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Analysis of the secondary structure of ITS transcripts in peritrich ciliates (Ciliophora, Oligohymenophorea): Implications for structural evolution and phylogenetic reconstruction

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ABSTRACT

Despite extensive previous morphological work, little agreement has been reached about phylogenetic relationships among peritrich ciliates, making it difficult to study the evolution of the group in a phylogenetic framework. In this study, the nucleotide characteristics and secondary structures of internal transcribed spacers 1 and 2 (ITS1 and ITS2) of 26 peritrich ciliates in 12 genera were analyzed. Information from secondary structures of ITS1 and ITS2 then was used to perform the first systematic study of ITS regions in peritrich ciliates, including one species of *Rhabdostyla* for which no sequence has been reported previously. Lengths of ITS1 and ITS2 sequences varied relatively little among taxa studied, but their G+C content was highly variable. General secondary structure models of ITS1 and ITS2 were proposed for peritrich ciliates and their reliability was assessed by compensatory base changes. The secondary structure of ITS1 contains three major helices in peritrich ciliates and deviations from this basic structure were found in all taxa examined. The core structure of peritrich ITS2 includes four helices, with helix III as the longest and containing a motif 5′-MAC versus GUK-3′ at its apex as well as a YU-UY mismatch in helix II. In addition, the structural motifs of both ITS secondary structures were identified. Phylogenetic analyses using ITS data were performed by means of Bayesian inference, maximum likelihood and neighbor joining methods. Trees had a consistent branching pattern that included the following features: (1) *Rhabdostyla* always clustered with members of the family Vorticellidae, instead of members of the family Epistylididae, in which it is now classified on the basis of morphology. (2) The systematically questionable genus *Ophrydium* closely associated with *Carchesium*, forming a clearly defined, monophyletic group within the Vorticellidae. This supported the hypothesis derived from previous study based on small subunit rRNA gene sequences that *Ophrydium* and its few relatives are morphologically anomalous vorticellids, not sufficiently distinct to be given familial status and should be placed within a more broadly defined family Vorticellidae. This study validated for the first time a secondary structure of ITS1 and ITS2 from peritrich ciliates and demonstrated its potential in helping to resolve deep phylogenetic relationships.

1. Introduction

The highly variable internal transcribed spacer regions (ITS1 and ITS2) lie between the regions coding for small subunit and large subunit rRNA genes. They can excise themselves during the maturation of the precursor of ribosomal RNA (rRNA) transcripts and thus play an important role in molecular processing (Maroteaux et al., 1985). Comparison of ITS sequences from various organisms reveals conserved regions, which are potentially involved in rRNA biogenesis, and yields new information about the evolutionary divergence of the corresponding region of the genome (Voronov et al., 2006).

Information from ITS regions has been used to infer phylogenetic relationships of organisms at different levels of divergence (Goertzen et al., 2003; Gottschling and Plötner, 2004; Young and Coleman, 2004). Despite this extensive use, ITS sequences have not been considered appropriate for resolving relationship at high taxonomic ranks, mostly due to excessive INDELs (insertions–deletions), saturation, and/or intragenomic variation (Fabry et al., 1999; Vollmer and Palumbi, 2004). However, it has been argued that despite a relatively rapid rate of primary sequence divergence, conserved structural elements within the secondary structures of the ITS regions facilitate the accurate alignment of truly homologous nucleotides (Mai and Coleman, 1997; Álvarez and Wendel, 2003; Coleman, 2003, 2007; Gottschling and Plötner, 2004; Wolf et al., 2005).

Many studies have been carried out among different eukaryotic taxa to construct phylogenies inferred from the information of ITS

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secondary structures (Liu and Schardl, 1994; Vonder Schulenburg et al., 1999; Gontcharov and Melkonian, 2005; Voronov et al., 2006; Feliner and Rosselló, 2007; Tippery and Les, 2008). However, knowledge of secondary structure in ciliates is still very scant, comprising only two previous studies (Coleman, 2005; Miao et al., 2008).

Peritrich ciliates are a large group of highly diverse organisms, comprising 1/7 of all known ciliates (approximately 1000 species described so far) that occur in marine, limnetic and terrestrial habitats (Fig. 1A–H). Morphological characteristics, such as mode of development of the feeding stage (solitary, Fig. 1A–C or colonial, Fig. 1E–H), structure of stalk (Fig. 1A–C, E–H, arrows) or lorica (Fig. 1D, arrowhead), appearance of oral area, and pattern of silverline system, are routinely used to identify species and to deduce the systematic relationships among them (Corliss, 1979). Among these characteristics, features of stalk and silverline system are widely used in peritrich taxonomy at the levels of family and genus. Historically, peritrichs have been assigned to the subclass Peritrichia Stein, 1859 within the class Oligohymenophora (Lynn, 2008). The Peritrichia was thought to be a monophyletic assemblage composed of the orders, Sessilida and Mobilida, based on the characteristics of oral apparatus (Lom, 1964; Lynn, 2008). Recent analyses using SSrRNA gene sequences (Gong et al., 2006) revealed, however, that mobilids are not allied with sessilids and that their distinctive similarity in oral morphology is a convergent characteristic. Removal of mobilids from the Peritrichia (Zhan et al., 2009) leaves the former sessilids are the only true, monophyletic group of taxa within the subclass.

Several phylogenetic studies of "true" peritrichs have been conducted using ssu rRNA gene sequences (Miao et al., 2001, 2004; Williams and Clamp, 2007; Utz and Eizirik, 2007; Li et al., 2008). But in analyses to date, the relationship between and within three major clades of peritrichs (III, families Astylozoidae–Opisthonectidae–Vorticellidae; II, families Zoothamniidae–Epistylididae and I, composed of three morphologically diverse species – *Epistylis galea*, *Opercularia microdiscum* and *Campanella umbellaria*) remain to be demonstrated. Clade III, comprised mostly of the Vorticellidae, the biggest family in the subclass is the most confusing of the major clades of peritrichs, offering examples of radical differences between phylogenies and classifications determined by morphology and those based on SSrRNA sequences. *Ophrydium versatile*, which is included currently in the family Ophrydiidae by its distinctive morphology, is nested within the family Vorticellidae and appears to be closely related to some species of *Vorticella* in SSrRNA trees (Miao et al., 2004; Williams and Clamp, 2007; Li et al., 2008). In SSrRNA trees by Miao et al. (2004) and Clamp and Williams (2006), *Vorticella microstoma* is deeply divergent from its congeners and clusters with species of *Opisthonecta* and *Astylozoon*, both of which are type genera of separate families in morphologically based classifications, and the SH test strongly rejected inclusion of *Vorticella microstoma* in a monophyletic genus *Vorticella* (Utz and Eizirik, 2007).

In order to clarify structural evolution of ITS regions in peritrichs and address their phylogeny in a more comprehensive way, the ITS regions for 21 strains of peritrich ciliates were newly sequenced in the present work, including one species of *Rhabdostyla* which sequence was reported the first time. Then we compared ITS1 and ITS2 secondary structures of 26 peritrich ciliates spread among 12 genera and 5 families, to see if it would lead to a unified model in spite of length and sequence differences. This also allowed us to acquire a relatively broad perspective of internal transcript spacer evolution for peritrich ciliates. Our study had the following objectives: (1) describing ITS1 and ITS2 secondary structures in peritrich ciliates; (2) comparing ITS1 and ITS2 secondary structures of peritrich ciliates with the general model proposed for eukaryotes; (3) testing previously proposed taxonomic and phylogenetic schemes of peritrich ciliates with data from ITS sequence variation; and (4) providing information from taxa not

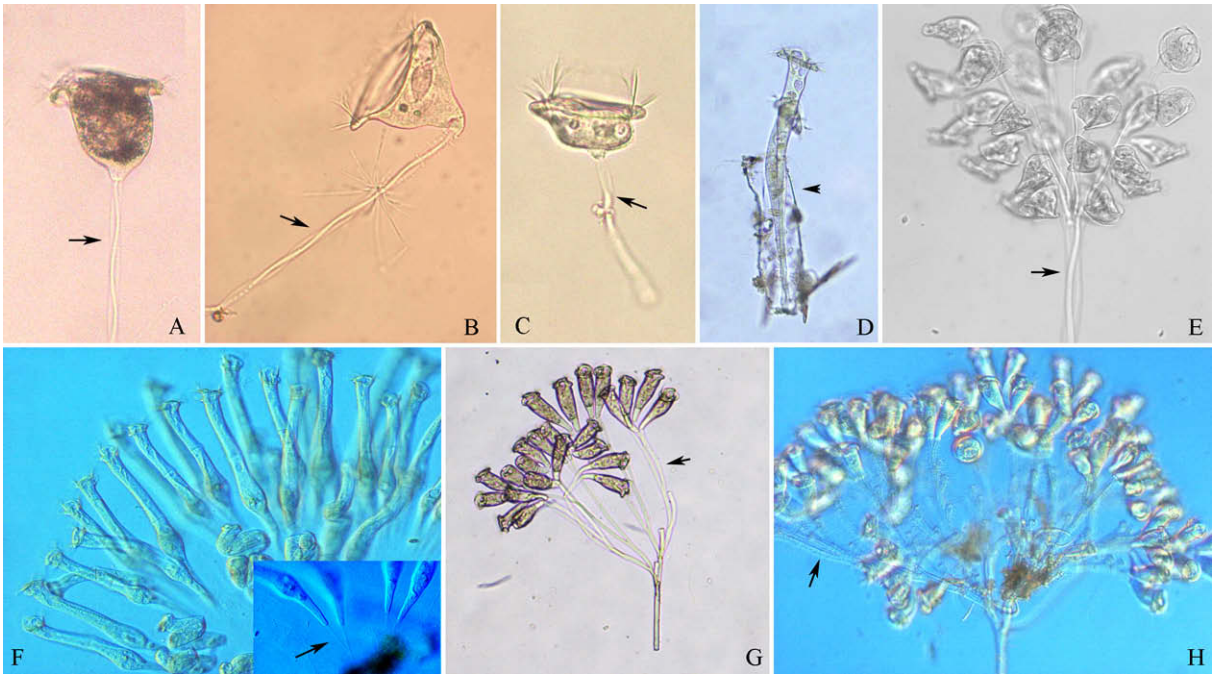

Fig. 1. Morphological diversity of peritrich ciliates. (A) *Vorticella campanula*; (B) *Pseudovorticella orientalis*; (C) *Pseudovorticella foissneri*; (D) *Thuricola valvata*; (E) *Carchesium polypinum*; (F) *Ophrydium eichornii*; (G) *Epistylis* sp.; (H) *Zoothamnium duplicatum*. Arrows (A–C, E) indicate the helically contractile stalk, with spasmoneme enclosed in the secreted part, which is diagnostic for members of the family Vorticellidae. The only difference between *Carchesium* (E) and the other vorticellids shown here (A–C) is its colonial mode of development. Arrow marks the rigid stalk of *Ophrydium*. Arrow indicates the contractile stalk of *Zoothamnium* (H) and its morphological relatives (family Zoothamniidae). It contracts in a zigzag conformation within a single plane, unlike the stalk of vorticellids. *Epistylis* (G) and its morphological relatives (family Epistylididae) are distinguished by having a rigid stalk (G, arrow). Arrowhead (D) indicates the protective lorica that members of the genus *Thuricola* secrete around themselves.

included in previous phylogenetic reconstructions to obtain a broader analysis of peritrichs evolution.

2. Materials and methods

2.1. Collection and fixation of samples

All species collected in present study are from freshwater habitats at localities in USA and China (Table 1). Isolation of peritrichs from samples, culturing, and fixation were performed by the methods of Clamp and Williams (2006).

2.2. Extraction and sequencing of DNA

Genomic DNA was extracted according to methods described in Clamp and Williams (2006). The ITS1–5.8S–ITS2 region was amplified by polymerase chain reaction (PCR) using the ITS-F (5′-GT TCCCCTTGAACGAGGAATTC-3′), and ITS-R (5′-TACTGATATGCTTA AGTTCAGCGG-3′) primers that were complementary to conserved regions and encompassed the 3′ end of SSrRNA, the entire ITS1–5.8S–ITS2 region, and the 5′ end of large subunit rRNA (Goggin and Murphy, 2000). Cycling parameters were as follows: 1 cycle (94 °C 1 min); 30 cycles (94 °C for 15 s; 63 °C for 30 s; 72 °C for 1 min); and 1 cycle (72 °C for 5 min). Products of PCR reactions were cleaned by filtration using a QIAquick PCR Purification Kit (Qiagen Sciences, MD) and sequenced in both directions using an ABI 3730-XL DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence fragments were assembled into contiguous sequences and edited with the Sequencher 4.0 software package (GeneCodes Corp., Ann Arbor, MI).

2.3. Analyses of data

In addition to sequences of species collected in the present study (Table 1), sequences of ITS regions of all other peritrichs in the Genbank database were included in analyses of secondary structure and phylogenetic reconstruction. Boundaries of the ITS1, 5.8S and ITS2 regions were identified by comparison with sequences available from Genbank, via Rfam (available on the web http://www.sanger.ac.uk/Software/Rfam/) (Griffiths-Jones et al., 2005) and the European ribosomal RNA database (http://bioinformatics.psb.ugent.be/webtools/rRNA/) (Van de Peer et al., 2000; Wuyts et al., 2001).

2.4. Predicting secondary structures of ITS1 and ITS2

Consensus structures of ITS1 and ITS2 regions were predicted using the Alifold Sever (http://rna.tbi.univie.ac.at/cgi-bin/alifold.cgi), which predicts structures from an alignment of related RNA sequences (Hofacker et al., 2002). With the guidance of these consensus structures, the secondary structures of ITS sequences were predicted with mFOLD version 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) (Zuker, 2003) by screening for thermodynamically optimal and suboptimal secondary structures using the default values. Results for the various species were compared to reveal the folding pattern common to them all. This, in turn, established the conserved structural models for peritrichs that revealed evidence of homology useful for phylogenetic analysis. Once the conserved structural models of ITS1 and ITS2 in peritrich ciliates were established, compensating base changes (CBCs) also were examined. The frequencies of bases at each position and mutual information of base-paired regions in helices were calculated with the program RNA Structure Logo (http://www.cbs.dtu.dk/?gorodkin/appl/slogo.html) (Gorodkin et al., 1997) and structural motifs identified based on the structure logo. For each structural domain, the position, number of base pairs, unpaired bases in bulges and/or interior loops, and the GC contents of base-paired regions were investigated and compared.

2.5. Analyses of phylogeny

Phylogenetic analyses of 26 peritrich ciliates were performed with data from ITS regions. It was difficult to obtain an unambiguous alignment with any other available ITS sequences from other taxa in the class Oligohymenophorea, therefore no outgroup was included. Sequences of ITS regions expressed in secondary-structure format were aligned using 4SALE (Seibel et al., 2006) based on both the primary sequences and secondary structures. Aligned structural and sequence matrices were analyzed using three methods (Bayesian Inference, Maximum Likelihood and Neighbor Joining). Bayesian Inference (BI) analyses were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using the HKY+I+G (for ITS1) and HKY+G (for ITS2) models selected by

Table 1
Names and collection data of newly sequenced peritrich ciliates.

Species	Locality of collection	Collector	Date collected
Vorticella sp1 popI	Toolik Lake, AK	P. Rublee	4/26/2003
Vorticella sp1 popII	Lake Otisco, NY	J. Clamp	7/2/2001
Vorticella sp1 popIII	Hart Springs, FL	J. Clamp	8/2/2002
Vorticella sp1 popIV	Guam	C. Lobban	9/20/2008
Vorticella sp7	Jones Lake State Park, NC	J. Clamp & P. Sun	2/23/2008
Vorticella sp8	Durham, NC	J. Clamp	12/3/2008
Vorticella sp11 popI	Morehead City, NC	J. Clamp	8/11/2008
Vorticella sp11 popII	Jones Lake State Park, NC	J. Clamp & P. Sun	2/23/2008
Vorticella sp11 popIII	Great Smoky Mt. National Park, NC	P. Sun	5/6/2008
Vorticella sp12	Morehead City, NC	J. Clamp	8/11/2008
Vorticella sp13	Morehead City, NC	P. Sun	8/11/2008
Vorticella sp13	Durham, NC	P. Sun	9/11/2008
Vorticella sp15	Morehead City, NC	J. Clamp & P. Sun	8/11/2008
Vorticella sp18	Durham, NC	J. Clamp & P. Sun	1/27/2008
Carchesium polypinum popI	Eno River State Park, NC	J. Clamp	11/2/2008
Carchesium polypinum popII	Durham, NC	P. Sun	2/3/2009
Carchesium polypinum popIII	Guangzhou, China	J. Clamp	6/13/2009
Epistylis chrysemydis popI	Durham, NC	J. Clamp	3/8/2009
Epistylis hentscheli popI	Carrboro, NC	J. Clamp	6/28/2009
Campanella umbellaria popI	Morehead City, NC	J. Clamp	8/11/2008
Rhabdostyla sp5	Guam	C. Lobban	10/16/2008

MrModeltest 2 (Nylander, 2004) under the AIC criterion. Four simultaneous MCMCMC chains were run for 1,000,000 generations sampling every 100 generations. The first 2500 trees were discarded as burn-in and the 50% majority-rule consensus tree was determined to calculate the posterior probabilities for each node. The appropriate models HKY+I+G (for ITS1) and HKY+G (for ITS2) were determined by MrModeltest 2 for Maximum Likelihood (ML) analysis. The ML tree was constructed with the PhyML V3.0 program (via http://www.phylogeny.fr/phylo_cgi/phyml.cgi) (Guindon et al., 2005) which performed ML analysis with a heuristic searches and a 100-fold bootstrap analysis. The Neighbor-Joining (NJ) analysis was performed with the software package PAUP* 4.0b10 (Swofford, 2002), and the support for the internal branches was estimated, using the bootstrap method with 1000 replicates (Felsenstein, 1985).

3. Results

3.1. Characteristics of ITS primary sequence in peritrich ciliates

All together, sequences of 26 species were included in our analyses (Table 2). We sequenced the ITS1 and ITS2 regions of 21 populations representing 13 species of peritrich ciliates, and accession numbers are given in Table 2. Sequences of the ITS1 and ITS2 regions of other species were obtained from Genbank. Very little intrageneric variation in length was observed. Eighteen populations of seven species in the genera *Vorticella*, *Carchesium*, *Epistylis* and *Campanella* had ITS1 and ITS2 sequences of almost exactly same length. The length of ITS1 sequences varied from 136 bp in *Pseudovorticella paracratera* to 156 bp in *Vaginicola crystallina*, and the length of ITS2 sequence ranged from 165 bp in *Vorticella* sp7 and *Vorticella* sp15 to 175 bp in *Zoothamnium alternans*. By contrast, the G+C content of ITS1 and ITS2 sequences varied greatly among the species studied (Table 2). The average G+C content of ITS2 was comparatively higher (from 23.56% in *Campanella umbellaria* to 46.67% in *Vorticella* sp7) than that of ITS1 (from 18.06% in *Campanella umbellaria* and *Epistylis galea* to 35.51% in *Pseudovorticella punctata*).

3.2. Putative secondary structures of ITS1 transcripts in peritrich ciliates

Several deviations from the general pattern of secondary structure of ITS1 found in peritrich ciliates are proposed in Supplement I. These were the basis for reconstructing a general model of secondary structure, as shown in Fig. 2. In spite of these distinctive variations in ITS1 sequences, peritrich taxa shared a very similar basic pattern of secondary structure with homologous sequence segments having homologous locations. Therefore, a general secondary structural model could be set up, the main features of which are: (1) a loop with three helices; (2) helix I showing variation among the different taxa; (3) a highly conserved helix II containing a motif 5′-RCCUA versus YAGGU-3′ (Supplement III 5A–F); (4) a helix III that is longer than other two and highly conserved, containing a motif 5′-YYWARAWWUYY versus GGAUAUCUWGG-3′ at its basal portion (Supplement III 5G–L). Helix III was the most consistent structural feature of ITS1 in the taxa studied, with high conservation of base pairing at its base and relatively low variation

Table 2
Characteristics of ITS regions in peritrich ciliates. Species that were sequenced during the present study are shown in bold type.

Species	Accession No.	ITS1		ITS2	
		Length (nt)	GC content (%)	Length (nt)	GC content (%)
***Vorticella* sp1 popI**	GU082378	145	29.66	167	32.34
***Vorticella* sp1 popII**	GU586174	145	29.66	167	32.24
***Vorticella* sp1 popIII**	GU586175	145	29.66	167	32.24
***Vorticella* sp1 popVI**	GU586176	145	29.66	167	32.24
***Vorticella* sp7**	GU187057	139	30.22	165	46.67
***Vorticella* sp8**	GU586177	145	30.34	167	31.14
***Vorticella* sp11 popI**	GU586178	144	29.86	168	32.74
***Vorticella* sp11 popII**	GU586179	144	29.86	168	32.74
***Vorticella* sp11 popIII**	GU586180	144	29.86	168	32.74
***Vorticella* sp12**	GU586181	145	28.28	167	29.94
***Vorticella* sp13 popI**	GU586182	145	31.03	167	32.93
***Vorticella* sp13 popII**	GU586183	145	31.03	167	32.93
***Vorticella* sp15**	GU586184	140	30.71	165	48.48
***Vorticella* sp18**	GU586185	146	28.77	169	29.59
Pseudovorticella paracratera	EU340855	136	34.56	166	39.76
Pseudovorticella punctata	EU340856	138	35.51	167	44.31
***Carchesium polypinum* popI**	GU082379	145	28.28	167	37.72
***Carchesium polypinum* popII**	GU082380	144	31.25	167	41.32
***Carchesium polypinum* popIII**	GU082381	145	30.34	167	39.52
Epicarchesium abrae	EU340854	142	30.99	166	42.17
***Epistylis chrysemydis* popI**	GU586187	145	24.14	170	28.82
Epistylis chrysemydis popII	AF429887	145	24.14	–	–
***Epistylis hentscheli* popI**	GU586186	148	23.65	170	26.47
Epistylis hentscheli popII	AF429889	148	23.65	–	–
Epistylis plicatilis	AF429890	144	23.61	–	–
Epistylis urceolata	AF429891	140	23.57	–	–
Epistylis galea	AF429888	155	18.06	–	–
Zoothamnium alternans	EU340858	152	23.68	175	28.00
Zoothamnium nii	EU340859	146	26.03	167	31.74
Zoothamnium plumula	EU340860	138	29.71	168	40.48
Zoothamnopsis sinica	EU340861	141	27.66	167	31.14
Ophrydium versatile	AF429894	146	27.40	–	–
Vaginicola crystallina	AF429895	156	28.85	–	–
***Campanella umbellaria* popI**	GU586188	155	18.06	174	23.56
Campanella umbellaria popII	AF429885	155	18.06	–	–
Opercularia microdiscum	AF429893	148	30.41	–	–
***Rhabdostyla* sp5**	GU586189	156	26.28	167	31.74

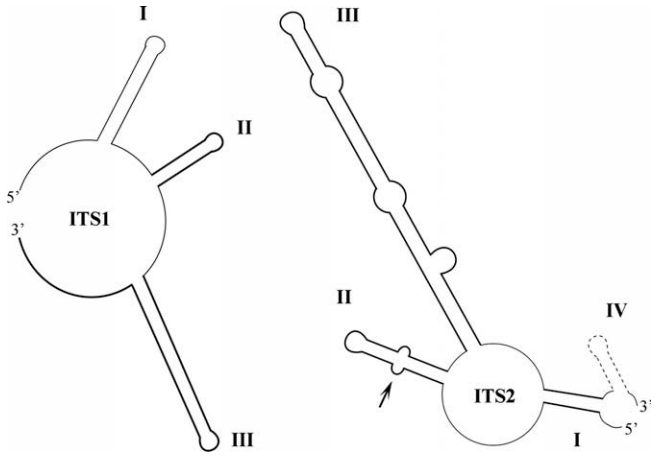

Fig. 2. The putative secondary structure models of ITS1 and ITS2 transcripts in peritrichs, supported by CBCs and hemi-CBCs that preserve the helix pairing. The domains, each with a stem-loop, are labeled I–III in ITS1 and I–IV in ITS2. Arrow indicates the pyrimidine–pyrimidine mismatch in Helix II of ITS2. Helix IV of ITS2 appeared only in one species and is marked with a dashed line. Lines in helices and unpaired region of ITS1 and ITS2 are in bold to suggest the relatively well-conserved nucleotide positions.

in length. Interestingly, the sequence flanking helix III is also strongly conserved in peritrichs, but unpaired (Fig. 2; Supplement I).

The estimated thermodynamic energy of putative secondary structures ranged from −34.17 kcal/mol to −47.56 kcal/mol (Table 3). Helix I showed comparatively variability in its primary sequence compared to the other two helices. Helix II was more conserved and helix III was highly conserved. Statistic of the deviations from the secondary structure given in Table 3: helix I is 8 bp long in *Vaginicola crystallina* ranging to 32 bp in *Carchesium polypinum* population 3 and helix II varies from 8 bp (*Epicarchesium abrae*) to 26 bp (*Vorticella* sp1, 12, 13); helix III is generally 20–30 bp long. Frequencies of bases at each position and mutual information in base-pair regions in helices II and III are shown in RNA structure logos (Supplement IV A and C).

Several compensatory base changes (CBCs) or hemi-CBCs were present within the helices of ITS1 among the species studied: four pairings in the Helix III (Supplement III G–L, arrows) and one in the relatively conserved region of helix II (Supplement III A–F, arrows). In fact, most of these changes concerned only one of the two paired positions. Although less stable than the Watson–Crick complementarities, GU appositions retain the RNA helical structure but their presence was usually low (5 at most) (Table 3).

3.3. Putative secondary structures of ITS2 transcripts in peritrich ciliates

Putative secondary structures of ITS2 transcripts are proposed in Supplement II, from which a general secondary structure consisting of four helices was constructed (Fig. 2). It is fundamentally similar to that of other eukaryotes in having the following features: (1) four helices, (2) a highly conserved helix II with a motif 5′-GYGAYUGA versus UCUYUCRY-3′ (highlighted in gray in Supplement III 5M–R) in its basal portion and bearing a pyrimidine–pyrimidine mismatch (5′-YU-UY-3′), and (3) helix III being the longest one and containing a motif 5′-MAC versus GUK-3′ at the apex (highlighted in gray in Supplement III S–X). In addition, helix I contained a motif 5′-GGUU versus AWCC-3′ in most species. In helix III, a highly conserved region was found in all species studied presenting as 5′-MYYAA versus UURRK-3′ (except three species), positioned in the middle of two conserved regions (indicated by boxes in Supplement III S–X) in most species. Helix IV occurred in only one species (*Vorticella* sp15) in which it was 10 bp long. The other helices in ITS2 differed in size from one another (Table 3):

Table 3
Numerical and statistical values of the secondary structures (ITS1 and ITS2) proposed in this study.

Species	ITS1					ITS2				
	Length (in nt) of each helix			G-U pairing	Δ*G* (25 °C, kcal/mol)	Length (in nt) of each helix			G-U pairing	Δ*G* (37 °C, kcal/mol)
	I	II	III			I	II	III		
Vorticella sp1	30	26	20	2	−40.95	16	14	64	3	−29.88
Vorticella sp7	12	14	24	3	−36.85	12	12	60	4	−40.13
Vorticella sp8	14	16	24	3	−45.25	14	14	60	4	−27.22
Vorticella sp11	14	18	22	1	−40.89	14	14	62	3	−33.12
Vorticella sp12	30	26	20	3	−39.29	14	14	64	4	−32.40
Vorticella sp13	30	26	20	3	−43.17	14	14	58	4	−27.98
Vorticella sp15	12	14	24	3	−37.07	12	12	62	5	−51.63
Vorticella sp18	14	18	24	1	−41.02	14	14	68	4	−36.00
Pseudovorticella paracratera	12	18	24	2	−38.60	22	14	54	3	−36.10
Pseudovorticella punctata	14	18	24	3	−47.56	22	14	58	5	−39.68
Epicarchesium abrae	20	8	24	5	−40.03	10	14	54	5	−32.93
Carchesium polypinum popI	30	14	20	2	−34.17	20	14	60	4	−34.31
Carchesium polypinum popII	30	16	20	2	−35.06	18	14	58	3	−34.68
Carchesium polypinum popIII	32	12	24	3	−39.59	18	14	60	3	−33.58
Epistylis hentscheli	16	10	24	2	−38.16	14	14	66	3	−34.96
Epistylis chrysemydis	10	18	24	3	−37.79	16	14	64	5	−33.23
Epistylis urceolata	18	10	24	3	−37.13	–	–	–	–	–
Epistylis plicatilis	20	10	24	2	−35.84	–	–	–	–	–
Epistylis galea	20	12	28	1	−37.21	–	–	–	–	–
Zoothamnium alternans	24	16	30	1	−40.76	26	16	62	4	−30.78
Zoothamnium nii	16	14	22	4	−38.13	28	14	54	3	−32.08
Zoothamnium plumula	20	10	24	3	−36.60	16	14	58	6	−40.46
Zoothamnopsis sinica	20	10	24	3	−39.14	28	14	54	2	−31.18
Ophrydium versatile	30	16	22	4	−35.41	–	–	–	–	–
Vaginicola crystallina	8	24	24	2	−43.70	–	–	–	–	–
Campanella umbellaria	20	12	28	1	−37.21	16	14	72	1	−29.67
Opercularia microdiscum	22	16	30	2	−44.66	–	–	–	–	–
Rhabdostyla sp5	20	16	30	1	−41.78	14	14	58	4	−31.26

helix I varied in length between 12 and 28 bp; helix II was from 12 to 16 bp; helix III was longest and generally 54–72 bp long.

The estimated thermodynamic energy of putative ITS2 secondary structures (Table 3) varied from −27.22 kcal/mol to −51.63 kcal/mol. Numerous CBCs and hemi-CBCs were found (indicated with arrows in Supplement III M–X), which supported our predictions of secondary structure. For example, the conserved motif 5′-GUGAUU-GAA versus UUCUCUCAC-3′ in helix II had changed to 5′-GCGAUU-GAA versus UUCUUUCGU-3′ in *Campanella umbellaria*. Similarly, the highly conserved motif 5′-CCUAA versus UUAGG-3′ in helix III had changed to 5′-AUUAA versus UUAGU-3′ in *Zoothamnopsis sinica*. As in ITS1, the incidence of G-U pairings in the proposed helices was low (2–6 bp), indicating high stability of the helices. Frequencies of bases at each position and mutual information on base-pair regions in Helices II and III are shown in RNA structure logos (Supplement IV B and D).

3.4. Alignment of sequences and ITS derived phylogeny

Primary sequences of ITS regions contained many variable regions that were difficult to align unambiguously. With the aid of structural information, the alignments were considerably improved based on adjustment of the secondary structures. Conserved stems identified in the secondary structural domains provided a consistent basis for correcting the alignments of variable loop regions for phylogenetic analyses (Fig. 3).

Phylogenetic relationships inferred from combined datasets of primary ITS sequences and secondary structures are shown in Fig. 4A and B. The phylogenetic results, though differing somewhat between ITS1- and ITS2-based results, provide consistent support for most clades. The following results were mainly based on ITS1 trees because the ITS2 sequences of six species included in ITS1 trees were not available. The constituent taxa of peritrichs grouped into three clades in all trees (Fig. 4A and B). *Epistylis galea*, *Campanella umbellaria*, and *Opercularia microdiscum* form a solid, monophyletic clade (I), which is basal to all other taxa of peritrichs. Branching off next was a clade (II) containing the families Zoothamniidae and Epistylididae. A connection of these two families was only hinted at Bayesian analyses, with high posterior probability in BI (1.00) and either with low support or none in ML and NJ analyses. The third clade (III) consisted of taxa presently in the family Vorticellidae except for three species, *Ophrydium versatile*, *Rhabdostyla* sp5, and *Vaginicola crystallina*.

In ITS1 trees, clade III included two stable clades whose association, however, was well-supported only in BI (1.00) and ML (0.92) trees based on ITS1 data (Fig. 4A). One clade consisted of *Vorticella*, *Carchesium*, *Ophrydium* and *Rhabdostyla*, grouping as a well supported, relatively stable assemblage (1.00 BI, 87 ML, 78 NJ) but with relationships among the four genera unresolved. Most interesting is that *Ophrydium* is clearly associated with *Carchesium* forming a monophyletic group, but this relationship is not well supported (79% BI, absent ML, 55% NJ). Species of *Vorticella* do not form a monophyletic association within this subclade of clade III in all trees based on ITS1 and ITS2 data, in direct conflict with traditional morphological taxonomy; the other consisted of *Pseudovorticella*, *Epicarchesium* and *Vaginicola*. *Rhabdostyla* was always associated with typical vorticellids although its position was somewhat different between ITS 1 and ITS2 trees.

4. Discussion

4.1. Alternative models of secondary structures of ITS2 in peritrich ciliates

Computer programs such as mFOLD find highly divergent secondary structures, even from the same sequence, independently of the settings, such as temperature. Initially, we submitted all ITS2 sequences to the most current version of mFOLD (Zuker, 2003) for generation of thermodynamically optimal and suboptimal secondary structures with default settings. We were able to set up a consensus structure which resembled the "hairpin" model reported by Yeh and Lee (1990) in yeast more than the "ring" model for general eukaryotic ITS2 (Coleman, 2007). We doubted these results and submitted ITS2 sequences to the older version of mFOLD (Zuker et al., 1999) with default settings (except for setting temperature at 25 °C). This produced a second consensus secondary structure, with almost exactly the same structural domains as the first one, which conformed to the "ring" model for eukaryotic ITS2.

It was difficult to judge which structural alternative was the suitable one: therefore, we included 10 bases from each of the flanking sequences (5.8S at the 3′ end and 28S at the 5′ end)

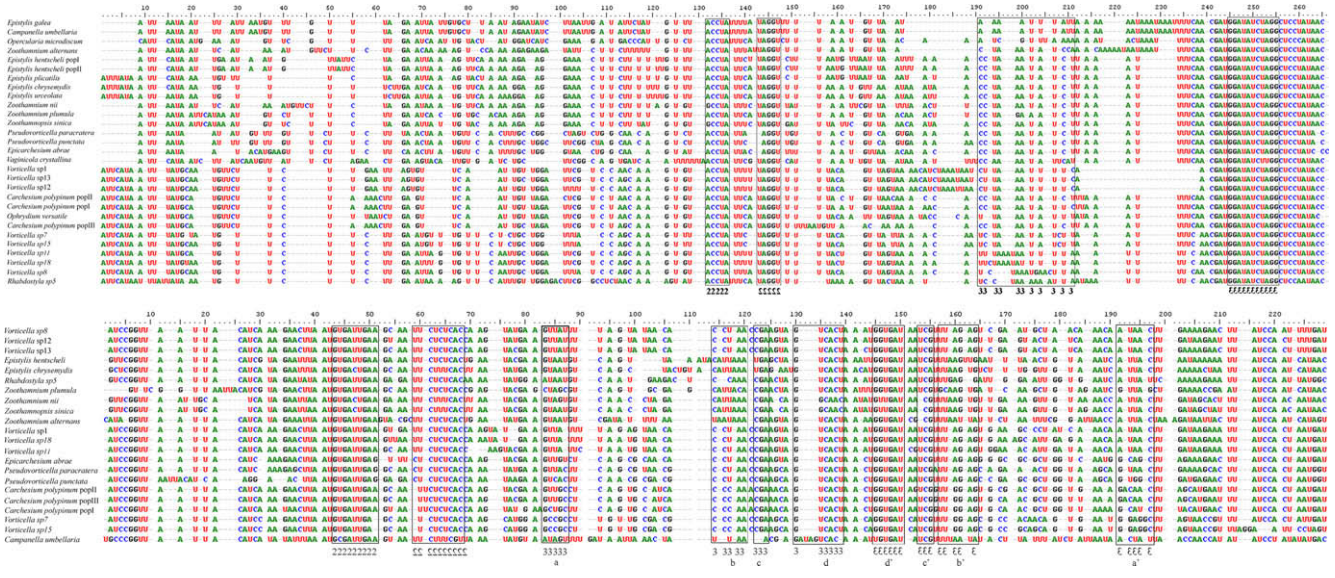

Fig. 3. Alignments of ITS1 (upper) and ITS2 (lower) sequences of peritrich ciliates, using 4SALE program. Names of species are listed on the left and dashes indicate indel events. The secondary structural motifs in helices II and III of ITS1 and ITS2 are boxed, with labels given beneath the alignments.

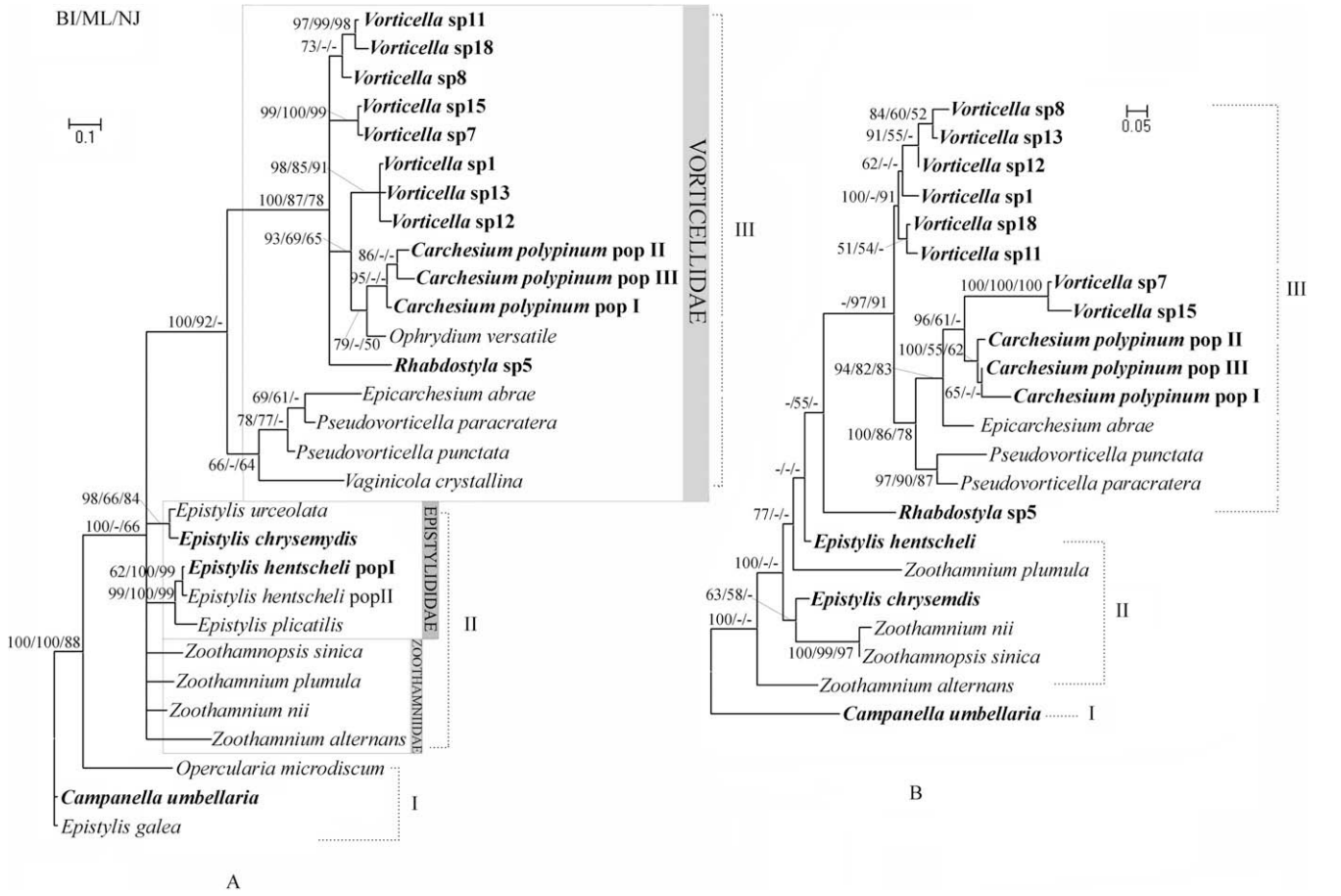

Fig. 4. Phylogenetic relationships derived from ITS1 and ITS2 regions in peritrichs. Each tree is a consensus tree: numbers given at nodes of branches are the posterior probability (BI) and bootstrap (ML and NJ) values, respectively. (A) Tree inferred from combined ITS1 primary sequences and secondary structures. (B) Tree inferred from combined ITS2 primary sequences and secondary structures. Scale bars correspond to 5/10 substitutions per 100 nucleotide positions. Systematic classification follows Lynn and Small (2002).

because these regions have been shown to have a significant influence on the folding of ITS2 sequences and thus may help to constrain the pattern of ITS2 secondary structures. With the flanking sequences added, the secondary structure with hairpin pattern was recovered for all species studied when folding was done at 37 °C, while the secondary structures with ring pattern changed to the hairpin pattern in half of the species when folded at 25 °C. This suggested that the secondary structure with hairpin pattern was more stable in peritrich ciliates and should be used in analyses.

The model of ITS2 secondary structure suggested here is supported by many CBCs or hemi-CBCs (see Supplement III M–X) as defined by Gutell et al. (1994). Many of the changes in the primary sequences are silent in terms of RNA structure, consistent with the analysis of all eukaryotic groups that revealed that ITS2 is an expandable RNA spacer but retains hallmarks among eukaryotes (Coleman, 2007). These hallmarks, such as the highly conserved apex of Helix III and the pyrimidine–pyrimidine bulge region of helix II, also were found in the ITS2 secondary structures of peritrich ciliates (Fig. 2; Supplement III, III M–X). The maintenance of these hallmarks validates the hypothesis of important functional role of these conserved regions. Furthermore, the identification of homologous alignment is somewhat ambiguous when based only on the primary sequences, which supports the existence of the helices.

Saccharomyces cerevisiae has been described as possessing both the "hairpin" model and "ring" model as alternative secondary structure models for its ITS2 secondary structure (Yeh and Lee, 1990; Joseph et al., 1999). The elements of both structural models are important in efficient processing, as indicated by the genetic study of Côté et al. (2002). They proposed a dynamic conformational model for the role of ITS2 in processing: initial formation of the ring structure may be required for essential, early events in processing complex assembly and followed by an induced transition to the hairpin structure that facilitates subsequent processing events. Sequences of scuticociliates examined by Miao et al. (2008) and their secondary structures are analogous to the "ring" model. Our work and the study on scuticociliates seem to suggest that ITS2 sequences have the ability to adopt either the ring or hairpin structures in ciliates, which also was observed in fungi (Joseph et al., 1999; Côté et al., 2002). In addition, the structural motifs of Helices II (5′-GYGAYUGA versus UCUYUCRY-3′) and III (5′-MAC versus GUK-3′) of ITS2 found in peritrich ciliates are extremely similar to those found by Miao et al. (2008) in scuticociliates (5′-GYGRUUGA versus UCYCYCRY-3′ in Helix II and 5′-GAAGUAGUCAC versus GUGAUCUC-3′ in Helix III), suggesting that these conserved regions are likely to play an important role in folding the secondary structure of ciliated ITS2.

4.2. Primary sequence of ITS in peritrich ciliates and structural evolution of ITS1

The evolution of eukaryotic ITS regions show two trends as indicated previously: increase in length and higher G+C contents (Goldman et al., 1983). However, the ITS regions of peritrich

ciliates are short and have a relatively low G+C contents as dinoflagellates and yeasts (Mai and Coleman, 1997; Joseph et al., 1999; Gottschling and Plötner, 2004).

ITS1 is generally less conserved than ITS2, making prediction of its secondary structure less consistent than that of ITS2, thus the significant subregions for ITS1 still are not fully identifiable. Studies of secondary structure of ITS1 are relatively few, but in most eukaryotes that have been investigated so far, it consists of an open loop with several helices (Liu and Schardl, 1994; Coleman et al., 1998; Goertzen et al., 2003). Within this general pattern, however, different numbers of ITS1 structural domains have been found in different eukaryotic taxa. For example, there are three in dinoflagellates (Gottschling and Plötner, 2004); four in yeast (*Saccharomyces cerevisiae*), scallops (Pectinidae) and volvocid flagellates (Van Nues et al., 1994; Coleman et al., 1998; Wang et al., 2007); three (Asteraceae), four (Boraginales), or five (Sapindaceae) in flowering plant (Gottschling et al., 2001; Goertzen et al., 2003; Harrington et al., 2009); six in desmid algae (*Staurastrum*) (Gontcharov and Melkonian, 2005); seven in Platyhelminthes (Digenea) (Vonder Schulenburg et al., 1999); and nine in Pine family (Pinaceae) (Kan et al., 2007). The insertions and deletions accounted for a large proportion of variability in the ITS1 region of peritrich ciliates, which did not impede the formation of conserved structural elements. Three structural domains were identified in the secondary structure of ITS1 in the present study. High conservation of the nucleotide sequence was found in the basal base pairings in helices II and III, while helix I was variable in both length and primary sequence.

As reported in other eukaryotes (Jacq, 1981; Walker et al., 1983), peritrich ciliates showed a higher degree of conservation in the 3′ end of ITS1 than those of its 5′ end with respect to both the primary nucleotide sequence and secondary structure. The GC content of paired nucleotide regions in the three helices was relatively low in peritrichs, a characteristic also observed in scallops by Wang et al. (2007). In addition, the unpaired sequence flanking helix III of ITS1 region was strongly conserved in peritrichs which also was found in the Asteraceae (Goertzen et al., 2003).

Several conserved motifs have been found in the ITS1 of a relatively wide variety of eukaryotic groups by previous investigators: a repeated motif 5′-CCAA-3′ in volvocids (Coleman et al., 1998), a nonpairing but highly conserved motif 5′-AAGGAA-3′ in flowering plants (Asteraceae, Boraginales, Sapindaceae) (Liu and Schardl, 1994; Gottschling et al., 2001; Goertzen et al., 2003; Harrington et al., 2009), the "Block D" motif in Coccinellid beetles (Vonder Schulenburg et al., 2001), conserved paired motifs 5′-UGAG versus CUCR-3′ and 5′-GGCGGC versus GUCGYC-3′ in dinoflagellates (Gottschling and Plötner, 2004), and an invariant GGAA teraloop in *Staurastrum* (Gontcharov and Melkonian, 2005). In the present study, two motifs could be identified in the ITS1 region of peritrichs: 5′-RCCUA versus YAGGU-3′ in helix II and 5′-YYWARAWWUYY versus GGAUAUCUWGG-3′ in helix III, which may have important functions in the maturation of rRNA in peritrichs since they are highly conserved among species studied. However, common motif of eukaryotes can not be identified, which suggested motifs listed above may not be universal but are limited to certain groups. And homology of certain helices in different groups of organisms should be more rigorously established in further studies.

4.3. Comments on two genera of peritrichs with ambiguous systematic positions: examples of the utility of ITS data in peritrich phylogeny

Compared with SSrRNA, ITS regions are variable which are more likely to accumulate mutations that might potentially record the divergence of relatively recently evolved groups among peritrichs. With the aid of conserved structural components, homology in diverse ITS sequences of peritrichs can be identified; thus, an original alignment based on primary sequences can be modified and used for reconstructing phylogeny. This is supported by the fact that bootstrap support values for most clades increased in comparison with the trees based only on primary sequences (Supplement V). In our study, three major clades were recovered in both ITS1 and ITS2 trees based on a combination of primary sequences and secondary structures (Fig. 4A and B). This corresponds well to phylogenies inferred from SSrRNA gene sequences in previous studies (Li et al., 2008; Miao et al., 2004), suggesting that deep phylogenetic signal has been retained in the ITS sequences of species studied.

Rhabdostyla is solitary and has a short, rigid stalk, which places it within the family Epistylididae in the current classification, along with colonial genus *Epistylis* that also has a rigid stalk (Fig. 1G, arrow) and a similar cellular morphology. The present study provides the first molecular data of *Rhabdostyla*, allowing this association to be tested. In clade III of our trees, *Rhabdostyla* occupied somewhat different positions in ITS1 and ITS2 trees but always was associated with vorticellids, which are traditionally defined by having a helically contractile stalk (Fig. 1A–C, E, arrows) and clearly separated from *Epistylis*. This opens up the question of whether the similar stalk morphologies of *Rhabdostyla* and *Epistylis* represent an instance of evolutionary convergence while also posing the challenge of determining whether *Rhabdostyla* is basal or derived in its association with vorticellids. Posterior probability and bootstrap values for the placements of *Rhabdostyla* are high in ITS1 trees but low or absent in ITS2 trees (Fig. 4A and B), indicating that more species of *Rhabdostyla* and species of vorticellids that appear to be closely related must be included in future studies to resolved its phylogenetic position.

Ophryidium and its few morphological relatives are presently assigned to the small family Ophrydiidae, which is characterized by possession of a short, non-contractile stalk, gelatinous extracellular covering, and unique contractile vacuole that discharges through a long canal (Fig. 1F). Previous studies derived from SSrRNA gene sequences (Miao et al., 2004; Williams and Clamp, 2007; Li et al., 2008) have placed *Ophrydium* in association with the clade containing vorticellids. Williams and Clamp (2007) cited examples of vorticellids that also have vestigial stalks and furthermore hypothesized that *Ophrydium* is a derived vorticellid that has progressively lost its stalk myoneme and much of its stalk. There are also a few species of *Vorticella* that secrete mucilaginous extracellular coverings, thus leaving the unusual contractile vacuole of ophrydiids as their only unique feature. In our ITS1 tree (Fig. 4A), *Ophrydium* again clustered with vorticellids. This supports the hypothesis based on SSrRNA gene sequences (Williams and Clamp, 2007) that *Ophrydium* is a morphologically anomalous vorticellid, not sufficiently distinct to be accorded familial status, that should be placed within a more broadly defined family Vorticellidae.

To summarize, ITS sequences were highly variable in peritrich ciliates. However, our investigation shows the resolving power of ITS sequences to extend well beyond the genus level within the subclass Peritrichia once sequences alignments incorporates the now available structure models. Although still far from a fully documented phylogeny, our sampling of species spread over relatively few, but quite divergent genera allows remote events like the basal split (Fig. 4A and B, clade I) to be tracked and outlines other two major clades (Fig. 4A and B, clade II and III). This work forms the basis for a more complete analysis of peritrich phylogeny in the future because a stable, realistic phylogeny will be achieved only by including sequences of a variety of genes from a representative sample of related taxa.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.02.030.

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