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Broad Taxon Sampling of Ciliates Using Mitochondrial Small Subunit Ribosomal DNA

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Abstract. Mitochondrial SSU-rDNA has been used recently to infer phylogenetic relationships among a few ciliates. Here, this locus is compared with nuclear SSU-rDNA for uncovering the deepest nodes in the ciliate tree of life using broad taxon sampling. Nuclear and mitochondrial SSU-rDNA reveal the same relationships for nodes well-supported in previously-published nuclear SSU-rDNA studies, although support for many nodes in the mitochondrial SSU-rDNA tree are low. Mitochondrial SSU-rDNA infers a monophyletic Colpodea with high node support only from Bayesian inference, and in the concatenated tree (nuclear plus mitochondrial SSU-rDNA) monophyly of the Colpodea is supported with moderate to high node support from maximum likelihood and Bayesian inference. In the monophyletic Phyllopharyngea, the Suctoria is inferred to be sister to the Cyrtophora in the mitochondrial, nuclear, and concatenated SSU-rDNA trees with moderate to high node support from maximum likelihood and Bayesian inference. Together these data point to the power of adding mitochondrial SSU-rDNA as a standard locus for ciliate molecular phylogenetic inferences.

Key words: Ciliophora, Colpodea, mitochondria, phylogeny, Phyllopharyngea, SSU-rDNA.

INTRODUCTION

Ciliates are a diverse clade of microbial eukaryotes with an estimated 10,000 described morphospecies with highly variable morphologies (Foissner *et al.* 2008, Lynn 2008). Molecular phylogenetic inferences for most of the ciliate tree of life have relied on sequencing the nuclear small subunit rDNA (nSSU-rDNA) locus (e.g., Utz *et al.* 2010, Vd'ačný *et al.* 2010, Yi *et al.* 2010, Bachy *et al.* 2012, Zhan *et al.* 2013). These nSSU-rDNA studies, along with numerous morphological observations, have led to the ciliates currently being classified into 12 major clades, or classes (Lynn 2008, Adl *et al.* 2012).

There is little additional molecular data for, or against, the proposed major ciliate clades from non-linked loci, or from loci that are likely not subject to paralogy (Israel *et al.* 2002, Dunthorn and Katz 2008,

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Yi *et al.* 2012). Recently, mitochondrial SSU-rDNA (mtSSU-rDNA) sequences were shown to effectively uncover deep nodes within the Colpodea (Dunthorn *et al.* 2011, 2012a), as well as in shallower nodes among isolates of the morphospecies *Chilodonella uncinata* (Katz *et al.* 2011). Here we evaluate the efficacy of mtSSU-rDNA for inferring deep nodes among diverse ciliate taxa. Our broad taxon sampling of mtSSU-rDNA from across the ciliates tree of life results in support for some of the deep nodes that were inferred also from morphology and nSSU-rDNA.

METHODS

Taxon sampling and classification

Isolates of twelve ciliate morphospecies were newly sequenced for mtSSU-rDNA, 5 of which were also sequenced for nSSU-rDNA (Table 1). DNA for *Acineta* sp., *Chlamydodon exocellatus*, *Chlamydodon triquetrus*, *Didinium* sp., *Dysteria* sp., and *Heliophrya* sp. came from Snoeyenbos-West *et al.* (2004). DNA for *Vorticella astyliformis* came from Foissner *et al.* (2009). Isolates of *Coleps* sp., *Colpidium* sp., and *Metafolliculina* sp. are from collections made by Katz and colleagues from the environment, and isolates for *Spirostomum* sp. and *Stentor* sp. were purchased from Connecticut Valley Biological Supplies (Southampton, MA). Additional nuclear and mitochondrial SSU-rDNA sequences came from GenBank (Table 1). *Plasmodium falciparum* was used as outgroup. Classification follows Adl *et al.* (2012).

Sequencing and phylogenetic analyses

DNA was extracted, amplified and sequenced for mtSSU-rDNA and nSSU-rDNA following Dunthorn *et al.* (2011) and Katz *et al.* (2011). Sequences were analyzed and polymorphisms confirmed using overlapping sequence reads in SeqMan (DNAStar, Inc., Madison, WI). New mtSSU-rDNA sequences were added to the alignment of Dunthorn *et al.* (2011) by pairwise alignments in MacClade v4.05 (Maddison and Maddison 2005). Nuclear SSU-rDNA sequences 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. Both these alignments were further adjusted, and ambiguously aligned positions masked, in MacClade. A third alignment was made by concatenating the mtSSU-rDNA and nSSU-rDNA alignments.

Phylogenetic analyses of these three alignments used the GTR-I-Γ model of substitution. Maximum likelihood (ML) analyses were carried out in RaxML-HPC v7.2.5 (Stamatakis 2006). Support came from a majority rule consensus tree of 1000 bootstrap replicates. Bayesian inference (BI) analyses were carried out using with MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003). Posterior probability was estimated using four chains running 20 million generations and sampling every 1000 generations. The first 25% of sampled trees were considered burn-in trees and were discarded pri-

Table 1. Taxon sampling and GenBank accessions for ciliates used in this study. New sequences are bolded.

Taxon	mtSSU GenBank #	nSSU GenBank #
***Acineta* sp.**	**KF639897**	AY332718
Aristerostoma sp.	HM246398	EU264563
Bardeliella pulchra	HM246399	EU039884
Bresslauides discoideus	HM246400	EU039885
Bryometopus atypicus	HM246401	EU039886
Bursaria spec. (“muco”)	HM246402	EU039889
Bursaria truncatella	HM246403	U82204
Chilodonella uncinata Poland	HM246404	JN111976
Chilodonella uncinata USA ATCC	JN111981	AF300281
Chilodonella uncinata USA SC1	JN111980	JN111979
Chilodonella uncinata USA SC2	JN111983	JN111977
Chilodonella uncinata USA WH	JN111982	JN111978
Chlamydodon exocellatus	**KF639898**	AY331790
Chlamydodon triquetrus	**KF639899**	AY331794
***Coleps* sp.**	**KF639900**	**KF639909**
***Colpidium* sp.**	**KF639901**	**KF639910**
Colpoda aspera	HM246405	EU039892
Colpoda cucullus	HM246406	EU039893
Colpoda henneguyi	HM246407	EU039894
Colpoda lucida	HM246409	EU039895
Cyrtolophosis mucicola Austria	HM246411	EU039899
Cyrtolophosis mucicola Brazil	HM246412	EU039898
Didinium nasutum	**KF639902**	U57771
***Dysteria* sp.**	**KF639903**	AY331797
Hausmanniella discoidea	HM246413	EU039900
Heliophrya erhardi	**KF639904**	AY007445
Ichthyophthirius multifiliis	JN227086	U17354
Ilsiella palustris	JQ026522	JQ026521
Maryna n. sp.	JQ026524	JF747218
Maryna umbrellata	JQ026523	JF747217
***Metafolliculina* sp.**	**KF639905**	**KF639911**
Ottowphrya dragescoi	HM246414	EU039904
Paramecium primaurelia	K01750	AF100315
Paramecium tetraurelia	X15917	X03772
Plasmodium falciparum	X95275	AL844501
Platyophrya bromelicola	HM246415	EU039906
Platyophrya-like sp.	HM246416	EU039905
Rostrophrya sp.	HM246417	EU039907
Sagittaria sp.	HM246418	EU039908
Sorogena stoianovitchae	HM246419	AF300285
***Spirostomum* sp.**	**KF639906**	**KF639912**
***Stentor* sp.**	**KF639907**	**KF639913**
Tetrahymena pyriformis	AF160864	M98021
Tetrahymena thermophila	AF396436	X56165
Tillina magna	HM246410	EU039896
Vorticella astyliformis	**KF639908**	GQ872427

or to constructing a 50% majority rule consensus trees. Trees were visualized with FigTree v1.3.1 (Rambaut 2006). For ML analyses we consider bootstraps values < 70% low and unsupported, 70–94% moderately supported, and ≥ 95% highly supported (Hillis and Bull 1993); for BI analyses we consider posterior probabilities ≤ 94% low and unsupported, and ≥ 95% highly supported (Alfaro *et al.* 2003).

RESULTS AND DISCUSSION

A broadly sampled mtSSU-rDNA ciliate tree of life

Almost all that we know of the deepest relationships within the ciliate tree of life come from morphological observations of a few key characters, and from molecular analyses of the nSSU-rDNA locus (Dunthorn and Katz 2008, Lynn 2008). Using these complementary data, ciliates have been classified into 12 major clades (e.g., Adl *et al.* 2012): Heterotrichea and Karyorelictea (both in the Postciliodesmatophora), and other lineages in the Intramacronucleata. Within the Intramacronucleata, the Armophorea, Cariacothrix, Litostomatea, and Spirotrichea are thought to be sister to the clade that contains Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Plagiopylea, and Prostomatea (Riley and Katz 2001, Lynn 2003, Gong *et al.* 2009, Phadke and Zufall 2009, Vd'ačný *et al.* 2010, Adl *et al.* 2012, Dunthorn *et al.* 2012b, Orsi *et al.* 2012).

Mitochondrial SSU-rDNA here infers some of the same, or similar, deep relationships that nSSU-rDNA infers within the ciliate tree of life (Fig. 1). The Heterotrichea are sister to all other sampled ciliates, but node support is low (< 50% ML bootstrap/ 82% Bayesian posterior probability). The rest of the taxa, all in the Intramacronucleata, form a monophyletic group with low to high node support (53/100). The Oligohymenophorea is not monophyletic, with only one intervening node being low to highly supported (68/100). Within the Oligohymenophorea, *Tetrahymena* is not monophyletic as *Colpidium* sp. nests within it, but node support is low (51/90). The two sampled *Paramecium* (Oligohymenophorea) species are sister to *Didinium* (Litostomatea) with variable node support (50/98). With the same taxon sampling as mtSSU-rDNA, the nSSU-rDNA (Fig. 2) and concatenated (Fig. 3) trees largely infer the same topologies for well-supported nodes. The Heterotrichea is monophyletic with high node support, while the Oligohymenophorea is monophyletic with low node support. In both the nSSU-rDNA and concatenated trees, Litostomatea is sister to the Heterotrichea, which would render the Intramacronucleata non-monophyletic, but in both trees this relationship has low node support. There are no mtSSU-rDNA sequences from the Karyorelictea, thus conclusions cannot be drawn the monophyly of the Postciliodesmatophora.

mtSSU-rDNA supports the monophyly of the Colpodea

In previous studies, monophyly of the Colpodea was supported initially in nSSU-rDNA analyses (Stechmann *et al.* 1998, Lynn *et al.* 1999, Lasek-Nesselquist and Katz 2001). A later nSSU-rRNA analysis based on broader taxon sampling both within this taxon, as well as among close outgroups, inferred a non-monophyletic Colpodea with low node support (Dunthorn *et al.* 2008). Subsequent nSSU-rRNA studies did not include enough outgroups to be effective tests (Dunthorn *et al.* 2009; Bourland *et al.* 2011, 2012; Foissner *et al.* 2011; Quintela-Alonso *et al.* 2011). Effective testing for monophyly was also precluded in two mtSSU-rDNA analyses of the Colpodea due to low taxon sampling among potential outgroups (Dunthorn *et al.* 2011, 2012a).

With new mtSSU-rDNA sequences and the increased taxon sampling of potential outgroups, we find that molecules once again infer a monophyletic Colpodea (Fig. 1). This monophyly is not supported by ML bootstraps, but highly supported by BI posterior probability (59/95). This result supports the morphological hypothesis that ciliates with a LkM fiber in their somatic ciliature should be united into a single taxon (Lynn 1976, Small and Lynn 1981, Foissner 1993). With the same taxon sampling, nSSU-rDNA (Fig. 2) and concatenated (Fig. 3) trees also infer a monophyletic Colpodea, with strong node support coming only from the concatenated tree (91/100).

mtSSU- and nSSU-rDNA are congruent for the Phyllopharyngea

The Phyllopharyngea are recognized by phyllae surrounding the cytopharyngeal apparatus, and include free-living and symbiotic species (Lynn 2008). Some members also have sucking tentacles (Matthes 1988, Lynn 2008). The first nSSU-rDNA analysis using broad taxon sampling of the morphologically defined subgroups within the Phyllopharyngea inferred the Suctoria as a clade sister to the Cyrtophoria (= Phyllopharyngia) (Snoeyenbos-West *et al.* 2004). Additionally, within the Cyrtophoria, *Chilodonella* was inferred to be sister to the clade formed by *Chlamydodon* and *Dysteria*, thus

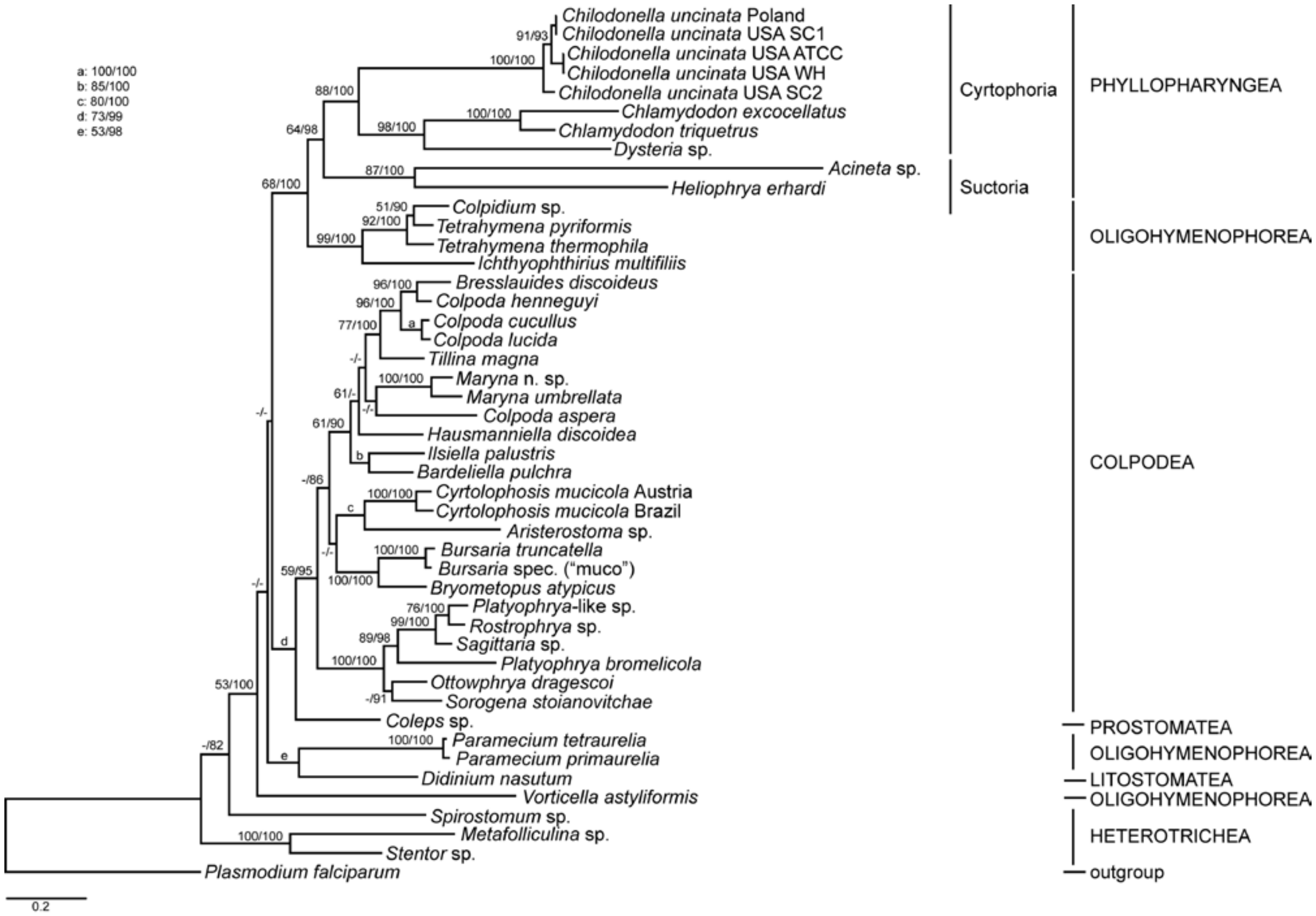

Fig. 1. Mitochondrial SSU-rDNA tree inferred from an alignment of 790 included characters. The most likely ML tree is shown; the BI tree was the same for well-supported nodes. Node support is shown as: ML bootstraps/BI posterior probability. Values ≤ 50 are shown as "-".

rendering the Chlamydodontida (which includes *Chilodonella* and *Chlamydodon*) non-monophyletic (Snoeyenbos-West *et al.* 2004). Later nSSU-rDNA analyses with additional taxon sampling confirmed this result (Li and Song 2006a, b; Gong *et al.* 2008; Gao *et al.* 2012).

With the new taxon sampling, we find that mtSSU-rDNA likewise infers *Acineta* sp. and *Heliophrya erhardi*, both in the Suctoria, as a clade sister to the other sampled Phyllopharyngea, which are all in the Cyrtophoria (Fig. 1). There is moderate to high node support for these clades: 87/100 for the Suctoria, and 88/100 for the Cyrtophoria. Within the Cyrtophoria, mtSSU-rDNA also infers *Chilodonella* to be sister to the clade formed by *Chlamydodon* and *Dysteria* with high node support (98/100). For the Phyllopharyngea, the exact same mtSSU-rDNA topology for the sampled taxa is inferred in the nSU-rDNA (Fig. 2) and concatenated (Fig. 3) trees with high node support.

Synthesis

Analyses of independent loci are essential to infer robust evolutionary relationships. Here we find a high level of congruence in analyses of both nuclear and mitochondrial SSU-rDNA sequences, which gives greater confidence in our interpretation of the evolutionary history of ciliates. However, the mtSSU-rDNA sequences fail to provide high node support for deep ciliate relationships, and we suggest that sequencing of this mitochondrial locus be used at least initially for relationships among shallower nodes.

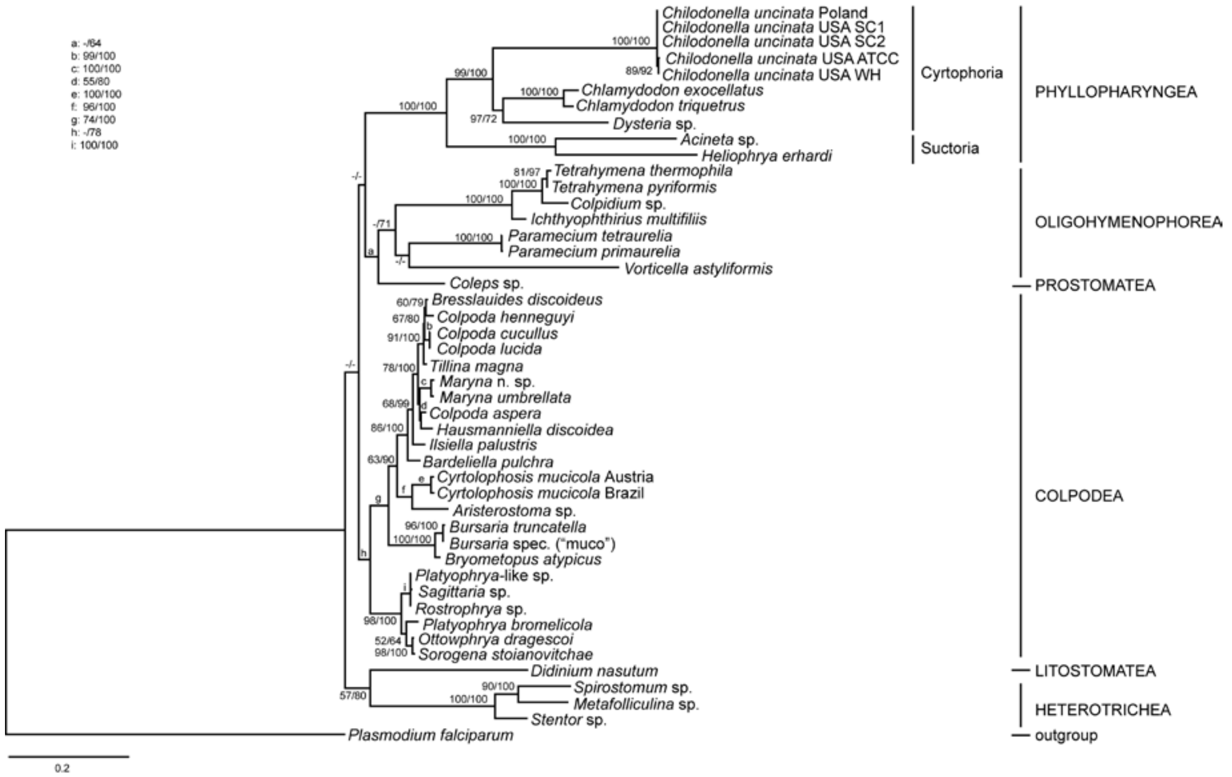

Fig. 2. Nuclear SSU-rDNA tree inferred from an alignment of 1543 included characters. The most likely ML tree is shown; the BI tree was the same for well-supported nodes. Node support is as in Fig 1.

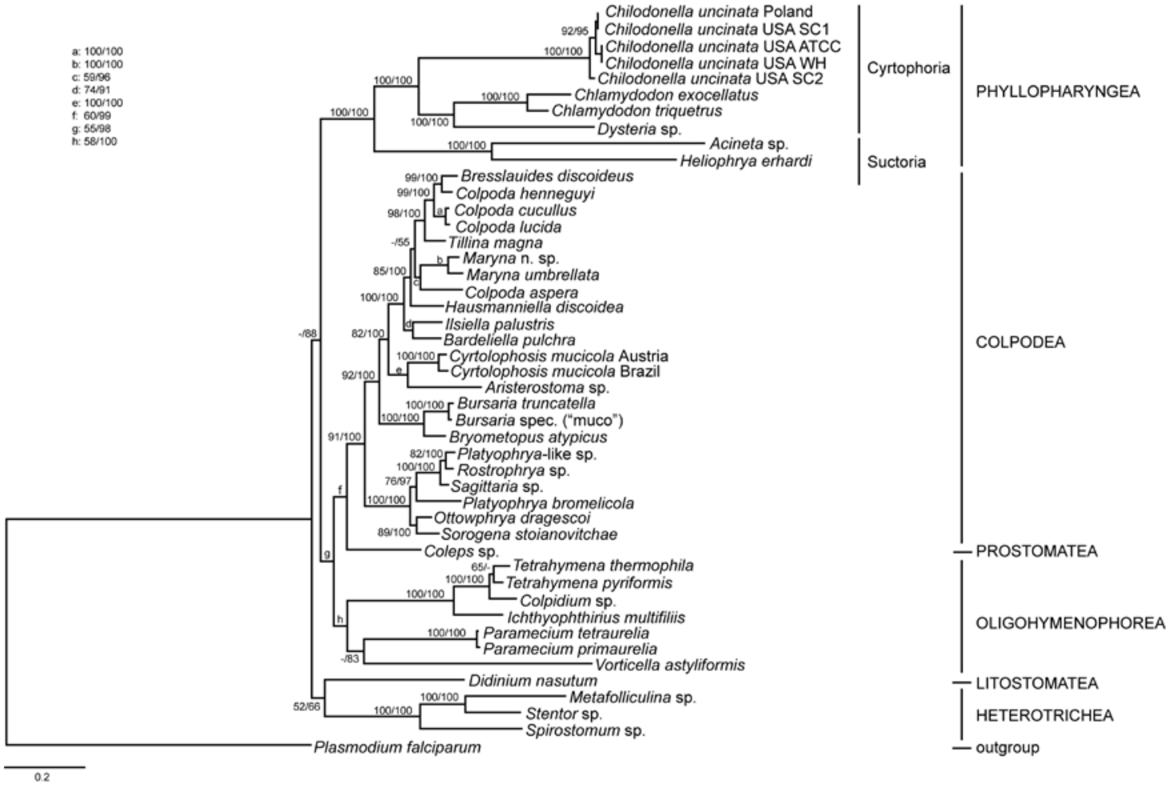

Fig. 3. Concatenated mitochondrial and nuclear SSU-rDNA tree inferred from an alignment of 2333 included characters. Most likely ML tree is shown; the BI tree was the same for well-supported nodes. Node support is as in Fig 1.

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