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
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* (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.
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 ZZ2878, Colpidium campylum X56532, Cyclidium porcatum Z29517, Dexiotriches pangi AY212805, Epistylis chrysemydis AF335514, Frontonia leucas AM072622, Frontonia vernalis U97110, Glaucoma scintillans AJ511861, Ichthyophthirius multifilis U17354, Lembadion bulbium AF255358, Mesanophrys carcin AY103189, Ophryoglena catenula U17355, Paramecium nephridiatum AF100315, Paramaecium primaurelia AF100315, Paramecium tetraurelia X03772, Paramaecium woodruffi AF255362, Paranophrys magna AY103191, Pararhodina 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 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%), Peniculiales (BI 0.98, LS 100%, NJ 100%, MP 100%), and Strongylocentrotinae (BI 0.97, LS 100%, NJ 100%, MP 100%). Bootstrap values were generated 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 strain names): Frontonia tchibisovae QD-pop2 (1748bp, DQ883820), F. lynnii QD-pop1 (1747bp, DQ196403) and F. didieri QD-pop1 (1744bp, DQ885986) (Fig. 2). The GC contents (F. tchibisovae 45.25%; F. lynnii 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 lynnii 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%), Peniculiales (BI 0.98, LS 100%, NJ 100%, MP 100%), and Strongylocentrotinae (BI 0.97, LS 100%, NJ 100%, MP 100%).

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

<table>
<thead>
<tr>
<th></th>
<th>F. tchibisovae</th>
<th>F. lynnii</th>
<th>F. didieri</th>
<th>F. vernalis</th>
<th>F. leucas</th>
<th>A. dohrni</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. tchibisovae</td>
<td>–</td>
<td>98.30</td>
<td>92.70</td>
<td>93.50</td>
<td>93.68</td>
<td>91.92</td>
</tr>
<tr>
<td>F. lynnii</td>
<td>–</td>
<td>–</td>
<td>91.62</td>
<td>91.92</td>
<td>92.11</td>
<td>90.45</td>
</tr>
<tr>
<td>F. didieri</td>
<td>92.70</td>
<td>91.62</td>
<td>–</td>
<td>90.22</td>
<td>96.75</td>
<td>–</td>
</tr>
<tr>
<td>F. vernalis</td>
<td>93.50</td>
<td>91.92</td>
<td>90.22</td>
<td>94.74</td>
<td>89.76</td>
<td>89.81</td>
</tr>
<tr>
<td>F. leucas</td>
<td>93.68</td>
<td>92.11</td>
<td>96.75</td>
<td>89.76</td>
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<td>–</td>
</tr>
<tr>
<td>A. dohrni</td>
<td>91.92</td>
<td>90.45</td>
<td>94.74</td>
<td>89.81</td>
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</table>
Fig. 2. Small subunit rRNA (SS rRNA) gene sequence alignment of *Frontonia tchibisovae* (*F.* *tchib*), *F. lynnii*, *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-Hymenosomatia 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).
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-
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-
Phylogenetic relationships of *Frontonia*-related Penicilia

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%.

- **Peritrichia**
  - *Pseudovorticella punctata*
  - *Vorticella fusca*
  - *Carchesium polypinum*
  - *Zoothamnium arbuscula*
  - *Epistyliis chrysemydis*
  - *Zoohamnopsis sinaca*
  - *Campanella umbellaria*
  - *Colpidium campylum*
  - *Glaucoma scintillans*
  - *Tetrahymena hegewischi*
  - *Tetrahymena australis*
  - *Ophryoglena catenula*
  - *Ichthyophthirius multifiliis*

- **Hymennostomatia**
  - *Dexiotrichides pangi*
  - *Urocentrum turbo*
  - *Uronema elegans*
  - *Paranophrys magna*
  - *Paraurocena longum*
  - *Cohnilembus verminus*
  - *Mesanophrys carcini*
  - *Cyclidiurn porcatum*
  - *Paramecium nephridiatum*
  - *Paramecium woodruffi*
  - *Paramecium tetrarelia*
  - *Paramecium primaurelia*
  - *Apofrontonia dohrni*

- **Scuticociliata**
  - *Frontonia didieri*
  - *Frontonia ichibisovae*
  - *Frontonia lynnii*
  - *Frontonia leucas*
  - *Frontonia vernalis*
  - *Lembadion bullinum*
  - *Loxodes striatus*

- **Oligohymenophorea**

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 congener 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).
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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REFERENCES


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