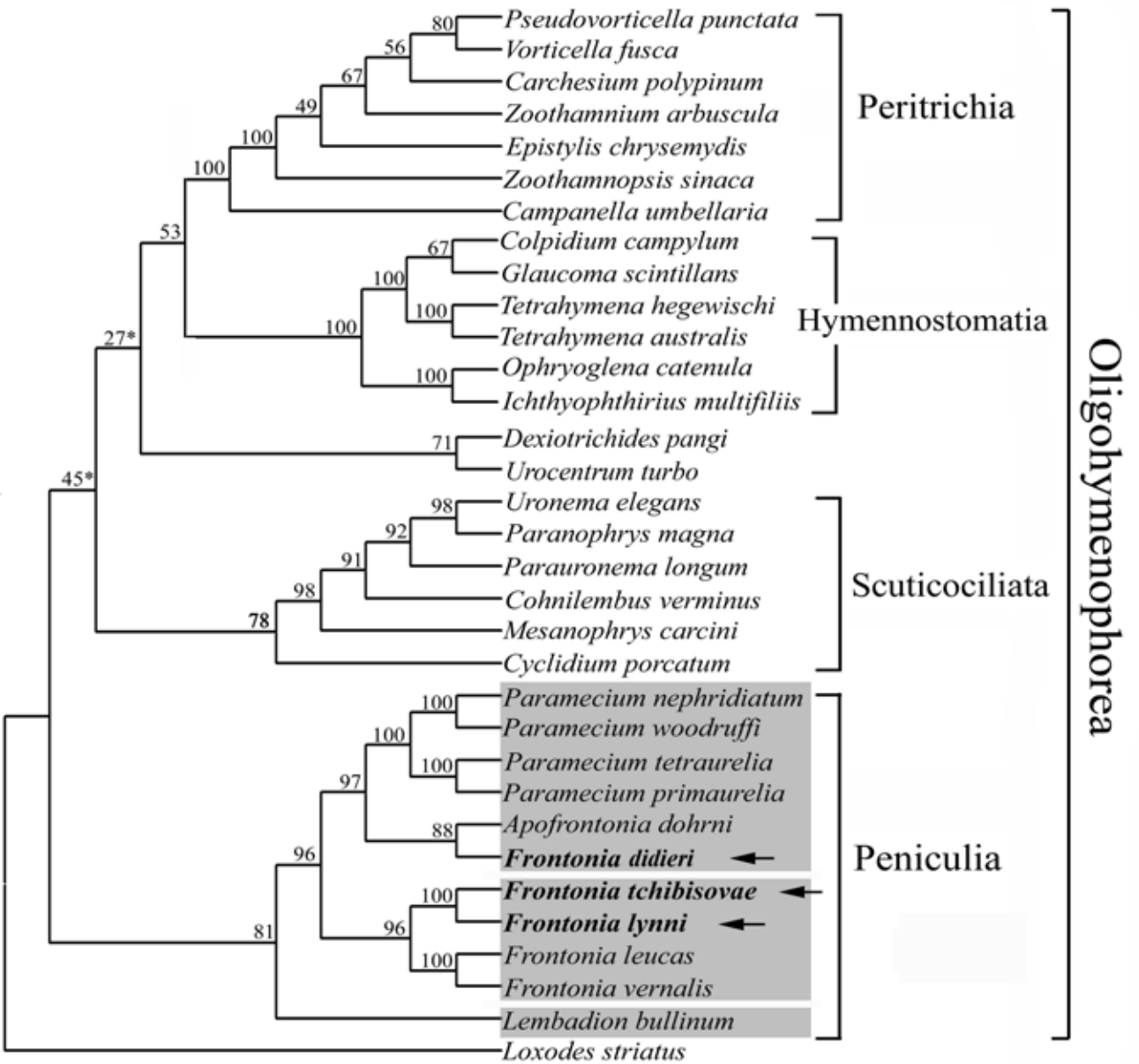

Fig. 5. A Maximum Parsimony (MP) tree of the oligohymenophorean ciliates inferred from the SS rRNA gene sequences. The numbers at the forks indicate the percentage of times that specific branch pattern occurred in 1,000 trees. Species sequenced in this work are highlighted *in Bold* and denoted by arrows. Asterisks refer the bootstrap values less than 50%.

structured peniculi, (3) large oral apparatus occupying at least half of ventral side, and (4) several scattered contractile vacuoles. Our result supports the separation of *Apofrontonia* from *Frontonia*, confirming the reports by Foissner and Song (2002) and Fokin *et al*. (2006). It is worth noting that *Frontonia didieri* clusters together with *Apofrontonia* rather than with its congeners inferred from the SS rRNA gene sequence. It exhibits that morphotypes of *Frontonia* might belong to different paraphyletic groups and hence conflicts with the separation of *Apofrontonia* from the family Frontoniidae, in which the genus *Frontonia* locates (Fokin *et al*. 2006).

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Since the general morphology, especially the features of the oral apparatus and buccal cavity unambiguously assigns *Frontonia didieri* into the genus *Frontonia*, the systematic arrangement using incomplete information might not be consistently reliable. More molecular data will be helpful to reveal the details of the intra- and inter-generic relationships of these organisms.

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