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Expanding Character Sampling for Ciliate Phylogenetic Inference Using Mitochondrial SSU-rDNA as a Molecular Marker

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Molecular systematics of ciliates, particularly at deep nodes, has largely focused on increasing taxon sampling using the nuclear small subunit rDNA (nSSU-rDNA) locus. These previous analyses have generally been congruent with morphologically-based classifications, although there is extensive non-monophyly at many levels. However, caution is needed in interpreting these results as nSSU-rDNA is just a single molecular marker. Here the mitochondrial small subunit rDNA (mtSSU-rDNA) is evaluated for deep ciliate nodes using the Colpodea as an example. Overall, well-supported nodes in the mtSSU-rDNA and concatenated topologies are well supported in the nSSU-rDNA topology; e.g., the non-monophyly of the Cyrtolophosidida. The two moderately- to well-supported incongruences between the loci are the placement of the Sorogenida and *Colpoda aspera.* Our analyses of mtSSU-rDNA support the conclusion, originally derived from nSSU-rDNA, that the morphological characters used in taxonomic circumscriptions of the Colpodea represent a mixture of ancestral and derived states. This demonstration of the efficacy of the mtSSU-rDNA will enable phylogenetic reconstructions of deep nodes in the ciliate tree of life to move from a single-locus to a multi-locus approach.

Key words: Ciliophora; Colpodea; mitochondrial SSU-rDNA; nuclear SSU-rDNA; phylogeny

Introduction

Whether it is better to increase the number of sampled taxa or the number of characters to improve the accuracy of phylogenetic inference is a central debate in molecular systematics (Cummings and Meyer 2005; Graybeal 1998; Hedtke et al. 2006; Hillis 1998; Hillis et al. 2003; Poe and Swofford 1999; Rannala et al. 1998; Rokas and Carroll 2005; Rokas et al. 2003). Each approach has its strengths and weaknesses, and it is generally advantageous to increase both when inferring the tree of life of any group of organisms. But this has not always been possible in all clades—such as in ciliates (Ciliophora Doflein, 1901).

In ciliates, molecular phylogenetic inferences to test morphologically-based hypotheses of deep relationships have relied primarily on expanding taxon sampling using just the nuclear small subunit ribosomal DNA (nSSU-rDNA) locus (e.g., Agatha and Strüder-Kypke 2007; Dunthorn et al. 2008, 2009; Gong et al. 2009; Schmidt et al. 2007a,

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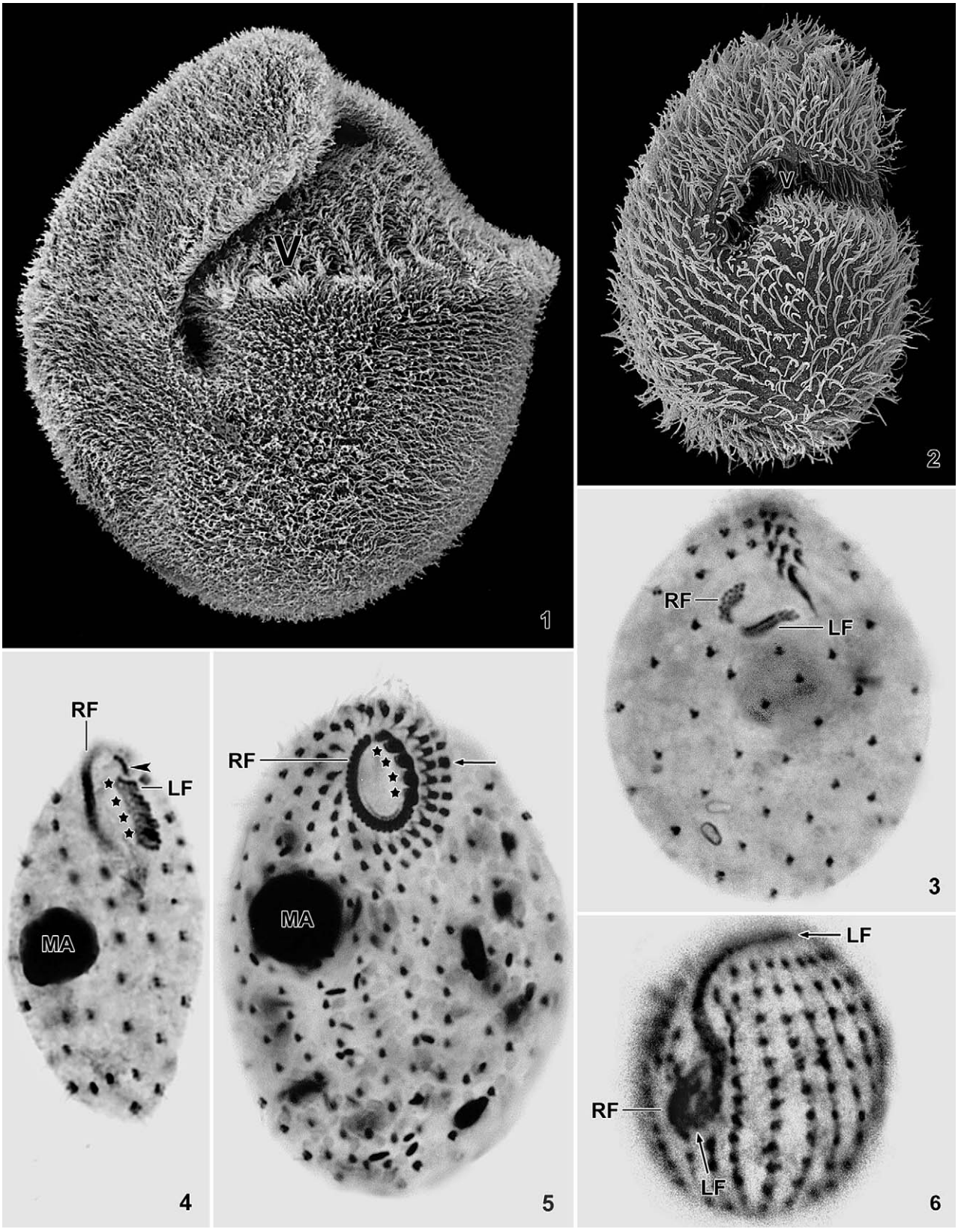
2007b; Strüder-Kypke et al. 2006; Williams and Clamp 2007; Yi et al. 2008). For these deep ciliate nodes, nSSU-rDNA gene trees are concordant with many morphological hypotheses, but there are a number of discrepancies (Dunthorn and Katz 2008; Foissner et al. 2004; Lynn, 2003, 2008). Because of this single-locus approach, we do not know if nSSU-rDNA molecules are elucidating ciliate evolution or just misleading us.

As additional molecular markers, nuclear protein-coding loci are problematic because their extensive paralogy and heterogeneous rates of evolution can lead to spurious phylogenetic relationships (Israel et al. 2002; Katz et al. 2004; Zufall and Katz 2007; Zufall et al. 2006). Moreover, the genome architecture of some ciliates enables generation of macronuclear protein families from alternatively processed and scrambled micronuclear chromosomes (Katz and Kovner 2010), processes that will further confound phylogenetic inferences. In contrast, for shallower ciliate nodes there are a number of available molecular markers from both nuclear protein-coding loci (Catania et al. 2008; Przyboś et al. 2006; Snoke et al. 2006; Ye and Romero 2002) and mitochondrial loci (Barth et al. 2008; Catania et al. 2008; Chantangsi and Lynn 2008; Chantangsi et al. 2007; Gentekaki and Lynn 2009; Lynn and Strüder-Kypke 2006; Snoke et al. 2006; Strüder-Kypke and Lynn 2010).

One ciliate lineage in which nSSU-rDNA genealogies have been compared to morphological hypotheses is the Colpodea Small & Lynn, 1981 (Figs 1-6). The Colpodea is diagnosed by a LKm (left kinetodesmal) fiber and unique silverline patterns (Foissner 1993; Lynn 2008). This primarily terrestrial group contains diverse oral morphologies and potentially arose 900 MYA (Lynn 2008; Wright and Lynn 1997). The Colpodea may or may not be an ancient asexual clade (Dunthorn et al. 2008; Dunthorn and Katz 2010; Foissner 1993). The almost 200 described species are monographed with an extensive morphological classification (Foissner 1993). Nuclear SSU-rDNA analyses have challenged some aspects of this morphologically-based classification (Dunthorn et al. 2008, 2009; Foissner and Stoeck 2009; Lasek-Nesselquist and Katz 2001; Lynn et al. 1999). In light of the nSSU-rDNA data, modified hypotheses of morphological evolution have been proposed (Dunthorn et al. 2008, 2009; Foissner and Kreutz 1998; Lasek-Nesselquist and Katz 2001; Lynn et al. 1999).

Here we move molecular systematics for deep ciliate nodes towards increasing character sampling by sequencing a broad sample of the Colpodea for another molecular marker: the mitochondrial small subunit rDNA (mtSSU-rDNA) locus. We test if well-supported nodes in the SSU-rDNA topology are congruent with well-supported nodes in the nSSU-rDNA topology, as well as if mtSSU-rDNA provides more resolution in nodes that are unsupported in the nSSU-rDNA topology. Our approach generates additional molecular characters for analyses that are not only from an independent locus but also from a separate genome. Hence, analyzing both nSSU-rDNA and mtSSU-rDNA has the potential to substantially increase our power for inferring deep nodes in the ciliate tree of life and mapping morphological changes within this microbial eukaryotic clade.

Figures 1-6. Morphological variation within the Colpodea. Some of the species sequenced for the mtSSU-rDNA here are shown in scanning electron micrographs (1,2) and in protargol silver preparations (3-6), exhibiting somatic and oral ciliary patterns. **1)** *Bresslauides discoideus* and **2)** *Colpoda cucullus* are closely related in the gene trees, but differ in overall size (up to 600 μm vs. 150 μm), vestibulum (very large vs. small), oral ciliary structures (hausmanniellid vs. colpodid), and habit (predaceous vs. bacteriovorous). **3)** *Colpoda aspera* and **6)** *Bardeliella pulchra* are also closely related in the gene trees but with no node support, even though they are classified in different families (Colpodidae and Bardeliellidae). In *B. pulchra* (Fig. 6) the left oral ciliary field (LF) is greatly and uniquely modified to a very long, vertically oriented ribbon, while minute and horizontally oriented in *C. aspera* (Fig. 3), as in all other *Colpoda* species. **4**) *Cyrtolophosis mucicola* and **5)** *Platyophrya bromelicola* are in the Cyrtolophosidida I and II (Fig. 7), respectively. Cyrtolophosidida was originally characterized by a shared outer membrane of the micronucleus and macronucleus, a “simple” dikinetidal right oral ciliary field (RF), and a strand of brick-shaped adoral organelles in the left oral ciliary field (asterisks). The molecular data indicate that these features are either an ancestral state of the Colpodea, or evolved convergently at least twice. There are differences between the Cyrtolophosidida I and II: *C. mucicola* has a minute, vertically oriented organelle (Figure 4, arrowhead), while *P. bromelicola* has a membrane-like ciliary condensation left of the adoral organelles (Figure 5, arrow). LF – left oral ciliary field, MA – macronucleus, RF – right oral ciliary field, V – vestibulum.


V
1
V
2
RF
LF
3
RF
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MA
4
RF
MA
5
LF
RF
LF
6

Table 1. Taxon sampling for nuclear and mitochondrial SSU-rDNA. GenBank numbers for new sequences are in bold. Measurements for previously published mtSSU-rDNA sequences were made for only the part of the sequence that would have been amplified by the primers used here.

Taxon	mtSSU			nucSSU
	sequence length (bp)	% GC content	GenBank #	GenBank #
Aristerostoma sp. ATCC #50986	1031	36.95	**HM246398**	EU264563
Bardeliella pulchra	1139	34.5	**HM246399**	EU039884
Bresslauides discoideus	1063	34.71	**HM246400**	EU039885
Bryometopus atypicus	1082	35.49	**HM246401**	EU039886
Bursaria spec. ("muco")	1071	32.77	**HM246402**	EU039889
Bursaria truncatella	1100	32.45	**HM246403**	U82204
Chilodonella uncinata	894	25.5	**HM246404**	AF300281
Colpoda aspera	1149	31.77	**HM246405**	EU039892
Colpoda cucullus	1094	31.44	**HM246406**	EU039893
Colpoda henneguyi	1083	31.39	**HM246407**	EU039894
	1082	31.15	**HM246408**[1]	
Colpoda lucida	1126	30.46	**HM246409**	EU039895
Colpoda magna	1152	33.68	**HM246410**	EU039896
Cyrtolophosis mucicola Austria	968	30.89	**HM246411**	EU039899
Cyrtolophosis mucicola Brazil	969	33.33	**HM246412**	EU039898
Hausmanniella discoidea	1076	32.99	**HM246413**	EU039900
Ottowphrya dragescoi	964	31.33	**HM246414**	EU039904
Paramecium primaurelia	994	34.91	K01750	AF100315
Paramecium tetraurelia	992	34.98	X15917[2]	X03772
Platyophrya bromelicola	970	32.89	**HM246415**	EU039906
Platyophrya-like sp.	1003	34.2	**HM246416**	EU039905
Rostrophrya sp.	993	34.54	**HM246417**	EU039907
Sagittaria sp.	1004	35.66	**HM246418**	EU039908
Sorogena stoianovitchae	1001	33.87	**HM246419**	AF300285
Tetrahymena pyriformis	1038	32.15	AF160864	M98021
Tetrahymena thermophila	1037	30.95	AF396436	X56165

[1]Not used in phylogenetic analyses.
[2]Labeled as *Paramecium aurelia* in GenBank.

Results

Characteristics of Gene Sequences

Twenty-one morphospecies were sequenced here for the mtSSU-rDNA locus (Table 1). The amplified products are of variable size and G-C content. For all sequences, the average number of base pairs is 1070, with a minimum of 894 in *Chilodonella uncinata* and a maximum of 1152 in *Colpoda magna*. Towards the five-prime end there is considerable variation in length due to insertions and deletions. The average G-C content is 32.9%.

Intra-isolate genetic variation in the mtSSU-rDNA locus was not found, except in *Colpoda henneguyi* (Table 1). The distance between the two *C. henneguyi* sequences is 2.69%; this same isolate had two different nSSU-rDNA sequences with a distance 0.12% (Dunthorn et al. 2008). As the DNA was extracted from a non-clonal culture, these differences may be due to within population variation (or presence of cryptic species) as opposed to within individual variation. The distance between two *Cyrtolophosis mucicola* isolates—one from Austria, the other from Brazil—is 10.05%; while their distance in nSSU-rDNA is 1.71% (Dunthorn et al. 2008). The nSSU-rDNA data from Dunthorn et al. (2008) and the mtSSU-rDNA here suggest that these two *C. mucicola* isolates may represent cryptic species.

Mitochondrial SSU-rDNA Analyses

Ambiguously aligned positions were removed in two ways. First, by eye in MacClade (Maddison and Maddison 2005), resulting in an alignment that includes 823 unmasked characters, of which 491 are parsimony-informative. Second, with the program Gblocks (Castresana 2000; Talavera and Castresana 2007) set to default parame-

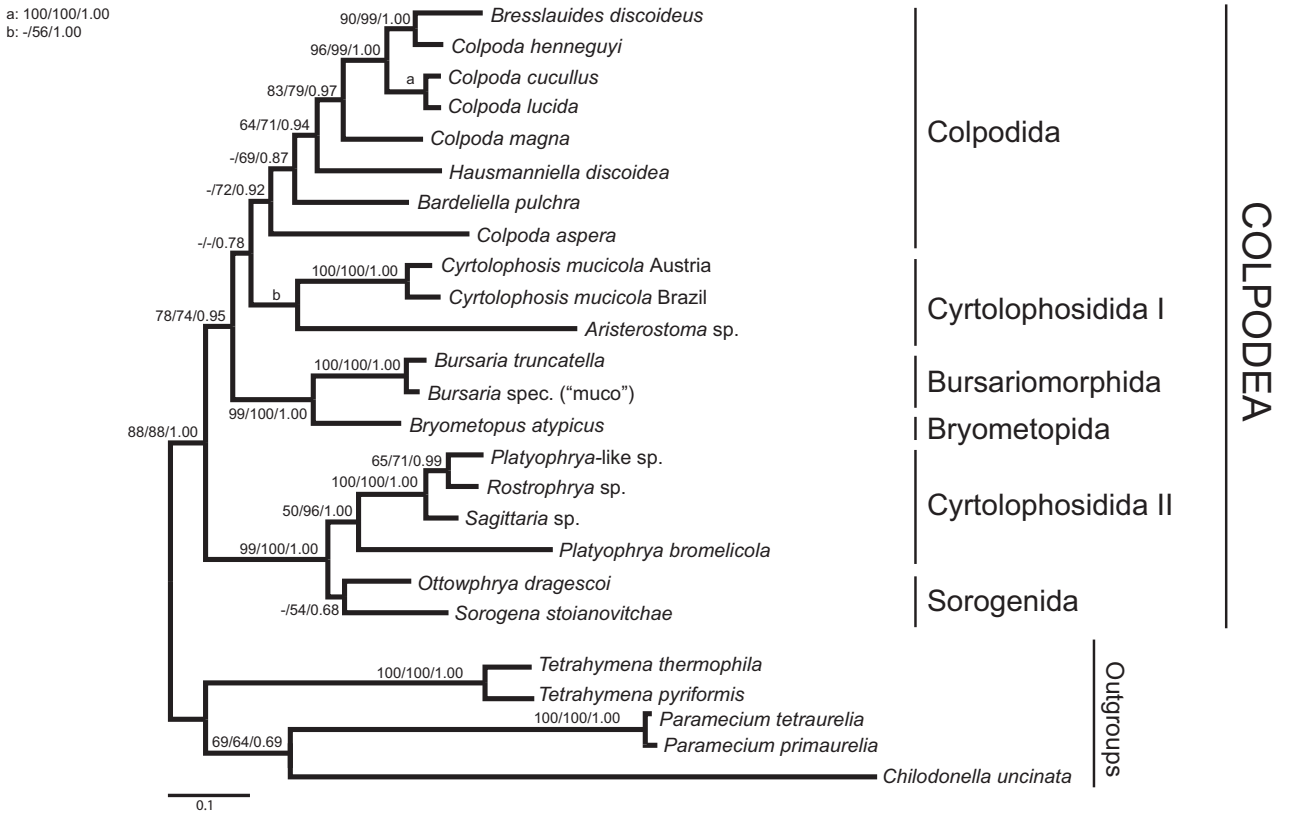

Figure 7. Mitochondrial SSU-rDNA topology of the Colpodea. The most likely ML tree and its branch lengths are shown. The Bayesian tree inferred using MrBayes and the ML tree are identical in topology. Node support is as follows: MP bootstrap/ML bootstrap/BI (MrBayes) posterior probability. Support <50% is shown as '-'.

ters, resulting in an alignment that includes 731 unmasked characters, of which 410 are parsimony-informative. Initial maximum likelihood analyses using the GTR-I-Γ model of evolution showed that the resulting topologies were congruent (data not shown); therefore, the alignment masked by eye was used for further phylogenetic analyses. Bayesian inference was first performed using the GTR-I-Γ model of evolution as implemented in MrBayes (Huelsenbeck and Ronquist 2003); hereafter referred to as the MrBayes tree.

There is little difference among the MP, ML, and MrBayes topologies for well-supported nodes, and the ML and MrBayes trees are identical. Here we present the most likely ML tree with node support from all three methods (Fig. 7). In all analyses the Colpodea is monophyletic, with moderate to full node support (MP bootstrap 88/ML bootstrap 88/MrBayes posterior probability 1.00). However, mtSSU-rDNA does not provide a valid test of monophyly here because of the current limited taxon sampling of outgroup lineages.

Mitochondrial SSU-rDNA does not support the monophyly of the Cyrtolophosidida (Fig. 7). The Cyrtolophosidida falls into two clades with moderately supported intervening nodes (78/74/0.95): Cyrtolophosidida I, which includes those taxa in the Cyrtolophosididae, is sister to the Colpodida with no (i.e., < 50) to low node support (-/-/0.78); and Cyrtolophosidida II, which includes the remaining sampled Cyrtolophosidida. Sorogenida is monophyletic with no to low node support (-/54/0.68), and is sister to Cyrtolophosidida II with high to full node support (99/100/1.00). Bryometopida and Bursariomorphida are sister to each other with high to full node support (99/100/1.00). Colpodida is monophyletic in the mtSSU-rDNA topology, though with no to moderate node support (-/72/0.92). Within the shallow ciliate nodes in the Colpodida, *Colpoda* is not monophyletic as *Bresslauides* nests within it with high to full node support (90/99/1.00). Both *Bardeliella* and *Hausmanniella* also nest within *Colpoda* in the mtSSU-rDNA topology but with only no to moderate node support (-/69/0.87 and 64/71/0.94). *Colpoda aspera* is sister to the clade formed by *Bardeliella*, *Bresslauideus*, *Hausmanniella*, and the other sampled *Colpoda* with no to moderate node support (-/72/0.92).

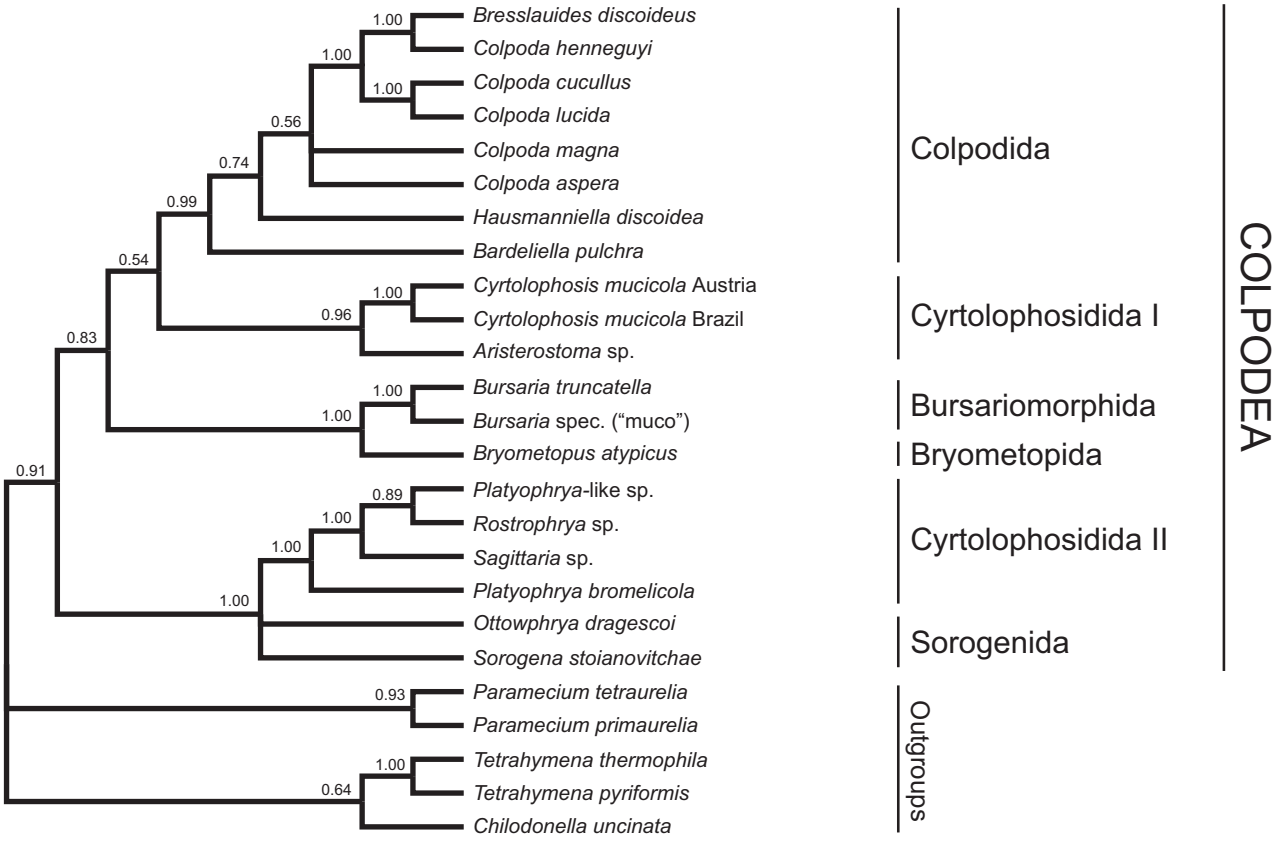

Figure 8. Mitochondrial SSU-rDNA topology of the Colpodea. Bayesian tree and node support inferred using PhyloBayes.

To account for the possibility of model and rate variation, the mtSSU-rDNA alignment was also analyzed using a second method of Bayesian inference that used a Dirichlet processes of different GTR matrices for a model of evolution as implemented in PhyloBayes (Lartillot and Philippe 2004; Lartillot et al. 2009); hereafter referred to as the PhyloBayes tree. The PhyloBayes tree is largely congruent with the ML and MrBayes tree for relationships within the Colpodea (Fig. 8). The PhyloBayes tree differs in: Sorogenida does not form a resolved clade; and *C. aspera* forms a clade with the remaining *Colpoda* plus *Bresslauideus*, although node support is low for this relationship (0.56 posterior probability).

Nuclear SSU-rDNA Analyses

To test whether truncated taxon sampling will affect the topology of the Colpodea, taxon inclusion in nSSU-rDNA alignment from Dunthorn et al. (2009) was decreased to the same sampling as that of mtSSU-rDNA here. This alignment includes 1631 characters, of which 438 are parsimony-informative. The MP, ML and MrBayes topologies are identical, except that in the ML tree *C. aspera* and *H. discoidea* are sister to each other, but there is no node support for this relationship. Here we present the most likely ML tree with node support from all three methods of analysis (Fig. 9).

The nSSU-rDNA topology for the Colpodea here (Fig. 9) is the same as those previously published analyses based on larger taxon sampling (Dunthorn et al. 2008, 2009), except for the low MP and MrBayes, but moderate ML, node support for the clade formed by Cyrtolophosidida I and Colpodida (57/90/0.57). The low support for this same clade in the mtSSU topologies (Figs 7, 8), may be due to the lower taxon sampling here, and may increase as more taxa are sampled for the mtSSU-rDNA.

Topology Testing

Overall, the nSSU- and the mtSSU-rDNA topologies are congruent for well-supported nodes, except in the placement of the Sorogenida and

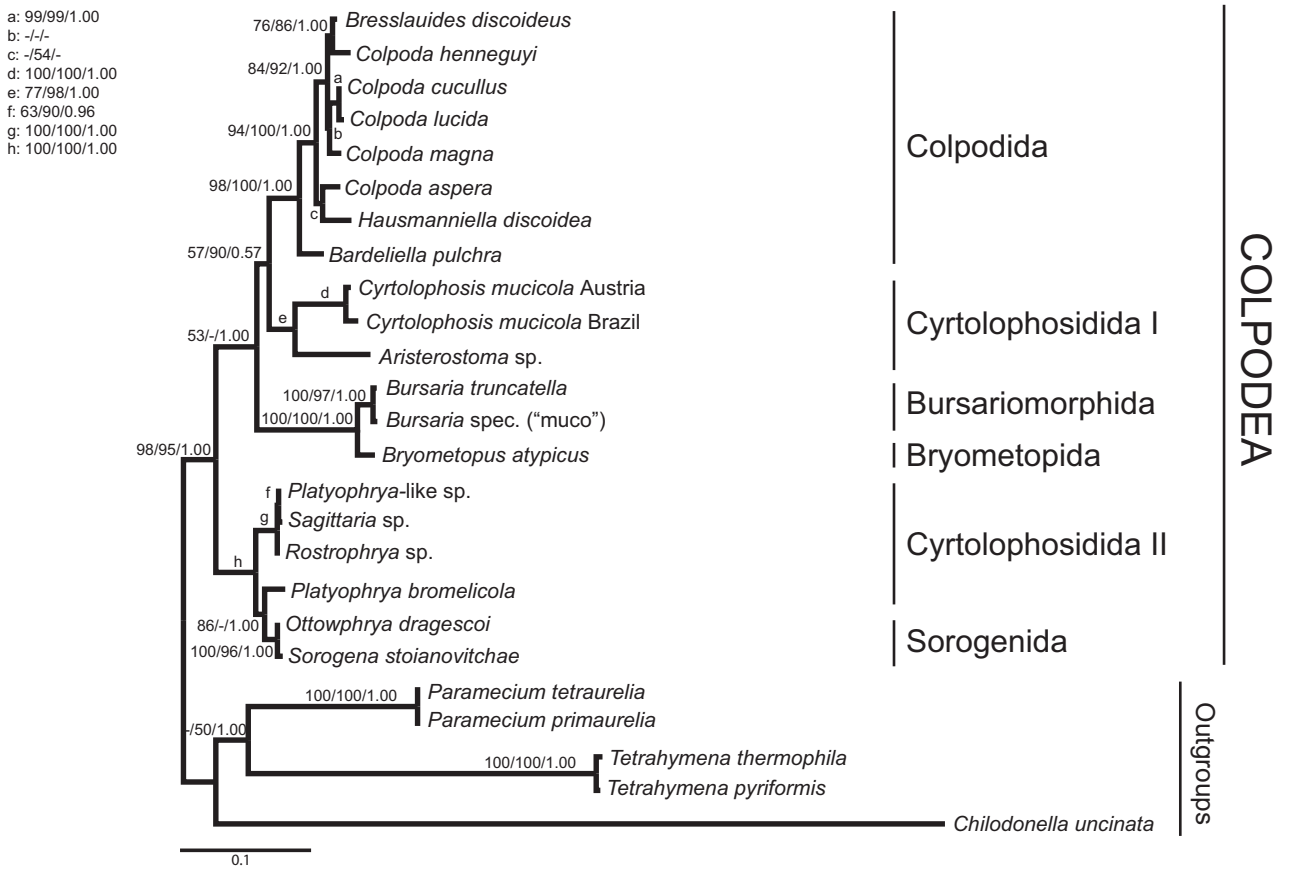

Figure 9. Nuclear SSU-rDNA topology of the Colpodea. The most likely ML tree and its branch lengths are shown. The Bayesian tree inferred using MrBayes and the ML tree are identical in topology. Node support is as follows: MP bootstrap/ML bootstrap/BI (MrBayes) posterior probability. Support <50% is shown as '-'.

C. aspera. To further compare the loci, Approximately Unbiased (AU) tests were carried out on the mtSSU-rDNA alignment where the ambiguously aligned positions were removed by eye, the mtSSU-rDNA alignment where the positions were removed by GBlock, and on the nSSU-rDNA alignment (Table 2).

In the mtSSU-rDNA topology the Sorogenida is sister to the Cyrtolophosidida II (Fig. 7), while in the nSSU-rDNA topology the Sorogenida is sister only to *Platyophrya bromelicola* (Fig. 9). The mtSSU-rDNA alignments could not reject the constrained topology where the Sorogenida was forced to be sister to *P. bromelicola*, as in the nSSU-rDNA topology ($p = 0.159$ and 0.283). Likewise, the nSSU-rDNA topology could not reject the constrained topology where the Sorogenida was forced to be sister to the clade formed by *Platyophrya*/*Sagittaria*/*Rostrophrya*/*Platyophrya*-like, as in the mtSSU-rDNA topology ($p = 0.365$). Therefore, the phylogenetic placement of the Sorogenida remains ambiguous.

In the mtSSU-rDNA topology *C. aspera* is sister to the clade formed by *Bardeliella*, *Hausmanniella*, *Bresslauides*, and the remaining *Colpoda* (Fig. 7), while in the nSSU-rDNA topology *C. aspera* is sister to just *Hausmanniella* (Fig. 9). All alignments were able to reject the constraint that all *Colpoda* form a monophyletic clade, as in the morphological classification ($p = 0.005$, 0.035, 0.009). However, the alignments were not able to reject the constraint that all *Colpoda* plus *Bresslauides* form a monophyletic clade ($p = 0.371$, 0.521, 0.572). Therefore, the phylogenetic placement of *C. aspera* remains ambiguous at least in relation to *Bardeliella* and *Hausmanniella*.

Concatenated Analyses

Given the overall congruence between the topologies and the results of the AU tests, a concatenated alignment of the nSSU- and mtSSU-rDNA sequences was compiled in order to further evaluate phylogenetic relationships in the Colpodea. This

Table 2. Approximately Unbiased test results. The unconstrained topologies were able to reject only the topologies where all sequences from *Colpoda* were constrained to be monophyletic.

Locus	Topology constraints	Log-likelihood	AU vaule (p)
mtSSU-rDNA[1]	unconstrained	−10252.4378	0.758
	Ottowphrya/Platyophrya/Sorogena monophyletic	−10263.65672	0.159
	Colpoda monophyletic	−10294.26615	0.005
	Colpoda/*Bresslauides* monophyletic	−10256.70544	0.371
mtSSU-rDNA[2]	unconstrained	−8368.18005	0.706
	Ottowphrya/*Platyophrya*/*Sorogena* monophyletic	−8375.782309	0.283
	Colpoda monophyletic	−8396.789226	0.035
	Colpoda/*Bresslauideus* monophyletic	−8370.52048	0.521
nSSU-rDNA[1]	unconstrained	−7952.422245	0.553
	Platyophrya/*Platyophrya*-like/*Rostrophrya*/*Sagittaria* monophyletic	−7954.886298	0.365
	Colpoda monophyletic	−7984.953972	0.009
	Colpoda/*Bresslauides* monophyletic	−7952.848102	0.572

Ambiguously aligned positions removed by [1]eye or [2]GBlocks.

alignment includes 2454 characters, of which 929 are parsimony-informative. There was little difference in the MP, ML, and MrBayes topologies for well-supported nodes, and the ML and MrBayes trees are identical. Here we present the most likely ML tree with node support from all three methods (Fig. 10).

The nSSU-rDNA, mtSSU-rDNA, and concatenated topologies are largely congruent with each other for almost all relationships, except for the placement of Sorogenida and *C.aspera* (Figs 7–10). Like the mtSSU-rDNA topology (Fig. 7), Sorogenida is sister to Cyrtolophosidida II in the concatenated topology (Fig. 10). *Colpoda aspera* forms clade with the remaining *Colpoda* and *Bresslauides* with no node support (-/-/-). The clade formed by Cyrtolophosidida I and Colpodida has low MP and MrBayes, but moderate ML, node support in the concatenated topology (-/84/0.87). This low MP and MrBayes support may likewise be due to low taxon sampling (see above).

To account for the possibility of model and rate variation, the concatenated alignment was likewise analyzed using the Dirichlet processes of different GTR matrices in PhyloBayes (Lartillot and Philippe 2004; Lartillot et al. 2009). The PhyloBayes tree is largely congruent with the ML and MrBayes tree for relationships within the Colpodea (Fig. 11). The PhyloBayes tree differs in that the Sorogenida forms a paraphyletic lineage at the base of the Cyrtolophosidida II.

Discussion

Phylogenetic Relationships in the Colpodea

The potential problem that individual gene trees may not necessarily reflect the species tree affects all organisms (Doyle 1992, 1997; Maddison 1997). So in ciliates there have been critiques that nSSU-rDNA gene trees may not provide an accurate inference of phylogeny where there are discrepancies between data from morphology and molecules (Agatha 2004; Dunthorn et al. 2008; Foissner et al. 2004; Schmidt et al. 2007a). On the other hand, alternative hypotheses, or re-interpretations, of morphological evolution given the topology of nSSU-rDNA gene trees have been suggested (Dunthorn et al. 2008; Dunthorn and Katz 2008; Lynn et al. 1999; Strüder-Kypke and Lynn 2003). To help resolve this, here we present additional and independent molecular data from the mtSSU-rDNA locus for testing hypotheses of relationships and morphological evolution for deep ciliate nodes.

When there are discrepancies between morphology and nSSU-rDNA analyses within the Colpodea

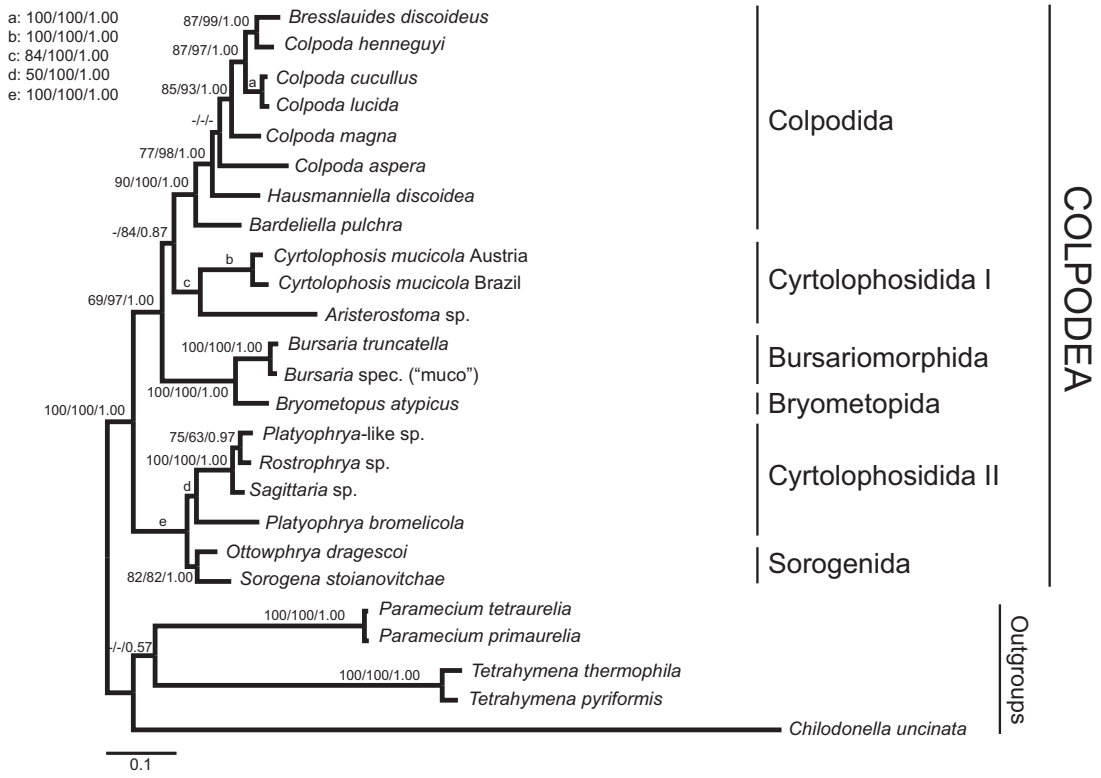

Figure 10. Concatenated nuclear and mitochondrial SSU-rDNA topology of the Colpodea. The most likely ML tree and its branch lengths are shown. The Bayesian tree inferred using MrBayes and the ML tree are identical in topology. Node support is as follows: MP bootstrap/ML bootstrap/BI (MrBayes) posterior probability. Support <50% is shown as '-'.

for deep nodes, the mtSSU-rDNA genealogy largely supports inferences made from analyses of nSSU-rDNA. For example, in our mtSSU-rDNA (Figs 7, 8), nSSU-rDNA (Fig. 9), the concatenated (Figs 10, 11) analyses, as well as those from previous nSSU-rDNA analyses (Dunthorn et al. 2008, 2009), the Cyrtolophosidida falls out into two separate clades separated by moderate to well-supported nodes: Cyrtolophosidida I and Cyrtolophosidida II. These two Cyrtolophosidida groups differ in details of their oral structures (Dunthorn et al. 2008; Foissner et al. 2002; Figs 4, 5).

Bryometopida and Bursariomorphida form a clade in analyses of nSSU-rDNA (Dunthorn et al. 2008; Lynn et al. 1999; Fig. 9). The mtSSU-rDNA (Figs 7, 8) and concatenated (Figs 10, 11) topologies support this relationship with high to full node support. Dunthorn et al. (2008), Foissner and Kreutz (1998), and Lynn et al. (1999) note that the Bryometopida and Bursariomorphida do share a number of morphological characters: apical oral structures, ventral clefts, adoral organelles that are conspicuous, and cysts with emergence pores.

Colpodida and Cyrtolophosidida I also form a clade in both the current analysis and previous nSSU-rDNA analyses (Dunthorn et al. 2008, 2009; Fig. 9). The mtSSU-rDNA (Figs 7, 8) and concatenated (Figs 10, 11) topologies likewise support this relationship with no to moderate node support. There is currently no known morphological character that unites these two groups, as the Cyrtolophosidida I may retain the ancestral morphological condition of the Colpodea, while the Colpodida exhibits numerous derived morphologies (Dunthorn et al. 2008).

Not all relationships are congruent between nSSU-rDNA and mtSSU-rDNA for deep nodes. The Sorogenida nests within the Cyrtolophosidida II in the current and previous nSSU-rDNA analyses with no to full node support (Dunthorn et al. 2008, 2009; Fig. 9). In contrast, the mtSSU-rDNA (Figs 7, 8) and the concatenated (Figs 10, 11) analyses place the Sorogenida sister to the Cyrtolophosidida II, likewise with no to full node support. Some possible explanations for this incongruence are differential rates of evolution between the loci or incomplete lineage sorting. In the

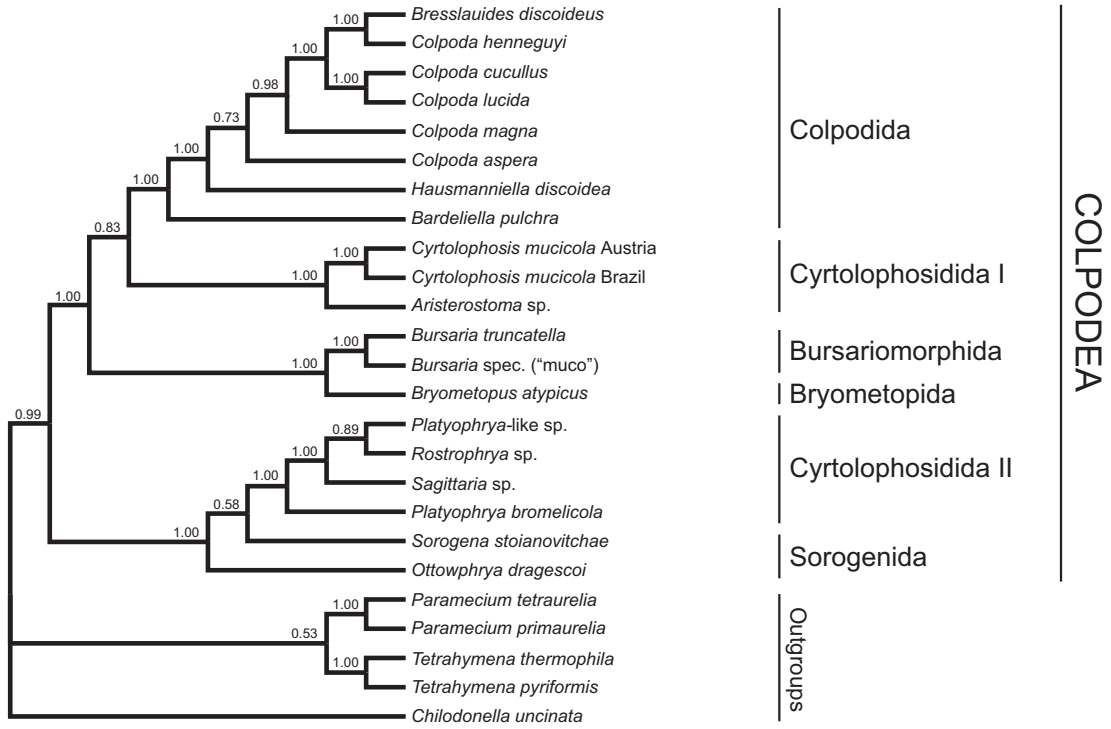

Figure 11. Concatenated nuclear and mitochondrial SSU-rDNA topology of the Colpodea. Bayesian tree and node support inferred using PhyloBayes.

AU tests, the unconstrained nSSU-rDNA topology could not reject the constrained topology that matched the mtSSU-rDNA relationship; likewise the unconstrained mtSSU-rDNA topologies could not reject the constrained topology, following the nSSU-rDNA relationship (Table 2). The phylogenetic placement of the Sorogenida thus remains unresolved. Although additional taxon sampling of previously unsequenced Cyrtolophosidia II species is needed to resolve the position of these taxa, at least a close relationship between Sorogenida and Cyrtolophosidida II is supported in that both having brick-shaped organelles on the left side of the oral structure as well as pleurotelokinetal stomatogenesis (partial re-organization of parental oral structures during cell division) (Dunthorn et al. 2008).

Within the shallow relationships in the Colpodida, node support is variable and there is a lack of resolution for many relationships from both molecular markers (Figs 1–3, 6–11). *Colpoda* is not monophyletic as *Bresslauides* nests within in the nSSU-rDNA, mtSSU-rDNA, and concatenated topologies here with high to full node support (Figs 7–11) and in a previous nSSU-rDNA analysis (Dunthorn et al. 2008). The non-monophyly of *Colpoda* in relation to *Bresslauides* is also supported by AU tests of both loci in that the topologies could reject the constraint that only the *Colpoda* isolates form a monophyletic clade (Table 2). Dunthorn et al. (2008) suggest that *Bresslauides* was taxonomically split off from *Colpoda* because of potential apomorphies (e.g., large semicircular right oral polykinetids, larger size, and feeding on other ciliates) that arose from within the *Colpoda* clade (Figs 1–3).

It should be noted that *Bresslauides* is not sister to the other sampled member of the Hausmanniellidae (i.e., *Hausmanniella*), suggesting that either the diagnostic characters for the Hausmanniellidae arose convergently or that the independent nSSU- and mtSSU-rDNA markers are both misleading in the same way. Although *Colpoda henneguyi*, *C. cucullus* (Fig. 4), and *C. lucida* are morphologically so similar that they were separated only recently (Foissner 1993); however, in the mtSSU-rDNA topology (Fig. 7) *C. henneguyi* is sister to *Bresslauides*. This relationship may be unlikely given that *Bresslauides* is much larger than the three *Colpoda* (200 – 600 μm vs. 60–150 μm) and has a different lifestyle (eats other ciliates vs. bacteriovorous). Either both nSSU- and mtSSU-rDNA are misleading at this depth in the ciliate tree of life, or morphological evolution in the *Colpoda* may

be extremely fast, resulting in multiple convergent morphologies.

In the current and previous analyses using nSSU-rDNA (Dunthorn et al. 2008, 2009; Fig. 9), *C. aspera* is sister to *Hausmanniella* with no node support. The mtSSU-rDNA topology from MP, ML, and MrBayes analyses (Fig. 7) places *C. aspera* basal to *Hausmanniella* and *Bardeliella* with no to moderate node support. This is an odd placement for *C. aspera* given that its horizontally oriented left oral ciliary field is quite unlike those in *Bardeliella* and *Hausmanniella* (Figs 2, 3, 6). However, in the PhyloBayes tree (Fig. 8) and in the concatenated analyses (Figs 10, 11), *C. aspera* forms a clade with the remaining *Colpoda* plus *Bresslauides* with no to full node support. Furthermore, in the AU tests the mtSSU-rDNA and the nSSU-rDNA alignments could not reject the monophyletic clade formed by *Colpoda* and *Bresslauides* (Table 2). The phylogenetic placement of *C. aspera* thus remains ambiguous.

The rate of substitution appears to be faster in mtSSU-rDNA than in nSSU-rDNA, such as in *C. henneguyi* and *C. mucicola*. This discrepancy in rates can be explained by a number of possible factors: smaller effective population size of the nuclei *vs*. mitochondria; homogenizing effects on nSSU-rDNA due to meiotic recombination, although there is debate as to whether the Colpodea are sexual (Dunthorn et al. 2008; Dunthorn and Katz 2010; Foissner 1993); elevated rates of mutations in the mitochondrial genome; and/or differing levels of functional constraints between the SSU-rDNAs of the two genomes.

Mitochondrial SSU-rDNA As a Ciliate Molecular Marker

Nuclear SSU-rDNA has remained the primary locus for molecular phylogenetic inferences of deep ciliate nodes since it was first sequenced by Sogin and Elwood (1986) and Lynn and Sogin (1988). Although congruent in many aspects with morphologically-based classifications, nSSU-rDNA topologies have been used to break up or reshuffle large taxa, as well as recognize new clades (Affa'a et al. 2004; Gong et al. 2009; Greenwood et al. 1991; Lynn 2003, 2008; Lynn and Strüder-Kypke 2002; Stoeck et al. 2007; Strüder-Kypke et al. 2006; Strüder-Kypke and Lynn 2003; van Hoek et al. 2000b; Yi et al. 2008). The resulting reliance on just nSSU-rDNA to infer deep nodes in the ciliate tree of life stands in contrast to the increasing repertoire of both low- and high-copy loci available for many other microbial and macro-organismic eukaryotic clades, as well as the number of loci used to reconstruct relationships within and among closely related ciliate species.

Here we show that the mtSSU-rDNA locus can infer well-supported nodes for the depths of the ciliate tree of life that were analyzed (i.e., in the Colpodea). Furthermore, most of the nodes in the individual (Figs 7, 8) and concatenated (Figs 10, 11) analyses are congruent with those that are well supported in previous nSSU-rDNA analyses (Dunthorn et al. 2008, 2009; Foissner and Stoeck 2009; Lasek-Nesselquist and Katz 2001; Lynn et al. 1999), as well as the truncated taxon sampling here (Fig. 9). We did not find that mtSSU-rDNA provides more resolution than nSSU-rDNA, as unsupported nodes remained unsupported in the Colpodea.

Future molecular phylogenetic inferences of deep nodes in the ciliate tree of life can now use a two-locus approach—with the nuclear and mitochondrial SSU-rDNA. This increasing of character sampling will help bring ciliate molecular systematics up to current practices in other eukaryotic clades where the use of multiple, independent molecular markers is both standard and expected. While we provide a two locus-approach for deep ciliate nodes in the Colpodea, in other eukaryotic clades it has been shown that the use of many more molecular markers drastically improves phylogenetic resolution (Philippe et al. 2005; Rokas et al. 2003). We anticipate that phylogenomic analyses are on the horizon for ciliate systematics, particularly for those lineages that can be cultured to obtain sufficient amounts of RNA for transcriptome analyses. At the same time, analysis of both nSSU-rDNA and mtSSU-rDNA will remain a powerful approach for those wishing to survey large numbers of taxa and/or those starting with limited numbers of cells.

Methods

Taxon sampling and terminology: Sequences were obtained from genomic DNA from earlier phylogenetic studies (Dunthorn et al. 2008, 2009; Riley and Katz 2001), as well as from GenBank. In total, our sampling includes 25 isolates from 24 morphospecies for mtSSU-rDNA (Table 1). One of us (W.F.) provided and identified most of the species used in this study. Of these, 20 are from the Colpodea. Exemplars from five of the seven orders within the Colpodea as recognized by Foissner (1993) are in included (Figs 1-6). For nSSU-rDNA, we started with the alignment from Dunthorn et al. (2009). Two *Paramecium* species, two *Tetrahymena* species, and *Chilodonella uncinata* are included as outgroups. Initial analyses included mtSSU-rDNA hydrogenosome sequences from Armorphorea accessions in GenBank; these were excluded from the final analyses since they exhibited extreme rate heterogeneity com-

pared to the rest of the sequences (possibly due to the evolution from mitochondria to hydrogenosome). When possible, both nSSU-rDNA and mtSSU-rDNA were from the same source DNA. Terminology follows Foissner (1993) and Lynn (2008). Classification follows Foissner (1993), with the addition of the labelling of Cyrtolophosidida clades 1 and 2 in the trees following Dunthorn et al. (2008).

DNA amplification and sequencing: Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen, CA). Mitochondrial SSU-rDNA was amplified with the 5' primer (TGT GCC AGC AGC CGC GGT AA) and the 3' primer (CCC MTA CCR GTA CCT TGT GT) from van Hoek et al. (2000a). Phusion polymerase (New England BioLabs, MA) was used with the following cycling conditions: 3:00 at 98 °C; 40 cycles of 0:15 at 98 °C, 0:15 at 67 °C, 1:15 at 72 °C; 10:00 extension at 72 °C.

Amplified products were cleaned with microCLEAN (The Gel Company, San Francisco, CA), and cloned with the Zero Blunt TOPO kit (Invitrogen, Carlsbad, CA). Positive clones were identified by PCR screening with AmpliTag Gold polymerase and vector primers (Applied Biosystems, Foster City, CA), and minipreped using Qiaprep Spin Miniprep kit (Qiagen). Clones were sequenced with the Big Dye terminator kit (Applied Biosystems), using vector primers. Up to eight colonies were sequenced in the forward direction; up to five of these were also sequenced in the reverse direction. Sequences were run on an ABI 3100 automated sequencer.

Alignments and removal of ambiguous positions: Mitochondrial sequences were determined and edited from overlapping sequence reads in SeqMan (DNAStar, Inc., Madison, WI). Vector and primer nucleotides were trimmed off. Sequences were aligned using Clustal X (Thompson et al. 1994), and further edited by eye in MacClade v4.05 (Maddison and Maddison 2005). Removal of ambiguously aligned positions was performed in two different ways: by eye in MacClade, and using Gblocks v0.91b (Castresana 2000; Talavera and Castresana 2007) set to default parameters (min conserved = 13, min flank = 21, max nonconserved = 8, min block = 10, gap = none). The two resulting alignments were analysed separately. For the nSSU-rDNA, the taxon sampling in the alignment from Dunthorn et al. (2009) was truncated so that it included the same taxa as the mtSSU-rDNA alignment.

Genealogical analyses: Pairwise distances for within and among samples were calculated as uncorrected "p" distances in PAUP* v4.0b8 (Swofford 2002). For all datasets the GTR-I-Γ evolutionary model was the best fitted model selected by AIC in MrModeltest v2 (Nylander 2004). Maximum parsimony (MP) analyses were carried out in PAUP* v4.0b8 (Swofford 2002), with all characters equally weighted and unordered. The TBR heuristic search option was used, running 100 random additions with MulTree option on, and support came from 1000 bootstrap replicates. Maximum likelihood (ML) analyses were carried out in RaxM-HPC v7.2.5 (Stamatakis et al. 2008). Support came from a majority rule consensus tree of 1000 multiparametic bootstrap replicates carried out in RaxM-HPC.

Bayesian Inference (BI) was carried out using two different algorithms. First with MrBayes v3.2.1 (Huelsenbeck and Ronquist 2003) using the GTR-I-Γ model. Posterior probability was estimated using four chains running 10 million generations sampling every 1000 generations. To determine if Bayesian analyses were run long enough, output files were examined using AWTY (Nylander et al. 2008). Second, to account to the possibility of model and rate variation, PhyloBayes v3.2e (Lartillot and Philippe 2004; Lartillot et al. 2009) was used with the QMM model (Dirichlet processes of GTR matrices). Posterior probability was estimated using one chain running at least 1.5 million generations sampling every cycle. For both methods the first 25% of sampled trees were considered burn-in trees and were discarded prior to constructing a 50% majority rule consensus trees. Trees were visualized with FigTree v1.3.1 (Rambaut 2006).

Topology testing: Three constrained ML analyses were carried out on the mtSSU-rDNA alignment masked by eye and the alignment masked using GBlocks: *Ottowphrya* + *Platyophrya* + *Sorogena* monophyletic, as in the nSSU-rDNA topology; *Colpoda* monophyletic, as in the morphological classification; and *Colpoda* + *Bresslauides* monophyletic. Resulting constrained topologies were then compared to the non-constrained ML topology using the AU test (Shimodaira 2002) as implemented in CONSEL v0.1j (Shimodaira and Hasegawa 2001). Likewise, three constrained ML analyses were carried out on the nSSU-rDNA dataset: *Platyophrya* + *Platyophrya*-like + *Rostrophrya* + *Sagittaria* monophyletic, as in the mtSSU-rDNA topology; *Colpoda* monophyletic, as in the morphological classification; and *Colpoda* + *Bresslauides* monophyletic. Resulting constrained topologies were then compared to the non-constrained ML topology using the AU test. For all constrains, internal relationships within the constrained groups was unspecified, and relationships among the remaining taxa were unspecified as well.

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