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Further analyses on the phylogeny of the subclass Scuticociliatia (Protozoa, Ciliophora) based on both nuclear and mitochondrial data

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ABSTRACT

So far, the phylogenetic studies on ciliated protists have mainly based on single locus, the nuclear ribosomal DNA (rDNA). In order to avoid the limitations of single gene/genome trees and to add more data to systematic analyses, information from mitochondrial DNA sequence has been increasingly used in different lineages of ciliates. The systematic relationships in the subclass Scuticociliatia are extremely confused and largely unresolved based on nuclear genes. In the present study, we have characterized 72 new sequences, including 40 mitochondrial cytochrome oxidase c subunit I (*COI*) sequences, 29 mitochondrial small subunit ribosomal DNA (mtSSU-rDNA) sequences and three nuclear small subunit ribosomal DNA (nSSU-rDNA) sequences from 47 isolates of 44 morphospecies. Phylogenetic analyses based on single gene as well as concatenated data were performed and revealed: (1) compared to mtSSU-rDNA, *COI* gene reveals more consistent relationships with those of nSSU-rDNA; (2) the secondary structures of mtSSU-rRNA V4 region are predicted and compared in scuticociliates, which can contribute to discrimination of closely related species; (3) neither nuclear nor mitochondrial data support the monophyly of the order Loxocephalida, which may represent some divergent and intermediate lineages between the subclass Scuticociliatia and Hymenostomatia; (4) the assignments of thigmotrichids to the order Pleuronematida and the confused taxon *Sulcigera comosa* to the genus *Histiobalantium* are confirmed by mitochondrial genes; (5) both nuclear and mitochondrial data reveal that the species in the family Peniculistomatidae always group in the genus *Pleuronema*, suggesting that peniculistomatids are more likely evolved from *Pleuronema*-like ancestors; (6) mitochondrial genes support the monophyly of the order Philasterida, but the relationships among families of the order Philasterida remain controversial due to the discrepancies between their morphological and molecular data.

1. Introduction

Ciliated protists (ciliates) are a large group of single-celled eukaryotes that are distributed in diverse habitats across the globe (Song et al., 2009). They are characterized by the presence of cilia (hair-like organelles), nuclear dimorphism (containing both germline and somatic nuclei within each cell), and special sexual reproduction (conjugation) (Corliss, 1979; Lynn, 2008), which make them important model organisms in the research areas of systematics, genetics, ecology, cell biology and epigenetics (Adl et al., 2012; Chen et al., 2018, 2019; Gao et al., 2017; Jiang et al., 2019; Li et al., 2019; Wang et al., 2017a, 2017b; Xu et al., 2019; Zhao et al., 2019). However, because of their complex morphological characters and high species diversity, the evolutionary relationships of many ciliate groups remain unknown or ambiguous, especially the groups that present particular difficulties in species identification (Sheng et al., 2018; Wang et al., 2019).

The subclass Scuticociliatia, which belongs to the class Oligohymenophorea, is one of the most confused groups in ciliates. Scuticociliates are either free-living in fresh or marine water or opportunistic or facultative parasites of aquatic animals, identified as causative agents of the disease “scuticociliatosis” (Fan et al., 2017). They are generally small in size (15–50 μm) with similar morphological

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Table 1
Primers for PCR amplification.

Genes	Primers	Sequences (5′–3′)	Reference
nSSU-rDNA	82F	GAAACTGCGAATGGCTC	Jerome et al. (1996)
	18SR	TGATCCTTCTGCAGGTTCACCTAC	Medlin et al. (1988)
COI	F298dT-S	GCNCAYGGTYTAATNATGGT	Strüder-Kypke and Lynn (2010)
	R1184dT-S	TADACYTCAGGGTGACCRAAAAATCA	Strüder-Kypke and Lynn (2010)
	COI-NEW-17-F1	TGGTNTTTTTTGTWGTWGTDCC	Present study
	COI-NEW-812-R2	GTWGTTTGTCAATGHCKRTCTA	Present study
	COI-NEW-81-F2	TATHGGHTCWAARGATGTDGCG	Present study
	COI-NEW-856-R1	GTTGHGATARAATWGGRTCHCC	Present study
mtSSU-rDNA	mtSSU-357-F2	AAACTTAACAGAATTGGCGGG	Present study
	mtSSU-1026-R1	GTACCTTGTGTCAACTTCACTC	Present study
	mtSSU-NEW-17-F1	GCGGGARTTTDTDMDAAYGGTGG	Present study

features *in vivo*, such as ciliary patterns, caudal cilium and posterior positioned contractile vacuole (Lynn, 2008; Small, 1967; Song et al., 2009), which makes species identification difficult. Recently, more taxa in the subclass Scuticociliatia have been investigated using a combination of morphological and molecular tools, e.g. live observation, silver staining preparations, and nuclear ribosomal DNA sequence analyses (Fan et al., 2014, 2017; Liu et al., 2016; Miao et al., 2010; Zhang et al., 2018). Furthermore, phylogenomic studies related to the subclass Scuticociliatia have been performed, but only two or three taxa were sampled (Feng et al., 2015; Gentekaki et al., 2017). Therefore, the phylogenetic relationships among taxa of scuticociliates remain extremely confused at present mainly because of the discordance between morphological characters and molecular evidence (Gao et al., 2012).

Phylogenetic studies within ciliates (including scuticociliates) have been mainly based on the single gene, nuclear small subunit ribosomal DNA (nSSU-rDNA) (Bai et al., 2018; Wang et al., 2018) or the linked loci of the nSSU-rDNA, ITS1-5.8S-ITS2 region and nuclear large subunit ribosomal DNA (nLSU-rDNA) (Gao et al., 2013, 2016; Hewitt et al., 2003; Huang et al., 2018; Luo et al., 2018; Snoeyenbos-West et al., 2002; Zhao et al., 2018). However, relying on single locus or multi-gene from the same genome or chromosome may not be sufficient enough to elucidate phylogenetic relationships among ciliate taxa, especially for those taxa with close relationships. In contrast, mitochondrial DNA possesses higher copy number and higher evolution rate compared to nuclear DNA (Rand, 2003). Given that, the mitochondrial DNA has been increasingly used for phylogenetic investigations in different lineages across the tree of life (Boore and Brown, 1998; Dunthorn et al., 2011, 2014; Strüder-Kypke and Lynn, 2010; Zhao et al., 2013; Zheng et al., 2018). The mitochondrial cytochrome oxidase c subunit I (*COI*) gene has been utilized as the DNA barcode to discriminate species of many animals (Folmer et al., 1994; Hajibabaei et al., 2006; Hebert et al., 2004; Smith et al., 2008). Within ciliates, *COI* is firstly employed for species identification in *Tetrahymena* and *Paramecium* (class Oligohymenophorea) and is demonstrated to be an appropriate DNA barcoding marker for species delimitation and phylogenetic analyses (Barth et al., 2006; Lynn and Strüder-Kypke, 2006; Zhao et al., 2013). Additionally, mitochondrial small subunit ribosomal DNA (mtSSU-rDNA) is also considered as an efficient molecular marker for phylogenetic inferences in ciliates (Wang et al., 2017b), especially among shallower nodes (Dunthorn et al., 2011, 2014; Katz et al., 2011).

In the present study, we expanded the molecular markers of *COI* and mtSSU-rDNA, providing 72 new sequences. Phylogenetic analyses based on both single gene and concatenated gene datasets of nuclear and mitochondrial data were performed and compared in order to further investigate and clarify the evolutionary relationships in the subclass Scuticociliatia. Moreover, we predicted the secondary structures of mtSSU-rRNA of *Uronema marinum* and compared the mtSSU-rRNA variable region 4 (V4) of the newly characterized sequences to assess the applicability of mtSSU-rRNA secondary structure in phylogenetic analyses in scuticociliates.

2. Materials and methods

2.1. Ciliate sampling and identification

In total 47 isolates of 44 morphospecies were selected in this study. Taxa information and collection sites are provided in Table S1. Species were identified by live microscopic observation and silver impregnations (Wilbert, 1975). Ciliate terminology and systematics are mainly according to Gao et al. (2016) and Lynn (2008).

2.2. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the optimized manufacturer's protocol. The primers of nSSU-rDNA were 82F (Jerome et al., 1996) and 18SR (Medlin et al., 1988). The *COI* fragments were obtained with the primers F298dT-S and R1184dT-S (Strüder-Kypke and Lynn, 2010). Nested polymerase chain reaction (PCR) was performed using the primers COI-NEW-17-F1 and COI-NEW-812-R2 or COI-NEW-81-F2 and COI-NEW-856-R1 (Table 1) when the first PCR amplification failed. The mtSSU-rDNA sequences were amplified with the primers mtSSU-357-F2 and mtSSU-1026-R1, which were designed in the present study according to the mtSSU-rDNA sequences of *Paramecium* and *Tetrahymena*. Similar to *COI* gene amplification, nested PCR was performed with the primers mtSSU-NEW-17-F1 and mtSSU-1026-R1 (Table 1). The PCR amplifications were performed using Q5® Hot Start High-Fidelity 2x Master Mix DNA Polymerase (NEB, Ipswich, MA) to minimize the errors during PCR (Wang et al., 2017a). Cycling parameters for PCR were according to Wang et al. (2018). PCR products were purified by Easy-Pure Quick Gel Extraction Kit (Transgen Biotech, China) and cloned using pEASY-T1 Cloning Kit (Transgen Biotech, China), or directly sequenced bidirectionally in Tsingke Biological Technology Company (Beijing, China). Where possible, both the *COI* gene and the mtSSU-rDNA were generated from the same genomic DNA as nSSU-rDNA.

2.3. Phylogenetic analyses and topology testing

The newly characterized *COI* gene sequences were predicted into amino acid sequences by MegAlign v7.1.0 (DNAStar). Newly characterized sequences were combined with relevant sequences downloaded from the GenBank database (Table 2). The sequences of nSSU-rDNA and *COI* were aligned using the MAFFT algorithm on the GUIDANCE2 Server (http://guidance.tau.ac.il/ver2/) and trimmed both ends by BioEdit v7.2.5 (Hall, 1999). The mtSSU-rDNA sequences were aligned using MAFFT v7 (https://mafft.cbrc.jp/alignment/server/) with data from Wang et al., 2017b as a structural alignment and then manually adjusted by SeaView v4 (Gouy et al., 2010). The final alignments of nSSU-rDNA, *COI* and mtSSU-rDNA used for phylogenetic analyses included 1814, 309 and 897 sites, respectively. The three

Table 2
GenBank accession numbers of nSSU-rDNA, *COI* and mtSSU-rDNA sequences used in present study. Newly characterized sequences are in bold.

Species	Accession No.			Species	Accession No.		
	nSSU-rDNA	*COI*	mtSSU-rDNA		nSSU-rDNA	*COI*	mtSSU-rDNA
Ancistrum crassum	HM236340	**MH605544**	–	*Paramecium biaurelia*	KU729877	DQ912534	KP973694
Cardiostomatella vermiformis	AY881632	**MH605533**	–	*Paramecium caudatum*	KX302699	FN424190	KX302680
Cinetochilum ovale	FJ870103	**MH605540**	–	*Paramecium jenningsi*	HE662760	FJ905139	KT950248
Cohnilembus verminus	HM236339	**MH605563**	**MH577598**	*Paramecium primaurelia*	AF100315	FJ905141	K01750
Colpoda lucida	EU039895	FJ905159	HM246409	*Paramecium sexaurelia*	FJ003978	FJ905154	KP973683
Colpoda magna	EU039896	FJ905160	HM246410	*Paramecium tetraurelia*	X03772	DQ912542	K01751
Cristigera media	FJ868180	**MH605569**	–	*Paramesanophrys typica*	**MH574792**	**–**	**MH577595**
Cristigera pleuronemaoides	KF256816	**MH605546**	**MH577581**	*Paranophrys magna*	JN885089	–	**MH577599**
Cyclidium glaucoma	KY476313	FJ905125	–	*Parauronema longum*	HM236338	**MH605542**	**MH577578**
Cyclidium varibonneti	KF256817	**MH605558**	–	*Peniculistoma mytili*	KU665371	KU665383	–
Dexiotricha cf. *granulosa*	KF878931	–	–	*Philaster apodigitiformis*	FJ648350	**MH605532**	–
Dexiotricha elliptica	KF878932	**MH605568**	–	*Philaster sinensis*	KJ815049	**MH605552**	–
Dexiotricha sp.	–	FJ905124	–	*Philasterides armatalis*	FJ848877	**MH605534**	**MH577575**
Entodiscus borealis	AY541687	FJ905123	–	*Plagiopyliella pacifica*	AY541685	FJ905121	–
Entorhipidium sp.	–	FJ905122	–	*Platyophrya bromelicola*	EU039906	FJ905158	HM246415
Eurystomatella sinica	JX310021	**MH605566**	**MH577601**	*Pleuronema* cf. *setigerum*	FJ848875	**MH605539**	–
Falcicyclidium fangi	FJ868183	**MH605538**	–	*Pleuronema coronatum* pop. 1	JX310014	**MH605531**	**MH577574**
Falcicyclidium citriforme	KF256819	**MH605550**	**MH577586**	*Pleuronema coronatum* pop. 2	AY103188	**MH605561**	**MH577594**
Frontonia magna	FJ876953	KJ475343	KX302681	*Pleuronema elegans*	KF840518	**MH605554**	–
Hippocomos salinus	JX310012	**MH605543**	**MH577579**	*Pleuronema grolierei*	KF840519	**MH605549**	**MH577584**
Histiobalantium comosa	KU665372	KU665391	–	*Pleuronema setigerum*	FJ848874	**MH605537**	**–**
Histiobalantium minor	JX310013	**MH605548**	**MH577583**	*Porpostoma notata*	HM236335	–	**MH577596**
Maryna umbrellata	JF747217	FJ905161	JQ026523	*Protocyclidium citrullus*	KF256820	**MH605545**	**MH577580**
Mesanophrys carcini pop. 1	JN885085	**MH605547**	**MH577582**	*Protocyclidium sinica*	KF256822	–	**MH577593**
Mesanophrys carcini pop. 2	JN885086	**MH605556**	**MH577591**	*Pseudocohnilembus hargisi*	JN885090	**MH605559**	**–**
Metanophrys orientalis	JN885084	**MH605551**	**MH577589**	*Pseudocohnilembus longisetus*	FJ899594	GQ500580	–
Metanophrys sinensis	HM236336	**MH605565**	**MH577600**	*Pseudocohnilembus persalinus* pop. 1	AY835669	GQ500579	**–**
Miamiensis avidus pop. 1	KX357144	–	**–**	*Pseudocohnilembus persalinus* pop. 2	GQ265955	ADP06862	**MH577587**
Miamiensis avidus pop. 2	JN885091	**MH605562**	**MH577597**	*Schizocalyptra aeschtae*	DQ777744	**MH605530**	**–**
Miamiensis avidus pop. 3	EU831192	EU831213	–	*Schizocalyptra* sp.	FJ848873	**MH605536**	–
Miamiensis avidus pop. 4	EU831195	EU831216	–	*Tetrahymena malaccensis*	–	DQ927303	DQ927303
Miamiensis avidus pop. 5	EU831193	EU831214	–	*Tetrahymena paravorax*	EF070253	DQ927304	DQ927304
Miamiensis avidus pop. 6	EU831196	MH078249	–	*Tetrahymena pigmentosa*	M26358	DQ927305	DQ927305
Miamiensis avidus pop. 7	EU831194	MH078247	–	*Tetrahymena pyriformis*	M98021	EF070300	AF160864
Miamiensis avidus pop. 8	EU831198	EU831218	–	*Uronema heteromarinum*	FJ870100	**MH605535**	**MH577576**
Miamiensis avidus pop. 9	KU720304	EU831227	–	*Uronema marinum*	GQ465466	**MH605541**	**MH577577**
Miamiensis avidus pop. 10	JN689229	GQ855300	**–**	*Uronema orientalis*	**MH574791**	**MH605553**	–
Miamiensis avidus pop. 11	EU831199	MH078246	**–**	*Uronema* sp. GD	JN885088	**MH605557**	**MH577592**
Miamiensis avidus pop. 12	AY642280	KX259258	**–**	*Uronemita filificum* pop. 1	**MH574793**	**MH605567**	**MH577602**
Miamiensis avidus pop. 13	EU831212	EU831233	–	*Uronemita filificum* pop. 2	EF486866	**MH605560**	–
Miamiensis avidus pop. 14	**–**	EU831226	**–**	*Uronemita parabinucleata*	KU199245	**MH605555**	**MH577590**
Philasterides dicentrarchi	JX914665	**–**	**–**	*Uronemita parafilificum*	HM236337	**MH605564**	–
Mytilophilus pacificae	KU665354	KU665373	–	*Uronemita sinensis*	JN885083	–	**MH577588**
Paramecium aurelia	–	X15917	X15917	*Wilbertia typica*	JX310022	–	**MH577585**

alignments were concatenated subsequently using SeaView v4. The most appropriate model for phylogenetic analyses of *COI* amino acid sequences was CpREV + I + G + F, selected under Akaike Information Criterion (AIC) by ProtTest v3.3 (Darriba et al., 2011). For nSSU-rDNA and mtSSU-rDNA, the best model was GTR + I + G as selected by Modeltest v3.4 (Posada and Crandall, 1998) and MrModeltest v2.2 (Nylander, 2004).

For the taxonomically confused species complex *Miamiensis avidus* and *Philasterides dicentrarchi,* sequence similarities of nSSU-rDNA and *COI* gene were calculated by BioEdit v7.2.5 (Hall, 1999) (Tables S4 and S5). Fourteen nSSU-rDNA sequences and 13 *COI* amino acid sequences of *M. avidus* and *P. dicentrarchi* were combined respectively with relevant sequences of the order Philasterida. *Cyclidium glaucoma* and *Histiobalantium comosa* were selected as outgroup species (Table 2). These two datasets were aligned and trimmed as described above. The best models of the *COI* amino acid and nSSU-rDNA datasets were CpREV + I + G and GTR + I + G, respectively.

For each dataset, maximum likelihood (ML) analysis was performed in CIPRES Science Gateway using RAxML-HPC2 on XSEDE v8.2.10 (Stamatakis, 2014). Support for the best-scoring ML tree came from 1000 bootstrap replicates. Bayesian inference (BI) analysis was carried out with MrBayes v3.2.6 on XSEDE (Ronquist et al., 2012) on the CIPRES Science Gateway. Markov chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations with sampling every 100 generations and a burn-in of 10,000 trees. MEGA v5 (Tamura et al., 2011) was used to visualize the tree topologies.

The approximately unbiased (AU) test (Shimodaira, 2002) was performed to test the monophyly of the focal group. The constrained ML trees were generated by enforcing the monophyly of the respective focal groups with unspecific internal relationships within the constrained or mtSSU-rDNA groups and among the remaining taxa. The site-wise likelihoods for the resulting constrained topologies and the non-constrained ML topology were calculated by local RAxML v8 (Stamatakis, 2014) and then calculated in CONSEL (Shimodaira and Hasegawa, 2001) to obtain *p*-values.

2.4. Secondary structure prediction

The secondary structure of mtSSU-rRNA of *Uronema marinum* was predicted based on the models of *Tetrahymena pyriformis* (M12714), *Paramecium tetraurelia* (K01751) (http://www.rna.ccbb.utexas.edu) and *Chilodonella uncinata* (JN111982) (Wang et al., 2017) using the Mfold website (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) with default settings. Four variable regions were

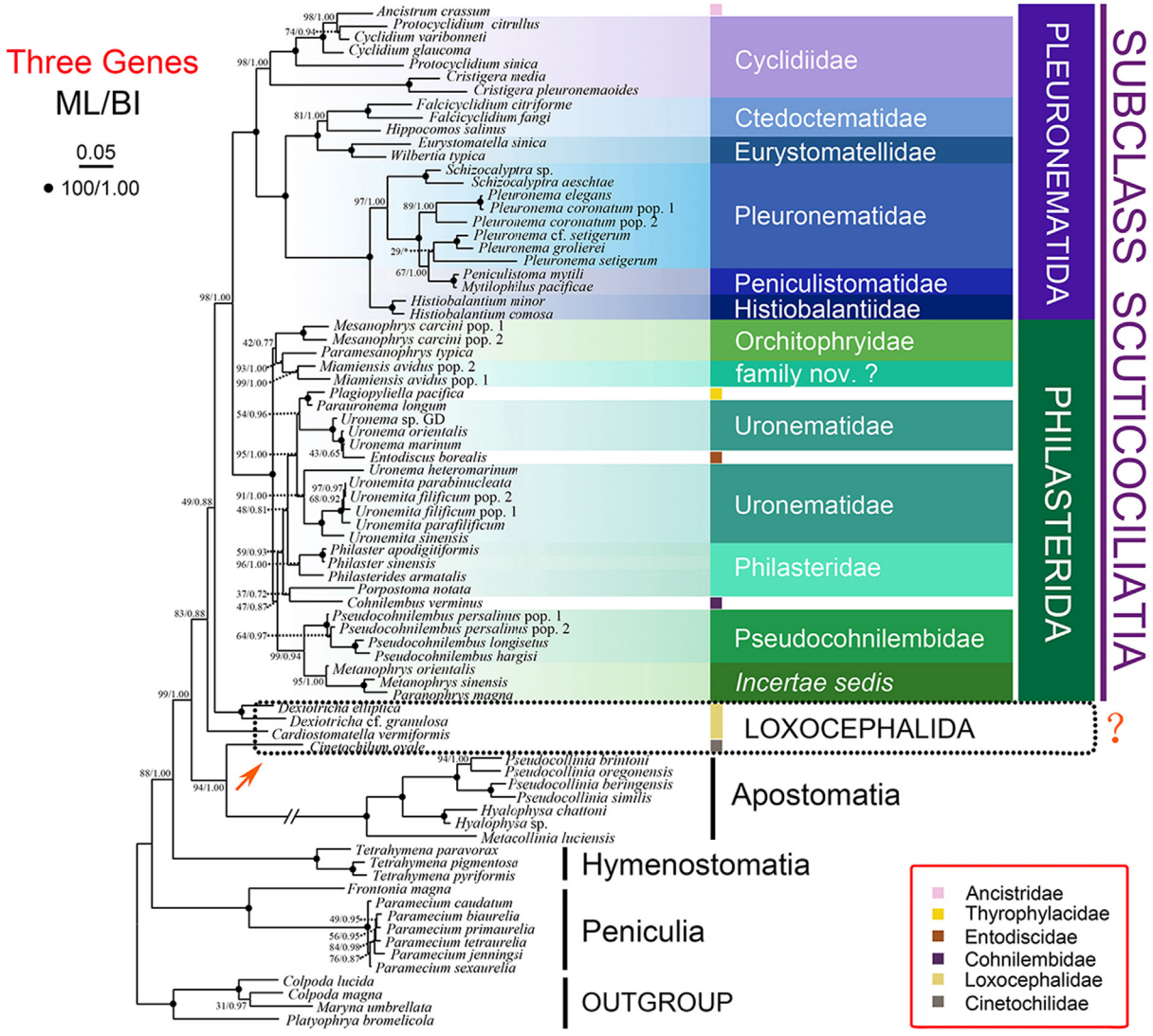

Fig. 1. Maximum likelihood (ML) tree based on the concatenated data of nSSU-rDNA, mtSSU-rDNA and *COI* sequences focusing on the subclass Scuticociliatia. Numbers at the nodes represent the bootstrap values of ML out of 1000 replicates and the posterior probability values of Bayesian analysis (BI). Asterisks (*) indicate the disagreement between ML and BI. Fully supported (100%/1.00) branches are marked with solid circles. The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

identified according to Schnare et al., 1986; Wang et al., 2017.

Additionally, we constructed the secondary structures of the mtSSU-rRNA V4 regions of all the pleuronematids and philasterids used in the present study. RnaViz v2.0 (Rijk and Wachter, 1997) was used to edit the structures for aesthetic purpose. The conserved structure model of V4 region was revealed by comparing the structures of the 29 taxa.

3. Results

3.1. Sequence summary and analyses

The newly characterized 72 sequences, including three nSSU-rDNA, 40 *COI* and 29 mtSSU-rDNA sequences from 47 isolates of 44 morphospecies, have been deposited in the GenBank database (Table 2). For scuticociliates, the overall pairwise sequence divergence of nSSU-rDNA is 0.1–18.2% (avg. 11.5%), while that of *COI* nucleotide and mtSSU-rDNA is 0.3–36.0% (avg. 24.0%), and 2.5–33.5% (avg. 21.0%), respectively (Tables S2 and S3). Among the species of Loxocephalida, the sequence difference of nSSU-rDNA ranges from 6.9% to 9.6% (avg. 8.3%), compared with 18.6% to 28.6% (avg. 24.9%) of *COI* nucleotide sequence. Within the order Pleuronematida, the sequence divergence is 0.1–18.2% (avg. 11.6%, nSSU-rDNA) vs. 1.2–34.4% (avg. 25.9%, *COI*) vs. 14.3–33.5% (avg. 27.1%, mtSSU-rDNA), while in Philasterida, it is 0.1–11.2% (avg. 7.1%) vs. 0.3–26.2% (avg. 18.1%) vs. 2.5–24.7% (avg. 14.7%), respectively.

3.2. Phylogeny based on the concatenated data of nSSU-rDNA, mtSSU-rDNA and COI (Fig. 1)

The topologies of the ML and BI trees are generally concordant, thus only the topology of ML tree with support values generated from both analyses is presented. Generally, the order Loxocephalida is polyphyletic because the family Cinetochilidae (represented by *Cinetochilum ovale*) clusters with Apostomatia with high support (ML/BI: 94/1.00). Both the orders Pleuronematida and Philasterida are fully supported monophyletic groups that are sister to each other (ML/BI: 98/1.00). Within the order Pleuronematida, seven families included in the analyses are divided into three major clades. The families Ancistridae (represented by *Ancistrum crassum*) and Cyclidiidae form one clade, the families Ctedoctematidae and Eurystomatellidae form the second clade in full support, and the families Pleuronematidae, Peniculistomatidae and Histiobalantiidae form the third clade in full support, which is

sister to the second clade. Five of the seven families are monophyletic, except for Cyclidiidae and Pleuronematidae. The family Cyclidiidae has Ancistridae nesting within it (ML/BI: 98/1.00), and the family Peniculistomatidae groups into the family Pleuronematidae (ML/BI: 67/1.00).

In contrast to the order Pleuronematida, the relationships among most families in the order Philasterida are unclear. For example, both the families Thyrophylacidae (represented by *Plagiopyliella pacifica*) and Entodiscidae (represented by *Entodiscus borealis*) group into the family Uronematidae, while *Miamiensis avidus* does not cluster with the putative relatives of Uronematidae, but groups in Orchitophryidae: it clusters with *Paramesanophrys typica* with high support (ML/BI: 93/1.00), which is then sister to two *Mesanophrys carcini* isolates with low bootstrap value (ML/BI: 42/0.77). The clade of *Metanophrys* and *Paranophrys* does not group with the putative relatives of Orchitophryidae, but groups with the monophyletic family Pseudocohnilembidae (ML/BI: 99/0.94). The family Philasteridae is not monophyletic either: a clade containing its two genera (*Philaster* and *Philasterides*) is sister to the Uronematidae-Thyrophylacidae-Entodiscidae assemblage (ML/BI: 48/0.81); the other genus of *Porpostoma* clusters with the family Cohnilembidae (represented by *Cohnilembus verminus*) in a poorly supported clade (ML/BI: 37/0.72).

3.3. Phylogeny based on nSSU-rDNA data (Fig. S1), COI (Fig. 2) and mtSSU-rDNA data (Fig. 3)

The topology of the nSSU-rDNA tree (Fig. S1) is basically consistent with the concatenated tree but differs in some low supported clades. For example, the clade of *Uronema* spp. (*Uronema* sp. GD, *U. orientalis* and *U. marinum*) + *Entodiscus* does not group with *Plagiopyliella* and *Parauronema longum*, but clusters with other Uronematidae species except for *Uronema heteromarinum* with low support (ML/BI: 48/0.62).

Similar to the concatenated tree, the order Loxocephalida is polyphyletic and both the orders Pleuronematida and Philasterida are monophyletic in the *COI* tree (Fig. 2). However, the main differences are: (1) *Schizocalyptra* branches earlier than the family Histiobalantiidae (ML/BI: 82/1.00), resulting the family Pleuronematidae polyphyletic; (2) *Pseudocohnilembus* spp. branch with the genus *Mesanophrys* rather than *Metanophrys* and *Paranophrys*; (3) two *Miamiensis avidus* populations do not cluster with each other; (4) the family Cinetochilidae clusters with other scuticociliates rather than falling into Apostomatia.

The mtSSU-rDNA tree (Fig. 3) has fewer species than the nSSU-rDNA and *COI* trees. Except for the subclasses, it does not reveal better resolution at order, family or genus levels. According to the mtSSU-rDNA phylogeny, the order Philasterida is monophyletic (ML/BI: 65/1.00) while the order Pleuronematida is not monophyletic. *Wilbertia typica* and *Eurystomatella sinica* do not cluster with each other, resulting the family Eurystomatellidae polyphyletic.

3.4. Comparison of secondary structure of mtSSU-rRNA

MtSSU-rDNA is a mitochondrial split gene in ciliates comprising rns_a and rns_b (Schnare et al., 1986; Swart et al., 2011). Here, the newly characterized mtSSU-rDNA sequence is 3′ part of rns_b and contains four variable regions of V3, V4, V5 and V9 (Fig. 4). The mtSSU-rRNA secondary structure of *Uronema marinum* is predicted, which is generally similar to the published structures of *Tetrahymena pyriformis*, *Paramecium tetraurelia* and *Chilodonella uncinata* (Konings and Gutell, 1995; Wang et al., 2017b). To be specific, the structures of V4 and V5 in *U. marinum* are concordant with that of *P. tetraurelia* and *C. uncinata*, respectively. The structure of V3 resembles that of *T. pyriformis* and *C. uncinata* and the structure of V9 is similar to structures of *P. tetraurelia* and *T. pyriformis*.

The mtSSU-rRNA secondary structures of scuticociliates are conserved in most parts, while the structure of V4 region is the most varied (ranging from 82 to 151 bp), which is used for species comparison in the present study. The secondary structures of mtSSU-rRNA V4 region of 29 isolates from 27 species and 20 genera are predicted and compared (Fig. 5). And a general model (Fig. 5X) is proposed based on these structures, containing three helices: a relatively conservative T-shaped helix (helix 1), a longer helix containing 0–2 bulges (helix 2), and a shorter helix (helix 3) adjacent to helix 2.

Comparison of the structures shows that the most remarkable divergence between the structures of the orders Pleuronematida and Philasterida is the absence or presence of helix 3: all the philasterids (except for *Cohnilembus verminus* and *Mesanophrys carcini* pop. 2) contain helix 3 while all the pleuronematids (except for *Histiobalantium minor*, which has a shorter one) miss helix 3. The species within the same genus share the identical secondary structure in *Protocyclidium*, *Uronema*, *Uronemita*, and *Metanophrys*. For the genera *Pleuronema* and *Mesanophrys*, the secondary structures are different between congeneric species. For instance, there is one bulge in helix 2 of *Pleuronema grolierei* while no bulge in that of *Pleuronema coronatum* (Fig. 5E and F). In addition, the V4 structures of the two isolates of *Mesanophrys carcini* are much different from each other in the helix 3 (no real helix 3 present in pop. 2).

4. Discussion

4.1. Comparison of nSSU-rDNA, COI and mtSSU-rDNA

The sequence divergence of nSSU-rDNA among scuticociliates involved in this study is much lower than that of mtSSU-rDNA sequence and *COI* nucleotide sequence (nSSU-rDNA: 0.1–18.2%, avg. 11.5%; mtSSU-rDNA: 2.5–33.5%, avg. 21.0%; *COI*: 0.3–36.0%, avg. 24.0%), which is consistent with previous studies on mitochondrial markers (Barth et al., 2006; Dunthorn et al., 2011, 2014; Lynn and Strüder-Kypke, 2006; Strüder-Kypke and Lynn, 2010; Wang et al., 2017; Zhao et al., 2013). Moreover, the average interspecific divergence varies considerably between different orders. The average divergence of the three genes within philasterids is the lowest, despite that more species are included in this order, which are consistent with the fact that philasterids are much more closely related to each other and their relationships are not resolved well (Gao et al. 2012).

Despite that mitochondrial DNA evolves faster than nuclear DNA in scuticociliates, the topologies revealed by nSSU-rDNA, mtSSU-rDNA and *COI* amino acid sequences are similar to each other (Figs. 2, 3 and S1). It has been demonstrated that nSSU-rDNA and mtSSU-rDNA reveal the same relationships for well-supported nodes in ciliates, although support for many nodes in the mtSSU-rDNA tree is low (Dunthorn et al., 2011, 2014; Wang et al., 2017). In the present study, the relationships revealed by *COI* amino acid sequences are much more consistent with those of nSSU-rDNA than those of mtSSU-rDNA, especially at the levels of subclass, order and family. Considering that *COI* gene shows the great potential as the barcoding maker to delimitate species (Barth et al., 2006; Dunthorn et al., 2011, 2014; Lynn and Strüder-Kypke, 2006; Strüder-Kypke and Lynn, 2010; Wang et al., 2017; Zhao et al., 2013), *COI* gene is a better choice than mtSSU-rDNA when in combination with nuclear genes to resolve the phylogenetic relationships of scuticociliates.

4.2. Systematics of loxocephalids

The loxocephalids display mixed morphological features and morphogenetic patterns that are found in both scuticociliates and hymenostomes (Fan et al., 2014). They were previously assigned in the order Philasterida (Small and Lynn, 1985), but later were proposed as an order (Jankowski, 1980; Li et al., 2006). Li et al. (2006) assigned this order which comprises the name-bearing type family Loxocephalidae and another family Cinetochilidae to the subclass Scuticociliatia. Whereas, some subsequent phylogenetic analyses based on rDNA

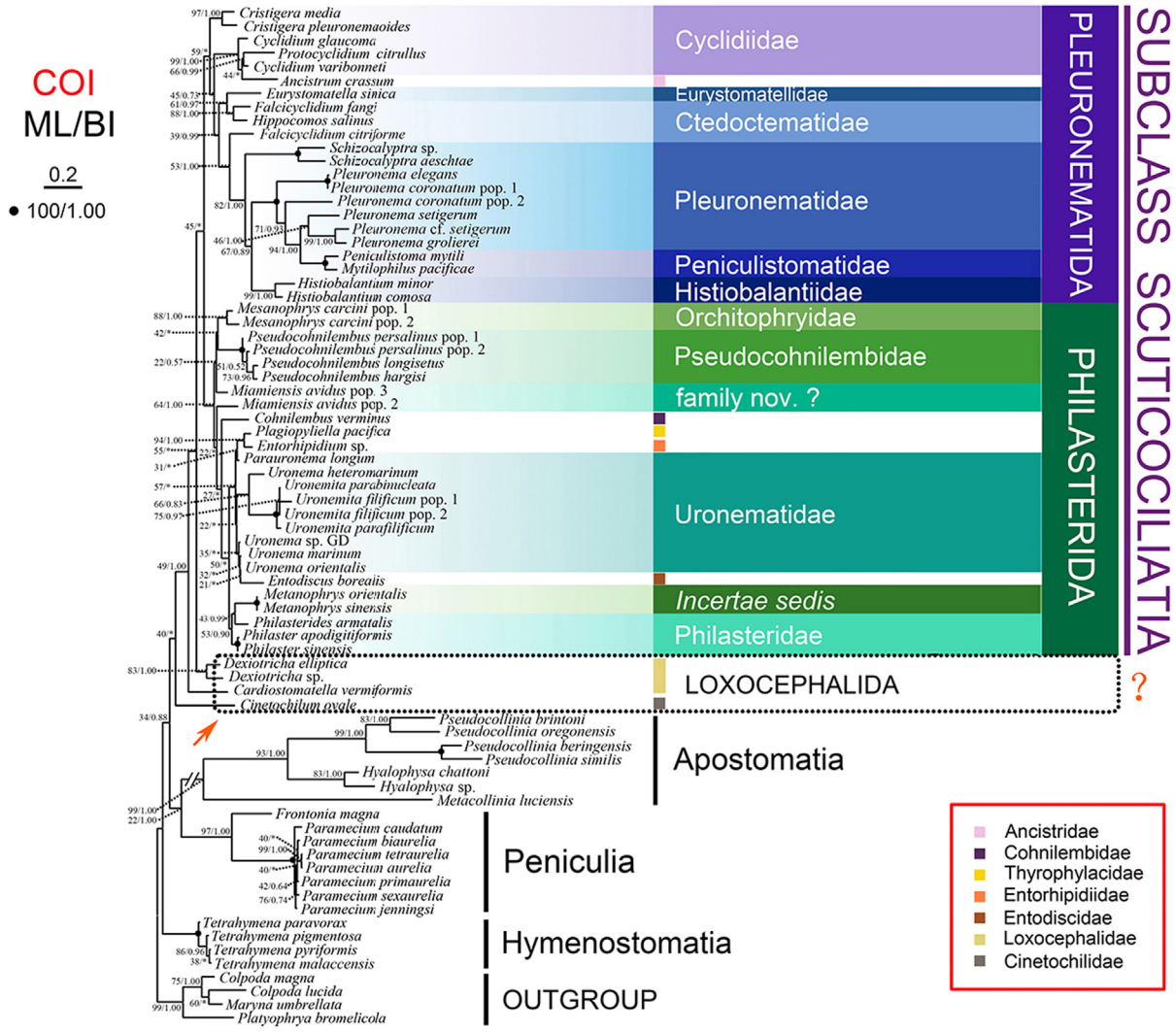

Fig. 2. Maximum likelihood (ML) tree based on the *COI* amino acid sequences focusing on the subclass Scuticociliatia. Numbers at the nodes represent the bootstrap values of ML out of 1000 replicates and the posterior probability values of Bayesian analysis (BI). Asterisks (*) indicate the disagreement between ML and BI. Fully supported (100%/1.00) branches are marked with solid circles. The scale bar corresponds to 20 substitutions per 100 nucleotide positions.

sequences support removing the loxocephalids from Philasterida, but do not support the monophyly of this order, because that one of its members (*Cinetochilum*) always falls with the subclass Apostomatia while others (e.g. *Sathrophilus* and *Pseudoplatynematum*) cluster with the subclass Astomatia (Gao et al., 2013, 2016). In the present study, we provide mitochondrial gene sequences of four loxocephalids and perform the phylogenetic analyses, which again support removing the loxocephalids from Philasterida (Figs. 1 and S1). However, the monophyly of Loxocephalida is not supported based on either nuclear or mitochondrial genes (Figs. 1, 2 and S1). Furthermore, the AU test rejects its monophyly based on concatenated data ($p = 0.025$, Table 3), which indicates that loxocephalids may represent several separate lineages.

According to phylogenomic studies, the subclass Scuticociliatia is most closely related to the subclass Hymenostomatia, compared to Peniculia and Peritrichia (Feng et al., 2015; Gentekaki et al., 2017). Unfortunately, no genomic data is available for the subclasses Astomatia and Apostomatia. However, based on the available nuclear and mitochondrial data, Astomatia and Apostomatia are more closely related to Scuticociliatia, especially with loxocephalids (Fig. 1; Gao et al. 2013). Considering the morphological and morphogenetic features, we think that loxocephalids may represent some divergent lineages occupying the intermediate position between Scuticociliatia and Hymenostomatia, especially closely relate to Astomatia and Apostomatia.

4.3. Phylogeny of the order Pleuronematida and its families

According to Lynn (2008), the order Pleuronematida comprises the ciliates having expansive oral region with paroral often prominently forming a curtain. Previous investigations showed a close relationship between thigmotrichids and pleuronematids based on nSSU-rDNA data (Antipa et al., 2016; Gao et al., 2013). Recently, Gao et al. (2016) incorporated thigmotrichids into the order Pleuronematida. In our phylogenetic trees, the representative of thigmotrichids, *Ancistrum crassum*, groups with Cyclidiidae with high support values in both trees based on *COI* gene and concatenated data (Figs. 1 and 2), and thus supports the idea of assigning thigmotrichids to Pleuronematida. Notably, the order Pleuronematida is monophyletic in the concatenated, nSSU-rDNA and *COI* trees but not in the mtSSU-rDNA tree, although the monophyly is not rejected according to AU test ($p = 0.671$, Table 3) in the latter case. However, only a limited number of mtSSU-rDNA sequences of pleuronematids have been obtained in the present work and more evidence is needed to draw a final conclusion on the phylogeny of the order.

Based on morphology, stomatogenesis, ecological niche, and sexual behavior, the family Peniculistomatidae is a divergent assemblage

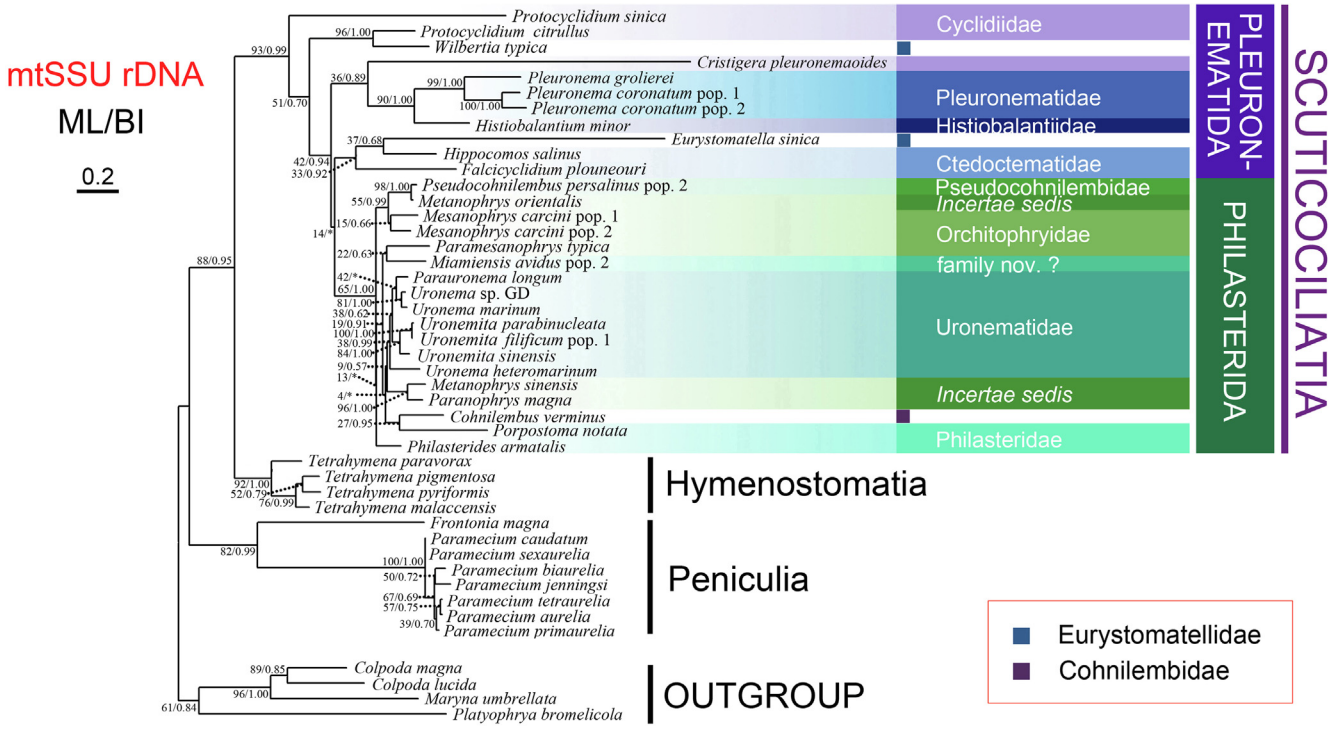

Fig. 3. Maximum likelihood (ML) tree based on the mtSSU-rDNA sequences focusing on the subclass Scuticociliatia. Numbers at the nodes represent the bootstrap values of ML out of 1000 replicates and the posterior probability values of Bayesian analysis (BI). Asterisks (*) indicate the disagreement between ML and BI. Fully supported (100%/1.00) branches are marked with solid circles. The scale bar corresponds to 20 substitutions per 100 nucleotide positions.

within the order Pleuronematida (Antipa et al., 2016). However, phylogenetic analyses inferred from nSSU-rDNA sequences suggest that Peniculistomatidae falls into the clade of *Pleuronema* (Antipa et al., 2016). Our analyses based on *COI* gene and concatenated data indicate a similar result that the genus *Pleuronema* and the family Pleuronematidae are both non-monophyly (Figs. 1 and 2). Additionally, the hypothesis that all isolates of Pleuronematidae cluster together is rejected by the AU test ($p = 3e-004$, Table 3) based on concatenated data; despite that the AU test does not reject the monophyly of *Pleuronema* ($p = 0.567$, Table 3). In fact, the well-developed oral region with long paroral membranes and three distinct membranelles M1-3 of Peniculistomatidae is quite similar to that of *Pleuronema* (Wang et al., 2009). We hypothesize that species in the Peniculistomatidae are more likely evolved from *Pleuronema*-like ancestors due to the results of adaptation to their endocommensal lifestyle in the mantle cavity of bivalve molluscs.

Foissner et al. (2009) argued that the genus *Sulcigera* proposed by Gajewskaja (1928) was possibly based on a misidentified *Histiobalantium* related species based on morphological features. This argument was supported later by Antipa et al. (2016) based on the phylogenetic analyses of nSSU-rDNA, and accordingly they transferred *Sulcigera comosa* to *Histiobalantium* and proposed the combination *H. comosa*. Such assignment and the affinity of the two species were further confirmed by our phylogenetic analyses based on the *COI* gene, in which the newly sequenced *H. minor* groups with *H. comosa* with full support (Fig. 2). Besides, the monophyly of the family Histiobalantiidae and its close relationship with Pleuronematidae is highly supported based on the phylogenetic analyses of concatenated, *COI* and mtSSU-rDNA data (Figs. 1–3).

The family Eurystomatellidae (Miao et al. 2010), comprising the genera *Eurystomatella* and *Wilbertia*, was assigned to the order Pleuronematida. It was characterized by an almost completely circular paroral membrane and showed a close relationship to the family Ctedoctematidae (Gao et al., 2013; Miao et al., 2010). In the present study, Eurystomatellidae is monophyletic based on the concatenated data (Fig. 1). However, according to mtSSU-rDNA data, the family is surprisingly non-monophyletic because *Wilbertia* forms a well-supported branch with a member of Cyclidiidae, *Protocyclidium citrullus*, while *Eurystomatella* groups with taxa of the family Ctedoctematidae (Fig. 3). In addition, the AU test ($p = 0.009$, Table 3) rejects the monophyly of Eurystomatellidae based on mtSSU-rDNA data. Gao et al. (2014) revealed that *Wilbertia* shares similar nLSU-rDNA sequence with another member of Cyclidiidae, *Cyclidium varibonneti*. Therefore, it is possible that *Wilbertia* might have some relationship with the family Cyclidiidae and thus the similar morphology of *Wilbertia* and *Eurystomatella* might be due to convergent evolution. However, more evidence from other genes might help to solve this confusion.

4.4. Phylogenetic relationships within the order Philasterida

The order Philasterida is a well-outlined lineage in both the phylogenies based on concatenated and mitochondrial data, however, the relationships among most families of the Philasterida remain unresolved (Figs. 1–3). For example, the genera *Mesanophrys*, *Metanophrys*, *Paranophrys* and *Paramesanophrys* were assigned to the family Orchitophryidae based mainly on morphological features such as the oral region in anterior 1/3 to 1/2 of body and scutica aligned along midventral postoral region (Lynn, 2008). They are opportunistic parasites that share general body shape (cylindrical with a pointed anterior end but no apical plate) and buccal ciliature (as reviewed in Pan et al. (2016)). The former three genera differ from each other mainly in terms of the relative location of paroral membrane to membranelle 2; while *Paramesanophrys* differs from the other three genera in that the paroral membrane extends anteriorly to the posterior end of membranelle 3 (Pan et al., 2016). However, the species in the family Orchitophryidae do not form a monophyletic clade in the phylogeny based on either the concatenated or the mitochondrial data, and even the congeners may not cluster together, e.g. two species of *Metanophrys* (Figs. 1 and 3).

Gao et al. (2012) proposed the separated clade containing *Metanophrys* and *Paranophrys* as *incertae sedis* at the familial level based on

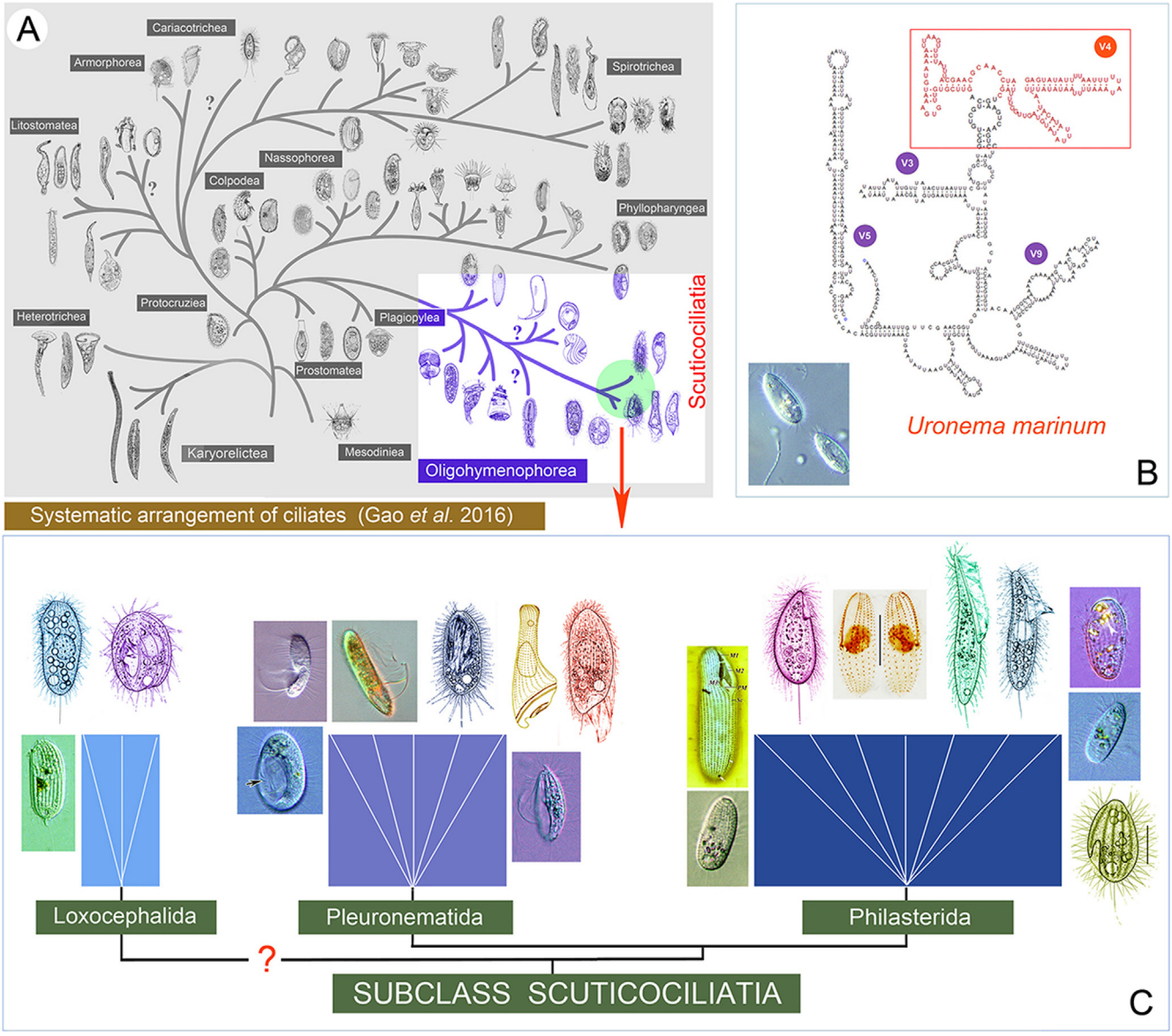

Fig. 4. Systematic relationships and secondary structure of the subclass Scuticociliatia. (A) Position of the scuticociliate groups in the phylogenetic tree (Gao et al., 2016). (B) Secondary structure of mtSSU-rRNA of the representative species, *Uronema marinum* (GenBank: MH577577). The variable region 4 (V4) is marked in red. (C) Morphological information of representatives of three orders. The question mark indicates the uncertain status of the order Loxocephalida. Illustrations are original from authors' group or from Liu et al. (2016); Miao et al. (2010); Pan et al. (2013, 2015); Song et al. (2009). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nuclear genes. Present study further confirmed the previous idea on the familial assignment of the two genera, since they cluster with different families of Philasterida in analyses using different data types: with Uronematidae (mtSSU rDNA), with Philasteridae (*COI*) and with Pseudocohnilembidae (concatenated data). It is worth noting that, no morphological features were found to explain the systematic position of *Metanophrys* and *Paranophrys*.

Despite that mitochondrial DNA is better to resolve the relationships of closely related ciliate taxa, the relationships within philasterids cannot be resolved well. One reason could be that *COI* and mtSSU rDNA data are not well sampled compared with nuclear gene data. However, more importantly, the morphological features used to identify philasterids are far too few, mainly depending on the buccal structures (Fan et al., 2017). It is hard to distinguish plesiomorphic and apomorphic characters. Moreover, most philasterids are opportunistic parasites, whose morphology could be highly variable or evolved convergently (Song et al., 2003). Therefore, one should take great care to identify taxa and to critically evaluate and interpret the discordances between molecular and morphological data of philasterids.

4.5. Controversial species of Miamiensis avidus and Philasterides dicentrarchi

The species delimitation of *Miamiensis avidus* and *Philasterides dicentrarchi* has been a *controversial issue for a long time*. *Miamiensis avidus* was originally described by Thompson and Moewus (1964), and *Philasterides dicentrarchi* was firstly reported by Dragesco et al. (1995). Both species have been reported for many times after they were firstly described (e.g. Jung et al. 2007; Gao et al. 2010). However, due to their extremely similar morphological characters (e.g. body size, buccal apparatus, number of somatic kineties), *P. dicentrarchi* is considered as a junior synonym of *M. avidus* (e.g. Song and Wilbert, 2000; Jung et al. 2007; Gao et al. 2010). Recently, the synonymy/conspecificity of the two taxa was rejected (De Felipe et al., 2017), mainly because that: (1)

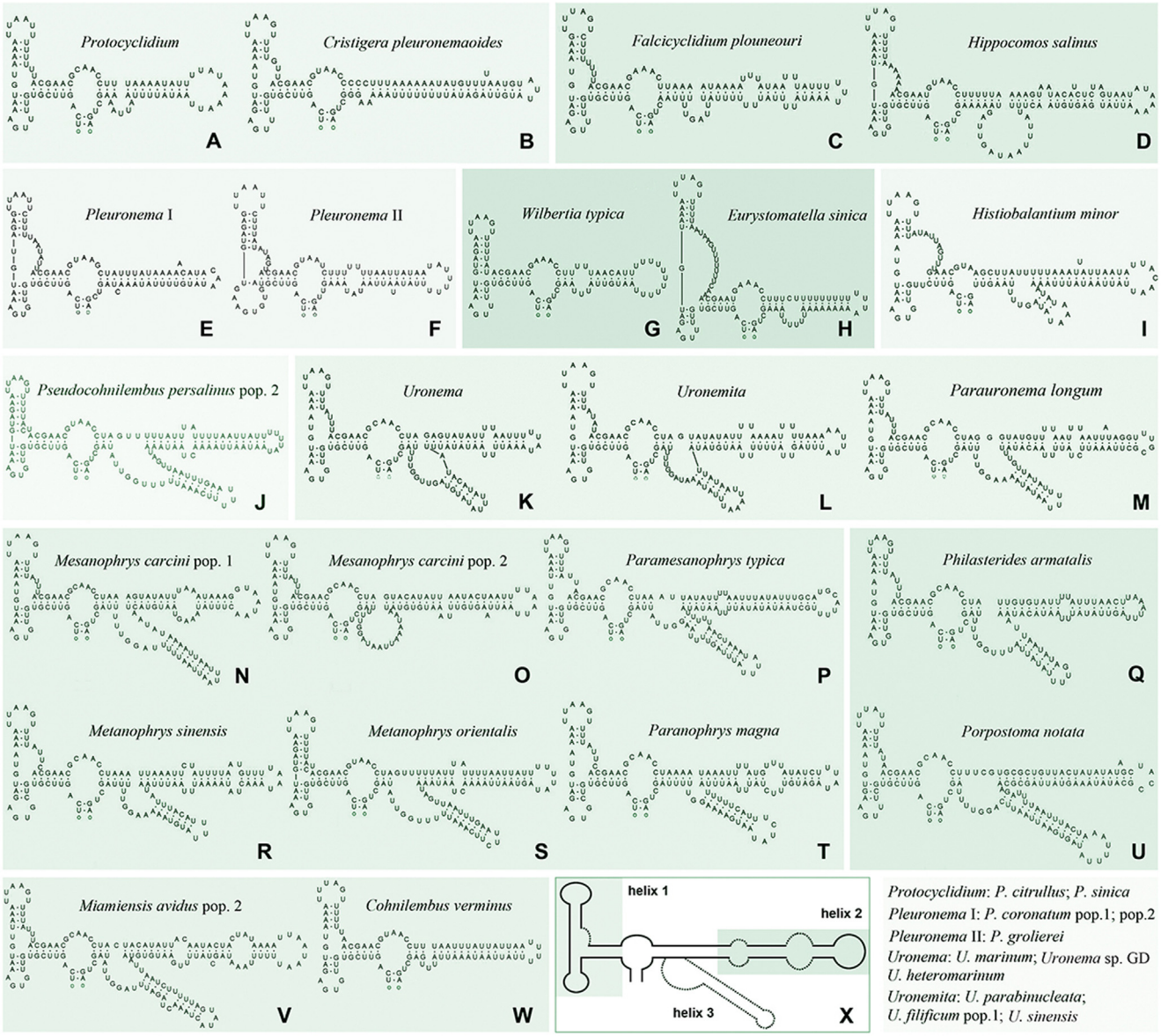

Fig. 5. Secondary structures of the variable region 4 (V4) of the mtSSU-rRNA of the order Pleuronematida (A–I) and Philasterida (J–W). The conserved structure model (X) is based on the structures of A–W and contains helix 1, 2 and 3.

Table 3
Approximately unbiased (AU) test results based on concatenated or mtSSU-rDNA (marked with *) data.

Topology constraints	AU value (*p*)
Loxocephalida	0.025
Pleuronematida*	0.671
Pleuronematidae	3e−004
Pleuronema	0.567
Eurystomatellidae*	0.009

$P < 0.05$ indicates the monophyly is rejected; $p > 0.05$ indicates the monophyly is not rejected.

M. avidus has a life cycle with three forms (macrostome, microstome and tomite), while *P. dicentrarchi* only has two forms (microstome and tomite); (2) *M. avidus* has a single continuous paroral membrane, while *P. dicentrarchi* has two clearly separated paroral membranes; (3) significant differences in the nSSU rDNA (3–4%), α-tubulin (11–19%) and β-tubulin gene sequences (15%).

However, whether the paroral membrane is continuous or separated could be variable within one population as stated in previous studies (Song and Wilbert, 2000; Thompson and Moewus, 1964) or observer dependent. Moreover, the different forms are morphologically distinct feeding stages, therefore, the transformations between different forms are induced by food or prey (Gómez-Saladin and Small, 1993). If the species cannot be cultured or the conditions are not appropriate, it will be failed to observe all the different forms. Therefore, it is not a consistent character for species identification. So far, *P. dicentrarchi* and *M. avidus* cannot be clearly and easily separated at the morphological level. Despite that they cannot be separated well based on morphological data, they are significantly differed at the molecular level. Based on previous studies in the nSSU rDNA, α-tubulin and β-tubulin gene sequences (De Felipe et al., 2017) as well as *COI* gene in the present study, the *P. dicentrarchi* and *M. avidus* complex can be divided into three groups, *M. avidus* pop.1, *M. avidus* pop.2 and all the other strains (Fig. S2). It indicates that the complex contains at least three cryptic species.

4.6. Secondary structure of the mtSSU-rRNA gene sequence

Given that the secondary structure of nSSU-rRNA variable regions is

more conserved than the primary nucleotide sequence, Wang et al. (2015) indicated that secondary structure information should be utilized in phylogenetic analyses. Recently, the secondary structures of mtSSU-rRNA V4 region were first applied in phylogenetic analyses and can play important roles in discrimination of closely related species within the class Phyllopharyngea (Wang et al., 2017). For the scuticociliates, analyses of the secondary structures of ITS2 and nSSU-rRNA V4 regions were applied in its phylogenetic studies, which revealed that ITS2 structures are conserved at the order level while nSSU-rRNA V4 structures are more variable (Gao et al., 2012, 2013, 2014). In the present study, we predict and compare the secondary structures of mtSSU-rRNA V4 region in scuticociliates, which indicates that the absence or presence of helix 3 can be generally used to discriminate the orders Pleuronematida and Philasterida. At the species or genus level, the structures are much more conserved compared to the highly variable primary nucleotide sequence and are in accordance with the phylogenetic results. For example, *Uronema* spp., *Uronemita* spp., and *Parauronema longum*, which form one clade, share similar mtSSU-rRNA V4 structures. However, for some groups, the secondary structure of mtSSU-rRNA V4 region could contribute to distinguish intraspecific and interspecific taxa. For instance, the structures between the two populations of *Mesanophrys carcini* differ in helix 2 and 3. They differ each other in both nuclear and mitochondrial genes (nSSU-rDNA: 65 bp; mtSSU-rDNA: 81 bp; *COI* nucleotide sequence: 110 bp), indicating that there might be cryptic species in *M. carcini*.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106565.

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