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Further consideration on the phylogeny of the Ciliophora: Analyses using both mitochondrial and nuclear data with focus on the extremely confused class Phyllopharyngea

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ABSTRACT

Most ciliate phylogenetic analyses have largely relied on the nuclear small subunit ribosome DNA (nSSU-rDNA) locus. However, single locus or multi-loci from the same genome or chromosome may not be sufficient enough to elucidate phylogenetic relationships among ciliate taxa. Therefore, in addition to nSSU-rDNA, the mitochondrial small subunit ribosome DNA (mtSSU-rDNA) was applied in this study. We expanded the taxon sampling especially within the class Phyllopharyngea. Phylogenetic analyses based on nSSU-rDNA and mtSSU-rDNA, independently, as well as concatenated were performed and revealed the following: (1) mtSSU-rDNA is more variable than nSSU-rDNA, and is better at elucidating relationships at lower levels, e.g. intra-/inter-specific or generic relationships; (2) the validity of the two genera *Mirodysteria* and *Spirodysteria* is challenged based on their similar morphology with *Dysteria* and the analyses from both mtSSU-rDNA and nSSU-rDNA; (3) *Brooklynella* is confirmed to be an intermediate taxon between Dysteriidae and Hartmannulidae, and may represent a distinct family; (4) *Trithigmostoma* should remain in Chilodonellidae; (5) the separation of *Paraspathidium* from Litostomatea is supported and it groups with prostomateans and plagiopyleans. In summary, results from mtSSU-rDNA corroborated those of nSSU-rDNA for highly supported clades, and the mtSSU-rDNA tree with its secondary structure gave topologies that could be explained by the morphology; therefore it can be useful in some cases towards better resolution of robust phylogenies.

1. Introduction

The ciliated protists are a large group of single-celled eukaryotes with high morphological diversity (Lynn, 2008). They are good model systems in a wide range of biological studies, including evolution, systematics, cell development, ecology, and genetics, and have been the source of many important discoveries (Lynn, 2008). Compared to their high diversity and the long history of morphological studies, molecular phylogenetic studies of ciliates have been limited since the sequencing of the nuclear small subunit ribosome DNA (nSSU-rDNA) in ciliates (Elwood et al., 1985). Phylogenetic analyses have been increasingly applied in recent study of ciliates and have helped to resolve a number of systematics problems (Chen et al., 2016; Feng et al., 2015; Gao et al., 2016b; Gentekaki et al., 2014, 2017; Lynn, 2008; Prescott, 1994; Sun et al., 2016; Zhao et al., 2013, 2016). However, evolutionary relationships of many groups remain unclear and phylogenetic results sometimes are inconsistent with morphologically based classifications. Thus, the debates surrounding whether certain morphological characters are ancestral or the weight of a given character for taxonomic assignment remain contested (Sun et al., 2016). However, molecular information of many taxa is unavailable and phylogenetic analyses to date have mainly focused on the single locus, nSSU-rDNA.

Phylogenetic studies based on additional molecular markers have been performed increasingly and demonstrated the robustness of multi-gene analyses (Feng et al., 2015; Gao et al., 2016a, b; Gentekaki et al., 2014, 2017; Huang et al., 2016). These additional molecular genes include ITS1-5.8S-ITS2 and nLSU-rDNA, which are not independent because they are in the same chromosome. Using protein-coding genes such as alpha-tubulin gene may be misleading due to the heterogeneous rates of protein evolution

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and divergent paralogs (Katz et al., 2004; Zufall et al., 2006). In contrast, previous studies have shown that mitochondrial genes can be helpful in illuminating phylogenetic relationships (Boore and Brown, 1998; Moore, 1995). However, mitochondrial genes have been used in limited groups of ciliates (Dunthorn et al., 2011, 2014; Przybos et al., 2013; Strüder-Kypke and Lynn, 2010; Zhao et al., 2013). For example, the mitochondrial SSU-rDNA (mtSSU-rDNA) has shown efficacy in ciliate molecular phylogenetic inference, especially for relationships among shallower nodes (Dunthorn et al., 2011, 2014; Katz et al., 2011). However, the mitochondrial genes have not been used on a broad scale partly because it is difficult to amplify the genes due to their high variability (Zhao et al., 2013). Because of this, the utility of this locus for more divergent relationships is unknown.

As a group within ciliates, the class Phyllopharyngea was firstly established by Small and Lynn (1981) based on the structure of the somatic kinetid (i.e. a distinctively shaped, laterally directed kinetodesmal fibril and subkinetal microtubules underlying the somatic monokinetids) and the presence of phyllae or leaf-like ribbons of microtubules surrounding the pharyngeal tube. It previously included four groups: cyrtophorians, rhynchodians, chonotrichians, and suctorians (Lynn, 2008; Puytorac, 1994). Gong et al. (2009) expanded the class Phyllopharyngea to include the synhymeniids as a subclass based on both the morphological and molecular data, which is now widely accepted. Despite Phyllopharyngea being a well-outlined group based on the morphological data and phylogenetic analyses using nSSU-rDNA, relationships within this group remain unclear even after expanding the taxon sampling (Gao et al., 2012; Qu et al., 2015a,b).

In this study, we characterized 48 new mtSSU-rDNA sequences and 10 new nSSU-rDNA sequences, which were obtained from 48 isolates of 46 species, mainly focusing on the class Phyllopharyngea. By combining nSSU-rDNA and mtSSU-rDNA data into the ciliate phylogenetic analyses, we aim to: (1) evaluate the efficacy of mtSSU-rDNA in inferring the phylogenetic relationships among phyllopharyngeans, especially the relationships that remain unresolved by nSSU-rDNA analyses; (2) find out more clues about evolutionary relationships within Phyllopharyngea; (3) assess the values of mtSSU-rRNA secondary structure in phylogenetic analyses.

2. Materials and methods

2.1. Taxon sampling and terminology

This study yields 48 new mtSSU-rDNA sequences and 10 new nSSU-rDNA sequences, which were obtained from 48 isolates of 46 morphological species (Table 1). Species identifications were made using live microscopic observation (Foissner and Stoeck, 2011) and protargol staining (Wilbert, 1975). Among them, morphological images of the key phyllopharyngean species were provided in Fig. S1. The genomic DNA investigated in previous studies was used in some taxa (Chen et al., 2016; Gao et al., 2012; Zhang et al., 2014). Both mtSSU-rDNA and nSSU-rDNA were obtained from the same DNA source when possible. Other sequences were obtained from GenBank (Table 1). Terminology follows Foissner et al. (1994) and Lynn (2008) with adjustments according to recent studies (Chen et al., 2016; Gao et al., 2016b, 2012; Zhang et al., 2014).

2.2. DNA extraction, PCR amplification and gene sequencing

Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen, CA). The nSSU-rDNA amplification referred to Yi and Song (2011), with the primers 18sF or 82F and 18sR (Lopez-Garcia et al., 2003; Medlin et al., 1988). The mtSSU-rDNA sequence fragment was amplified with the primers mtF and mtR (Table 2) (Dunthorn et al., 2014; van Hoek et al., 2000). Nested PCR was performed on the samples that failed to give results for the first PCR amplification using the primers mtF and mt400F with mt900R (Table 2). PCR was performed with ex*Taq* polymerase (TaKaRa Biomedicals, Japan) in the following protocol: 95 °C for 5 min, followed by 11 cycles of 94 °C for 15 s, 66 °C for 30 s with touchdown by 0.5 °C for each cycle, 72 °C for 75 s; 26 cycles of 94 °C for 15 s, 60 °C for 30 s, 72 °C for 75 s; and a final extension at 72 °C for 10 min. Purified PCR products were directly sequenced on an ABI 3700 sequencer (Sangon sequencing facility, Shanghai, China) bidirectionally using the primers mtF, mt900R and mt400F.

2.3. Phylogenetic analyses

The nSSU-rDNA sequences were aligned by GUIDANCE with default parameters in the GUIDANCE web server (Penn et al., 2010a,b) and then subsequent manual alignments were performed when necessary using BioEdit v7.2.5 (Hall, 1999). MtSSU-rDNA sequences were aligned based on the data from Dunthorn et al. (2014). The alignment was performed with SeaView v4 (Gouy et al., 2010) and then manually adjusted. The full length nSSU-rDNA and mtSSU-rDNA alignments were used for calculating sequence identity by BioEdit v7.2.5 (Hall, 1999) and compared for the taxa that are available for both gene sequences. The nSSU-rDNA and mtSSU-rDNA alignments were then concatenated using BioEdit v7.2.5 (Hall, 1999). The final alignments of the three datasets (i.e. 1526 sites of nSSU-rDNA (91 taxa in total), 1337 sites of mtSSU-rDNA (96 taxa in total), and 2863 sites of concatenated genes (91 taxa in total)) were used for phylogenetic analyses.

For each database, a GTR + I + G model was selected under AIC by the program MrModeltest v3.7 (Posada and Crandall, 1998) and was implemented for the Bayesian phylogenetic interference (BI) in MrBayes on XSEDE v3.2.3 (Ronquist and Huelsenbeck, 2003) in CIPRES Science Gateway (Miller et al., 2010; Posada and Crandall, 1998; Ronquist and Huelsenbeck, 2003). Four MCMC chains were run for 4,000,000 generations, sampling every 100 generations, and the first 25% trees were discarded as burn-in. The maximum likelihood (ML) tree was constructed with RAxML-HPC2 on XSEDE v8.1.11 (Stamatakis, 2006; Stamatakis et al., 2008) using a GTR + G model in CIPRES Science Gateway. The best scoring ML tree was assessed with 1000 bootstrap for support values.

The Approximately Unbiased (AU) test was performed based on mtSSU-rDNA data. Constrained ML trees enforcing the monophyly of the respective focal groups (Table 1) were generated with interrelationships among the constrained taxa with the remaining taxa not specified. The site-wise likelihoods for the resulting constrained topologies and the non-constrained ML topology were calculated using PAUP* 4.0b10 (Shimodaira, 2002; Swofford, 2002), which were analyzed by CONSEL v0.1 (Shimodaira and Hasegawa, 2001).

2.4. Secondary structure prediction

The mtSSU-rRNA sequence of *Chilodonella uncinata* was selected as an example to predict the secondary structure following the previous models of *Tetrahymena pyriformis* (M12714) and *Paramecium tetraurelia* (K01751) (http://www.rna.ccbb.utexas.edu). Default parameters were used for prediction on Mfold website (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). Rnaviz was used for aesthetic purposes (Rijk and Wachter, 1997). Based on the sequence alignment of mtSSU-rDNA and mtSSU-rRNA secondary structure of *Chilodonella uncinata*, the variability of certain segments was compared and variable regions were detected and

Table 1
GenBank accession numbers of the mtSSU-rDNA and nSSU-rDNA sequences used for phylogenetic analyses in this study. Newly characterized sequences are in bold.

Sampled species name	mtSSUrDNA Genbank No.	nSSUrDNA Genbank No.	Sampled species name	mtSSUrDNA Genbank No.	nSSUrDNA Genbank No.
Acineta sp.	KF639897	AY332718	*Heliophrya erhardi*	KF639904	AY007445
Aristerostoma sp.	HM246398	EU264563	*Heterohartmannula fangi*	**KX302654**	HQ605946
Bardeliella pulchra	HM246399	EU039884	*Ichthyophthirius multifiliis*	JN227086	U17354
Bresslauides discoideus	HM246400	EU039885	*Ilsiella palustris*	JQ026522	JQ026521
Brooklynella sinensis	**KX302686**	KC753483	*Maryna* sp.	JQ026524	JF747218
Bryometopus atypicus	HM246401	EU039886	*Maryna umbrellata*	JQ026523	JF747217
Bursaria spec. ("muco")	HM246402	EU039889	*Metafolliculina* sp.	KF639905	KF639911
Bursaria truncatella	HM246403	U82204	*Mirodysteria decora*	**KX302667**	JN867020
Chilodonella acuta	**KX302683**	KJ452458	*Odontochlamys alpestris biciliata*	**KX302678**	KC753484
Chilodonella parauncinata	**KX302672**	KJ509197	*Orthodonella* sp.1	**KX302650**	FJ998038
Chilodonella uncinata Poland	HM246404	JN111976	*Orthodonella* sp.2 pop1	**KX302653**	**KX302705**
Chilodonella uncinata USA ATCC	JN111981	AF300281	*Orthodonella* sp.2 pop2	KX354449	
Chilodonella uncinata USA SC1	JN111980	JN111979	*Ottowphrya dragescoi*	HM246414	EU039904
Chilodonella uncinata USA SC2	JN111980	JN111979	*Paracrytophoron tropicum*	**KX302694**	FJ998035
Chilodonella uncinata USA WH	JN111982	JN111978	*Parafurgasonia* sp.	**KX302682**	KC832955
Chlamydodon excocellatus	KF639898	AY331790	*Paramecium caudatum*	**KX302680**	**KX302699**
Chlamydodon paramnemosyne	**KX302679**	JQ904059	*Paramecium primaurelia*	K01750	AF100315
Chlamydodon salinus	**KX302692**	JQ904057	*Paramecium tetraurelia*	X15917	X03772
Chlamydodon triquetrus pop1	KF639899	AY331794	*Paraspathidium apofuscum*	**KX302657**	FJ875140
Chlamydodon triquetrus pop2	**KX302666**	**KX302700**	*Plagiopogon loricatus*	**KX302671**	KC771342
Chlamydonellopsis calkinsi pop1	**KX302656**	KC753487	*Plasmodium falciparum*	X95275	AL844501
Chlamydonellopsis calkinsi pop2	**KX302660**		*Platyophrya bromelicola*	HM246415	EU039906
Coleps sp.	KF639900	KF639909	*Platyophrya-like* sp.	HM246416	EU039905
Colpidium sp.	KF639901	KF639910	*Prorodon ovum*	**KX302665**	KM222104
Colpoda aspera	HM246405	EU039892	*Pseudochilodonopsis alveolata*	**KX302668**	KC753495
Colpoda cucullus	HM246406	EU039893	*Pseudochilodonopsis fluviatilis*	**KX302663**	JN867021
Colpoda henneguyi	HM246407	EU039894	*Pseudochilodonopsis* sp.1	**KX302674**	KC753498
Colpoda lucida	HM246409	EU039895	*Pseudochilodonopsis* sp.2	**KX302675**	KC753496
Cyrtolophosis mucicola Austria	HM246411	EU039899	*Rostrophrya* sp.	HM246417	EU039907
Cyrtolophosis mucicola Brazil	HM246412	EU039898	*Sagittaria* sp.	HM246418	EU039908
Didinium nasutum	KF639902	U57771	*Sorogena stoianovitchae*	HM246419	AF300285
Dystera derouxi pop1	**KX302685**	**KX302697**	*Spirodysteria kahli*	**KX302691**	KC753499
Dystera derouxi pop2	**KX302696**		*Spirostomum* sp.	KF639906	KF639912
Dysteria brasiliensis	**KX302658**	FJ870067	*Stentor* sp.	KF639907	KF639913
Dysteria compressa	**KX302687**	KC753491	*Tetrahymena pyriformis*	AF160864	M98021
Dysteria cristata	**KX302690**	KC753488	*Tetrahymena thermophila*	AF396436	X56165
Dysteria lanceolata	**KX302664**	KC753490	*Tillina magna*	HM246410	EU039896
Dysteria pectinata	**KX302661**	FJ870068	*Trichopodiella faurei* pop1	**KX302684**	EU515792
Dysteria sp.	KF639903	AY331797	*Trichopodiella faurei* pop2	**KX302662**	FJ870071
Ephelota gemmipara	**KX302649**	EU600180	*Trithigmostoma cucullulus*	**KX302673**	FJ998037
Ephelota sp.1	**KX302693**		*Trochilia petrani* pop1	**KX302659**	
Ephelota sp.2	**KX302652**	GQ265956	*Trochilia petrani* pop2	**KX302670**	JN867016
Ephelota sp.3	**KX302651**	KX302701	*Trochilioides recta*	**KX302669**	JN867017
Euplotes crassus	GQ903131	AJ310492	*Vortichella astyliformis*	KF639908	GQ872427
Euplotes minuta	GQ903130	EF094959	*Zosterodasys* sp.1	**KX302655**	**KX302702**
Euplotes vannus	**KX302695**	**KX302698**	*Zosterodasys* sp.2	**KX302689**	**KX302703**
Frontonia magna	**KX302681**	FJ876953	*Zosterodasys* sp.3	**KX302677**	**KX302704**
Hausmanniella discoidea	HM246413	EU039900	*Zosterodasys* sp.4	**KX302688**	**KX302706**

marked according to Schnare et al. (1986). The V4 regions of cyrtophorian taxa were predicted for further comparison.

3. Results

3.1. mtSSU-rDNA sequences and secondary structures

The mtSSU-rDNA of ciliates is discontinuous, comprising two distinct components, *rns*a and *rns*b (Schnare et al., 1986). In the present study, 48 mtSSU-rDNA sequences characterized from 48 isolates of 46 species belong to *rns*b and have been deposited in the GenBank database with the accession numbers KX302649-KX302696, and KX354449 (Table 1). For the newly characterized sequences, GC-content ranged from 22.77% to 43.04%.

The sequence identity of the nSSU-rDNA alignment is overall higher than that of the mtSSU-rDNA alignment (Table S1). The nSSU-rDNA sequence identities among species of the family Dysteriidae are from 0.672 to 0.976 while in the mtSSU-rDNA alignment they are from 0.438 to 0.979. The sequence identities between *Spirodysteria kahli* and other family members range from 0.792 to 0.976 (nSSU-rDNA) vs. 0.413 to 0.610 (mtSSU-rDNA). Between *Mirodysteria decora* and other genera, they are from 0.719 to 0.896 (nSSU-rDNA) vs. 0.438 to 0.510 (mtSSU-rDNA). For species within the family Hartmannulidae, sequences identities vary from 0.648 to 0.992 (nSSU-rDNA) vs. 0.255 to 0.482 (mtSSU-rDNA) while the sequence identities between *Brooklynella sinensis* and other hartmannulids are from 0.648 to 0.816 (nSSU-rDNA) compared to 0.345 to 0.482 (mtSSU-rDNA). For the family Chlamydodontidae, the sequence identities vary from 0.831 to 0.950

Table 2
Primers for PCR amplification (*newly designed primers for nest PCR).

Amplified fragment	Primers	Reference
nSSU-rDNA	18sF: 5-AACCTGGTTGATCCTGCCAGT-3	Medlin et al. (1988)
	82F: 5-GAAACTGCGAATGGCTC-3	Lopez-Garcia et al. (2003)
	18sR: 5-TGATCCTTCTGCAGGTTCACCTAC-3	Medlin et al. (1988)
mtSSU-rDNA	mtF: 5-TGTGCCAGCAGCCGCGGTAA-3	Dunthorn et al. (2014); van Hoek et al. (2000)
	mtR: 5-CCCA(C)TACCA(G)GTACCTTGTGT-3	
	*mt900R: 5-GAGCGTGATGGGCGGTGTGTGCA-3	
	*mt400F: 5-AAACTTAAAA(G)AAATTGGCGGGA-3	

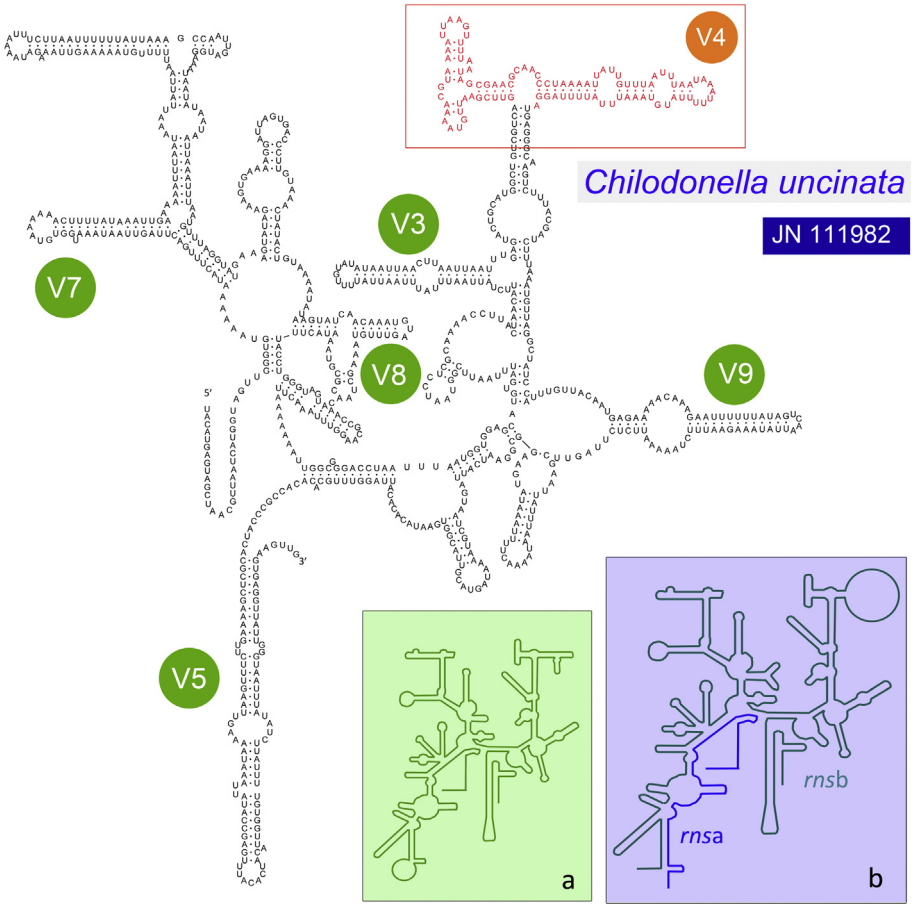

Fig. 1. mtSSU-rRNA secondary structure of *Chilodonella uncinata* (JN111982). The highly variable regions V4 is in red. (a) the mtSSU-rRNA secondary structure of *Paramecium tetraurelia*. (b) the mtSSU-rRNA secondary structure of *Tetrahymena pyriformis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(nSSU-rDNA) vs. 0.342 to 0.862 (mtSSU-rDNA). Among species in the family Chilodonellidae, identities range from 0.514 to 0.996 (nSSU-rDNA) vs. 0.474 to 0.998 (mtSSU-rDNA). Within this family, the sequence identities of *Odontochlamys* and other genera are from 0.539 to 0.890 (nSSU-rDNA) compared to 0.462 to 0.513 (mtSSU-rDNA); the sequence identities between *Trithigmostoma* and other genera range from 0.542 to 0.909 (nSSU-rDNA) compared to 0.474 to 0.603 (mtSSU-rDNA).

The mtSSU-rRNA secondary structures of cyrtophorian taxa are predicted and compared with the published secondary structure models (Konings and Gutell, 1995). Though mtSSU-rDNA has insertions of various lengths between *rns*a and *rns*b in different taxa, they do share almost the same secondary structure (e.g., *Chilodonella uncinata*, Fig. 1). The secondary structures of the *rns*b fragments obtained in this work are compared based on the mtSSU-rDNA alignment. The structures are conserved in most parts but some stems and loops are much more variable (Figs. 1 and 2). For example, the V4 region is AT rich and has the highest length variety (64–496 bp), with the longest found in the mtSSU-rRNA of *Trochilioides recta*. As shown in Fig. 2, the V4 structure typically has a Y-shaped helix on the left (hereafter referred to as helix 1) and a one- or two-stem helix on the right (helix 2). In the order Dysteriida, *Brooklynella* shares the similar structure of the long helix 2 with dysterids while the other hartmannulids have a short helix 2. By contrast, *Spirodysteria* has a much longer helix 2 and *Mirodysteria* has a bulge in helix 2. In Chlamydodontida, a bulge in helix 2 is also shared in the family Chlamydodontidae. For the family Chilodonellidae, the structures all have a conserved two-stem helix 2 structure except those of the genus *Chilodonella*, which have both types of helix 2 (*Chilodonella acuta* has a two-stem

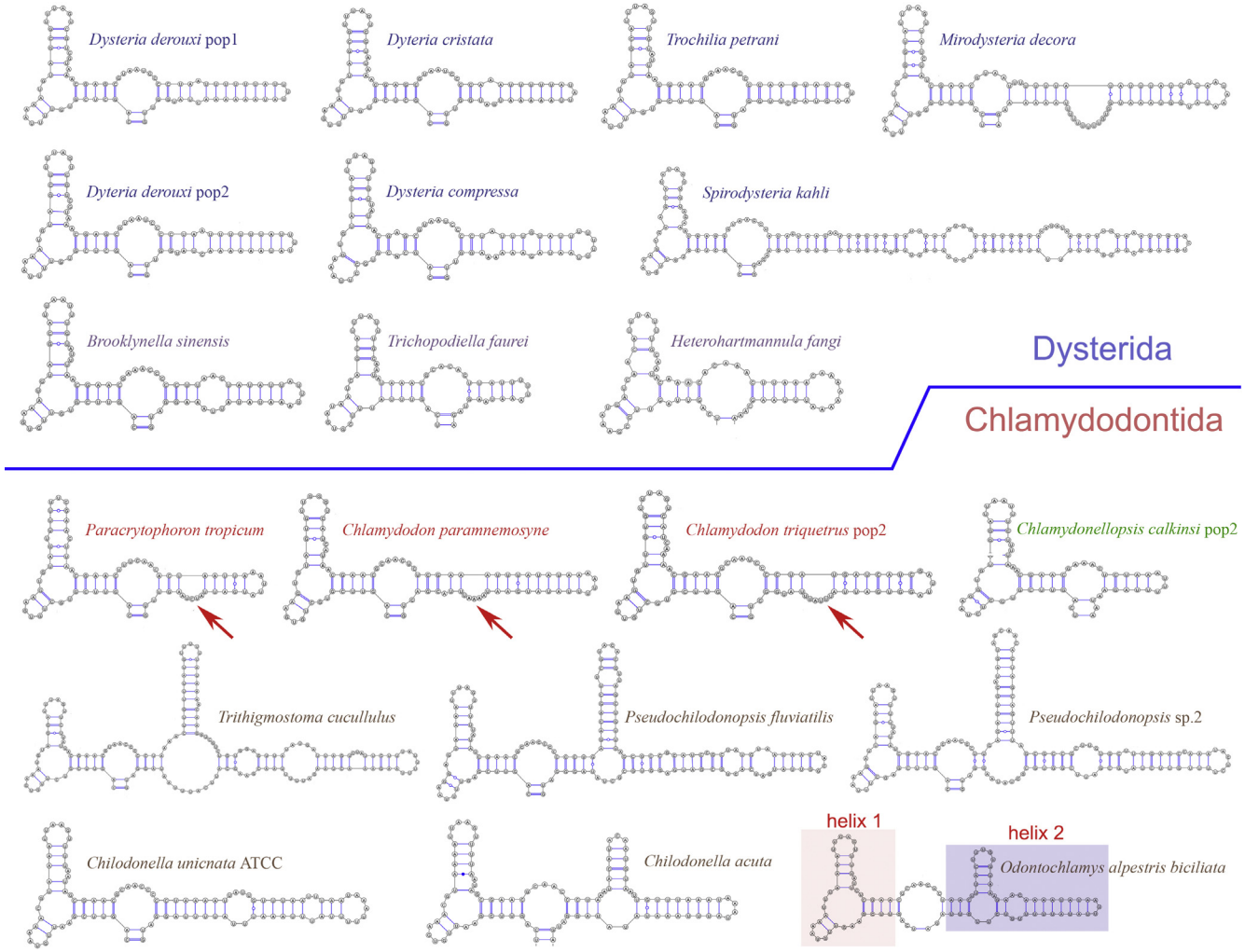

Fig. 2. Secondary structures of variable region 4 (V4) of mtSSU-rRNA focusing on the two orders Dysterida and Chlamydodontida. Color of the taxa's names corresponds to the phylogenetic trees. Arrows indicate the bulge in helix 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

helix 2). Two populations of *Chlamydonellopsis calkinsi* have similar V4 regions structures. Limited data in this study show that structures of conspecific populations are similar.

3.2. Phylogeny based on the concatenated data

For each of the database, BI and ML algorithms yield almost identical topologies, thus that the ML topology is presented with node supports from both algorithms (Figs. 3–5). Topologies of the three databases are similar, while most differences exist among deep nodes. The concatenated tree provides the highest support values (Fig. 3).

In the concatenated tree (Fig. 3), the class Phyllopharyngea is monophyletic (89% ML, 1.00 BI) and comprises the representatives from three subclasses, Cyrtophoria, Suctoria and Synhymenia. Each of the three subclasses independently forms a well-supported clade. Within Cyrtophoria, the order Dysteriida is monophyletic with full support while the order Chlamydodontida is found to be polyphyletic. Within Dysteriida, the genus *Dysteria* is paraphyletic with *Spirodysteria* nesting within it. *Mirodysteria* is positioned as sister to the clade comprising *Dysteria* and *Spirodysteria*. *Brooklynella sinensis* falls on a branch sister to Dysteriidae in the ML tree (65% ML) and is a sister to Hartmannulidae in the BI tree (0.91 BI). The other members of the family Hartmannulidae form one cluster with full support. Chlamydodontida exhibits full monophyly of each of its constituent families. Within the family Chlamydodontidae, *Paracyrtophoron tropicum* is sister to the genus *Chlamydodon* with full support. Within the family Chilodonellidae, *Odontochlamys* groups with *Chilodonella* (83% ML, 1.00 BI) and *Trithigmostoma* groups with *Pseudochilodonopsis* in ML analyses (41% ML), whereas it clusters with *Odontochlamys* and *Chilodonella* in BI analyses (0.52 BI).

For other taxa, each of the classes Oligohymenophorea, Colpodea, Heterotrichea and Spirotrichea form monophyletic groups. Prostomatea and Plagiopylea form one clade (55% ML, 1.00 BI), which sisters to Oligohymenophorea clade. The nassophorean species *Parafurgasonia* sp. is sister to Colpodea (33% ML, 0.99 BI). The litostomatean species *Didinium nasutum* is sister to all the other ciliates (62% ML, 0.99 BI).

3.3. Phylogeny based on nSSU-rDNA

The topology of the nSSU-rDNA tree (Fig. 4) is more similar to the concatenated tree than mtSSU-rDNA tree below the class level. The main differences of the nSSU-rDNA tree compared to the concatenated tree are: (1) the genus *Dysteria* is paraphyletic with both *Spirodysteria* and *Mirodysteria* nesting in; (2) *Trithigmostoma* forms falls sister to the rest of Chilodonellidae, instead of grouping with

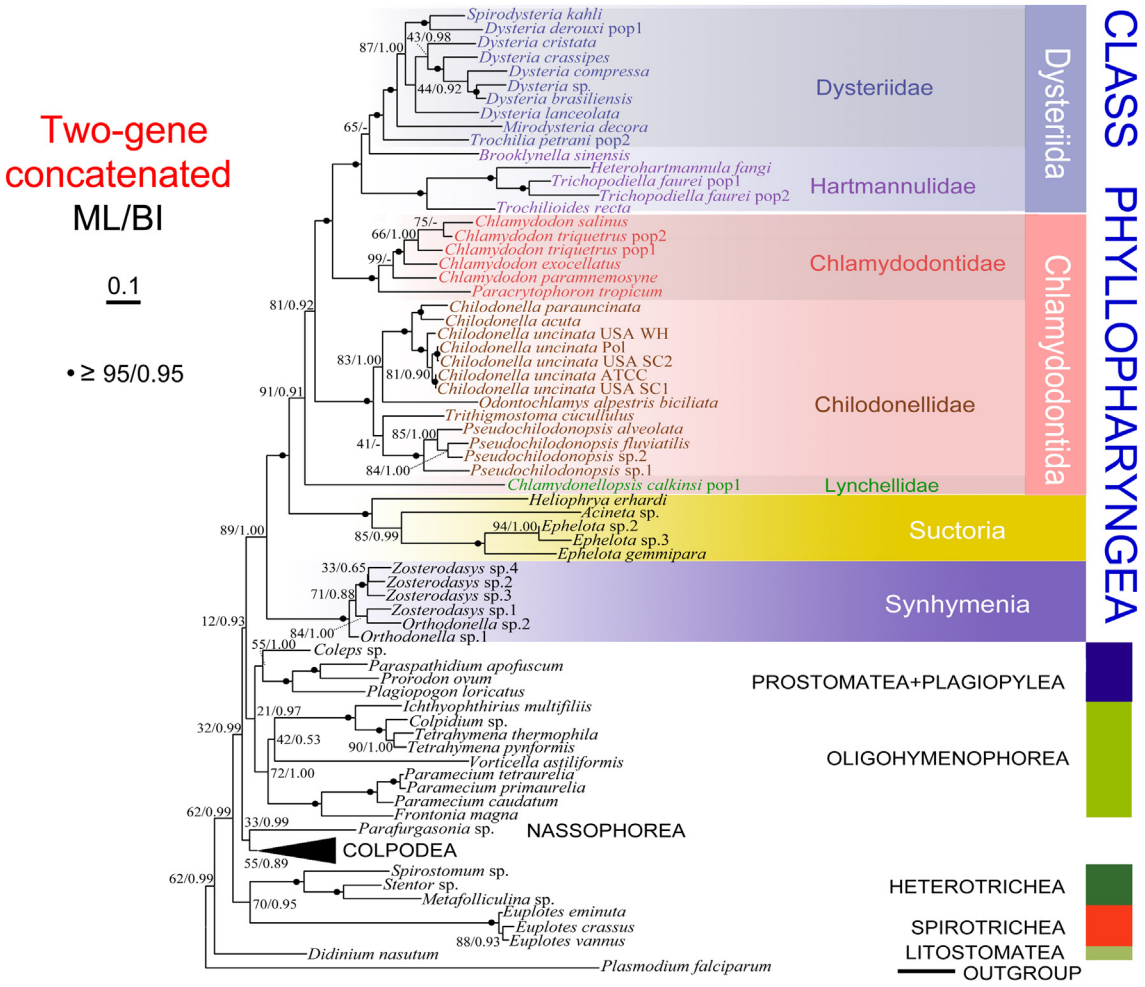

Fig. 3. Maximum likelihood (ML) tree based on two-gene concatenated sequence alignment. Numbers at the nodes represent the bootstrap values of ML out of 1000 replicates and the posterior probability values of Bayesian analysis (BI). Hyphen (-) indicates the disagreement between ML and BI. The scale bar corresponds to 10 substitutions per 100 nucleotide positions.

Pseudochilodonopsis; (3) in the subclass Suctoria, *Ephelota* specimen (*Ephelota* sp.) is not monophyletic as *Ephelota* sp.3 forms a sister relationship to all other suctorians; (4) the nassophorean species *Parafurgasonia* sp. nests within the class Colpodea, instead of being sister to this clade.

3.4. Phylogeny based on mtSSU-rDNA

The topology of the mtSSU-rDNA tree (Fig. 5) is similar to the concatenated tree at or above family levels, but with lower support values especially among the deep nodes. The main differences of the mtSSU-rDNA tree compared to the concatenated tree are: (1) *Brooklynella* clusters in the Hartmannulidae clade both in the ML and BI analyses (45% ML, 0.98 BI); (2) within the family Hartmannulidae, *Trichopodiella faurei* pop2 does not cluster with *T. faurei* pop1 but, instead, groups with *Heterohartmannula fangi*; (3) *Odontochlamys* falls outside of the rest of Chilodonellidae, instead of grouping with *Chilodonella*; (4) *Trithigmostoma* groups with *Pseudochilodonopsis* in the ML tree (54% ML), whereas it groups with *Chilodonella* in the BI tree (0.81 BI); (5) the nassophorean species *Parafurgasonia* sp. nests within the Prostomatea + Plagiopylea clade instead of being sister to Colpodea; (6) Prostomatea + Plagiopylea is sister to the subclass Synhymenia (54% ML, 0.99 BI), instead of being sister to Oligohymenophorea, resulting Phyllopharyngea not being monophyletic; (7) *Vorticella* is separated from other oligohymenophoreans and *Didinium*, a litostomatean, groups into Oligohymenophorea (72% ML, 0.52 BI); (8) the monophyly of Heterotrichea is not supported because *Spirostomum* sp. clusters with Spirotrichea (83% ML, 1.00 BI).

4. Discussion

4.1. Comparisons between nSSU-rDNA and mtSSU-rDNA

This study reveals that mtSSU-rDNA is much more variable among the species studied than nSSU-rDNA (Table S1). It is consistent with a previous study that the mtSSU-rDNA sequences differ by up to 8.0% among the five isolates of the ciliate morphospecies *Chilodonella uncinata*, although these isolates have nearly identical nSSU-rDNA sequences (Katz et al., 2011). Our results indicate that mtSSU-rDNA is potentially able to elucidate relationships at lower level (i.e. intra- or interspecies), where nSSU-rDNA might be too conserved to discriminate groups. This increased resolution at shallower time scales comes at a cost, however, it is more difficult to align mtSSU-rDNA sequences due to their increased variability, especially when the taxa are distantly related. Therefore, precision of alignment is extremely important when studying mtSSU-rDNA sequences in phylogenetic contexts.

Most of the highly supported clades in the nSSU-rDNA tree also receive high support values in the mtSSU-rDNA tree. Moreover, the

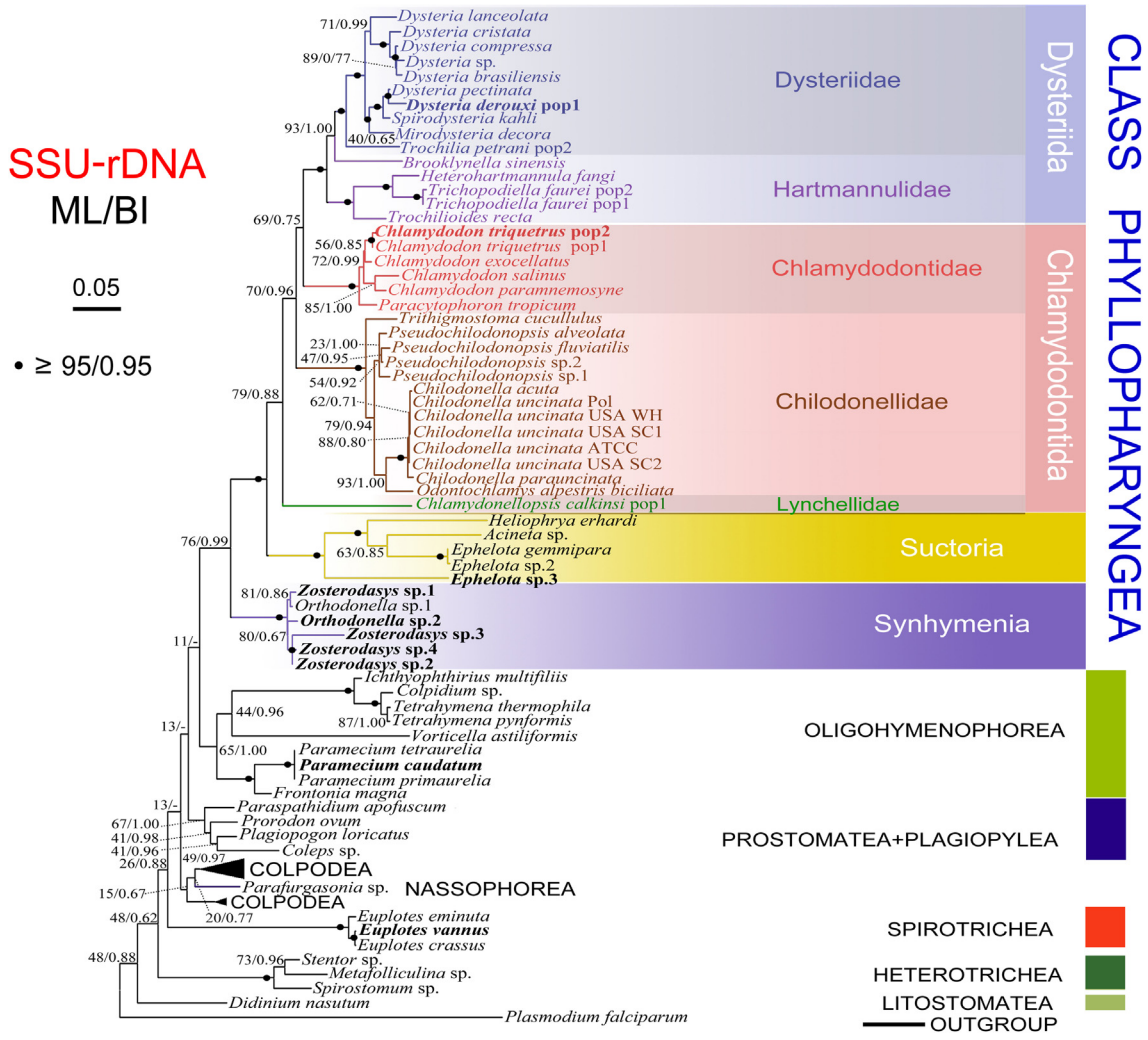

Fig. 4. Maximum likelihood (ML) tree based on nSSU-rDNA sequence alignment. Numbers at the nodes represent the bootstrap values of ML out of 1000 replicates and the posterior probability values of Bayesian analysis (BI). Hyphen (-) indicates the disagreement between ML and BI. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

mtSSU-rDNA tree gives more reasonable relationships in some taxa. For example, the genus *Ephelota* is recovered as a monophyletic group in the mtSSU-rDNA tree while it is split in the nSSU-rDNA tree (Figs. 4 and 5). Similarly, the class Colpodea, which is revealed as paraphyletic in the nSSU-rDNA tree, forms one clade in the mtSSU-rDNA tree (Figs. 3 and 4). In addition, the relationships within the genus *Chilodonella* are poorly resolved in the nSSU-rDNA tree, whereas they are clearly reflected in the mtSSU-rDNA tree, which is consistent with previous work (Katz et al., 2011). However, there are also some relationships that are more reasonable based on morphological data in the nSSU-rDNA trees. For example, each of the classes Phyllopharyngea, Oligohymenophorea, and Heterotrichea is revealed monophyletic in the nSSU-rDNA trees (Gao et al., 2016b), while they are not in the mtSSU-rDNA trees. Generally, mtSSU-rDNA performs better in resolving shallower nodes among closely related species, while nSSU-rDNA is more reliable in recovering relationships among deeper nodes at higher levels, e.g. the class-level relationships. It is worth mentioning that concatenated analyses of mtSSU-rDNA and nSSU-rDNA inferred better-resolved phylogenies (Fig. 3), with more robust support and more consistent with the morphological data. Therefore, we suggest that more mtSSU-rDNA sequences should be characterized and analyzed, concatenated with nSSU-rDNA, in the future to reconstruct more reliable ciliate phylogeny.

4.2. Phylogenetic relationships within Dysteriida

According to Lynn (2008), there are four families in Dysteriida: Dysteriidae, Hartmannulidae, Kyaroikeidae, and Plesiotrichopidae. However, only species within Dysteriidae and Hartmannulidae have been studied using molecular data. The main taxonomic problem within Dysteriida lies in the non-monophyletic *Dysteria* and the unstable position of *Brooklynella.*

Previous studies based on nSSU-rDNA indicate that the genus *Dysteria* is paraphyletic, with *Spirodysteria* and *Mirodysteria* being nested within the genus (Chen et al., 2016). In the present study, this paraphyly is confirmed by the phylogenetic analyses based on mtSSU-rDNA and concatenated data (Figs. 3 and 5), though the AU test based on mtSSU-rDNA data does not reject its monophyly ($p = 0.114$, Table 3). Our results are consistent with the morphological data. Morphologically, *Mirodysteria* shares a highly similar body shape with *Dysteria,* but *Mirodysteria* has loosely arranged conspicuous right kinety fragments and distinct dorsal spines (Pan et al., 2011). *Spirodysteria* is also morphologically similar to *Dysteria* but with highly characteristic spirally twisted body shape (Gong et al., 2007). It seems that *Mirodysteria* and *Spirodysteria* represent highly specialized *Dysteria*-like morphology and their characteristic morphologies are more likely due to the results of adaptation to their peculiar lifestyles (Chen et al., 2016). There-

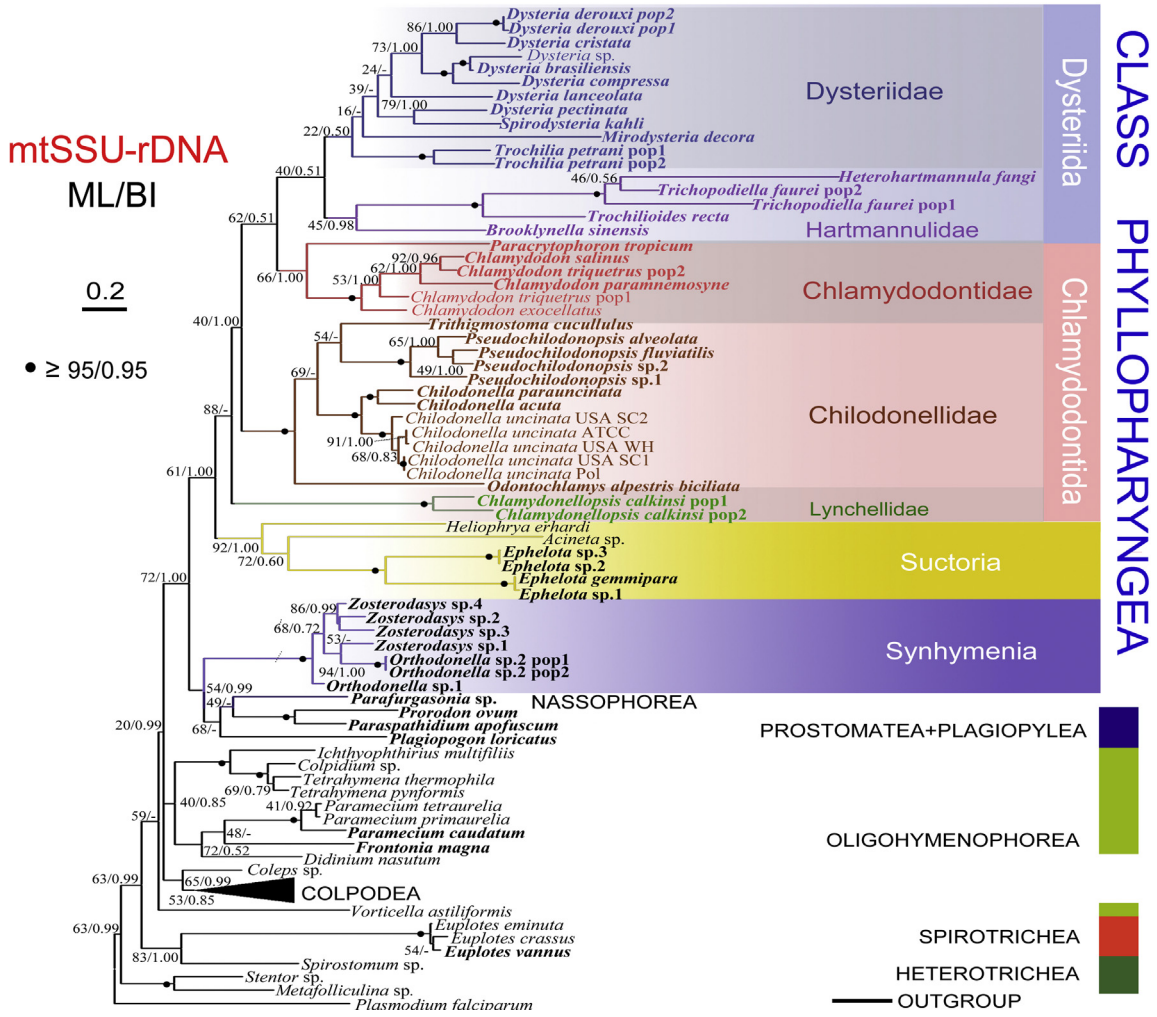

Fig. 5. Maximum likelihood (ML) tree based on mtSSU-rDNA sequence alignment. Numbers at the nodes represent the bootstrap values from of ML out of 1000 replicates and the posterior probability values of Bayesian analysis (BI). Hyphen (-) indicates the disagreement between ML and BI. The scale bar corresponds to 20 substitutions per 100 nucleotide positions.

Table 3
Approximately Unbiased test results based on the mtSSU-rDNA data. Rejected monophyly ($p < 0.05$) is highlighted in gray.

Topology constraints	−Ln likelihood	AU value (p)
Unconstrained		
Dysteria	59078.53104922	0.114
Brooklynella + Hartmannulidae	59067.82772824	0.510
Brooklynella + Dysteriidae	59073.30188888	0.052
Chlamydodontidae + Chilodonellidae + Lynchellidae	59085.93594528	0.026
Chlamydodontidae + Lynchellidae	59079.48553340	0.057
Trithigmostoma + Lynchellidae	59067.83064430	0.001
Phyllopharyngea	59071.46992904	0.148

fore, the validity of the two genera *Mirodysteria* and *Spirodysteria* is challenged by their similar morphology and the phylogenetic results from both mtSSU-rDNA and nSSU-rDNA trees. Further investigations with more taxa from *Mirodysteria* and *Spirodysteria*, especially the type species, are needed to confirm their validity.

Phylogenetic analyses based on mtSSU-rDNA reveal a close relationship between the genus *Brooklynella* and the family Hartmannulidae (Fig. 5). The genus *Brooklynella* was first established by Lom and Nigrelli (1970) with the type species *B. hostilis*, which was assigned in the family Hartmannulidae. Another *Brooklynella* species, *B. sinensis*, was recently described by Gong and Song (2006a), and the definition of this genus was also given based on the data available. Present and previous phylogenetic analyses based on nSSU-rDNA reveal that *B. sinensis* groups with Dysteriidae in high support values (Chen et al., 2016), while *B. sinensis* clusters with Hartmannulidae in the mtSSU-rDNA trees (Fig. 5). Our study also revealed that its V4 region secondary structure of mtSSU-rRNA resembles those of the genus *Dysteria* more with a longer helix 2 (Fig. 2). Neither the grouping of *Brooklynella* with Dysteriidae nor with the family Hartmannulidae is rejected by the AU test based on mtSSU-rDNA data (Table 3). The intermediate position of *B. sinensis* was also suggested by Gong and Song (2006a) based on its morphology of possessing continuous ventral ciliature in the left field (like hartmannulids) as well as cilia-free postoral kineties and reduced number of nematodesmal rods (like dysteriids). Therefore, the genus *Brooklynella* may represent a taxon at the family level, but such definition will require more molecular evidence from more species, including the type species.

4.3. Phylogenetic relationships within Chlamydodontida sensu Lynn, 2008

According to Lynn (2008), the families Chlamydodontidae, Chilodonellidae and Lynchellidae in the present study belong to

the same order, Chlamydodontida. Alternatively, Puytorac (1994) assigned them into two orders: Chlamydodontida, with a juxtaposed heteromerous macronucleus (Chlamydodontidae, Lynchellidae), and Chilodonellida, with a centric heteromerous macronucleus (Chilodonellidae). However, neither classification was supported by previous phylogenetic analyses based on nSSU-rDNA, despite the finding that each of the three families was monophyletic (Chen et al., 2016; Gao et al., 2012). The phylogenies of both individual loci and the concatenated dataset (Figs. 3 and 5) show that the order Dysteriida always forms a sister clade with the family Chlamydodontidae, thus rendering the order Chlamydodontida *sensu* Lynn (2008) paraphyletic. The three families clustering together are also rejected by the AU test based on mtSSU-rDNA data, though the grouping of Chlamydodontidae and Lynchellidae is not rejected (Table 3).

We propose that dysteriids originally evolved from a chlamydodontid-like ancestor. Morphologically, Dysteriida is mainly different from Chlamydodontida *sensu* Lynn, 2008 in that: (1) dysteriids attach to substrates by a non-ciliated adhesive region or by a flexible podite (vs. by thigmotactic ventral somatic cilia); (2) ventral kineties are posteriorly shortened from right to left (vs. ventral kineties terminate at the margin or posterior end of the cell) (Lynn, 2008; Pan et al., 2013; Qu et al., 2015a). Dysteriids therefore seem to be highly specialized chlamydodontids based on their morphology and the molecular findings reported here.

For the family Chilodonellidae, there remains some uncertainty about the phylogenetic position in *Trithigmostoma*. The three trees present three different topologies with regard to the genus *Trithigmostoma*. The nSSU-rDNA tree shows similar topologies with previous studies based on nSSU-rDNA data that *Trithigmostoma* sisters to the remainder of Chilodonellidae (Gao et al., 2012), whereas *Trithigmostoma* either groups with *Pseudochilodonopsis* or *Chilodonella* in the mtSSU-rDNA and concatenated trees, respectively. Morphological studies have also been uncertain about *Trithigmostoma*. *Trithigmostoma* was classified in Chilodonellidae mainly because of its oral ciliary structure found in the type species *T. cucullulus* (Jankowski, 1967). However, *Trithigmostoma* has continuous left and right kinetic rows, which is different from the typical gap between left and right somatic kineties of Chilodonellidae (Lynn, 2008). It is even inferred that *Trithigmostoma* have plesiomorphic morphology relative to lynchellids since the family Lynchellidae features no gap between the left and right kineties (Chen et al., 2016; Gong and Song, 2006b). However, *Trithigmostoma* never forms a sister relationship with lynchellids, and the AU test based on nSSU-rDNA also rejects their grouping ($p = 0.001$). In addition, morphological data shows that both *Trithigmostoma* and *Odontochlamys* have non-fragmented preoral kineties, indicating that they could be closer to *Chilodonella* (Foissner et al., 1991). Therefore, *Trithigmostoma* is proposed to stay in the family Chilodonellidae based on the molecular data from nSSU-rDNA and mtSSU-rDNA as well as the main morphological data, although this genus possesses distinct somatic kinety morphology.

4.4. Phylogeny of Synhymenia

Synhymeniids were once considered as a nassophorean group but were then transferred into the class Phyllopharyngea, which is supported in the present study as well as previous molecular studies based on nSSU-rDNA sequences (Gong et al., 2009; Zhang et al., 2014). The mtSSU-rDNA tree shows a different topology, in which synhymeniids do not group in the Phyllopharyngea, but group with Nassophorea + Prostomatea + Plagiopylea though this relationship is poorly supported (Fig. 5). However, the AU test does not reject the clustering of synhymeniids with other phyllopharyngeans ($p = 0.148$). The concatenated tree also reveals that synhymeniids cluster with other phyllopharyngeans (Fig. 3), which is consistent with the morphological data that synhymeniids and other phyllopharyngeans share a most-recent common ancestor (Gong et al., 2009). Therefore, we agree to include synhymeniids in the class Phyllopharyngea (Adl et al., 2012; Gong et al., 2009). It is worth mentioning that, as a subclass, there are three different spellings of this taxon: Synhymenia (Adl et al., 2012), which is a hemi-homonym of the genus name; Synhymeniidia (Gong et al., 2009), which reminds on the order name Synhymeniida; and Synhymeniia (Zhang et al., 2014), which can be easily confused by the one-letter difference with the genus name. We recommend the spelling "Synhymenia", because the definition is more comprehensive compared to other names (Adl et al., 2012).

4.5. Phylogeny of the ambiguous taxa Paraspathidium

Paraspathidium has been regarded as a haptorid within the class Litostomatea, based on its haptorid-like shape and suite of morphological characters (Foissner, 1997; Long et al., 2009). However, it has a variable body shape, uniform holotrichous-arranged somatic cilia, and kinetosomes, similar to plagiopyleans, meanwhile it resembles prostomateans due to its dikinetid perioral corona and contractile vacuole complex, encircling the cytostome (Foissner, 1997; Lynn, 2008). Previous studies based on a single gene (nSSU-rDNA, nLSU-rDNA, or alpha-tubulin gene) as well as concatenated data of rDNA and alpha-tubulin clearly rejected its assignment in the Litostomatea (Gao et al., 2016b; Zhang et al., 2010, 2012). The present phylogenetic analyses based on mtSSU-rDNA and concatenated data reveal the same result, that *Paraspathidium* groups with prostomateans and plagiopyleans.

4.6. Secondary structure of mtSSU-rRNA sequence

Difference in the V4 region secondary structures provides useful information to discriminate taxa within Phyllopharyngea, and the V4 region should be included in the phylogenetic analyses. Prior to this study, this portion of the sequence is typically removed during phylogenetic inference since it is too variable to be aligned. However, the secondary structures are more conserved than the primary sequences and can be more easily and less ambiguously aligned (Wang et al., 2015). Moreover, due to its high variation, closely related taxa can often be discriminated based on subtle difference in the secondary structures. For example, the genus *Chilodonella* has two types of helix 2, which indicates that *Chilodonella uncinata* could be more recently derived from the lineage. In general, the V4 region is likely to be an interesting region for future investigation. It is worth noting that *Trochilioides recta* has the longest V4 region and this species possesses an intron in the nSSU-rDNA sequence (Gao et al., 2012). The V4 region of its mtSSU-rDNA was also found to have an insertion since no homologous part could be aligned with it. The distinctive structure of the rDNA of this species will provide an interesting model for the future studies of the evolutionary relationship between mitochondrial and nuclear DNA.

4.7. Conclusion

Ciliate phylogenetics have been effectively elucidated using nSSU-rDNA or multi-nuclear-loci analysis, however, inner relationships within certain taxa are still elusive, e.g. the class Phyllopharyngea. In the present study, the mitochondrial small subunit ribosome DNA (mtSSU-rDNA) was first applied in the phylogenetic analyses of the class Phyllopharyngea. We expanded the taxon sampling by providing 48 new mtSSU-rDNA sequences and 10 new nSSU-rDNA sequences, which were mainly focusing on the class Phyllopharyngea. Phylogenetic analyses based on mtSSU-

rDNA data as well as combined data indicate the efficacy of including mtSSU-rDNA in inferring the phylogenetic relationships among phyllopharyngeans, especially among close related taxa. Present phylogenetic analyses also provide some new insights into the evolutionary relationships among phyllopharyngeans, e.g. the validity of genera *Mirodysteria* and *Spirodysteria* was questioned, the positions of *Brooklynellla* and *Trithigmostoma* were confirmed, *Paraspathidium* is supported to be in the group of prostomateans and plagiopyleans, etc. Besides, the mtSSU-rRNA secondary structure are predicted and compared, which showed values in phylogenetic analyses and could be included for clarifying generic or specific relationships. Further phylogenetic analyses including mtSSU-rDNA or other genes from mitochondrial genome for a larger group of ciliate taxa are needed to elucidate a more robust genealogical relationship of ciliates.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.04.018.

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