



Further insights into the phylogeny of two ciliate classes Nassophorea and Prostomatea (Protista, Ciliophora)



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ABSTRACT

The Nassophorea and Prostomatea are two of the key classes in understanding the morphological diversification and higher classification of the phylum Ciliophora. However, their phylogenetic relationships with other ciliate groups within the subphylum Intramacronucleata remain elusive. In this study, we investigated the small and large subunit (SSU and LSU) rRNA gene-based phylogeny of these groups with sequences of additional taxa including several key species. The results show that: (1) the class Nassophorea remains polyphyletic, with the microthoracids clustering with the Phyllopharyngea, whereas the nassulids represent a basal group of the CONthreeP superclade in the SSU tree; (2) the Prostomatea is not depicted as a monophyletic group in phylogenetic trees, and the monophyly of this class is marginally rejected by statistical tree topology tests; (3) the nassulid genus *Parafurgasonia* is more closely related to the family Colpodidiidae than to *Furgasonia*; (4) *Paranassula*, which was previously thought to be a nassulid, is phylogenetically related to the oligohymenophorean peniculids in both the SSU and LSU trees; (5) the microthoracid genus *Discotricha* does not group with the other microthoracids in either SSU or LSU trees; (6) the family Plagiocampidae is closely related to the prostome parasite *Cryptocaryon irritans* and to the family Urotrichidae in the order Prorodontida; and (7) the family Placidae, represented by *Placus salinus*, is sister to the family Holophryidae in the order Prorodontida. Based on the present data, we consider the genus *Discotricha* to be an unclassified taxon within the CONthreeP. We also propose resurrecting the order Paranassulida and classifying it within the subclass Peniculia, class Oligohymenophorea. Primary and secondary structure signatures for higher taxa within Phyllopharyngea and Nassophorea are supplied.

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1. Introduction

Ciliates (phylum Ciliophora) are morphologically diverse and are excellent model organisms for understanding the morphological diversification, evolution and systematics of protists (e.g. Dunthorn et al., 2008; Gao et al., 2013; Vd'ačný et al., 2011). It was generally recognized that the 11 classes within this phylum were monophyletic based on morphology, ultrastructure and molecular (especially SSU rRNA) inferences (Lynn, 2008), although the new class Cariacotrichea, recognized originally from environmental SSU rRNA gene sequence data, was recently established for a novel clade from the anoxic Cariaco Basin (Orsi et al., 2011). However, recent phylogenetic studies using broad taxon sampling and/or multiple gene markers have demonstrated that several of the classes

recognized by Lynn (2008) may be paraphyletic or polyphyletic (Dunthorn et al., 2008; Gong et al., 2009b; Zhang et al., 2012). Three such examples are classes Nassophorea, Phyllopharyngea and Prostomatea, which could be crucial taxa in understanding the evolution of phenotypes (e.g. oral ciliation and kinetid fiber systems) and the systematics of the subphylum Intramacronucleata (Gao et al., 2012; Gong et al., 2009b; Lynn, 2008; Zhang et al., 2012).

The class Nassophorea sensu Lynn, 2008 comprises three orders, Synhymeniida, Nassulida and Microthoracida (Lynn, 2008). SSU rRNA-based phylogenies have shown that the order Synhymeniida clusters strongly with the class Phyllopharyngea rather than with the other two orders of Nassophorea (Dunthorn et al., 2008; Gong et al., 2009b; Kivimaki et al., 2009). Based on this, and the fact that the synhymeniids and Phyllopharyngea share a morphological synapomorphy (subkinetid homologue), Gong et al. (2009b) revised the higher classification by incorporating the synhymeniids as a subclass of the class Phyllopharyngea. The former phyllopharyngeans (i.e. cyrtophorians, chonotrichians, rhynchodians and suctorians) were then reclassified as members

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of the newly established subclass “Subkinetalia” (Gong et al., 2009b). It was also shown that the order Microthoracida might represent a basal lineage of the newly defined Phyllopharyngea, although the systematic position of this order remained uncertain owing to the lack of sufficient data (Gong et al., 2009b).

The class Prostomatea was thought to be an ancestral group because of its “primitive” morphological features, e.g. the radial symmetry and apical location of the cytostome (Corliss, 1979). Two orders have been recognized based on the position of the mouth and the presence of a brosse and toxicysts, namely Prostomatida and Prorodontida (Dragesco et al., 1974; Lynn, 2008). However, this arrangement is far from certain and the systematics of Prostomatea has been vigorously debated for several decades (Bardele, 1999; Corliss, 1979; Hiller, 1992, 1993; Lynn and Small, 2002; Stechmann et al., 1998). Availability of SSU rRNA gene sequences of morphologically identified prostomes is limited to a single order, the Prorodontida (Foissner et al., 2008; Kim et al., 2007; Stechmann et al., 1998; Wright and Colorni, 2002; Yi et al., 2012). Consequently, the highly biased taxon sampling of prostomes in phylogenetic analyses might raise problems. For example, a recent study has shown that two litostomean genera, *Cyclotrichium* and *Paraspathidium*, should be placed within the Prostomatea, albeit with poor support (Zhang et al., 2012).

In this study, we have newly sequenced 22 SSU and LSU gene sequences from 15 nassophorean, prostome and phyllopharyngean species, including for the first time representatives of the genera *Paranassula*, *Parafurgasonia* and *Plagiocampa* (Table 1). Phylogenetic analyses were carried out on this expanded dataset in order to better elucidate the evolutionary relationships and higher classification of these key classes of ciliates.

2. Materials and methods

2.1. Ciliate collection and identification

A total of 15 ciliate species were collected and identified. Information on the species names, strain numbers, sampling locations, habitats, and sequence information of collection are shown in Table 1. Specimens were observed in vivo and stained with protargol (Wilbert, 1975). Identifications of specimens were made by referring to published guides (Foissner et al., 1994; Gong et al., 2009a; Lynn and Small, 2002; Xu et al., 2005; Vd'ačný and

Tirjaková, 2012a,b). Terminology and systematic classification were according to Lynn (2008) and Gong et al. (2009b).

2.2. DNA extraction, gene amplification and sequencing

Genomic DNA was extracted using REExtract-NAMP Tissue PCR Kit (Sigma, St. Louis, USA) with modifications suggested by Gong et al. (2007). The PCR amplifications were performed using a TaKaRa ExTaq DNA Polymerase Kit (TaKaRa Biomedicals, Japan). Primers used for SSU rRNA gene amplification were Euk A (5'-AAC CTG GTT GAT CCT GCC AGT-3') and Euk B (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') (Medlin et al., 1988) covering nearly the full length of the gene. Primers for partial LSU rRNA gene amplification were 28S-F3: 5'-ACC CGC TGA ACT TAA GCA T-3' and 28S-R3: 5'-CAT TCG GCA GGT GAG TTG TTA CAC-3' (Moreira et al., 2007). PCR conditions, product purification and cloning were performed as previously described (Zhang et al., 2012). Genes were sequenced in both directions on an ABI 3700 sequencer (Sangon sequencing facility, Shanghai, China), using the M13-47 and M13-48 primers.

2.3. Phylogenetic analyses and hypothesis testing

SSU and LSU rRNA gene sequences were aligned using ClustalW implemented in BioEdit 7.0.0 (Hall, 1999), and were further modified manually using SeaView (Gouy et al., 2010). The final alignment used for subsequent phylogenetic analyses included 115 species and 1,610 positions for SSU, and 31 species and 1,048 positions for LSU. The GTR+I+ Γ model was selected as the best fit by MrModeltest 2 (Nylander, 2004) for Bayesian inference (BI) analyses for both genes. BI analyses were performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains using the default settings, with a sampling frequency of 0.01. Convergence of the chain length was confirmed from the standard deviation of split frequencies (<0.01). The chain lengths were 1,500,000 and 1,000,000 generations for SSU and LSU datasets, respectively. For each analysis, 25% of generations were discarded as burn-in. The remainder was used to generate consensus trees and to calculate the posterior probabilities (PP) of all branches using a majority-rule consensus approach. For the ML analyses, selection of the best model was performed using program jModeltest (Darrriba

Table 1
Species identified and newly sequenced in this study.

Species	Strain	Sampling location	Habitat	Accession number	
				SSU	LSU
Unidentified synhymeniid	LHA07091204	Clear Water Bay, Hong Kong	Marine water	FJ868194	KC832965
<i>Discotricha papillifera</i>	FXP20081011-01	Jiaozhou Bay, Qingdao, northern China	Marine water		KC832966
<i>Dysteria derouxi</i>	JJM2008052701	Jiaozhou Bay, Qingdao, northern China	Marine water		KC832960
<i>Leptopharynx costatus</i>	GJ061204-01	South China Normal University, Guangzhou, southern China	Fresh water pool		KC832958
<i>Nassula labiata</i>	LWW09052701	Daya Bay, Huizhou, southern China	Marine water	KC832949	KC832957
<i>Orthodonella</i> sp.1	FXP07052501	Jiaozhou Bay, Qingdao, northern China	Marine water	FJ998038	
<i>Orthodonella</i> sp.2	FXP2007052504	Jiaozhou Bay, Qingdao, northern China	Marine water	KC832952	KC832963
<i>Parafurgasonia</i> sp.	S011	Zulfi, Saudi Arabia	Dry soil	KC832955	
<i>Paranassula</i> sp.1	LWW06121404	Daya Bay, Huizhou, southern China	Marine water	KC832956	KC832964
<i>Paranassula</i> sp.2	FXP2007122008	Mangrove wetland, Shenzhen, southern China	Marine water	FJ998039	
<i>Placus salinus</i>	FXP09051102	Weifang, Northern China	Flounder culture pond, marine water	KC832954	KC832959
<i>Plagiocampa</i> sp.	PHB09022602	Jiaozhou Bay, Qingdao, northern China	Marine sandy beach	KC832950	KC832962
<i>Zosterodasys agamaliievi</i>	FXP2007122601	Daya Bay, Huizhou, southern China	Marine water	FJ998040	KC832961
<i>Zosterodasys</i> sp.1	CXR08040810	Mangrove wetland, Shenzhen, southern China	Marine water	KC832951	
<i>Zosterodasys</i> sp.2	CXR08040807	Mangrove wetland, Shenzhen, southern China	Marine water	KC832953	

et al., 2012). The GTR+I+ Γ model was again selected for both SSU and LSU rRNA datasets. Using these settings, ML trees were constructed with the RAxML program (Guindon and Gascuel, 2003). The reliability of internal branches was assessed using a nonparametric bootstrap method with 1000 replicates for both genes.

In order to assess how different the tree topologies of alternative hypotheses may be, approximately unbiased (AU) tests (Shimodaira, 2002) were performed using CONSEL 0.1 (Shimodaira and Hasegawa, 2001) for the SSU dataset. For each hypothesis, RAxML (Shimodaira, 2002; Stamatakis et al., 2008) was used to search for the best constrained ML trees in which the related taxa consistently formed a monophyletic group. The site-wise likelihoods were calculated for each tree topology with RAxML, using the same model as mentioned above.

2.4. Primary and secondary structure signatures

The alignment of SSU rRNA gene sequences was visually inspected to identify the signature fragments for “Subkinetalia”, Synhymeniia, Microthoracia, Nassulida and Discotrichidae. The variable region 2 with substantial indels were identified, and their secondary structures were depicted using mfold with default settings (Zuker, 2003), and edited with RnaViz 2.0 (De Rijk and De Wachter, 1997) for aesthetic purposes under the eukaryotic SSU model of Van de Peer et al. (2000).

3. Results

The 15 ciliate species were isolated from diverse habitats and geographically distant locations (Table 1). These included freshwater ponds, Yellow Sea coastal waters off cities in northern China (Qingdao and Weifang), South China Sea coastal/fresh waters off cities in southern China (Guangzhou, Hong Kong, Huizhou and Shenzhen), and dry soils from Saudi Arabia. Most of these organisms were identified to generic or species level based on their morphology, except for one synhymeniid isolate. Twelve SSU and 10 LSU rRNA gene sequences representing 11 nassulid and prostome genera were newly obtained. Phylogenetic analyses were performed separately for SSU and LSU rRNA genes. However, there were few species with both genes available for analysis of the concatenated SSU and LSU data.

3.1. SSU rRNA phylogeny (Fig. 1)

In the SSU rRNA-based trees, the ML and Bayesian analyses show similar topologies (Fig. 1). All the newly obtained synhymeniid sequences form a monophyletic group representing the subclass Synhymeniia, which is strongly supported (100% ML, 1.00 BI) (Fig. 1). The monophyly of the genus *Orthodonella* is highly supported, whereas the genus *Zosterodasys* is paraphyletic with *Z. agamalievi* and an unidentified *Zosterodasys* species basal to the subclass Synhymeniia. Sequences of two unidentified *Orthodonella* species (sp.1 and 2) differed only by 1 base pair. The sequence of *Orthodonella* sp.1 is identical to the only previously published *Orthodonella* sequence (GenBank number EU286809). These three sequences have a similarity of 98.5–98.6% to that of *O. apoharmatus*. Both the ML and BI trees show *Zosterodasys* sp.2 as a basal branch to other synhymeniids.

The order Microthoracida is not monophyletic. One clade of this order is placed basally to the cluster of synhymeniids and the group “Subkinetalia” sensu Gong et al. (2009b), forming a monophyletic assemblage with moderate support (ML 60%, BI 1.00). The family Discotrichidae, represented by *Discotricha* and one unidentified microthoracid, branches as a basal lineage to the clade comprising “Subkinetalia”, synhymeniids, other microthoracids,

the Oligohymenophorea, Prostomatea, and Plagiopylea, although support for the placement of *Discotricha*-clade is rather low (15% ML, 0.76 BI).

Instead of grouping with synhymeniids and/or microthoracids, the order Nassulida appears as a monophyletic assemblage with moderate to high support (ML 79%, 1.00 BI), that is placed basally in the superclade comprising Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Plagiopylea, and Prostomatea, the so-called CONthreeP in the latest system of Adl et al. (2012). The newly sequenced *Parafurgasonia* sp. shows a closer relationship with the genus *Colpodidium* (ML 95%, BI 1.00) than with other nassulids (Fig. 1).

The placement of two unidentified *Paranassula* species, which have hitherto been considered as typical nassulid taxa, is entirely unexpected (Fig. 1). Sequences of the two *Paranassula* species have a similarity of 99.4%, differing from each other by only 10 bases. In both analyses they are grouped together with *Paramecium* and *Frontonia*, classical representatives of the order Peniculida within the class Oligohymenophorea, with maximum support (ML 100%, BI 1.00; Fig. 1).

The monophyly of the class Prostomatea is not supported in the SSU trees, although several monophyletic groups within this class can be recognized (Fig. 1). The class Plagiopylea appears to be monophyletic with full support and is sister to the family Colepidae (ML 41%, BI 0.98). The species *Placus salinus* clusters with the family Holophryidae, represented by the genera *Holophrya* and *Pelagothrix*, with variable support (BI 0.99, ML 45%). The newly sequenced *Plagiocampa* clusters with *Cryptocaryon irritans*, a representative of the family Cryptocaryonidae, with high support (87%, BI 1.00), and then with *Urotricha* sp., the only sequenced species of the family Urotrichidae (ML 51%, BI 0.72). This clade forms a sister group with two litostomean (?) species, *Paraspathidium flacus* and *Cyclotrichium ovum*, with moderate to high support (ML 66%, BI 0.99). *Balanion masanensis* appears to be an early branching lineage of the prostomes. These prostome clades are thus sister groups to the classes Oligohymenophorea and Plagiopylea, forming a superclade with variable support (ML 51%, BI 0.97).

3.2. LSU rRNA phylogeny (Fig. 2)

Since there are few ciliate LSU sequences available, the LSU trees reveal relatively limited phylogenetic information for the classes Phyllopharyngea, Nassophorea and Prostomatea, compared to the SSU tree. Nevertheless, several aspects are noteworthy. In the LSU tree, a monophyletic assemblage that comprises a “subkinetalian” (*Dysteria derouxi*) and two synhymeniids (*Zosterodasys* and *Orthodonella*) is recovered, although this topology is only weakly supported (ML 55%, BI 0.64). The microthoracid *Leptopharynx costatus* groups with the nassulids (*Nassula* and *Zosterograptus*), with variable support (ML 53%, BI 0.99; Fig. 2). However, another microthoracid species, *Discotricha papillifera*, groups with the colpodean *Bresslauides*, albeit with weak support (ML 40%, BI 0.56). As in the SSU phylogeny, the nassulid *Paranassula* sp.1 clusters with the peniculid *Paramecium* in the LSU tree (ML 98%, BI 1.00). The monophyly of Prostomatea is not recovered in the LSU tree which is also consistent with the SSU tree: *Placus salinus* groups with the synhymeniid “Subkinetalia” clade with low support values (ML 48%, BI 0.55; Fig. 2), and *Plagiocampa* sp. clusters with the litostomean (?) genera *Cyclotrichium* and *Paraspathidium* (ML 83%, BI 1.00; Fig. 2).

3.3. Hypothesis testing (Table 2)

AU tests were performed on the SSU rRNA gene dataset to test the robustness of phylogenetic associations of particular interest (Table 2). At the 5% significance level, the alternative hypothesis

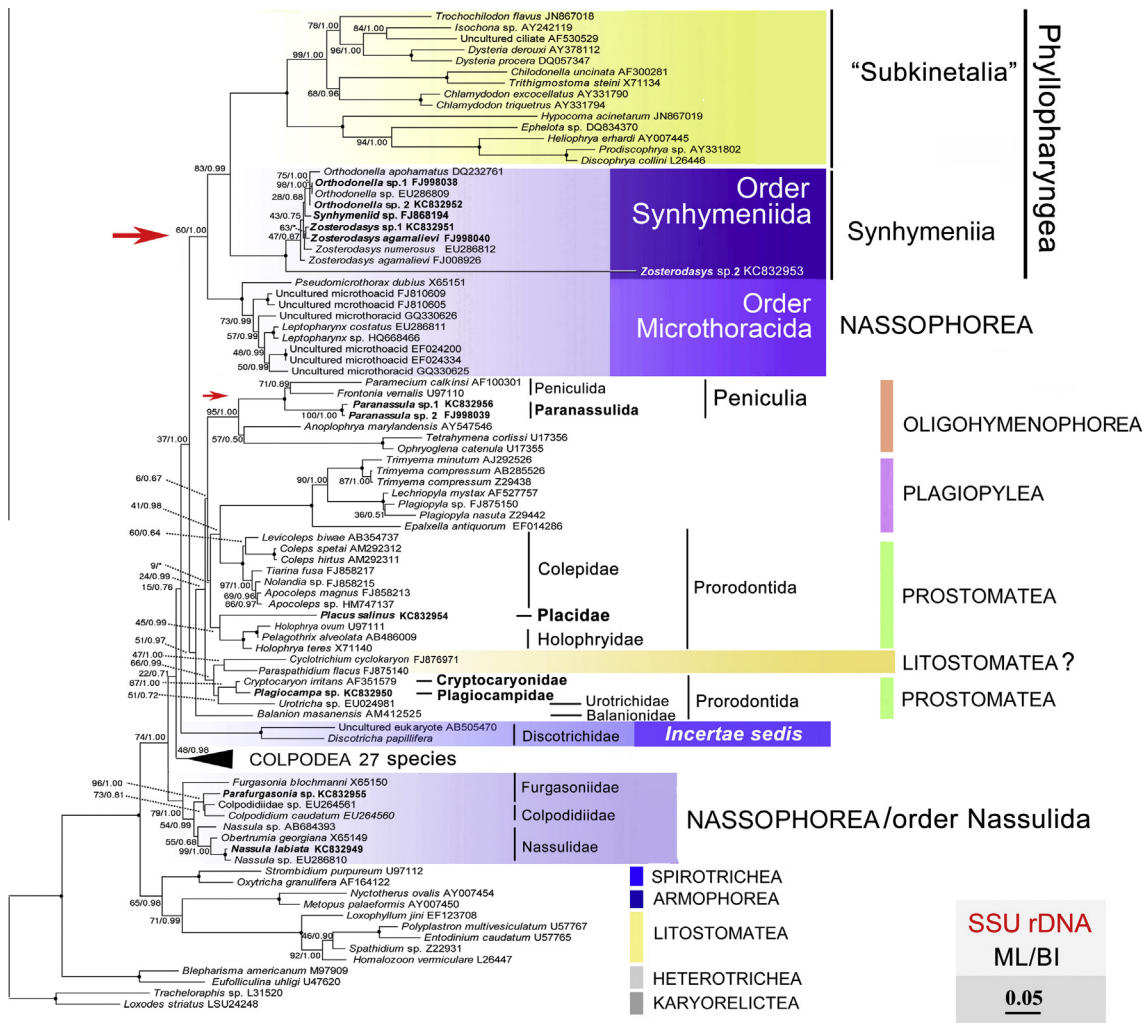


Fig. 1. Maximum likelihood (ML) tree inferred from SSU rRNA gene sequences of representative ciliate taxa and species newly sequenced in this study. Numbers at the nodes represent ML and Bayesian support values respectively. Newly sequenced species are in bold. The clade “Subkinetalia” comprises Cyrtophoria, Chonotrichia, Rhynchodia, and Suctorina (Gong et al., 2009b). The family Discotrichidae, which belonged to the order Microthoracida, is classified as *incertae sedis* in the superclade CONthreeP. Note the medium to high support values for the clustering of the subclasses Microthoracia, Synhymeniia and “Subkinetalia” (larger arrow), and the strong support for *Paranassula* grouping with Peniculia (smaller arrow) rather than with the core nassulids. The scale bar corresponds to 0.05 expected substitutions per site.

that Microthoracida and Discotrichidae form a monophyletic assemblage was rejected ($P = 0.043$), the hypothesized monophyly of “Subkinetalia” + Discotrichidae ($P = 0.062$), and Prostomatea ($P = 0.053$) was also marginally rejected. However, the hypothesized monophyly of Nassulida + Synhymeniida + Microthoracida + Discotrichidae was not rejected ($P = 0.182$). The following hypotheses were likewise not rejected: Nassulida forming a monophyletic group with others, e.g. Nassulida + Synhymeniida, Nassulida + Microthoracida, Nassulida + Microthoracida + Discotrichidae ($P > 0.16$, Table 2); the monophyly of the genus *Zosterodasys* ($P > 0.40$, Table 2); the forced grouping of the nassulids *Furgasonia blochmanni* and *Parafurgasonia* (Table 2).

3.4. Signatures of primary and secondary structures (V2 region) of SSU rRNA (Figs. 3 and 4)

Based on the alignment of 115 SSU rRNA gene sequences of ciliates, signature sites for the four groups (“Subkinetalia”, synhymeniids, nassulids and microthoracids) were identified (Figs. 3 and 4). Specific sites or fragments can be identified in each of these groups, except for the “Subkinetalia” (Fig. 3). Synhymeniia is featured by many signature fragments, e.g. A(c/g)C(c/a)(g/-)TGC at

sites 272–280, CAGACCGGGTC at sites 473–483, GCTG(c/t)TA at sites 1329–1336, CC(-/a)TAGCA at sites 1340–1347, and AGT at sites 1486–1488. Characteristic single nucleotide polymorphisms (SNPs) are detected in this group at sites 240 (A vs. T), and 1239 (T vs. A). Microthoracids exclusively share fragments at sites 277–280 (CTGC) and 1237–1245 (CTATGGGTA). Nassulida have several signature regions at sites 473–483 (CAA(a/c) (c/t)CG(g/t)GGC), 489–495 (GTGCTTA) and 1333–1336 (CTAA). Although there are no distinctly conserved regions in “Subkinetalia”, characteristic SNPs are observed at sites 209 (T/C vs. A/G), 461 (T/C vs. G) and 1474 (A/C vs. G).

The crown sister groups of “Subkinetalia” and Synhymeniia share no specific sites except for a consistent deletion of T/C at site 153. Nevertheless, the monophyletic group comprising the “Subkinetalia”, synhymeniids and microthoracids is characterized by sites 1407–1409 (CCG), contrasting to CTG shared by all other ciliates groups (Fig. 3). No common sites are exclusively shared by the Nassophorea sensu Lynn, 2008 (i.e. synhymeniids, Nassulida, microthoracids and discotrichids). However, some regions conserved in both microthoracids and nassulids, for instance (t/c)GA at sites 180–182, CTATGGGTA at sites 1237–1245, and CACT at sites 1465–1468, may be variable in “Subkinetalia” and/or

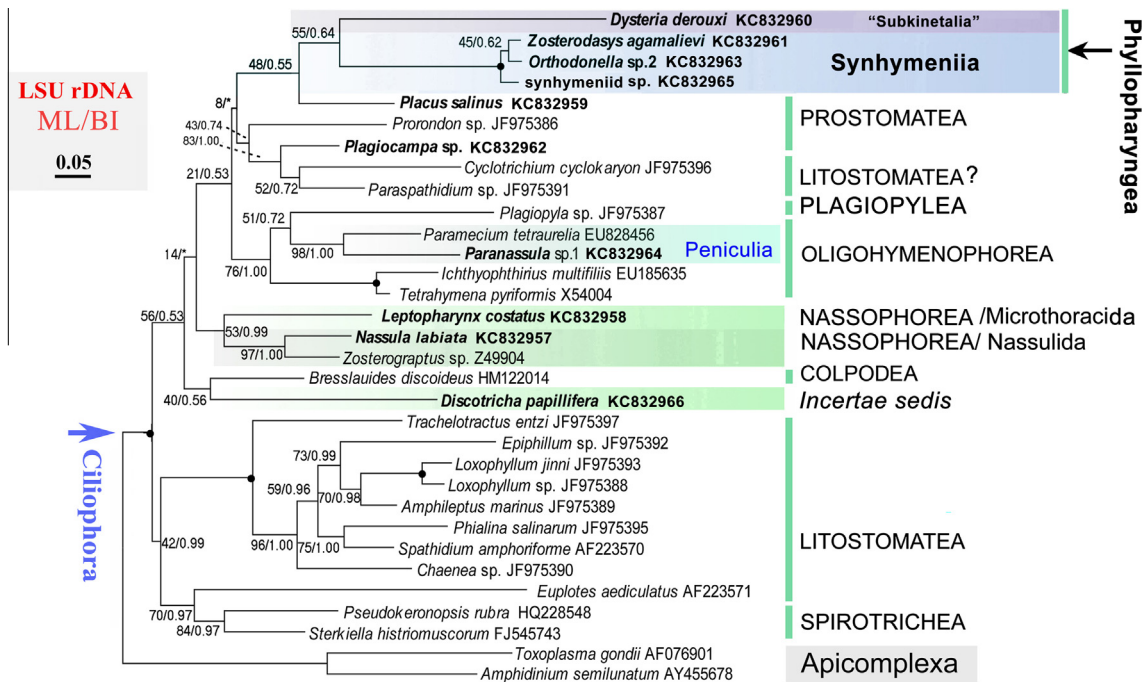


Fig. 2. Maximum likelihood (ML) tree of eight classes of ciliates inferred from LSU rRNA sequences. Numbers at the nodes represent ML and Bayesian support values respectively. New sequences are shown in bold. *Discotricha papillifera*, a member previously assigned in the order Microthoracida, is classified as *incertae sedis* in the superclade CONthreeP. The arrow refers to the full support for the phylum Ciliophora. The scale bar corresponds to 0.05 expected substitutions per site.

Table 2

Results of approximately unbiased tests comparing the best maximum likelihood tree with the best constrained trees obtained under alternative hypotheses of monophyletic assemblages. Result in which P -values < 0.05 are shown in bold.

Alternative hypothesis	P -value
Class Prostomatea	0.057
Nassulida + Microthoracida + Synhymeniida + Discotrichidae	0.182
Nassulida + Synhymeniida	0.163
Nassulida + Microthoracida	0.439
Nassulida + Microthoracida + Discotrichidae	0.184
Microthoracida + Discotrichidae	0.043
"Subkinetalia" + Discotrichidae	0.062
<i>Zosterodasys</i> sp.1 + <i>Z.</i> sp.2 + <i>Z. agamaliievi</i> FJ998040 + <i>Z. agamaliievi</i> FJ008926 + <i>Z. transversus</i>	0.473
<i>Furgarsonia blochmanni</i> + <i>Parafurgarsonia</i> sp.	0.297

synhymeniids. Sites 278–280 (TGC) are conserved in both synhymeniid and microthoracid sequences, but differ from the corresponding sites of both nassulids and "Subkinetalia". No conserved sites were specifically shared by Microthoracida and Discotrichidae. Among the nassulids, 11 homologous sites are exclusively shared by *Parafurgarsonia* sp. and the Colpodiidae species, whereas no homologous sites are specifically shared by *Furgarsonia blochmanni* and *Parafurgarsonia* sp.

Although there are few signature sites in the primary structure of SSU rRNA, deletion of nucleotides are much more characteristic in the "Subkinetalia" and synhymeniids, and is most typically reflected in variable region 2 (V2, Fig 4) on the rRNA secondary structures. Compared with rRNA sequences of other groups in Fig. 4 (i.e. synhymeniids, microthoracids, nassulids and discotrichids), helices 10 and E10-1 in the V2 region in "Subkinetalia" are generally shorter (Fig. 4), and both "Subkinetalia" and synhymeniids have fewer sites in helix 11. "Subkinetalia", synhymeniids and microthoracids are coincidentally characterized by having shorter sequences in helix E10_1 than nassulids and most of other ciliates groups (Fig. 4, corresponding to sites 228–233 in Fig. 3)

4. Discussion

Increased sampling of taxa has unveiled missing evolutionary links between Phyllopharyngea and nassophoreans, changing our view of the diversification, morphological evolution and classification of cyrtos-bearing ciliates (Gong et al., 2009b). The Nassophorea however, remains non-monophyletic, with subgroups being frequently placed as an early branch to the CONthreeP superclade, i.e. Colpodea, Oligohymenophorea, microthoracids, Phyllopharyngea, Plagiopylea, and Prostomatea, stressing their importance in assessing the phylogenetic relationships among major groups within the subphylum Intramacronucleata. The present work extends this line of study by sequencing and analyzing additional genes and taxa of Nassophorea and Prostomatea, thus providing further insights into the phylogeny and classification of nassophoreans (e.g. microthoracids, Paranassulidae and Furgarsoniidae) and prostomates (e.g. Plagiocampidae and Placidae).

4.1. Microthoracids and Discotricha

In the SSU rRNA tree, the family Discotrichidae, represented by *Discotricha papillifera*, is separated from other microthoracids (e.g. *Leptopharynx* and *Pseudomicrothorax*, hereafter referred to as "microthoracids s. str."). This topology is partly supported by the LSU phylogeny (Fig. 2). The AU test rejects the monophyly of the microthoracids s. str. + *Discotricha* ($P = 0.043$), suggesting that *Discotricha* is divergent from the two families of the order Microthoracida (i.e. Leptopharyngidae and Microthoracidae). This phylogenetic divergence is also supported by phenotypic data, as *Discotricha* clearly differs from the other microthoracids by its non-ciliated (vs. ciliated) dorsal surface, its cirrus-like polykinetids (vs. kineties) on the ventral side, having extrusomes without (vs. with) anchor-like tips and by its marine (vs. freshwater and/or terrestrial) habitat (Foissner, 1997; Lynn, 2008; Tuffrau, 1954; Wicklow and Borror, 1977). Thus the characters used to assign *Discotricha*, along with the families Leptopharyngidae and Microthoracidae, to the

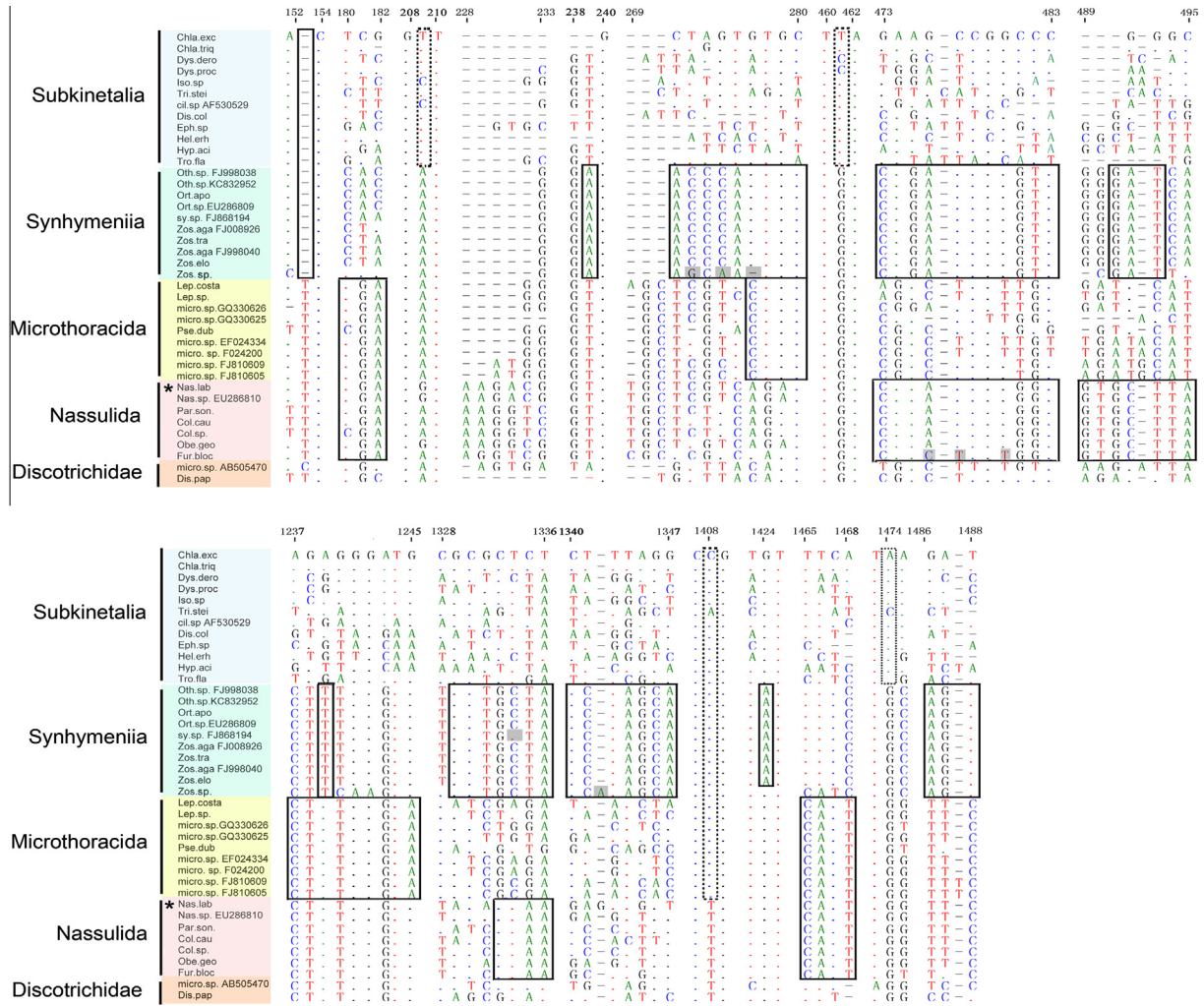


Fig. 3. Partial alignment of SSU rDNA showing signature sites for “Subkinetalia”, Synhymeniia, Microthoracida, Nassulida and Discotrichidae. The fragments were extracted from a full alignment based on 115 ciliate SSU rDNA sequences, from which the tree shown in Fig. 1 was derived. Homologous sites/regions specifically shared by individual group or a combination of groups are boxed with solid lines, with the few exceptions shaded in gray; the exclusively variable regions of “Subkinetalia” and Phyllopharyngea + Microthoracida are dash-boxed. Numbers at the beginning and end of lines indicate position within the complete sequence of *Nassula labiata* (asterisk), to which all the homologous sites/regions are referred.

order Microthoracida, i.e. the sparse somatic ciliation and the three adoral polykinetids, are probably either a result of convergent evolution or they represent conserved old plesiomorphies. Nevertheless, since the position of *Discotricha* is still unresolved, we consider it to be *incertae sedis* within the superclade CONthreeP.

The microthoracids other than discotrichids group with synhymeniids and “Subkinetalia” in the SSU trees with moderate to high support (ML 60%, BI 1.00), which is consistent with previous investigations (Gong et al., 2009b). However, the AU test does not reject the hypothesized monophyly of the microthoracids s. str. + nassulids ($P = 0.182$). The SSU rDNA sequences of the microthoracids s. str. and nassulids exclusively share two homologous regions (Fig. 3). Furthermore, the LSU trees do not support the clustering of the microthoracids s. str. with synhymeniids and “Subkinetalia” (Fig. 2). These findings suggest it is still premature to reclassify the microthoracids within the class Phyllopharyngea.

4.2. The genera *Paranassula* and *Parafurgasonia* and the newly redefined class Nassophorea

Four families, i.e. Nassulidae, Furgasoniidae, Paranassulidae and Colpodidiidae, have long been classified in the order Nassulida

(Breiner et al., 2008; Lynn and Small, 2002). In our study, both the SSU and LSU phylogenies suggest that the genus *Paranassula* is closely related to the peniculids and hence a member of the class Oligohymenophorea (Fig. 1 and 2). This is noteworthy because *Paranassula* has long been considered as a member of the class Nassophorea (order Nassulida, family Paranassulidae), due to its well-developed cyrtos and polykinetid oral ciliation (Lynn, 2008). As such, the morphological classification and evolution of *Paranassula* should be reconsidered. Two morphological characters can be recognized in the separation of *Paranassula* from typical nassulids: (1) the presence of just two polykinetids that are restricted to a shallow oral cavity in *Paranassula* (vs. many polykinetids transversely arranged on the ventral surface in typical nassulids) (Carey, 1992); (2) the presence of a paroral kinety in *Paranassula* (vs. absent in typical nassulids) (Lynn and Small, 2002). As the cyrtophorians and nassulids are well known for having a conspicuous cyrtos, the peniculid-related and cyrtos-bearing *Paranassula* indicates that the presence of a conspicuous cyrtos is a convergent character which might have evolved several times within the phylum Ciliophora (Gong et al., 2009b). Furthermore, the morphological and SSU rDNA sequence similarities (about 82.5–87.8%) between *Paranassula* and peniculids suggest that

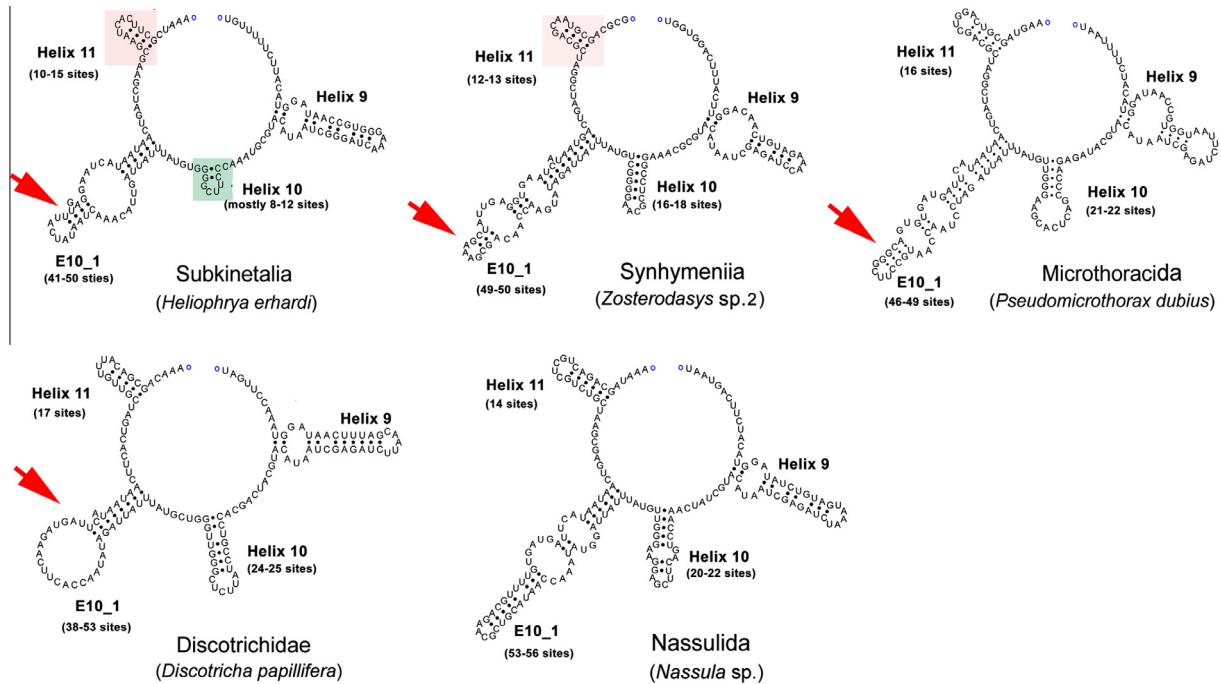


Fig. 4. Secondary structures of the small subunit ribosomal RNA gene in the V2 region of the five genera showing the progressively shortening of helices 10 and 11 in “Subkinetalia” and Synhymeniia. Arrows note the shortened E10_1 in “Subkinetalia”, Synhymeniia, Microthoracida and Discotrichidae compared to Nassulida. The range of helix lengths in each group is according to the SSU rDNA sequences used in building the tree as shown in Fig. 1.

Paranassula could represent an order level taxon within the subclass Peniculia (class Oligohymenophorea).

The genus *Parafurgasonia*, which was considered a typical member of the family Furgasoniidae (Lynn, 2008), exhibits a closer relationship to the family Colpodidiidae (e.g. *Colpodidium*) than to the family Furgasoniidae represented by *Furgasonia blochmanni* in the SSU tree (Fig. 1). The proposed divergence of *Parafurgasonia* from Furgasoniidae is supported by the absence of homologous sites specifically shared by *Parafurgasonia* and *Furgasonia*. Morphologically, *Parafurgasonia* is quite unlike *Colpodidium* in having a distinct cyrtos (vs. absent in *Colpodidium*) and a single oral polykinety (vs. one long and several reduced oral polykineties in *Colpodidium*), whereas the only character shared between these two taxa is the presence of a paroral kinety which, coincidentally, is absent in both *Nassula* and *Obertrumia*. Furthermore, the early branching furgasoniid taxon, *Furgasonia*, is also characterized by having a distinct paroral kinety and three oral polykineties (Eisler, 1988). This suggests that the loss of the paroral kinety and development/extension of oral polykineties could have occurred during the diversification of the core nassulids. Despite the morphological differences, the AU test does not reject the hypothesized monophyly of *Furgasonia* + *Parafurgasonia* ($P = 0.297$; Table 2). We therefore conclude that further taxonomic revision of the family Furgasoniidae should await more data.

4.3. A higher phylogenetic diversity in the genus *Zosterodasy*

With the addition of LSU sequences and greater taxon sampling, this study supports the conclusion from a previous study that the synhymeniids represent a subclass (Synhymeniia) of the class Phyllopharyngea (Gong et al., 2009b). Furthermore, our current SSU rRNA data show that two populations of the same species (i.e. *Z. agamalievi*) are divergent by 32 sites in rRNA sequences and do not form a monophyletic group in the phylogenetic trees (Fig. 1). A recent study has shown that, for peritrich and oligotrich ciliate species, having extremely high copy number of rDNA in a

single cell generally results in highly divergent rDNA sequences (Gong et al., 2013). Therefore, it is reasonable to postulate that high intragenomic rDNA polymorphisms could also occur in *Z. agamalievi*, which might lead to the non-monophyletic topology of these two populations.

It is also surprising that *Zosterodasy* sp.2 shares rather low SSU sequence identities (79.3–79.7%), but still clusters as a basal lineage with its congeners. The identical tree topologies and similar nodal support were obtained when the long-branching sequence of *Zosterodasy* sp.2 was excluded from the dataset (data not shown). This could be due to the fact that the SSU rDNA sequences of *Zosterodasy* sp.2 shares the most signature sites (e.g. 239, 272, 274, 1239; Fig. 3) with other synhymeniids. Nevertheless, although the high rDNA sequence variations observed at individual level can be naturally extrapolated to population/species level (Gong et al., 2013); such large differences of rDNA sequences between populations have yet to be confirmed.

4.4. Prostome taxa

Several subclades of the class Prostomatea can be recognized in the SSU-based ML and Bayesian trees (Fig. 1). However, the phylogenetic relationships among these subclades and other classes (e.g. Plagiopylea, Oligohymenophorea) are not resolved in the ML tree, but occasionally supported in the Bayesian tree. Some morphological characters may favor a close relationship between prostomes and oligohymenophoreans. For example, based on electron microscopic studies of morphogenesis, it has been suggested that the prostome ciliates should be classified close to the oligohymenophoreans (Bardele, 1999; Baroin-Tourancheau et al., 1992; Fleury et al., 1992). Having been supported in some molecular phylogenetic investigations but rejected in others, the monophyly of the class Prostomatea has long been uncertain (Lynn, 2003; Stechmann et al., 1998; Strüder-Kypke et al., 2006; Wright and Colorni, 2002). In the present study, the monophyly of the class Prostomatea was not supported by the SSU trees. The AU test marginally rejected the

hypothesized monophyly of the currently available prostome sequences. However, we conclude that there are still insufficient data to determine whether or not the class Prostomatea is monophyletic since the branching order of the four prostome clades is still unresolved. One possible reason is the under-sampling of representative taxa, that is molecular data are available for only 12 of 37 prostome genera. Alternatively, as the prostome SSU sequences do not share any specific homologous sites in the alignment (data not shown), it is highly likely that the prostome rDNA operons also show a non-strictly concerted evolution (considerable sequence variation within the macronuclear genome) as previously reported for peritrich and oligotrich ciliates (Gong et al., 2013), which further exhibits the constraints of using SSU rRNA alone in resolving the genealogy of prostomes.

The family Placidae has long been considered a member of the order Prorodontida (Corliss, 1979; de Puytorac, 1994; Lynn, 2008; Lynn and Small, 2002). Historically, however, its relationship with morphologically similar families was not clarified. In our study, *Placus* is sister to the *Holophrya*–*Pelagothrix* clade (Fig. 2), indicating that Placidae is closely related to the family Holophryidae, which is consistent with Lipscomb et al. (2012). This supports the kinship between *Placus* and holophryids proposed by Borrer (1972) based on morphological/morphogenetic characters and feeding behavior, and the assignment of this genus to the family Placidae (Small and Lynn, 1985). Likewise, the notion that *Placus* and *Plagiocampa* are closely related (Noland, 1937) should be rejected, despite the fact that they both possess a dense ciliary structure composed by dikinetids on the border of the oral slit, which were called adoral organelles, or brosse, in the later taxonomic studies (Foissner, 2000; Xu et al., 2005). In our SSU tree (Fig. 1), *Plagiocampa* clusters with *Cryptocaryon* with high support (ML 90%). The well-known parasite *Cryptocaryon* has variously been assigned to the families Ichthyophthiriidae, class Oligohymenophorea (Corliss, 1979), Cryptocaryonidae, class Prostomatea (Wright and Colorni, 2002), and Holophryidae, class Prostomatea (Lynn, 2008). Our findings are consistent with those of Wright and Colorni (2002), i.e. *Cryptocaryon* belongs to the family Cryptocaryonidae, which is sister to the order Plagiocampida.

5. Remarks on classification

Based on previous findings (Gong et al., 2009b) and results obtained in this study, we generally accept the latest classification by Adl et al. (2012) that the class Phyllopharyngea comprises five subclasses: Cyrtophoria, Chonotrichia, Rhynchodia, Suctorina and Synhymeniia. Nevertheless, name “Subkinetalia”, coined by Gong et al. (2009b) to refer to the superclade comprising the subclasses Cyrtophoria, Chonotrichia, Rhynchodia and Suctorina, that share a synapomorphic character (the presence of subkinetal microtubules), is still biologically meaningful. Sequence signatures for these taxa not given in previous work are supplied as follows:

SSU rRNA sequence signatures for the subclade “Subkinetalia”. Deletions occur in region E10-1 and helices 10 and 11 of the V2 region (Fig. 4). Sequences are more variable in the semi-conserved regions, i.e. sites 269–280, 473–483, and 1237–1245. There are three signature SNPs (single nucleotide polymorphisms), i.e. at sites 209 (T/C), 461 (C/T), and 1474 (A/C).

SSU sequence signatures of the subclass Synhymeniia. Five signatures are specifically shared by the synhymeniids, i.e. at sites 272–280 (A(c/g)C(c/a)(g/-)TGC), 473–483 (CAGACCGGGTC), 1329–1336 (GCTG(c/t)TA), 1340–1347 (CC(-/a)TAGCA) and 1486–1488 (AGT). Characteristic SNPs are detected at sites 239 (A vs. T), 1239 (T vs. A), and 1424 (A vs. G) (Fig. 3). The SSU sequence signatures for Synhymeniia appear to be abundant, indicating a characteristic evolutionary history of this taxon. Nevertheless, this

could also be due to the under-sampling, as only sequences from two closely related genera are included.

Based on the present study, we propose the removal of the genus *Discotricha* from the order Microthoracida (Class Nassophorea), and its assignment to an as-yet unclassified taxon within the clade CONthreeP. Thus, the redefined order Microthoracida comprises only two families, Leptopharyngidae and Microthoracidae.

Class Nassophorea Small and Lynn, 1981. Order Microthoracida Jankowski, 1967.

Diagnosis: Phyllopharyngeans with body mostly bilaterally flattened and rigid cell surface; buccal apparatus with several membranelles on right side of cytostome; fibrous trichocysts present; somatic ciliature on both sides of body, often consisting of dikinetids.

Two families included: Leptopharyngidae and Microthoracidae.

SSU sequence signatures for the order Microthoracida. Signatures at sites 277–280 (CTGC) and 1237–1245 (CTATGGGTA).

Order Nassulida Jankowski, 1967.

In the system of de Puytorac (1994), the order Paranassulida Deroux in de Puytorac et al. (1993) was considered as a member of the class Nassophorea, while the genus *Paranassula* only represented a family (Paranassulidae Fauré-Fremiet, 1962) within the order Nassulida (Lynn, 2008). According to our molecular phylogenetic analyses, we propose resurrecting the order Paranassulida and classifying it within the subclass Peniculia, class Oligohymenophorea. Three families remain in the order Nassulida, i.e. Nassulidae, Furgasoniidae, and Colpodidiidae,

SSU sequences signatures for the order Nassulida. Having no deletions in the helix 10, E10-1 and helix 11 of V2 region (Fig. 4). Three signatures at positions 473–483 (CAA(a/c)(ct)CG(g/t)GGC), 489–495 (GTGCTTA), and 1333–1336 (CTAA).

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