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Insights into the phylogenetic and taxonomy of philasterid ciliates (Protozoa, Ciliophora, Scuticociliatia) based on analyses of multiple molecular markers

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ABSTRACT

Scuticociliates are a rich assemblage of species with mostly unresolved phylogenetic relationships, especially in the order Philasterida. In the present work, 48 new sequences for three linked genes are characterized and phylogenetic trees are constructed to assess the inter- and intra-generic relationships of philasterids. Results reveal the following: (1) the combined three-gene tree provides more resolution in nodes than in the SSU-rDNA topologies; (2) the family Orchitophryidae is non-monophyletic as it is split into two parts and *Paranophrys magna*, *Metanophrys* sp. and *Metanophrys sinensis* are designated *incertae sedis* at the familial level; (3) Uronematidae is non-monophyletic and *Homalogastra setosa* is designated *incertae sedis*; (4) Parauronematidae becomes a junior synonym of Uronematidae and the clade containing *A. haemophila*, *Miamiensis avidus*, and *Glauconema trihymene* might stand for a new family; (5) *Parauronema* being a junior synonym of *Uronema* is supported and *P. longum* should be removed from the genus *Parauronema*; (6) *Uronema* is not monophyletic and molecular analyses reveal that *Uronema* sp. QD shares a more recent common ancestor with *Uronemella* species than with other *Uronema* species; (7) *Metanophrys* is polyphyletic; (8) multiple samples of two highly controversial species, viz., *Mesanophrys pugettensis* and *M. chesapeakensis* have identical ITS1-5.8S-ITS2 region sequence and we propose they should be synonymous with *M. carcini*, and (9) there may be cryptic species in *M. carcini and M. avidus*.

1. Introduction

Scuticociliates are a rich assemblage of species that is generally small in size, share a basic pattern of silverline system and infraciliature, and show similar characters *in vivo* (Fan et al., 2010, 2011a,b; Lobban et al., 2011; Small, 1967; Song et al., 2003). There is considerable variation in classifications of the ciliates within the subclass Scuticociliatia. This is because classifications of scuticociliates have been largely dependent on the infraciliature, mainly the structure of buccal apparatus and general appearance in morphology, and only a few diagnostic characteristics in the scuticociliates can be used to define taxa (Fig. 1; Corliss, 1979; Lynn, 2008; Lynn and Small, 2002).

Philasterida, which is comprised of over 70 genera, is the most controversial group of scuticociliates. In Corliss's revision based on infraciliature, Philasterida was divided into 12 families (Corliss, 1979) while in Lynn's revision it was divided into 16 families (Lynn, 2008). In order to investigate further the evolutionary relationships among the philasterids, molecular phylogenetic analyses based on SSU-rDNA sequences have been increasingly used in recent few years (Foissner et al., 2009; Gao et al., 2010; Li et al., 2010; Miao et al., 2009; Yi et al., 2009; Zhang et al., 2010a, 2011). However, the relationships among most families of the Philasterida remain unresolved as molecular and morphological interpretations conflict: ciliates assigned to the same family with similar oral structures are in separated molecular clades and ciliates assigned to different families with different oral features sometimes fall in the same molecular clade (Lynn and Strüder-Kypke, 2005). Therefore, greater taxon sampling as well as additional molecular markers are needed to provide a better resolution of the phylogenetic positions of scuticociliates.

Due to the poor resolution of phylogenies based on single genes, multigene analyses are proving useful in inferring better-resolved phylogenies within ciliates (Hewitt et al., 2003; Snoeyenbos-West et al., 2002; Yi et al., 2011). Considering the advantage of being accommodated by a single model of sequence evolution for a combined analysis, the linked loci ITS1-5.8S-ITS2 region, LSU-rDNA and SSU-rDNA can be combined to perform multi-gene analyses. Such combined phylogenetic analyses within scuticociliates are relatively rare and the ITS1-5.8S-ITS2 region analyses have been reported only twice (Goggin and Murphy, 2000; Miao et al., 2008) while the LSU-rDNA locus has never been used for this group.

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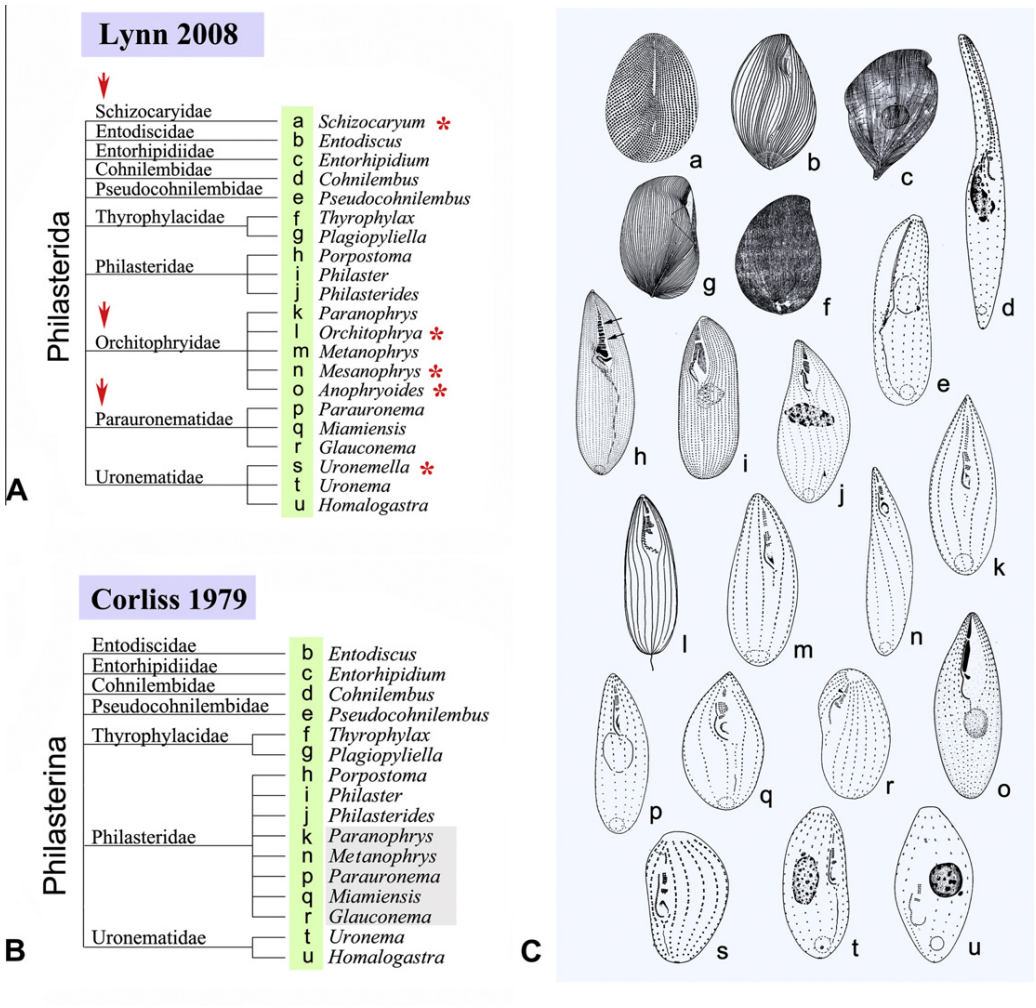

Fig. 1. Classifications of philasterid ciliates. (A) Classification scheme of Lynn (2008); (B) Classification scheme of Corliss (1979); (C) Infraciliature of the philasterid genera appeared in the present work (a, from Berger (1961); b and c, from Profant (1965); d, e, h, j, k, m, n, p, q, r, s, t, from Song et al. (2009); f, from Lynn and Berger (1973); g, from Lynn and Berger (1972); i, from Miao et al. (2009); l, from Bouland et al. (1987); o, from De Puytorac and Grolière (1979); u, from Buitkamp (1977)). Arrows in Fig. A indicate the newly suggested families in Lynn's system, while additional genera marked with "*". Note that the family assignment of five genera in Corliss' system (Fig. B, marked in gray) was changed in Lynn's arrangement.

Based on the background above, we have substantially improved the taxonomic sampling of SSU-rDNA, ITS1-5.8S-ITS2 region and LSU-rDNA, providing 48 new sequences, enabling us to include 19 philasterids on LSU trees for the first time. This study also represents the first attempt to reconstruct generic level relationships within Philasterida with molecular characters from multiple genes. Moreover, morphological data are discussed in light of the molecular phylogenetic analysis to elucidate evolutionary history of philasterid ciliates.

2. Materials and methods

2.1. Ciliate collection and identification

Nineteen taxa in this study were selected to represent the morphological diversity of Philasterida (Table 1). Microscopical observations and silver impregnations were performed according to Wilbert (1975). Terminology and systematics follow Lynn (2008).

2.2. DNA extraction and gene sequencing

Total genomic DNA was extracted from cells using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) as described by Zhang et al. (2010b). The PCR amplifications of SSU-rDNA were performed with the universal primers (Medlin et al., 1988). A fragment of approximately 500 bp containing the ITS1, 5.8S ribosomal gene, and ITS2 was amplified using primers ITS-F (5′-GTA GGT GAA CCT GCG GAA GGA TCA TTA-3′) and ITS-R (5′-TAC TGA TAT GCT TAA GTT CAG CGG-3′) (Shang, 2004). The PCR amplifications of part of the LSU-rDNA (ca. 1800 bp) were amplified using modified primers 28S-1F (5′-ACC/G CGC TGA/G AT/CT TAA GCA T-3′) and 28S-3R (5′-AAC CTT GGA GAC CTG AT-3′) from Moreira et al. (2007). Sometimes, ITS1-5.8S-ITS2 and partial LSU-rRNA gene were amplified together using the primers ITS-F and 28S-3R. Cycling parameters for PCR amplifications were as follows: 5 min initial denaturation at 94 °C; 40 cycles of 30s at 95 °C, 1 min at 56–60 °C, and 1–2 min at 72 °C; with a final extension of 10 min at 72 °C. Purified PCR product of the appropriate size was inserted into the pMD™18-T vector (Takara Biotechnology, Dalian Co., Ltd.) and sequenced on an ABI-PRISM 3730 automatic sequencer (Applied Biosystems).

2.3. Dataset assembly and alignments

Newly-characterized sequences were combined with relevant sequences obtained from the NCBI GenBank (Table 1). Seven

Table 1
List of philasterid species for which SSU-rDNA, ITS1-5.8S-ITS2 region and LSU-rDNA were newly sequenced in the present work.

Species	Collection site	SSU-rDNA		ITS1-5.8S-ITS2		LSU-rDNA	
		Accession number	Length (bp)	Accession number	Length (bp)	Accession number	Length (bp)
Cohnilembus verminus	Gao et al. (2012)	–	–	JN885093	555	JN885111	1869
Mesanophrys carcini GD	Clear Water Bay, Hong Kong	JN885086	1701	JN885104	538	JN885113	1871
Mesanophrys carcini QD	Jiaozhou Bay, Qingdao	JN885085	1756	JN885094	541	JN885112	1870
Metanophrys sinensis	Gao et al. (2012)	–	–	JN885092	593	JN885114	1872
Metanophrys sp. QD	Jiaozhou Bay, Qingdao	JN885084	1735	JN885110	565	JN885129	1864
Miamiensis sp. QD	Mariculture pond, Weifang	JN885091	1760	JN885095	554	JN885115	1866
Paranophrys magna QD	Mariculture pond, Weifang	JN885089	1756	JN885105	548	JN885116	1862
Parauronema cf. *virginianum* QD	Jiaozhou Bay, Qingdao	JN885082	1754	JN885106	536	JN885117	1856
Parauronema longum	Gao et al. (2012)	–	–	JN885096	540	JN885118	1862
Parauronema virginianum QD	Jiaozhou Bay, Qingdao	JN885087	1758	JN885109	536	JN885128	1856
Philaster apodigitiformis	Miao et al. (2009)	–	–	JN885097	520	JN885119	1863
Philasterides armatalis	Gao et al. (2012)	–	–	JN885098	521	JN885120	1865
Porpostoma notata	Gao et al. (2012)	–	–	JN885099	532	JN885121	1862
Pseudocohnilembus hargisi GD	Nansan Island, Zhanjiang	JN885090	1648	JN885100	472	JN885122	1822
Uronema heteromarinum	Gao et al. (2012)	–	–	JN885101	466	JN885123	1847
Uronema marinum	Pan et al. (2010)	–	–	JN885102	536	JN885124	1856
Uronema sp. GD	Daya Bay, Guangdong	JN885088	1758	JN885107	532	JN885125	1832
Uronema sp. QD	Jiaozhou Bay, Qingdao	JN885083	1753	JN885108	528	JN885126	1863
Uronemella parafilificum	Pan et al. (2010)	–	–	JN885103	534	JN885127	1863

Table 2
Dataset assembled in the present analyses.

Dataset	No. of taxa	Description
1	69	SSU-rDNA sequences including all available scuticociliates
2	39	ITS1-5.8S-ITS2 sequences including all available scuticociliates
3	24	LSU-rDNA sequences including all available scuticociliates
4	19	Concatenation of the aligned SSU-rDNA, ITS1-5.8S-ITS2, and LSU-rDNA sequences including all available philasterid species
5	19	SSU-rDNA sequences in Dataset 4
6	19	ITS1-5.8S-ITS2 sequences in Dataset 4
7	19	LSU-rDNA sequences in Dataset 4

datasets were evaluated in the present analyses (Table 2). Sequences of Dataset 1 were aligned using Hmmer Package version 2.3.2. In summary, the secondary structure-based SSU-rDNA sequence alignment of oligohymenophoreans downloaded from the European Ribosomal Database (Wuyts et al., 2002) was used as the "seed" alignment to build a profile hidden Markov model (HMM) using Hmmer Package version 2.3.2. The resulting HMM profile was then used to create an alignment of the 69 sequences using Hmmalign within the package. The resulting alignment was further modified manually using BioEdit 7.0.0 (Hall, 1999) and 1677 characters were used for subsequent phylogenetic analyses. Sequences of Datasets 2, 3, 5, 6, and 7 were aligned using Muscle v3.7 with default parameters (Dereeper et al., 2010, 2008; Edgar, 2004). Resulting alignments were further edited by eye using BioEdit 7.0.0. The final alignments of Datasets 2–7 were 631, 1931, 4128, 1666, 571, 1891 positions in length, respectively.

2.4. Phylogenetic analyses

Bayesian inference (BI) analyses were performed with MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) using the GTR + I + G model selected by MrModeltest v.2.2 (Nylander, 2004) according to the AIC criterion. Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains using the default settings: chain length 1,000,000–3,000,000 generations, with trees sampled every 100 generations. The first 25% of sampled trees were discarded as burn-in. All remaining trees were used to calculate posterior probabilities (PPs) using a majority rule consensus. Maximum likelihood (ML) trees were constructed with PhyML v.2.4.4 (Guindon and Gascuel, 2003) using the best model according to the AIC criterion selected by Modeltest v.3.4 (Posada and Crandall, 1998). The reliability of internal branches was assessed using nonparametric bootstrapping with 1000 replicates. Phylogenetic trees were visualized with TreeView v.1.6.6 (Page, 1996) and MEGA v.4 (Tamura et al., 2007).

2.5. Topology testing

To test the monophyly of the focal group against competing phylogenetic hypotheses, the Approximately Unbiased (AU) tests was used (Shimodaira, 2002). ML trees were generated with a constraint block, enforcing the monophyletic constraint of the respective focus group in PAUP (Swofford, 2002), under the same model as estimating the global ML tree. The three best scoring trees (i.e. with lowest-Ln likelihood score) that met the constraint of each alternative hypothesis were used for comparison (Table 3). The site-wise likelihoods were calculated for each tree topology using PAUPUP (Calendini and Martin, 2005) interface relying on PAUP* DOS version and were then subjected to the AU test (Shimodaira, 2002) as implemented in Consel (Shimodaira and Hasegawa, 2001).

2.6. Predicting secondary structures of ITS2

Consensus structures of ITS2 region 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

Table 3
Approximately unbiased test results. *P* values >0.05 are highlighted in gray.

Topology constraints	Dataset 1		Dataset 2		Dataset 3		Dataset 4	
	Ln likelihood	AU (p)	Ln likelihood	AU (p)	Ln likelihood	AU (p)	Ln likelihood	AU (p)
Unconstrained	18586.22252	1.000	7614.36233	0.975	13646.58972	0.890	21198.33936	0.784
Parauronema spp.	18966.31677	2e−009	7641.90186	0.304	14120.31991	3e−005	21915.83679	8e−007
The second best tree	18977.40293	3e−005	7642.42076	0.185	14120.96023	2e−005	21917.68082	2e−008
The third best tree	18993.04726	2e−031	7642.42076	0.144	14123.68109	1e−005	21918.36738	3e−009
Uronema spp.	18929.40597	5e−006	7673.71103	0.001	14157.47251	4e−006	21961.83379	6e−059
The second best tree	18933.18654	2e−005	7676.99328	0.004	14159.87515	1e−005	21961.83379	6e−059
The third best tree	18939.69922	1e−071	7684.51548	0.007	14163.29934	6e−006	21961.83379	6e−059
Metanophrys spp.	18738.48037	4e−006	7689.06064	0.001	–	–	21205.29649	0.566
The second best tree	18744.08789	2e−042	7689.12249	5e−004	–	–	21208.07772	0.349
The third best tree	18754.70852	3e−045	7693.73942	0.001	–	–	21208.37366	0.404
Uronematidae	18992.88816	1e−046	7715.61279	7e−006	14115.03446	0.001	21908.70432	6e−083
The second best tree	18995.44445	3e−060	7721.45455	0.001	14115.04482	0.001	21909.79918	4e−084
The third best tree	18995.44445	7e−062	7729.86322	4e−008	14115.61929	0.001	21909.79918	4e−084
Parauronematidae	19126.81379	7e−014	7699.74550	4e−004	14180.50216	2e−006	22062.66040	3e−006
The second best tree	19128.89362	6e−054	7703.46505	3e−019	14181.52245	8e−005	22062.66040	3e−006
The third best tree	19137.40024	4e−008	7708.89556	0.001	14183.36702	4e−006	22062.83951	7e−008
Orchitophryidae	18683.81538	0.001	7664.50717	0.024	13655.40522	0.294	21230.95705	0.003
The second best tree	18686.86611	0.001	7665.03129	0.020	13657.86080	0.146	21231.22243	0.006
The third best tree	18688.32657	0.002	7665.58565	0.020	13659.05170	0.240	21231.22243	0.006

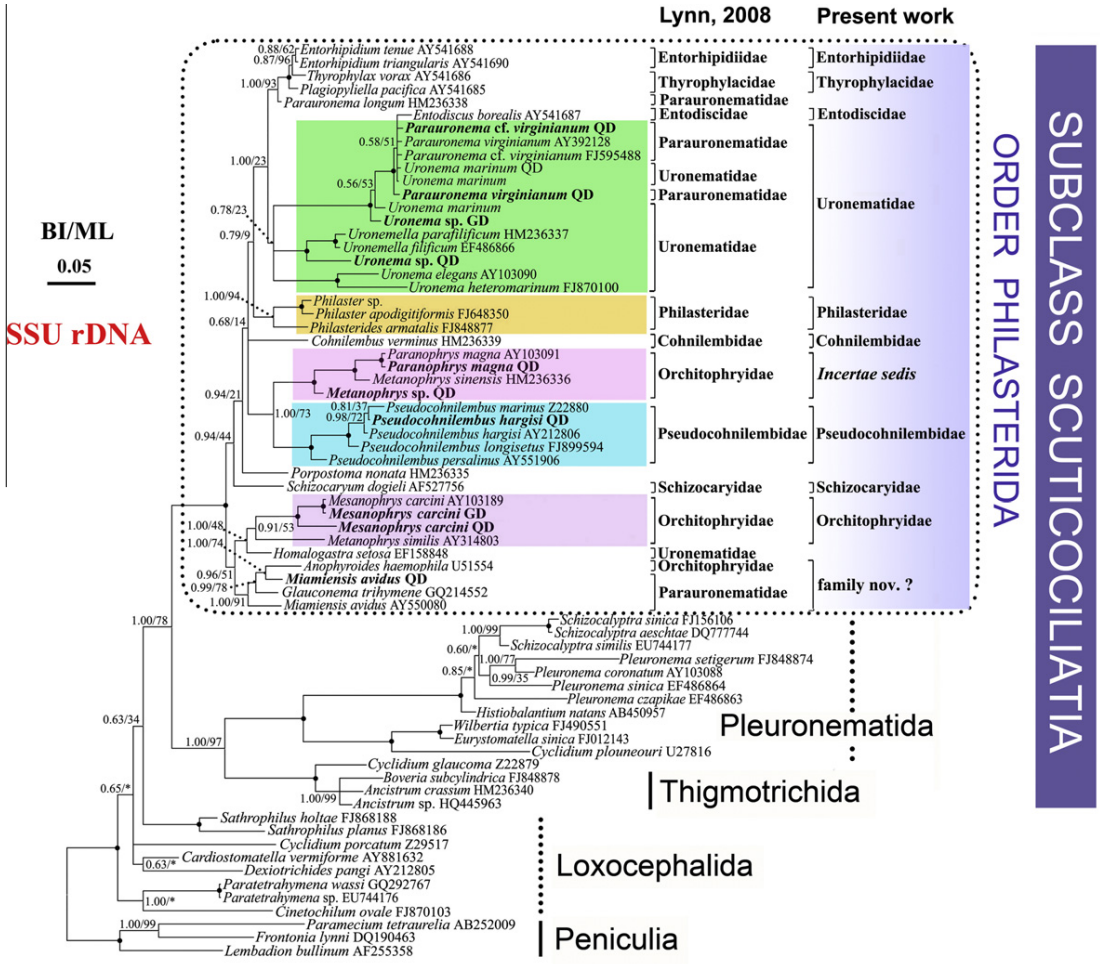

Fig. 2. Phylogeny of the subclass Scuticociliatia inferred by Bayesian analysis of SSU-rDNA sequences. Numbers at nodes represent the posterior probability of Bayesian analysis and the bootstrap values of maximum likelihood out of 1000 replicates. Fully supported (1.00/100%) branches are marked with solid circles. Asterisks (*) indicate the disagreement between Bayesian and ML. The scale bar corresponds to five substitutions per 100 nucleotide positions. Newly sequenced species in this work are in bold.

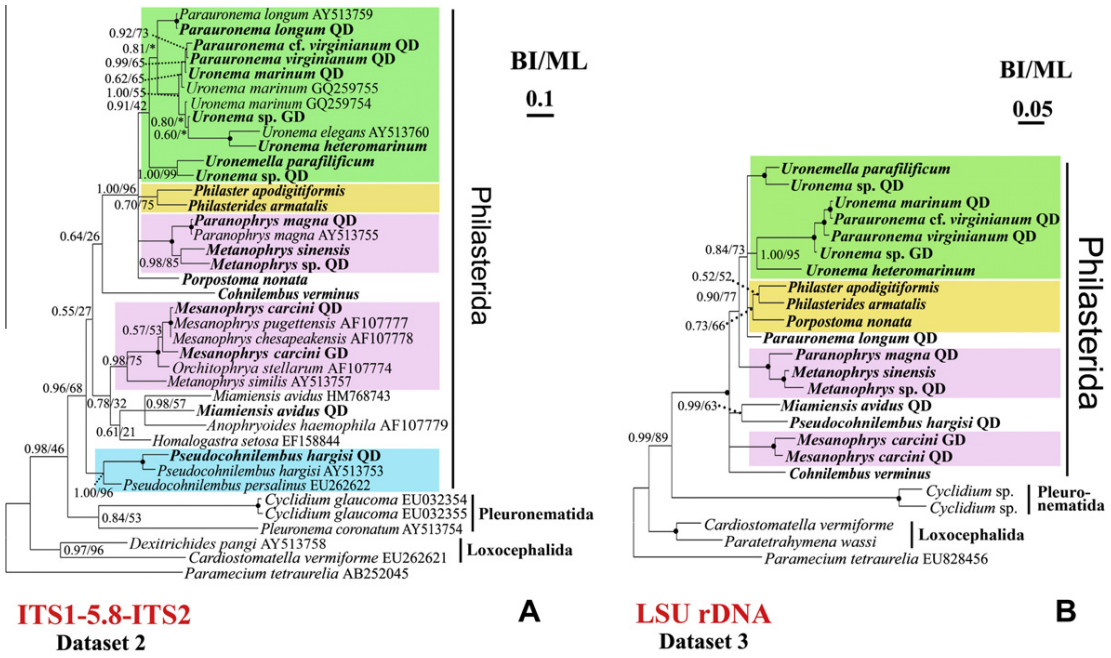

Fig. 3. Phylogeny of the subclass Scuticociliatia inferred by Bayesian analysis of ITS1-5.8S-ITS2 region sequences (A) and LSU-rDNA sequences (B). Numbers at nodes represent the posterior probability of Bayesian analysis and the bootstrap values of maximum likelihood out of 1000 replicates. Fully supported (1.00/100%) branches are marked with solid circles. Asterisks (*) indicate the disagreement between Bayesian and ML. The scale bar corresponds to 10/5 substitutions per 100 nucleotide positions. Newly sequenced species in this work are in bold.

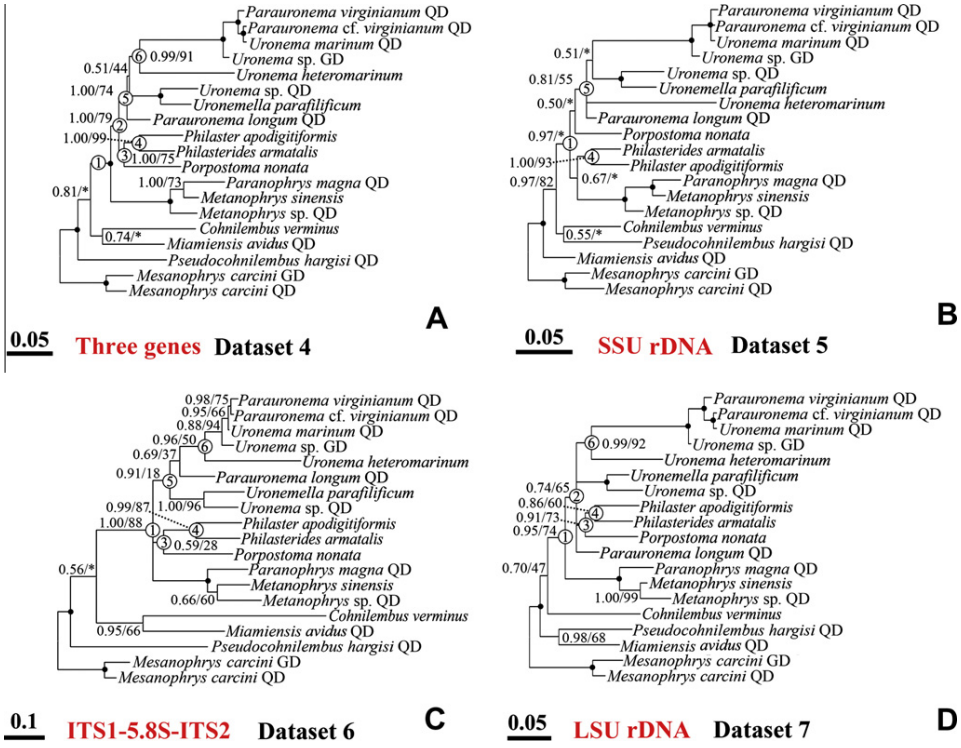

Fig. 4. Phylogeny of the 19 philasterids inferred by Bayesian analysis. Numbers at nodes represent the posterior probability of Bayesian analysis and the bootstrap values of maximum likelihood out of 1000 replicates. Fully supported (1.00/100%) branches are marked with solid circles. Asterisks (*) indicate the disagreement between Bayesian and ML. The scale bar corresponds to 10/5 substitutions per 100 nucleotide positions. Circled numbers in B–D refer to node numbers corresponding to six nodes with high support in A in order to evaluate the influence of combining genes on nodal support (Table 4).

structures, the secondary structures of ITS2 sequences were predicted with Mfold version 3.2 (http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003) using default settings. Results for the various species were compared to reveal the folding pattern common

Table 4
Bootstrap support to nodes in Fig. 4.

Nodes	Three genes	SSU-rDNA	ITS1-5.8S-ITS2	LSU-rDNA
1	1.00/100	0.97/*	1.00/88	0.95/74
2	1.00/79	*/*	*/*	0.74/65
3	1.00/75	*/*	0.59/28	0.91/73
4	1.00/99	1.00/93	0.99/87	0.86/60
5	1.00/74	0.81/55	0.91/18	*/*
6	0.99/91	*/*	0.96/50	0.99/92
Alteration over all nodes		−0.21/−25	−0.54/−168	−0.54/−80

to them all, which in turn, established the conserved structural models for philasterids. The structures were edited for esthetic purposes with RnaViz 2.0 (Rijk and Wachter, 1997).

3. Results

3.1. Sequences deposition and analyses

A total of 10 SSU-rDNA, 19 ITS1-5.8S-ITS2 regions, and 19 LSU-rDNA sequenced in our analyses have been deposited in the GenBank data base (Table 1). The nucleotide sequences of SSU-rDNA, ITS1-5.8S-ITS2 region and LSU-rDNA among 19 philasterids share identities of 83.9–99.3% (avg. 90.77%), 58.5–99.3% (avg. 73.61%), and 82.1–99.4% (avg. 88.21%), respectively (Supplementary Tables S1 and S2). Despite of the low sequence identities of ITS1-5.8S-ITS2 region, the identities of 5.8S-rDNA is very high (93.2–100.0%, avg. 97.24%), while the identities of ITS2 region is very low (54.2–99.1, avg. 76.20%) and ITS1 even lower (23.6–99.2, avg. 50.98%; Supplementary Tables S2 and S3).

3.2. Phylogenetic analyses based on Dataset 1 (SSU-rDNA, 69 taxa)

All available SSU-rDNA sequences of scuticociliates are included in our phylogenetic analyses. The resulting topologies of the phylogenetic trees generated using two algorithms (MrBayes and PhyML) are generally concordant; therefore, only one topology is presented with support values from both analyses indicated on branches (Fig. 2). The order Philasterida is a well defined monophyletic group (1.00 BI, 78% ML) and is sister to the clade containing thigmotrichids and pleuronematids. Within the order Philasterida, all 20 available genera representing 10 families are included (Figs. 1 and 2). *Parauronema longum* clusters with the clade formed by Entorhipidiidae and Thyrophylacidae (1.00 BI, 93% ML; Fig. 2). Except for *P. longum*, other species of *Parauronema*, *Uronema* and *Uronemella* form one clade with low support (0.78 BI, 23% ML); this clade also contains *Entodiscus borealis*. Two *Philaster* species cluster with *Philasterides armatalis* with high support (1.00 BI, 94% ML). Five species of *Pseudocohnilembus* form a fully supported monophyletic group. The family Orchitophryidae is divided into three parts: (1) *Paranophrys magna*, *Metanophrys sinensis* and *Metanophrys* sp. QD forming a fully supported clade which is sister to the clade of *Pseudocohnilembus*, (2) *Mesanophrys carcini* and *Metanophrys similis* forming a poorly supported (0.91 BI, 53% ML) clade which then groups with *Homalogastra*, and (3) *Anophryoides haemophila* grouping with *Miamiensis* spp. and *Glauconema trihymene*. *Cohnilembus verminus*, *Porpostoma notata*, and *Schizocaryum dogieli* each branches separately.

3.3. Phylogenetic analyses based on Dataset 2 (ITS1-5.8S-ITS2, 39 Taxa) and Dataset 3 (LSU-rDNA, 24 Taxa)

Although the ITS1-5.8S-ITS2 and LSU-rDNA trees have fewer species than the SSU RNA trees, analyses of these loci show similar results compared to SSU-rDNA trees (Fig. 3). The differences are that in LSU-rDNA trees *Porpostoma notata* clusters with the clade of *Philaster apodigitiformis* and *Philasterides armatalis* with low support (0.73 BI, 66% ML), which in turn falls in the group of *Parauronema* species, *Uronema* species and *Uronemella* species with moderate support (0.84 BI, 73% ML).

3.4. Comparison of phylogenetic analyses based on 19-taxa datasets (Datasets 4–7, Table 2)

Given the overall congruence between the topologies, a concatenated alignment of the SSU-rDNA, ITS1-5.8S-ITS2 region, and LSU-rDNA sequences is compiled to evaluate further phylogenetic relationships in philasterids. The topologies of the combined three-gene trees with the 19 taxa are basically consistent with the topologies inferred from the single-gene analyses, and the combined data provides better support for relationships than the single-gene analyses (Fig. 4, Table 4): (1) In the phylogenetic trees based on Datasets 4–6, all the *Parauronema*, *Uronema* and *Uronemella* species form a monophyletic group with variable support (1.00 BI, 74% ML; 0.81 BI, 55% ML; 0.91 BI, 18% ML), while they form a polytomy with the clade of *Philaster apodigitiformis*, *Philasterides armatalis*, and *Porpostoma notata* in the phylogenetic trees based on Datasets 7 (0.74 BI, 65% ML); (2) *P. apodigitiformis*, *P. armatalis*, and *P. notata* cluster together with variable support (1.00 BI, 75% ML; 0.59 BI, 28% ML; 0.91 BI, 73% ML), except in the phylogenetic trees based on Datasets 5; (3) all the four phylogenetic trees show that *Paranophrys magna*, *Metanophrys sinensis* and *Metanophrys* sp. QD form a fully supported clade, which is distant from the clade of two *Mesanophrys* species; (4) the relationships of *Cohnilembus verminus*, *Pseudocohnilembus* and *Miamiensis* are unstable among the four phylogenetic trees.

In order to evaluate the influence of combining genes on node support, we select six nodes with high support in three gene combined trees (Fig. 4A). Three, five, and five of the six nodes can be found in trees inferred from Datasets 5–7, respectively (Fig. 4B–D). Considering all the six nodes, the bootstrap values in the combined three-gene tree are higher than in the three single-gene trees (Table 4).

3.5. Putative secondary structures of ITS2 in philasterids

Putative secondary structures of the ITS2 transcript of the 26 species are presented in Supplementary Figure, from which a general secondary structure was constructed (Fig. 5). As the secondary structure preserved the pairing potential with a compensatory base change (CBC) or hemi-CBC (compensatory change on only one side of a helix pairing) (Coleman, 2003), these taxa shared a very similar pattern of secondary structure with homologous sequence segments having similar locations in spite of distinct sequence variation. The generally putative secondary structure model consists of: (1) a closed loop with three helices (helix I, II, and III); (2) helix I is highly conserved with a motif 5′-GGA vs.

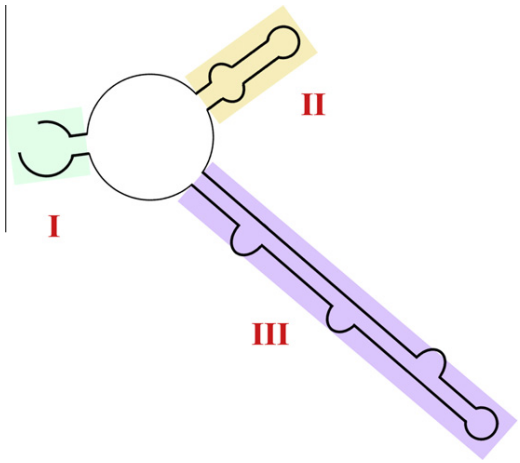

Fig. 5. The putative secondary structure model of the ITS2 transcript in philasterids, supported by CBCs and hemi-CBCs that preserve the helix pairing. The three domains, each with a stem-loop, are labeled I–III. Lines in helices and unpaired region are in bold to suggest the relatively well-conserved nucleotide positions.

UCC-3′; (3) helix II is conserved containing a motif 5′-GYGRUUGA vs. UCUCYCRY-3′ at its base; (4) helix III is the longest of the three and bears three bulge loops (Fig. 5). The three helices differ in size from one another: helix I is 3 bp long in most species while 5 bp long in *Uronema elegans* and 4 bp long in *Metanophrys* sp., *M. sinensis*, *Mesanophrys carcini* GD, *M. carcini* QD, and *Orchitophrya stellarum*; helix II was from 7 to 11 bp; helix III was longest and generally 35–38 bp long.

4. Discussion

4.1. Comparison of sequences and topologies based on SSU-rDNA, ITS1-5.8S-ITS2 region and LSU-rDNA

Here we show that either ITS-5.8S-ITS2 region or LSU-rDNA can infer well-supported nodes of the philasterids. Furthermore, most of the nodes in the individual gene trees (Fig. 4C and D) are congruent with those that are well supported in the SSU-rDNA analyses (Figs. 2 and 3B). Though ITS1-5.8S-ITS2 region and LSU-rDNA do not provide more resolution than SSU-rDNA, the combined three-gene trees do provide better support in nodes that were poorly supported in the SSU-rDNA gene topologies (e.g. nodes 1, 2, 3 in Fig. 4A). These results demonstrate that phylogenetic analysis of concatenation of the SSU-rDNA, ITS1-5.8S-ITS2 region, and LSU-rDNA sequences is an efficient way to solve the relationships among scuticociliates. However, many taxa are not sequenced the ITS1-5.8S-ITS2 region and LSU-rDNA and we anticipate a better resolution among scuticociliates with when the ITS1-5.8S-ITS2 region and LSU-rDNA are sequenced from additional taxa.

4.2. Taxonomic assignment of species in the family Orchitophryidae

Based on the molecular data reported here, the family Orchitophryidae is polyphyletic. The hypothesis that all Orchitophryidae species clustered together is rejected by the AU test based on Datasets 1, 2, and 4 (Table 3). The family Orchitophryidae was proposed for the genus *Orchitophrya* which was described originally as a parasitic astome of echinoderms (Cepede, 1907). *Orchitophrya* was later placed in the family Paranophryidae, which was created by Small and Lynn (1985) based on the possession of scuticociliate-like oral organelle complexes that are similar to other members of the Paranophryidae (Bouland et al., 1987). As *Orchitophrya* is the type genus of Orchitophryidae, the two families became one family named Orchitophryidae, comprising six mostly marine genera: *Orchitophrya*, *Anophryoides*, *Paranophrys*, *Metanophrys*, *Mugardia*, and *Mesanophrys* (Lynn, 2008).

In the present analysis, five of the six Paranophryidae genera are included. *Anophryoides haemophila* always clusters with *Miamiensis* while *Metanophrys sinensis*, *Metanophrys* sp. QD and *Paranophrys magna* always form a fully supported monophyletic group to the exclusion of a clade containing *Metanophrys similis*, *Mesanophrys* spp. and *Orchitophrya stellarum*. As *Orchitophrya* is the type genus of Orchitophryidae, we propose to remove *A. haemophila*, *M. sinensis*, *Metanophrys* sp. QD and *P. magna* from the family Orchitophryidae. The position of *A. haemophila* is discussed in the following section. However, considering that there is no molecular information of the type species of *Paranophrys* and *Metanophrys*, it would not be appropriate to create a new family for this group. For the time being, *M. sinensis*, *Metanophrys* sp. QD and *P. magna* are designated *incertae sedis* at the familial level.

4.3. Phylogenetic assignment of the families Uronematidae and Parauronematidae

Neither of the families Uronematidae and Parauronematidae is monophyletic in our trees (Figs. 2 and 3). The hypotheses that all Uronematidae species and Parauronematidae species clustered together respectively are also rejected by the AU test ($AU < 0.001$) based on all the data (Table 3). The family Uronematidae was proposed by Thompson (1964) for the genus *Uronema*. According to the classification schemes of Corliss (1979) and Lynn (2008), *Homalogastra* and *Uronemella* were also assigned to Uronematidae. However, based on our phylogenetic analysis, *Homalogastra setosa* does not cluster with *Uronema* and *Uronemella* and instead shows a close relationship with *Mesanophrys* spp. and *M. similis* (Fig. 2) and is closer to *Miamiensis* and *Anophryoides haemophila* (Fig. 3A). Thus, we designate *H. setosa* as *incertae sedis* due to its unresolved position relative to different datasets.

The family Parauronematidae was proposed by Small and Lynn (1985) to contain the genera of *Parauronema*, *Miamiensis*, *Glauconema* and *Potomacus*. However, *Parauronema* spp. do not cluster with the putative relatives, viz. *Miamiensis* and *Glauconema* and was synonymized with *Uronema* (Foissner, 1971). Likewise, the family Parauronematidae, whose type genus is *Parauronema*, should become the junior synonym of Uronematidae with the genera under Parauronematidae being assigned to Uronematidae. However, the current study shows that *Miamiensis* and *Glauconema* do not group with Uronematidae but show a close relationship with *A. haemophila*. Morphologically, *M. avidus* and *A. haemophila* are very similar in buccal apparatus, body size, and body shape and they differ only in paroral membrane (two parts vs. one part) and fewer somatic kineties (12 or 13–14 vs. 16–18). Considering these, the clade containing *A. haemophila*, *Miamiensis avidus*, and *Glauconema trihymene* might stand for a new family if it continues to be well-supported with additional information of more taxa especially type species.

4.4. Phylogenetic analyses of Parauronema

All *Parauronema* spp. do not cluster together and the monophyly of this genus is rejected by the AU test based on our Datasets 1, 3, and 4 (Table 3). In our analyses (Figs. 2–4), *P. virginianum* always clusters in the *Uronema* clade, which is consistent with their very similar morphology, the only exception being a two-rowed M1 (vs. one-rowed). Additionally, both *U. marinum* and *P. virginianum* have the same number of somatic kineties and inconspicuous extrusomes (Song et al., 2009). This observation suggests that the

difference of M1 does not warrant a separation at generic level which was also supported by Petz et al. (1995) who argued that *U. marinum*, the type species of *Uronema*, sometimes also had a paired basal body in M1, i.e. a very short second row. We thus agree with Foissner (1971) who synonymized *Parauronema* with *Uronema*.

Parauronema longum was first reported by Song (1995) and was put in *Parauronema* mainly because of its apical plate and two-rowed M1. However, based on both morphological data and SSU-rDNA topology, it was suggested that *P. longum* be removed from the genus *Parauronema* and to be assigned as *incertae sedis* within the clade containing the families Entorhipidiidae and Thyrophylacidae (Gao et al., 2012). The phylogenies based on ITS1-5.8S-ITS2 region, LSU-rDNA and the combined three-gene trees also show the same result (Figs. 3 and 4A). Comparison of the ITS2 secondary structure also indicates that helix II is 7 bp long in *P. longum* while it is 9 bp long in *P. virginianum* (Supplementary Figure). The result is consistent with the hypothesis that the structure of M1 should not be used as a diagnostic character at generic level.

4.5. Phylogenetic analyses of Uronema and Uronemella

Uronema and *Uronemella* are not only morphologically similar to each other but also show a close relationship in the phylogenetic trees (Figs. 2–4). The hypothesis that all *Uronema* spp. cluster together is rejected by the AU test (AU < 0.01) based on Datasets 1, 2, 3, and 4 (Table 3), supporting the paraphyly of the genus. Prior to this study, SSU-rDNA had only been available from three *Uronema* species (*U. marinum*, *U. elegans* and *U. heteromarinum*) and two *Uronemella* species (*U. filificum* and *U. parafilificum*) and analyses of these sequences revealed that *Uronema* was not monophyletic (Gao et al., 2012; Pan et al., 2010). In the present study, we isolate another two *Uronema* species and sequence their SSU-rDNA. The ITS1-5.8S-ITS2 region and LSU-rDNA of four *Uronema* species and one *Uronemella* species are also sequenced. Our phylogenetic trees reveal that the genus *Uronema* is paraphyletic, as *U. marinum* and *Uronema* sp. GD cluster in the clade formed by *Parauronema virginianum* and *Entodiscus borealis*, *U. elegans* and *U. heteromarinum* form a separate clade while *Uronema* sp. QD clusters with *Uronemella* clade (Figs. 2–4). Noticeably, *Uronema* sp. QD is assigned in *Uronema* as its features *in vivo* are very similar to *Uronema* spp., namely oval body shape, and non-thigmotactic locomotion. However, *Uronema* sp. QD is also very similar to *Uronemella* spp., vis. post-equatorially positioned cytostome. Moreover, *Uronema elegans* and *U. heteromarinum* also have subequatorial cytostome, being distinct from other *Uronema* spp., which might explain why they form a separate clade. This suggests that the position of cytostome might be a very important character to identify these genera while using characters *in vivo* to distinguish these two genera is misleading. Hence, based on molecular data and the post-equatorially positioned cytostome, *Uronema* sp. QD should be assigned to the genus *Uronemella*.

There is also a hypothesis that *Uronema* may just be a large, diverse genus, and the three lineages are the tips of related lineages within the large clade. The species included in the analyses are still too few to reveal deep relationships.

4.6. Phylogenetic analyses of Metanophrys

The present phylogenetic analyses indicate the genus *Metanophrys* is non-monophyletic, which was supported in previous study (Gao et al., 2012). The monophyly of *Metanophrys* is evaluated using the AU tests. The results show that *M. sinensis* clustering with *Metanophrys* sp. is not rejected based on the concatenated three genes data while three *Metanophrys* species clustering together is rejected based on SSU-rDNA and ITS1-5.8S-ITS2 region data (Table 4). Based on the phylogenetic data, *M. similis* is divergent with its congeners, which is consistent with their morphology. *Metanophrys* was defined with the length of the paroral membrane extended anteriorly to about half way along M2, which can be thus separated from the morphologically closely-related genus *Mesanophrys* and *Paranophrys* (it stretched only to the posterior end of M2 in *Mesanophrys* and it extended anteriorly to the anterior end of M2 in *Paranophrys*) (Song et al., 2009). However, *M. similis* can be separated from *M. sinensis* in: (1) absence (vs. presence) of extrusomes; (2) 3-rowed membranelle 1 in *M. similis* (vs. 2-rowed in *M. sinensis*); (3) lower number of basal bodies in somatic kinety No. 1 (23–28 in *M. similis* vs. 31–39 in *M. sinensis*) and (4) different arrangement of the scutica (solitary and sparsely distributed in a long row in *M. similis* vs. basal bodies in pairs and closely packed in *M. sinensis*) (Song et al., 2002). Hence the level at which the paroral membrane terminates anteriorly is a species level, rather than a genus-level, character.

4.7. Phylogenetic analyses of Mesanophrys

Mesanophrys was proposed by Small and Lynn (1985) and was defined by two characters: (1) M2 as long as M1; (2) the paroral membrane terminates anteriorly at the level of M3. Four species of *Mesanophrys* have been described: *M. maggii*, *M. carcini*, *M. pugettensis*, and *M. chesapeakensis*. However, an examination of the literature reveals a very confused taxonomic history of this genus and its closely related ciliate genera. Based on morphological characters, Wiackowski et al. (1999) concluded that *M. carcini*, *M. pugettensis*, and *M. chesapeakensis* should be referred to *Mesanophrys maggii*. This hypothesis needed to be tested by new evidence, such as host specificity, life history or molecular information (Wiackowski et al., 1999). Subsequently, Goggin and Murphy (2000) submitted two ITS1-5.8S-ITS2 region sequences under the name of *M. pugettensis* and *M. chesapeakensis*, which provided a way to test whether *M. carcini*, *M. pugettensis*, and *M. chesapeakensis* should be considered the same species. We find that the two ITS1-5.8S-ITS2 region sequences of *M. pugettensis* and *M. chesapeakensis* are identical, and only have three nucleotide site changes compared to that of *M. carcini* QD. The three isolates form a fully supported clade (Fig. 3A), suggesting that they might be conspecific. However, due to the lack of adequate morphological information and the absence of molecular data, it is not currently possible to determine whether *M. maggii* and *M. carcini* are conspecific.

The three isolates of *M. carcini* cluster together but show some differences at molecular level. Comparison of the SSU-rDNA sequences shows that two nucleotide substitutions exist between that of *M. carcini* GD and of previous *M. carcini* (AY103189). However, another isolates from Qingdao (*M. carcini* QD), which corresponds well to the diagnostic characteristics of *M. carcini* and also has 10-rowed of somatic kineties as *M. carcini* GD does, differs from *M. carcini* GD with 65 nucleotides in the SSU-rDNA sequence, 33 nucleotides in the ITS1-5.8S-ITS2 region sequence and 69 nucleotides in the LSU-rDNA sequence, indicating that there might be cryptic species in *M. carcini*.

4.8. Phylogenetic analyses of Miamiensis

As a monotypic genus, *Miamiensis* was first reported by Thompson and Moewus (1964) for the marine facultative parasite *Miamiensis avidus*. Song and Wilbert (2000) redescribed *M. avidus* based on the bimorphic paroral membrane with monokinetid anterior and dikinetid posterior part as its main characteristic. In the present study, we isolate another strain of *M. avidus* that is very morphologically similar to *M. avidus* described by Song and Wilbert (2000) but differs in body size (20–30 × 15–22 μm vs. 25–40 × 15–20 μm) and number of somatic kineties (12 vs. 13 or

14). Moreover, the new isolate shows great differences at the molecular level with the strain from Korea (Jung et al., 2011): 68 nucleotide substitutions (sequence divergence 3.86%) between the SSU-rDNA sequences and 124 nucleotide substitutions (sequence divergence 21.79%) between the ITS1-5.8S-ITS2 region sequences. The current SSU-rDNA trees also show that *M. avidus* QD. does not cluster with *M. avidus* but with *Anophryoides haemophila* while together these taxa form a polytomy in the ITS1-5.8S-ITS2 region trees, indicating that there might be cryptic species in *M. avidus*.

5. Conclusion

The SSU-rRNA gene is a rich source of phylogenetic and taxonomic characters that makes it a powerful tool for evolutionary reconstruction, and therefore, it has shed much light on the phylogeny of scuticociliates; however, a number uncertainties remain within the group (Foissner et al., 2004; Gao et al., 2012; Lynn, 2003, 2008; Zhang et al., 2010a). Also, SSU-rDNA may not always be useful for resolving phylogenetic questions because of the possibility that specific regions of the gene may evolve at different rates in related clades. In this study, we apply two additional molecular markers (ITS-5.8S-ITS2 region and LSU-rDNA) to reconstruct relationships within scuticociliates especially philasterids. We find that phylogenetic analysis of concatenation of the SSU-rDNA, ITS1-5.8S-ITS2 region, and LSU-rDNA sequences provides higher support for relationships among scuticociliates. However, as the ITS1-5.8S-ITS2 and LSU-rDNA have only been sequenced from a small number of taxa, most of the relationships among scuticociliates are still unknown. At the same time, there is still considerable discrepancy among morphological studies in scuticociliates, as it is hard to distinguish plesiomorphy and apomorphy of morphological characters, which in turn leads to different criteria used to define the taxa among different researchers. In summary, more in-depth studies on morphology and more gene information are needed to critically evaluate the discordances between molecular and morphological data as well as to elucidate genealogical relationships of scuticociliates.

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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.2012.04.008.

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