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ScienceDirect
Molecular Phylogenetics and Evolution 46 (2008) 316–327
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Molecular phylogenetic analysis of class Colpodea (phylum Ciliophora) using broad taxon sampling

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Received 3 May 2007; revised 1 August 2007; accepted 9 August 2007
Available online 28 August 2007

Abstract

The ciliate class Colpodea provides a powerful case in which a molecular genealogy can be compared to a detailed morphological taxonomy of a microbial group. Previous analyses of the class using the small-subunit rDNA are based on sparse taxon sampling, and are therefore of limited use in comparisons with morphologically-based classifications. Taxon sampling is increased here to include all orders within the class, and more species within previously sampled orders and in the species rich genus *Colpoda*. Results indicate that the Colpodea may be paraphyletic, although there is no support for deep nodes. The orders Bursariomorphida, Grossglockneriida, and Sorogenida are monophyletic. The orders Bryometopida, Colpodida, and Cyrtolophosidida, and the genus *Colpoda*, are not monophyletic. Although congruent in many aspects, the conflict between some nodes on this single gene genealogy and morphology-based taxonomy suggests the need for additional markers as well as a reassessment of the Colpodea taxonomy.

Keywords: Colpodea; Ciliophora; Phylogeny; Small subunit rDNA

1. Introduction

Assessment of phylogeny based on morphological characters is limited in many microbial eukaryotes. In most amoebae and flagellates morphology provides little guidance and taxonomic resolution is not rich below the class and ordinal levels. In contrast, phylum Ciliophora Doflein, 1901 is relatively morphologically rich and has a well-described taxonomy (Lynn, 2003; Lynn and Small, 2002). Class Colpodea Small and Lynn, 1981 provides a particularly good opportunity to compare the power of morphology and molecular analyses in reconstructing the phylogeny of ciliates. The Colpodea is monographed and contains a number of somatic and oral characteristics that were used to establish an extensive classification (Foissner, 1993a). Because previous molecular investigations of the class are based on sparse taxon sampling (Lasek-Nesselquist and Katz, 2001; Lynn et al., 1999; Stechmann et al., 1998), molecular support for the groups established by Foissner (1993a) remains to be evaluated.

The Colpodea is one of eleven ciliate classes (Adl et al., 2005; Lynn, 2003). Although its position in the subphylum Intramacronucleata is established (Lynn, 2003), well-supported evidence for the sister class of the Colpodea remains elusive. With current taxonomic sampling, neither morphology nor molecules give convincing or consistent arguments because of homoplasy, low bootstrap support, and problems from both paralogy and rate heterogeneity in protein-coding genes (Lasek-Nesselquist and Katz, 2001; Lynn et al., 1999; Stechmann et al., 1998). The classes Nassophorea, Oligohymenophorea, Plagiopylea, and Prostomatea are the likely sister-group candidates.

Historically, members of the class Colpodea were placed in disparate groups based on oral structure differences

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1055-7903/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.ympev.2007.08.006

(Foissner, 1993a; Lynn et al., 1999). With Lynn's (1976, 1981) structural conservatism hypothesis, somatic kinety (kinetosomes and associated fibers) differences were found to be a appropriate guide to the deep divisions within the ciliates. The Colpodea were united because of their left kinetodesmal fiber (LKm fiber) (Foissner, 1993a). This fiber extends posteriorly and to the left of the posterior kinetosome of their somatic dikinetids. In contrast, Bardele (1981, 1989) argues against the monophyly of the class because of differences in the presence or absence of particles (ciliary plaques) in the membrane of the somatic cilia: they are present only in one order in the Colpodea (Colpodida) and are absent in the rest of the class.

The Colpodea are a group of primarily terrestrial ciliates (Foissner, 1993a). Besides the unique LKm fiber, the class Colpodea contains distinctive silverline patterns of regular meshes: 'colpodid', with large, rectangular meshes; 'platyophryid', meshes divided by median silverline between the kineties, or 'kreyellid', with minute irregular meshes (Foissner, 1993a). Members of the Colpodea also have somatic stomatogenesis, where parental oral structures are partially or completely reorganized before new oral structures develop during cell division (Foissner, 1993a, 1996). In general, a single 'germline' micronucleus is close to the single 'somatic' macronucleus; in at least some taxa in the order Cyrtolophosidida the micronucleus and macronucleus share an outer membrane of the nuclear envelope (Foissner, 1993a). Sex has only been demonstrated in *Bursaria truncatella* and is unreported in the rest of the class (Foissner, 1993a; Raikov, 1982).

Foissner (1993a) monographed about 170 species and established an extensive higher-level classification for the Colpodea. Subsequently, new genera and species have been described (Foissner, 1993b, 1993c, 1994, 1995, 1999, 2003; Foissner et al., 2002, 2003). Foissner (1993a) split the Colpodea into two subclasses: one with the order Bryometopida based on a 'kreyellid' silverline pattern; with the rest of the orders in another subclass, based on 'colpodid' and 'platyophryid' silverline patterns. These silverline patterns were later argued to be misleading, as SSU rDNA places the Bryometopida next to order Bursariomorphida (Lynn et al., 1999). In large part there is agreement over Foissner (1993a) orders and families among other classifications (e.g., Puytorac, 1994), except order Grossglockneriida is lumped with order Colpodida in Lynn and Small (1997, 2002).

Based on morphological characters, Foissner (1993a) offers several hypotheses for relationships among these Colpodea orders (Fig. 1). First, Colpodida and Grossglockneriida are sister taxa since they share merotelokinetal stomatogenesis (complete reorganization of parental oral structures), which is probably the derived condition (Fig. 1a, character 12). In contrast, the other orders have the possibly plesiomorphic state of pleurotelokinetal stomatogenesis (partial reorganization of parental oral structures) (Fig. 1a, character 1); this hypothesis is supported

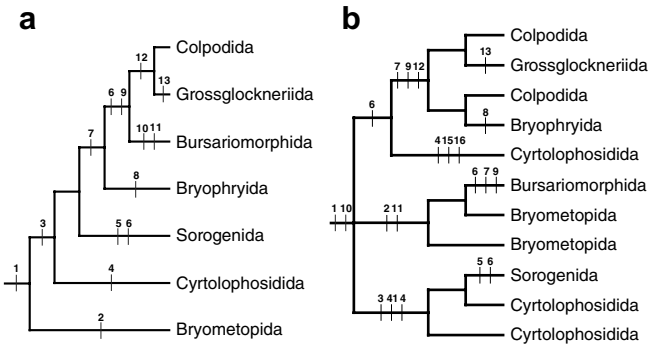

Fig. 1. Evolution among some morphological characters within the class Colpodea. (a) Hypotheses of relationships among orders and morphological character evolution modified from Foissner (1993a), where some characters are removed. (b) Possible alternative evolution of characters mapped out on the SSU rDNA gene tree found here; the deeper nodes in the Colpodea are not well supported and are thus shown as a polytomy. The character are: (1) Lkm fiber, pleurotelokinetal stomatogenesis, brick-shaped adoral organelles, flat vesitibulum, and 'kreyellid,' 'platyophryid,' or 'colpodid' silverline pattern; (2) 'kreyellid' silverline pattern; (3) 'platyophrid' or 'colpodid' silverline pattern; (4) shared micronuclear and macronuclear outer membrane of the nuclear envelope; (5) aerial sorocarps; (6) 'colpodid' silverline pattern; (7) deep vestibulum; (8) paroral formation with radial ciliary fields; (9) equidistantly spaced adoral organelles; (10) conjugation; (11) emergence pore in resting cysts; (12) merotelokinetal stomatogenesis; (13) feeding tube; (14) one paroral membrane segment; (15) two paroral membrane segments; (16) postoral pseudomembrane. See text for explanations of characters.

in previous molecular analyses (Lynn et al., 1999; Lasek-Nesselquist and Katz, 2001). Second, Bursariomorphida, Colpodida and Grossglockneriidae form a clade because of the possibly apomorphic equally-spaced rows of oral polykinetids (Fig. 1a, characters 9), as opposed to the possibly plesiomorphic brick-shaped adoral organelles (Fig. 1a, character 1); this morphologically-based hypothesis is not supported in previous molecular and morphological analyses (Foissner and Kreutz, 1998; Lasek-Nesselquist and Katz, 2001; Lynn et al., 1999). Third, Bryophryida, Bursariomorphida, Colpodida, and Grossglockneriida form a clade because of the possibly apomorphic deep vestibulum (depression in the cell with oral structures) (Fig. 1a, character 7), as opposed to the possibly plesiomorphic flat vestibulum (Fig. 1a, character 1).

Using limited taxon sampling with small-subunit rDNA (SSU rDNA), monophyly of the class Colpodea is strongly supported by least-squares (LS) and neighbor-joining (NJ), and weakly supported by maximum parsimony (MP) analyses in Lynn et al. (1999). In contrast, with just one additional taxon sampled, Lasek-Nesselquist and Katz (2001) find the class to be paraphyletic, with Nassophorea embedded within it—although support is weak from NJ, MP, and maximum likelihood (ML).

Here, we increase taxon sampling of the Colpodea using SSU rDNA sequences, including morphospecies from all seven orders, multiple morphospecies within most orders, and multiple morphospecies in the genus *Colpoda*. Our aim is to assess the following hypotheses: (1) the class Col-

Table 1
Taxon sampling within the Colpodea

Taxon	Collection site and notes	Culture conditions	No. of cells picked	No. of clones sequenced	Pairwise distance (%)	GB#
Bardeliella pulchra	Africa, Kruger National Park, floodplain	nc, npc	100	6	0.94	EU039884
Bresslauides discoideus 1	Dominican Republic, bromeliad	nc, npc	20	3	0	EU039885
Bresslauides discoideus 2	Dominican Republic, bromeliad	As above, but with food vacuoles	20	2	0	
Bryometopus atypicus	Africa, Kruger National Park, floodplain	nc, npc	80	4	0.23	EU039886
Bryometopus pseudochilodon	Brazil, Parana River floodplain	nc, npc	11	2	0.1	EU039887
						EU039888*
Bursaria sp. 1	Niger, floodplain	nc, npc	2,000	3	0.04	EU039889
Bursaria sp. 2	Namibia, Etosha pan	nc, npc	50	3	0.94	EU039890
						EU039891
Colpoda aspera	Jamaica, bromeliad	nc, pc	1,000	3	0	EU039892
Colpoda cucullus	Niger, floodplain	nc, pc	150	3	0	EU039893
Colpoda cucullus	Malaysia, rainforest	nc, pc	150	3	0	
Colpoda henneguyi	Niger, floodplain	c with 3 cells, pc	200	7	0.12	EU039894
Colpoda lucida	Niger, floodplain	nc, pc	250	2	0.2	EU039895
Colpoda magna	Salzburg, Austria, ephemeral pond	c, pc	500	3	0	EU039896
Colpoda minima	Niger, floodplain	nc, pc	200	2	0.24	EU039897
Cyrtolophosis mucicola 1	Brazil, Parana River floodplain	nc, npc	40	2	0.06	EU039898
Cyrtolophosis mucicola 2	Brazil, Parana River floodplain	nc, npc	200	2	0	
Cyrtolophosis mucicola 3	Salzburg, Austria, ephemeral pond	nc, pc	10,000	2	0	EU039899
Hausmanniella discoidea	Ecuador, bromeliad	nc, npc	30	3	0.02	EU039900
Ilsiella palustris	Brazil, Parana River floodplain	nc, npc	40	4	0.57	EU039901
Ilsiella palustris	Hawaii	nc, npc	30	3	0.54	
Mykophagophrys terricola	Borneo, rainforest	nc, npc	50	4	0.12	EU039902
Notoxoma parabryophryides	Borneo, rainforest	nc, npc	30	1	n/a	EU039903
Ottowphrya dragescoi	Galapagos Islands	nc, npc	10	6	0	EU039904
Platyophrya-like sp.	Venezuela, coastal marsh	nc, npc	60	6	0.1	EU039905
Platyophrya sp.	Jamaica, bromeliad	nc, npc	100	2	0.04	EU039906
Rostrophrya sp.	Venezuela, coastal marsh	nc, npc	20	2	0	EU039907
Sagittaria sp.	Venezuela, coastal marsh	nc, npc	50	2	0.12	EU039908

Species were identified using silver impregnation by W. Foissner. Type and voucher material of the new species and the newly investigated populations are deposited at the Oberoesterreichische Landesmuseum in Linz (LI), Austria. nc, nonclonal culture; c, clonal culture; npc, nonpure culture; pc, pure culture.
* Sequence with deletion; discussed in the paper but not used in the phylogenetic analyses.

podea is monophyletic, (2) orders within the class Colpodea are monophyletic, and (3) the genus *Colpoda* is monophyletic. We also discuss other features uncovered during characterization of SSU rDNA sequences: two distinct copies of SSU rDNA in one taxon, a group I intron in another, and evidence for sex in the Colpodea taxa sampled here. Furthermore, we evaluate alternative hypotheses of morphological evolution based on the SSU rDNA topology. Results from these analyses will further development of a predictive, tree-based framework for the taxonomy of the Colpodea.

2. Materials and methods

2.1. Taxon sampling and collection

To reconstruct an SSU rDNA genealogy of the Colpodea 27 collections representing 22 species were sampled for this study (Table 1). Most species sequenced were collected from soil, i.e., from nonflooded Petri dish cultures as described in Foissner et al. (2002). Some were from the water and mud occurring in the tanks of bromeliad plants (Foissner et al., 2003). Cells were either collected

from the raw culture (with other species in the dish) or were isolated into clonal culture (with one to few starter cells). With the addition of GenBank accessions from previous studies (Appendix A), the current sampling includes exemplars from all orders, 15 families, 18 genera, and 7 morphospecies in the genus *Colpoda*. Outgroup selection is based partially on previous analyses.

2.2. Identification

Species were identified according to the monograph of Foissner, 1993a,b,c, using live observations and various silver impregnation techniques. The new species collected here—*Bursaria* sp., *Platyophrya*-like, *Platyophrya* sp., *Rostrophyra* sp., and *Sagittaria* sp.—will be described in separate papers.

2.3. DNA extraction, amplification, cloning and sequencing

Between 10 and 10,000 cells were picked with a micropipette, washed, and placed into DNA lysis buffer. Genomic DNA was extracted using phenol/chloroform following standard protocols (Ausubel et al., 1993) or with a DNeasy Tissue kit (Qiagen, CA). Genomic DNA was amplified using universal 5′ and 3′ prime SSU rDNA primers (Medlin et al., 1988) with one of two polymerases. For some species Vent polymerase (New England BioLabs, MA) was used with the following cycling conditions: 4:00 at 95°; 32 cycles of 0:30 at 95°, 0:30 at 54°, and 2:00 at 72°; 10:00 extension at 72°. For others Phusion polymerase (New England BioLabs, MA) was used with the following cycling conditions: 0:30 at 98°; 36 cycles of 0:30 at 98°, 0:15 at 68°, 1:30 at 72°; 10:00 extension at 72°. Amplified products were cleaned with a low-melt gel and Ultrafree-Da columns (Millipore, MA), or with microCLEAN (The Gel Company, CA).

To assess within-sample variation, amplified products were cloned with the PCR-SMART Cloning kit (Lucigen, WI), or Zero Blunt TOPO kit (Invitrogen, CA). Positive clones were identified by PCR screening with AmpliTag Gold polymerase (Applied Biosystems, CA), and minipreped using Qiaprep Spin Miniprep kit (Qiagen, CA). Clones were sequenced with the Big Dye terminator kit (Applied Biosystems, CA), using 5′ and 3′ primers as well as two internal primers (Snoeyenbos-West et al., 2002). All sequences were run on an ABI 3100 automated sequencer.

Three samples required further methods. For the *Bryometopus pseudochilodon* indel found in this study, a 5′ primer (AAA CAG TTA TAG GCA GGC AAT TG) was designed that spanned both sides of the deletion to make sure the sequences containing the deletion were not an amplification artifact. Genomic DNA was amplified with this primer along with the universal 3′ primer, following the above protocol. For *Colpoda aspera* and *Cyrtolophosis mucicola* (from Austria), algal contaminant SSU rDNA sequences were removed by enzymatic digestion. Amplified products were cleaned with microCLEAN. Re-suspended DNA was incubated at 37° for 3 h with BamH1 (New England BioLabs, MA). The reaction was stopped with microCLEAN, and the DNA was cloned with the Zero Blunt TOPO kit and sequenced following the above protocol.

2.4. Genealogical analyses

Phylotypes were constructed from the consensus of the multiple sequence reads of the cloned products and edited in SeqMan (DNAStar). Pairwise distances for within samples were calculated as uncorrected distances in PAUP* v4.0b8 (Swofford, 2002). Phylotypes were aligned using Hmmer v2.1.4 (Eddy, 2001), with default settings. The training alignment for model building was all available ciliate SSU rDNA sequences downloaded from the European Ribosomal Database (Wuyts et al., 2004) and aligned according to their secondary structure. The alignment was further edited by eye in MacClade v4.05 (Maddison and Maddison, 2005), with ambiguously aligned regions and base-pair positions with more than five taxa having a gap masked. Remaining gaps were treated as missing data.

The GTR+I+G evolutionary model was estimated using hLTR 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 ten random additions with MulTree option on. Maximum likelihood (ML) analyses were carried out in RAxML v2.2.0 (Stamatakis, 2006) running 100 replicates. Support for MP and ML analyses came from 1000 bootstrap replicates using heuristic searches. Bayesian analyses was carried out using MrBayes v3.2.1 (Huelsenbeck and Ronquist, 2003) with support coming from posterior probability using four chains and running 10 million generations. Trees were sampled every 100 generations. The first 25% of sampled trees were considered 'burnin' trees and were discarded prior to tree reconstruction. A 50% majority rule consensus of the remaining trees was used to calculate posterior probability. Trees were imaged with TreeView v1.6.6 (Page, 1996).

3. Results

3.1. Pairwise distances within collections

SSU rDNA sequences from 27 collections representing 22 morphospecies show, for the most part, less than 0.50% average pairwise difference among clones within samples (Table 1). Sequences are deposited in GenBank numbers EU039884–EU039908. Six clones from *Bardeliella pulchra* show more variation with an average pairwise difference of 0.94%. Clones from the *Bursaria* sp. 2 collection contain two different phylotypes that are 0.94% different. The phylotypes of the *Ilsiella palustris* collection from Brazil are 0.57% different, while in the Hawaiian collection phylotypes are 0.54% different. The levels of within-collec-

tion variation are assumed to be a combination of intraspecific variation and experimental error. Contaminant SSU rDNA sequences were found in a few cases; for example: algae in *B. pulchra*, *Bresslauides discoideus*, *C. aspera*, *C. mucicola* from Austria, and *I. palustris* from Hawaii; fungi in *B. pseudochilodon*, and *Mykophagophrys terricola*; and a tetrahymenid ciliate from *Hausmanniella discoidea*.

Five species were collected more than once, allowing for some within species comparison (Table 2). There is no variation between the two *B. discoideus* collected from Dominican Republic. Between the Malaysian and Niger *Colpoda cucullus* collections the average pairwise difference among collections was 0.47%, with no phylotype shared between the sites. Although there is no difference between the two Brazilian collections of *C. mucicola*, the Brazilian and the Austrian collections are 1.71% different with no phylotype shared between the countries. The *I. palustris* collections are 0.64% different and likewise do not share phylotypes between the sites.

3.2. Deletion within one SSU rDNA copy in Bryometopus pseudochilodon

Two distinct SSU rDNA sequences were characterized from the *B. pseudochilodon* collection (Table 1). One sequence corresponds to the other full-length Colpodea sequences found here and from GenBank accessions. The second sequence is almost identical to the first except there is a 642 bp deletion towards the 5′ prime end of the SSU rDNA sequence and there are two nucleotide differences on the 5′ end. The deletion starts at nucleotide position 129 in *Escherichia coli* (GB# J01695) (Cannone et al., 2002). There is no evidence of elevated substitutions in the sequence with the deletion. This shorter sequence was uncovered in two separate amplifications using universal SSU rDNA primers, as well as from amplifications using a 5′ primer (see methods) that was designed to span either side of the deletion (data not shown). The deletion spanned multiple regions of the SSU rDNA molecule that are conserved in all extant organisms (Mears et al., 2002). We hypothesize that the deletion sequence is a macronuclear variant, which occurred in the process of macronuclear development and has been perpetuated during asexual divisions.

Table 2
Pairwise distance between collections for species sampled more than once

Taxon	Collection site	Pairwise distance (%)
Bresslauides discoideus	Dominican Republic 1 and 2	0
Colpoda cucullus	Malaysia and Niger	0.47
Cyrtolophosis mucicola	Brazil 1 and 2	0
Cyrtolophosis mucicola	Brazil 1 and Austria	1.71
Ilsiella palustris	Brazil and Hawaii	0.64

3.3. Intron in Cyrtolophosis mucicola

A 427 bp intron was found in all SSU rDNA clones from *C. mucicola* collected from Austria but not the *C. mucicola* collected from Brazil. The start residue is T and the ending residue is G, which is consistent with group I introns. Blast results also point to this sequence being a group I intron (E value = $1e^{-16}$ with the group I intron in *Fulgio septica*, GB# AJ555452.1; E value = $5e^{-13}$ with the group I intron in *Acanthamoeba* sp., GB# EF140633.1). There is no evidence for a homing endoculease gene in the intron. The insertion position of this intron in the SSU rDNA molecule corresponds to nucleotide 516 in *E. coli* (GB# J01695) (Cannone et al., 2002), which is a hotspot for group I intron insertions (Jackson et al., 2002).

3.4. SSU rDNA genealogy of the Colpodea

After a preliminary analysis using multiple exemplars from all eleven ciliate classes, only Colpodea sequences and close outgroups were chosen for more detailed analyses. The potential sister classes in this analysis as determined in the preliminary global ciliate analysis are the same as in previous studies: Nassophorea, Plagiopylea, Prostomatea, and Oligohymenophorea (data not shown). One phylotype from each sampled species was used in the alignment, except two representatives of *C. mucicola* (because they may underlie two species, see below) and *Bursaria* sp. 2 (because the other *Bursaria* sequences are relatively close).

The final SSU rDNA alignment used for comparing the morphological hypotheses of the Colpodea and its subgroups includes 59 sequences and has a length of 1582 unmasked nucleotides, of which 219 are parsimoniously informative. The most parsimonious tree from the MP analysis is 3349 in length, with a Consistency index of 0.3842, and a Homoplasy index of 0.6157. The most likely tree from the ML analysis has a log likelihood of −17450.098, while the most likely tree from the Bayesian analysis has a log likelihood of −17445.169.

Here, we present only the most likely Bayesian tree with node support from all three methods (Fig. 2, see Supplementary Figure. 1 for all node support values). The topologies of the MP- and ML-derived genealogies are mostly congruent with the Bayesian topology, except in three places. First, in the MP and ML analyses Cyrtolophodidia II (see below) is basal to the rest of the Colpodea + Oligohymenophorea + Plagiopylea + Prostomatea with no bootstrap support, and a paraphyletic Nassophorea is basal to this group with no bootstrap. Second, in the MP and ML analyses *Bryometopus pseudochilodon* is basal to the rest of its order with no bootstrap support, while in the Bayesian tree *Bryometopus sphagni* is basal. Third, in the MP and ML analyses the order Grossglockneriida forms an unsupported clade with *C. aspera, C. steinii,*

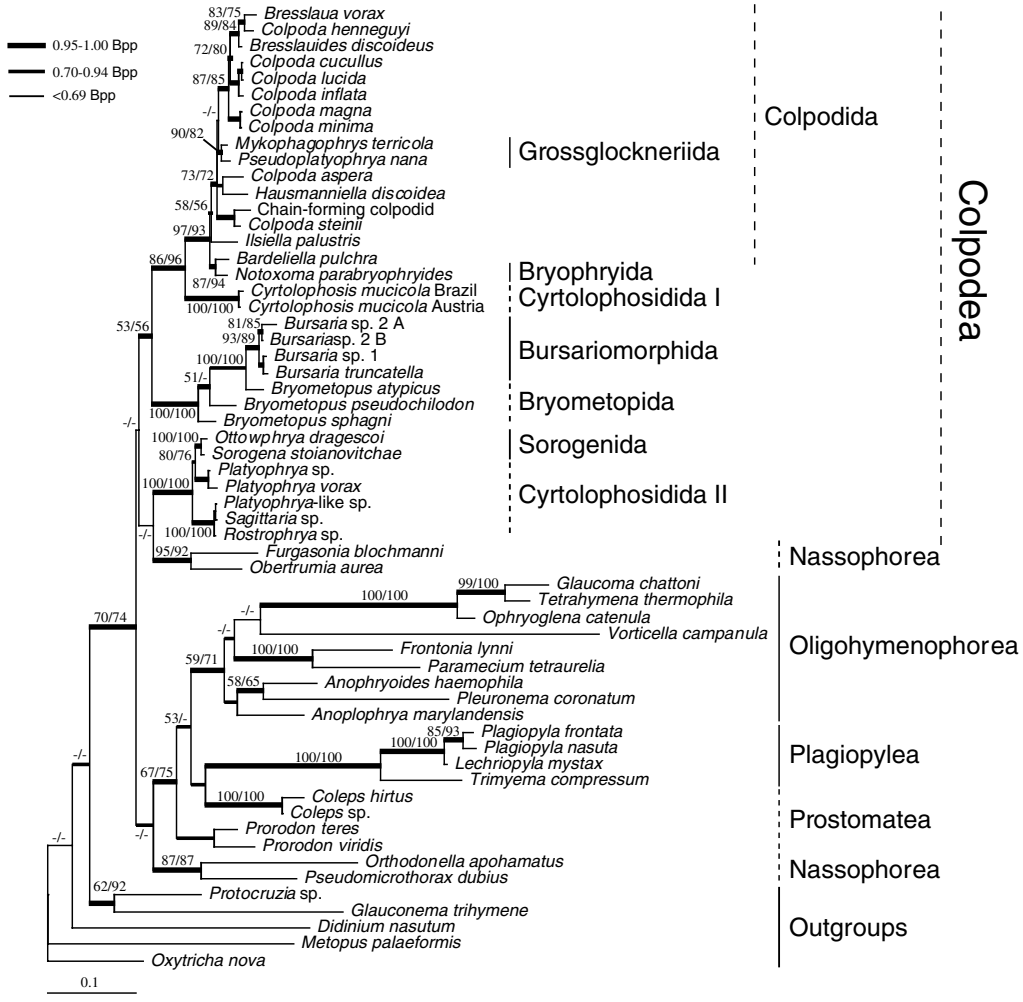

Fig. 2. SSU rDNA genealogy of the class Colpodea and potential sister classes. The most likely Bayesian tree is shown. Bayesian posterior probability support is shown by differences in thickness of branches. Numerical values from bootstrap support is shown next to the branches as: MP bootstrap/ML bootstrap. Values <50% are shown as '-'. Monophyletic classes and orders are labeled with a solid line, while nonmonophyletic ones labeled with a dashed line. All support values for all nodes are given in Supplementary Figure. 1.

Chain-forming colpodid, and *H. discoidea*, while in the Bayesian tree it does not.

In our analyses, there is no support for the monophyly of the Colpodea: monophyly of the class is weakly rejected by all three methods based on tree topologies and support values. *Furgasonia* and *Obertrumia* (both in the class Nassophorea) fall out sister to part of the order Cyrtolophosidida with no support from all three methods (-MP bootstrap/-ML bootstrap/-Bayesian posterior probability; support >50% or 0.5 is shown as '-'). Changing the number of outgroup classes does not significantly alter this nonmonophyletic topology as the deep nodes are not well resolved anyways and there is no support for any class to be sister to the Colpodea. The rest of the Colpodea forms a monophyletic clade with weak support from MP and ML but with high support from Bayesian analysis (53/56/0.99).

Support for relationships among the outgroups varied by method. The class Prostomatea is paraphyletic with only moderate support from Bayesian analysis (-/-/0.90), with the genus *Coleps* sister to a well-supported monophyletic class Plagiopylea (100/100/1.00). The monophyly of Oligohymenophorea is moderately to highly supported in ML and Bayesian analyses (59/71/1.00). The clade containing Prostomatea, Plagiopylea and Oligohymenophorea is moderately to highly supported by ML and Bayesian analysis (53/-/1.00).

Monophyly of the morphologically defined groups could be assessed with our single gene tree for every order within the Colpodea, except Bryophryida as only one morphospecies was sampled for this order. Sorogenida, with two genera, is monophyletic with full support (100/100/1.00). Bursariomorphida, with four taxa, is monophyletic also with high support (93/89/1.00). Grossglockneriida, with two genera, is likewise monophyletic with moderate to high support (90/82/1.00).

Order Cyrtolophosidida, with six sampled genera, is not monophyletic. The genus *Cyrtolophosis* falls sister to the

order Colpodida with moderate to high support (86/96/1.00). The remaining Cyrtolophosidida genera (*Ottowphrya*, *Platyophrya*-like, *Platyophrya*, *Rostrophrya*, and *Sagittaria*) form a paraphyletic group, with order Sorogenida nested within it, at the base of the class with high support (100/99/1.00).

Order Bryometopida, with one genus and three species sampled, is not monophyletic with full support from all three methods (100/100/1.00). Order Bursariomorphida is nested within this order, and it is sister to *Bryometopus pseudochilodon* with full support (100/100/1.00). To determine whether this topology is spurious due to the GenBank accession for *B. sphagni* missing about 500 bp from the 5′ end, the Bryometopida and Bursariomorphida sequences were realigned (with *Colpoda magna* and *C. mucicola* as outgroups) minus the 5′ end; the same topology was found with this alignment (data not shown). Order Colpodida is not monophyletic with high support (97/93/1.00), containing orders Grossglockneriida and Bryophryida. *B. pulchra* is sister to *Notoxoma parabryophryides* (order Bryophryida) with moderate to high support (87/94/0.98).

Monophyly of the genus *Colpoda* was assessed in this molecular analysis using eight morphospecies within the genus and numerous close outgroups. *Colpoda* is not monophyletic in the SSU rDNA genealogy with moderate to full support (73/72/1.00). Most *Colpoda* species form a sister group to the Grossglockneriida with no support from any method (-/-/-). *B. vorax, B. discoideus,* and *Colpoda henneguyi* form a clade with moderate to high support (89/84/1.00). This clade is in turn sister to most of the remaining *Colpoda* species with moderate to high support (72/80/0.97). To determine if the topology of the *Colpoda* phylotypes is robust, only Colpodida, Grossglockneriida, and Bryophryida phylotypes were realigned and remasked for a separate analysis; overall, the resulting ingroup topology is concordant with the full class analysis (data not shown).

4. Discussion

4.1. Comparisons between morphology and molecules

Here we compare the morphologically-based classification and well-supported SSU rDNA nodes. Furthermore, we evaluate the possible evolution of morphological characters in light of the SSU rDNA genealogy.

4.1.1. The class

We find no molecular support for the monophyly of the class Colpodea based on analyses of SSU rDNA sequences (Fig. 2). Conversely, the nonmonophyly of the class (with part of the class Nassophorea being sister to part of the order Cyrtolophosidida) is not well supported either. Similarly, the Nassophorea is also not monophyletic with respect to the Colpodea, though with no support. The nonmonophyletic relationships of the Colpodea with respect to the Nassophorea should not be given much weight, as there is neither support for this relationship nor for the Nassophorea even being sister to the Colpodea. In sum, the SSU rDNA genealogy here provides little support for class-level relationships within the subphylum Intramacronucleata in general, as seen elsewhere (Lynn, 2003).

These results do not pose a serious challenge to Lynn's (1976, 1981) structural conservatism hypothesis given the limited support at deep nodes. On the other hand, these results do challenge Bardele's (1981, 1989) use of ciliary plaques in his argument that the members in the Colpodea are not closely related.

4.1.2. The orders

Molecular support for monophyly could be assessed for all orders within the Colpodea except the Bryophryida. The SSU rDNA genealogy presented here does support much of the morphologically-based classification of the Colpodea, although there is some discordance at the ordinal level between morphology and molecules (Fig. 2).

The order Cyrtolophosidida is polyphyletic. Cyrtolophosidida I, containing the genus *Cyrtolophosis*, falls away from Cyrtolophosidida II, containing the most recent common ancestor of *Sagittaria* and *Platyophrya* and all of its descendants plus the order Sorogenida. This nonmonophyly of the Cyrtolophosidida suggests the need for a reevaluation of the character that was used to establish this group. Cyrtolophosidida was circumscribed based on the shared outer membrane of the nuclear envelope of the micronucleus and macronucleus (Foissner, 1985, 1993a). This character, however, has only been confirmed with transmission electron microscopy for six species: *Aristerostoma marinum* (Detcheva and Puytorac, 1979), *C. mucicola* (Detcheva, 1976; Didier et al., 1980), *Platyophrya sphagni* (Kawakami, 1991), *Platyophrya spumacola* (Dragesco et al., 1977), *Pseudocyrtolophosis alpestris* (Foissner, 1993a), and *Woodruffides metabolicus* (Golder, 1976). Njine (1979) states that nuclei in *Kuklikophrya ougandae* share an outer membrane (and presents a drawing of a stained cell showing this), but does not present an electron micrograph. *Platyophryides latus* is drawn with a shared outer membrane by Dragesco and Dragesco-Kernéis (1979), but Puytorac et al. (1992) show that the membranes are separate with their transmission electron micrographs. Foissner (1993a) argues that two taxa, *Sagittaria australis* and *Woodruffia australis*, have the shared outer membrane because of their thick silver-stained membranes. On the other hand, Díaz et al. (2000) show separate outer nuclear membranes in *Cyrtolophosis elongata*. Hence, the shared outer membrane of the nuclear envelope of the micronucleus and macronucleus is not only a weak character for the Cyrtolophosidida, but also one whose distribution is neither well known nor confirmed (Fig. 1b, character 4). Future transmission electron microscopy studies are much needed to confirm the presence or absence of this character in other species. Foissner et al. (2002) suggest those species with a separate outer

micronucleus and macronucleus membrane can be transferred to the clade *Plesiocaryon* or into the order Sorogenida (as was done with *Ottowphrya*).

There are morphological differences between the two Cyrtolophosidida groups. In Cyrtolophosidida I, there are two segments in the paroral (right oral) membranes, the anterior bearing tuft-like cilia (the unique feature of its family) (Fig. 1b, character 15). Only one paroral segment is present in taxa in Cyrtolophosidida II (Fig. 1b, character 14). These groups also differ in the presence of nonciliated kinety on the right margin of the adoral organelles in Cyrtolophosidida I (and its family), which is absent in Cyrtolophosidida II (Fig. 1b, character 16).

Although originally placed with the haptorid ciliates (Bradbury and Olive, 1980), the close relationship between the Sorogenida and the Cyrtolophosidida was soon recognized morphologically (Bardele et al., 1991; Foissner, 1985; Small and Lynn, 1981). This relationship was confirmed in a previous SSU rDNA analysis (Lasek-Nesselquist and Katz, 2001) and the SSU rDNA topology presented here. The Sorogenida was originally separated from the Cyrtolophosidida because it lacked the shared outer membrane of the nuclear envelope of the micronucleus and macronucleus (Foissner, 1985, 1993a)—although this character maybe is weak (see above)—and because of its slime mold-like aerial sorocarp in one life history stage (Fig. 1, character 5). Like the Cyrtolophosidida, the Sorogenida has brick-shaped organelles on the left slope of the vesibulum and pleurotelokinetal stomatogenesis (Fig. 1b, character 1) (Foissner, 1993a). The SSU rDNA genealogy suggests that the aerial sorocarp of *Sorogena* may represent a complex apomorphy arising from within Cyrtolophosidida II.

The order Bryometopida is paraphyletic in relation to the monophyletic Bursariomorphida in the SSU rDNA genealogy. The close relationship between Bryometopida and Bursariomorphida was also found by Foissner and Kreutz (1998) and Lynn et al. (1999). Although these two orders differ in their silverline pattern (Bryometopida having 'kryellid' to 'platyophryid,' Bursariomorphida having 'colpodid'), taxa in these two orders share an apical oral opening, a ventral cleft, conspicuous adoral organelles, and an emergence pore in their cysts (Foissner and Kreutz, 1998; Foissner, personal observations).

The order Colpodida is paraphyletic in our molecular analyses, though support is limited at many nodes. That the Grossglockneriida was close to the Colpodida has been proposed as they share the unique (in the Colpodea) merotelokinetal stomatogenesis (Fig. 1b, character 12), colpodid silverline pattern (Fig. 1b, character 6), and a simple oral polykinetid (Aescht et al., 1991; Foissner, 1993a). These two orders are even lumped together in some classifications (Lynn and Small, 1997, 2002). The question remained just how they were related: the SSU rDNA genealogy here suggests that the Grossglockneriida falls within the Colpodida, not sister to it. The position of Bryophryida within the Colpodida has not been hypothesized as the Bryophryida has a platyophryid silverline pattern and brick-shaped organelles on the left vestibulum (Fig. 1b, character 1). The Bryophryida and Colpodida do, though, share a deep vestibulum (Foissner, 1993a).

The use of differences in the type of division seems to be helpful at the ordinal level. As suggested by Foissner (1993a): pleurotelekinetal stomatogenesis is probably plesiomorphic within the Colpodea. Only orders Colpodida and the Grossglockneriida have merotelokinetal division (Foissner, 1993a). Stomatogenesis is undescribed in Bryophryida; assuming that its phylogenetic position found here is confirmed in future studies, then it is predicted that its division type should be merotelokinetal. On the other hand, the power of the silverline pattern for use in the systematics of the Colpodea at the ordinal level is debatable. While Foissner (1993a) uses differences in silverlines to help construct a higher-level classification, Foissner and Kreutz (1998) and Lynn et al. (1999) argue that this character is sometimes misleading. The results presented here are in agreement with Lynn et al. (1999) on the limitations of the use of silverline patterns at the ordinal level.

4.1.3. The genus Colpoda

In our molecular analyses the large genus *Colpoda* is paraphyletic not only in relation to genera within its own family, but also to other families in its order (Fig. 2). Most of the relationships among the *Colpoda* morphospecies in the SSU rDNA tree are not well supported; there is support for *Bresslaua* and *Bresslauides* nesting within the *Colpoda*. *Bresslaua* was originally separated from *Colpoda* based on a difference in vestibulum size (Kahl, 1931). However, Claff et al. (1941), Foissner (1985, 1993a), and Lynn (1979) find that *Bresslaua*'s voracious feeding behavior and its left-projecting vestibular wall (as opposed to right-projecting in *Colpoda*) are probably better characters to separate the genus from *Colpoda*. The SSU rDNA topology suggests that these characters may represent apomorphies arising from within a *Colpoda* clade. *Bresslauides* (and its family Hausmanniellidae) also falls within the *Colpoda* in the SSU rDNA tree. This genus was circumscribed based on the unique semicircular right oral polykinetid that was longer than the left as opposed to being equal in the Colpodidae (Foissner, 1987, 1993a). Because *Bresslauides* is not falling out with the other member of its family (*Hausmanniella*) sampled here, the character of a semicircularly curved right oral polykinetid may have evolved more than once.

4.2. Open questions with some species designations

The level of diversity among some SSU rDNA sequences from the morphospecies collected here suggests possible problems with some circumscriptions. *C. magna* and *C. minima* differ little in the SSU rDNA phylotypes, indicating a need for further genetic studies. These morphospecies species differ in size and kinety number, as well as the number of micronuclei, with one in *C. minima* and 2–

16 in *C. magna* (Foissner, 1993a). The low genetic distance between these two species and the lack of much morphological differences could point to these being nascent but "biological" species. Alternatively, *C. minima* and *C. magna* may represent morphological variation within a single species where a change in micronuclei and kinety number is correlated with size.

The *C. mucicola* morphospecies may represent two genetic species: there is a putative group I intron in the Austrian collection that is absent from the Brazilian collections, and there is greater than 1% pairwise distance between the Austrian and Brazilian collections. In contrast, the diversity in SSU rDNA phylotypes for the genus *Bursaria* from this study and GenBank accessions (1.31% average pairwise distance) supports the view that there are more than one species in the genus, although some argue for there being only one species.

We do not find the large sequence diversity in our *Colpoda* morphospecies as does Nanney et al. (1998). In our analysis we find a 2.79% average pairwise distance among the *Colpoda* morphospecies sampled here and from GenBank accessions, while Nanney et al. (1998) find an average "slack" value of 31.5% among their *Colpoda*. There are at least two reasons for this difference. First, our analyses were based on SSU rDNA, while theirs is based on 190 bp of the hyper-variable D2 region of the large subunit rRNA (LSU rDNA). Second, our analyses of distance used the uncorrected distance method in PAUP*, while theirs use string analyses in the program PHYLOGEN. Using our distance method, Nanney et al.'s (1998) data show an average pairwise distance of 20.73% for the D2 region of the LSU rDNA (data not shown). Despite the difference in levels of variation between the SSU rDNA and the short variable region of the LSU rDNA, the topology found by Nanney et al. (1998) among their five *Colpoda* morphospecies is congruent with our analyses (data not shown).

4.3. Evidence for sex

Conjugation (ciliate sex) is documented in all ciliate classes (Bell, 1988; Dini and Nyberg, 1993; Miyake, 1996; Sonneborn, 1957). In the Colpodea conjugation is only known in *B. truncatella* even though over the decades researchers have looked for conjugation in other species but have yet to observe it (Foissner, 1993a; Raikov, 1982). There are a few reports of possible conjugation in some species of *Colpoda*; because nuclear division or exchange was not shown these observations are possibly of "pseudoconjugation," where exchange of nuclei does not occur (Foissner, 1993a).

Assuming that Colpodea species behave genetically in a way similar to other eukaryotes, we could predict that if the Colpodea were asexual, allelic variation would be high within species (Mark Welch and Meselson, 2000; Normark et al., 2003). The low allelic values within most collections sampled here suggest that the Colpodea species are indeed having sex albeit covertly. There is an important caveat in this statement in that the number of clones sequenced per morphospecies in this study is relatively low (1–7 clones) and we could have missed some variants. Nevertheless, the results here are in opposition to Bowers et al. (1998), who present isozyme evidence for asexuality for three *Colpoda* species. Although cryptic sex is consistent with the low allelic values found here, further evidence of conjugation is much needed to confirm sex within the Colpodea beyond *B. truncatella*.

4.4. Group I intron in Cyrtolophosis mucicola

While group I introns are widespread in microbial eukaryotes (Bhattacharya et al., 1996; Haugen et al., 2003, 2005; Snoeyenbos-West et al., 2004; Wikmark et al., 2007), the putative group I intron found in the Austrian *C. mucicola* morphospecies is the fourth identification of this type of intron in ciliates. The other known species with group I introns are: *Tetrahymena thermophila* (Grabowski et al., 1981), *Acineta* sp. (Snoeyenbos-West et al., 2004), and *Tokophrya lemnarum* (Snoeyenbos-West et al., 2004). Undoubtedly there remain more of these introns to be uncovered in future sequencing projects of the various ciliate groups. We suggest that the intron in the Austrian *C. mucicola* is a product of a recent horizontal transfer into the SSU rDNA locus, as group I introns are known to be mobile over relatively short evolutionary time scales (Haugen et al., 2005; Simon et al., 2005) and because it was not found in other isolates of the species or other Colpodea sequences.

4.5. Reconciling morphology and molecules in the Colpodea

In large part morphology and the SSU rDNA genealogy agree in the hypothesized relationships within the ciliate class Colpodea, although the paraphyletic relationships among previously hypothesized closely related taxa was unexpected (Fig. 1). The SSU rDNA genealogy is based on a single gene and may not follow the actual species phylogeny (e.g., Doyle, 1992; Maddison, 1997). Further tests using other loci are needed to confirm the areas where there is discordance between morphology and molecules.

Acknowledgments

The authors thank Jessica Grant, Dan Lahr, Yonas Tekle, other members of the Katz lab, and Ben Normark for help in an early draft. We also thank Scott Miller and two anonymous reviewers for insightful comments and suggestions. Partial support for M.D. came from the mini-PEET grant from the Society of Systematic Biologists. Support for W.F. came from FWF Grant P-15017. And support for L.A.K. came from NSF Grants OCE 0221062, DEB 043115, and DEB 0092908.

Appendix A

GenBank accessions used in analyses for both previously sequenced Colpodea taxa and outgroups

	GB#
Colpodea	
Bresslaua vorax	AF060453
Bryometopus sphagni	AF060455
Bursaria truncatella	U82204
Chain-forming colpodid	AY398684
Colpoda inflata	M97908
Colpoda steinii	DQ388599
Platyophrya vorax	AF060454
Pseudoplatyophrya nana	AF060452
Sorogena stoianovitchae	AF300285
Outgroups	
Anophryoides haemophila	U51554
Anoplophrya marylandensis	AY547546
Apofrontonia dohrni	AM072621
Blepharisma americanum	M97909
Caenomorpha uniserialis	U97108
Cardiostomatella vermiforme	AY881632
Chilodonella uncinata	AF300281
Climacostomum virens	X65152
Coleps hirtus	U97109
Coleps sp.	X76646
Didinium nasutum	U57771
Diplodinium dentatum	U57764
Discophrya collini	L26446
Ephelota sp.	AF326357
Epidinium caudatum	U57763
Epistylis chrysemydis	AF335514
Eufolliculina uhligi	U47620
Euplotes crassus	AY007437
Frontonia lynni	DQ190463
Furgasonia blochmanni	X65150
Geleia simplex	AY187927
Glaucoma chattoni	X56533
Glauconema trihymene	AY169274
Gruberia sp.	L31517
Haleria grandinella	AY007443
Heliophrya erhardi	AY007445
Isotricha intestinalis	U57770
Loxodes magnus	L31519
Loxophyllum utriculariae	L26448
Metopus contortus	Z29516
Metopus palaeformis	AY007450
Nyctotherus ovalis	AY007454
Obertrumia aurea[*]	X65149
Ophrydium versatile	AF401526
Ophryoglena catenula	U17355
Orthodonella apohamatus	DQ232761
Oxytricha nova	X03948
Paramecium tetraurelia	X03772
Parduczia orbis	AY187924
Pleuronema coronatum	AY103188
Prorodon teres	X71140
Prorodon viridis	U97111
Protocruzia sp.	AF194409
Pseudomicrothorax dubius	X65151
Schizocaryum dogieli	AF527756
Spirostomum ambiguum	L31518
Stentor roeseli	AF357913
Strombidium purpureum	U97112
Stylonychia lemnae	AF164124
Tetrahymena thermophila	X56165
Tokophrya lemnarum	AY332720
Trachelorapbis sp.	L31520
Trithigmostoma steini	X71134
Uronema elegans	AY103190
Vorticella campanula	AF335518

[*] In GenBank as *Obertrumia georgiana*, which is a junior synonym.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.08.006.

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