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

Qianqian Zhang a,b,1, Zhenzhen Yi c,1, Xinpeng Fan d, Alan Warren e, Jun Gong a,c,*, Weibo Song b, *

a Yantai Institute of Coastal Zone Research, Chinese Academy of Science, Yantai 264003, China
b Laboratory of Protozoology, College of Life Science, South China Normal University, Guangzhou 510631, China
c School of Life Science, East China Normal University, Shanghai 200062, China
d Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK

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 Colpodiidae 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 microthoracic 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 Placididae, 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 Peniculida, class Oligohymenophorea. Primary and secondary structure signatures for higher taxa within Phyllopharyngea and Nassophorea are supplied.

Keywords: Ciliophora, Phylogeny, 18S rRNA, 28S rRNA, Nassophorea, Prostomatea

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; Vidačny 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 Cariacotrichaea, 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, choanotrichians, rynchodians and suctorians) were then reclassified as members

* Corresponding authors. Address: Yantai Institute of Coastal Zone Research, Chinese Academy of Science, Yantai 264003, China (J. Gong), Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China (W. Song).
E-mail addresses: jgong@yic.ac.cn (J. Gong), wsong@ouc.edu.cn (W. Song).
1 Co-first authors.
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 Prostomata 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). Availibility 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 litostomatean genera, Cyclotrichium and Paraphrynia, 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 Paramussula, 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 species were made by referring to published guides (Foissner et al., 1994; Gong et al., 2009a; Lynn and Small, 2002; Xu et al., 2005; Vďačný 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 REDExtract-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 (<0.1). 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 (Darriba et al., 2012).
et al., 2012). The GTR+I+Γ model was again selected for both SSU and LSU rRNA datasets. In 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 consisted of 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”, Synhymenia, 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 nassophorean and prostomate 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 Synhymenia, which is strongly supported (100% ML, 1.00 BI) (Fig. 1). The monophyly of the genus Orthodona is highly supported, whereas the genus Zosterodasys is paraphyletic with Z. agamalevi and an unidentified Zosterodasys species basal to the subclass Synhymenia. Sequences of two unidentified Orthodonna species (sp.1 and 2) differed only by 1 base pair. The sequence of Orthodona sp.1 is identical to the only previously published Orthodonna sequence (GenBank number EU286809). These three sequences have a similarity of 98.5–98.6% to that of O. aphantomurus. 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 CONthriep in the latest system of Adl et al. (2012). The newly sequenced Parafurgasonia sp. shows a closer relationship with the genus Colpodium (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 (ML100%, 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 Colepiidae (ML 41%, BI 0.98). The species Placus salinus clusters with the family Holophryidae, represented by the genera Holophrya and Pelagophrya, with variable support (BI 0.95, 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 Urotrocha sp., the only sequenced species of the family Urotrichidae (ML 51%, BI 0.72). This clade forms a sister group with two litostomatean (?) species, Paraphryidae placus 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 protoneme 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” (Dystera derouxii) and two synhymeniids (Zosterodasys and Orthodonna) is recovered, although this topology is only weakly supported (ML 55%, BI 0.64). The microthoracid Leptopharynx costatus groups with the nassulids (Nassula and Zosterograpthus), with variable support (ML 53%, BI 0.99; Fig. 2). However, another microthoracid species, Discotricha papillifera, groups with the colpodean Breslauideae, 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 Paraphryidae (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


that Microthoracida and Discotrichidae form a monophyletic assemblage was rejected ($P = 0.043$), the hypothesized monophyly of "Subkinetalia" + Discotrichae ($P = 0.062$), and Prostomatea ($P = 0.053$) was also marginally rejected. However, the hypothesized monophyly of Nassulida + Synhymeniida + Microthoracida + Discotrichae 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 + Discotrichae ($P > 0.16$, Table 2); the monophly 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). Synhymeniids is featured by many signature fragments, e.g. A/c/g/c/a/g/-/GCG at sites 272–280, CAGACCGGCTc at sites 473–483, GC(c/t)GTA at sites 1329–1336, 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 (CTG) and 1237–1245 (CTATGGGTA). Nassulida have several signature regions at sites 473–483 (CAA(c/t)(c/t)CG(g/t)GGC), 489–495 (GTGGCTTA) 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 Synhymeniids 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
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 Microthoracia and Discotrichidae.

Among the nassulids, 11 homologous sites are exclusively shared by *Parafurgasonia* sp. and the Colpodidiidae species, whereas no homologous sites are specifically shared by *Furgasonia blochmanni* and *Parafurgasonia* sp.

Increased sampling of taxa has unveiled missing evolutionary links between Phyllopharyngea and nassophores, 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 nassophores (e.g. microthoracids, Paranassulidae and Furgasoniidae) and prostomates (e.g. Plagiocampidae and Placidae).

**4.1. Microthoracids and Discotricha**

In the SSU rRNA tree, the family Discotrichidae, represented by *Discotricha papillifera*, a member previously assigned in the order Microthoracida, is classified as incerta 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>
<thead>
<tr>
<th>Alternative hypothesis</th>
<th>P-value</th>
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<tbody>
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<td>Class Prostomatea</td>
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<tr>
<td>Nassulida + Microthoracida + Synhymeniida + Discotrichidae</td>
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</tr>
<tr>
<td>Nassulida + Synhymeniida</td>
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<tr>
<td>Nassulida + Microthoracida</td>
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<tr>
<td>Microthoracida + Discotrichida</td>
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</tr>
<tr>
<td>Furgasonia blochmanni + Parafurgasonia sp.</td>
<td>0.297</td>
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</table>

**4. Discussion**

Increased sampling of taxa has unveiled missing evolutionary links between Phyllopharyngea and nassophores, 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 nassophores (e.g. microthoracids, Paranassulidae and Furgasoniidae) and prostomates (e.g. Plagiocampidae and Placidae).
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 polykintetid 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...
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, coincidently, 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 Zosterodasys

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 rDNA data show that two populations of the same species (i.e. Z. agamalievi) are divergent by 32 sites in rDNA 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 Zosterodasys 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 Zosterodasys sp.2 was excluded from the dataset (data not shown). This could be due to the fact that the SSU rDNA sequences of Zosterodasys 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 (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–Pelagotricha 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 Borror (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, 1997) 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 (Foisson, 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, which has variously been assigned to the families Ichthyophthiridae, class Oligohymenophorea (Corliss, 1979), Cryptocaryonidae, class Prostomata (Wright and Colorni, 2002), and Holophryidae, class Prostomata (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: Cytophoria, Chonotrichia, Rynchododia, Suctoria and Synhymenia. Nevertheless, name “Subkinetalia”, coined by Gong et al. (2009b) to refer to the superclade comprising the subclasses Cytophoria, Chonotrichia, Rynchododia and Suctoria, 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 subclass “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 Synhymenia**. Five signatures are specifically shared by the synhymenids, i.e. at sites 272–280 (A(c)/c(g)c(a)/g(j)-TGC), 473–483 (C(AG)C(C/G)G(TC), 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 Synhymenia 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, Leptopharyngiidae and Microthoracidae.

**Class Nassophorea Small and Lynn, 1981.**

**Order Microthoracida Jankowski, 1967.**

Diagnosis: Phyllopharynges 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: Leptopharyngiidae 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 Penicilia, 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 (C(A/a)c(CT)CG(t/g)TGC), 489–495 (GTGCTTA), and 1333–1336 (CTAA).

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**References**


