

Congruence and indifference between two molecular markers for understanding oral evolution in the Marynidae *sensu lato* (Ciliophora, Colpodea)

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Abstract

Our understanding of the evolution of oral structures within the Colpodida is confounded by the low number of morphological characters that can be used in constructing hypotheses, and by the low taxon and character sampling in molecular phylogenetic analyses designed to assess these hypotheses. Here we increase character sampling by sequencing the mitochondrial SSU-rDNA locus for three isolates of the Marynidae *sensu lato*. We show that the inferred mitochondrial and nuclear SSU-rDNA trees, as well as concatenated and constrained analyses, are congruent in not recovering a monophyletic Marynidae. However, due to low node support, the trees are indifferent to whether the morphological characters used to unite the Marynidae are the result of retention of ancestral states or convergence. In light of this indifference and an increased amount of nuclear and mitochondrial SSU-rDNA data, alternative hypotheses of oral evolution in the Colpodida are presented.

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Introduction

Using Lynn's (1976, 1981) structural conservatism hypothesis, various ciliate lineages were united into the Colpodea Lynn and Small, 1981 based on the presence of the LK_m fiber (Small and Lynn 1981). Some hypotheses about morphological evolution within the clade have since been proposed and molecular phylogenetic relationships have been inferred (Bourland et al. 2011; Dunthorn et al. 2008, 2009, 2011; Foissner 1985, 1993; Foissner and Kreutz 1998; Foissner and Stoeck 2009; Foissner et al. 2011;

Lasek-Nesselquist and Katz 2001; Lynn 2008; Lynn and Small 2002; Lynn et al. 1999; Quintela-Alonso et al. 2011; Small and Lynn 1985). Overall, the molecular data suggest that our use of morphological data – particularly from the oral structures – can be misleading in inferring relationships among colpodeans because of the retention of ancestral conditions and convergence of different character states (Dunthorn et al. 2011).

The molecular data, however, have not always been a panacea for the colpodeans. While deep nodes in this clade are beginning to be resolved with high node support, many shallow nodes remain unsupported or uninvestigated (Dunthorn et al. 2011; Quintela-Alonso et al. 2011). Thus, molecules have yet to shed much light on morphological evolution for some taxa. One example of this is the Marynidae Poche, 1913,

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a taxon recognized by a suite of unusual features (Foissner 1993): the presence of oral structures located in the posterior pole area of the cell, a preoral calix (=a large, cup-shaped preoral area), and a postoral uvula (=a small, but densely ciliated postoral area).

Recently, Bourland et al. (2011) sequenced *Maryna ovata*, and showed that it did not form a monophyletic clade with the previously sampled Marynidae, *Ilsiella palustris*. However, only one of two intervening nodes between these two species was moderately supported, with 76% bootstrap by Maximum Likelihood (ML) and a posterior probability of 100% by Bayesian Inference (BI). As monophyly was rejected by an S–H test ($p < 0.05$), Bourland et al. (2011) concluded that the Marynidae *sensu lato* (s.l.), as circumscribed in Foissner (1993), had been united based on convergent oral character states. They moved *Ilsiella* into a new taxon, Ilsiellidae, and kept *Maryna* and other close relatives in the Marynidae *sensu stricto* (s.str.). Given their topology, Bourland et al. (2011) also presented a hypothesis of oral evolution within the Colpodida in which the *Colpodal/Maryna* oral ciliary pattern originates from a cyrtolophosidid ancestor via a bardeliellid and bryophryid stage.

In congruence with Bourland et al. (2011), Foissner et al. (2011) found the Marynidae s.l. to be non-monophyletic. But, using isolates of *Maryna umbrellata*, *Maryna* sp. and *Pseudomaryna* sp. in the analyses that contained all sequenced Colpodea, there was only one intervening node with high support from BI. When they limited taxon inclusion to just the Colpodida and increased the number of included nucleotide positions, there was still no support in the intervening nodes between *Ilsiella* and *Maryna/Pseudomaryna* from both ML and BI analyses. This lack of node support limits confidence as to whether the Marynidae s.l. is monophyletic, and whether *Ilsiella* may or may not be best placed into a different taxon.

Both Bourland et al. (2011) and Foissner et al. (2011) used sequences only from the nuclear small subunit rDNA (nSSU-rDNA). The nSSU-rDNA gene trees might not be tracking accurately the species phylogeny; this would prevent accurate assessment of the evolution of oral features within the Colpodida. To increase character sampling, we sequenced mitochondrial small subunit rDNA (mtSSU-rDNA) from the Marynidae to provide data from an additional and independent molecular marker.

Material and Methods

Sampling, terminology, and classification

Three colpodean isolates were sequenced for this study (Table 1). The DNA used for amplifying mtSSU-rDNA from *Maryna* sp. and *Maryna umbrellata* was the same used to amplify nSSU-rDNA in Foissner et al. (2011). The DNA for *Ilsiella palustris* was newly collected for this study from Hawaii. Morphological terminology, and classification for other taxa, follows Foissner et al. (2011). By Marynidae s.l.,

Table 1. Taxon sampling. New isolates are in bold.

Taxon	mtSSU GenBank #	nSSU GenBank #
<i>Bardeliella pulchra</i>	HM246399	EU039884
<i>Bresslauides discoideus</i>	HM 246400	EU039885
<i>Colpoda aspera</i>	HM246405	EU039892
<i>Colpoda cucullus</i>	HM246406	EU039893
<i>Colpoda henneguyi</i>	HM246407	EU039894
<i>Colpoda lucida</i>	HM246409	EU039895
<i>Tillina magna</i> ^a	HM246410	EU039896
<i>Cyrtolophosis mucicola</i>	HM246411	EU039899
<i>Hausmanniella discoidea</i>	HM246413	EU039900
<i>Ilsiella palustris</i> ^b	–	EU039901
<i>Ilsiella palustris</i>	JQ026522	JQ026521
<i>Maryna umbrellata</i>	JQ026523	JF747217
<i>Maryna</i> sp.	JQ026524	JF747218

^aSubmitted to GenBank as *Colpoda magna*.

^bNot used in phylogenetic analyses.

we mean the taxon as circumscribed by Foissner (1993). By Marynidae s.str., we mean the taxon as circumscribed by Bourland et al. (2011) and followed by Foissner et al. (2011).

There are multiple options for describing inferred relationships in molecular trees. Here we follow Farris (1974) in his definition of monophyly. Rather than likewise following Farris' (1974) definitions for paraphyly and polyphyly, we lump these two concepts into simply “non-monophyly”. We therefore can focus on what non-monophyly can imply: i.e., retention of shared ancestral morphological states, or convergence in morphological states.

Amplification, sequencing, and alignments

Primers and amplifications followed Dunthorn et al. (2011) for mtSSU-rDNA, and Foissner et al. (2011) for nSSU-rDNA. Overlapping sequences from individual forward, reverse and internal sequencing reactions of the same clones were quality checked and combined using CondonCode Aligner v.3.0 (CondonCode Corporation, Dedham, MA). Vector and primer nucleotides were trimmed off. Sequences were added to the alignments of Dunthorn et al. (2011) and Foissner et al. (2011), and ambiguously aligned positions were removed by eye in MacClade v4.05 (Maddison and Maddison 2005). The masking for the mtSSU-rDNA alignment was originally checked using Gblocks v0.91b (Castresana 2000; Talavera and Castresana 2007) by Dunthorn et al. (2011). Here we also checked the removal of nucleotide sites using GUIDANCE

and Bayesian topologies for moderately to highly supported nodes (Fig. 2). For non-Marynidae sequences, the nSSU-rDNA topology is congruent with previously published trees (Bourland et al. 2011; Dunthorn et al. 2008, 2009, 2011; Foissner et al. 2011; Quintela-Alonso et al. 2011) for moderately to highly supported nodes. Thus, the limited taxon inclusion does not appear to have an effect. As in the mtSSU-rDNA tree, the Marynidae s.l. are not monophyletic. *Ilsiella* branches sister to all Colpodida, except *Bardeliella*. The two *Maryna* sequences, which are sister to each other with full node support (100/100), form a clade with *Hausmanniella* and *Colpoda aspera*, although the node for this larger clade is not supported (<50/71). As the intervening nodes between *Maryna* and *Ilsiella* are not moderately to fully supported, the nSSU-rDNA tree provides little confidence in the non-monophyly of the Marynidae.

Concatenated tree

As with the single gene trees described above, the inferred ML and Bayesian topologies from the concatenated alignment of 2506 sites were identical for moderately to well-supported nodes (Fig. 3). Nodes in this tree are congruent with those moderately to highly supported nodes in a previously published concatenated topology (Dunthorn et al. 2011). The Marynidae s.l. are not monophyletic, although, as above, there is little confidence in this as none of the intervening nodes are moderately to fully supported. *Ilsiella* branches in a position similar to the nSSU-rDNA tree.

Constrained analyses

The morphological hypothesis that the Marynidae are monophyletic was further evaluated by constraining the three relevant lineages into a single clade in ML inferences of the mitochondrial, nuclear and concatenated alignments. Shimodaira–Hasegawa (S–H) tests were able to significantly reject monophyly for all three alignments ($p = 0.000$). This suggests that Marynidae s.l. may truly not be monophyletic given the gene and taxon sampling available here. Yet, this S–H test provides no information on branching order of the members of Marynidae s.l., nor on how to interpret character evolution within this group.

Discussion

Molecules and the Marynidae s.l.

Morphological studies have laid most of the groundwork for our view of ciliate evolution and taxonomy. As with Lynn's (1976, 1981) structural conservatism hypothesis, these characters have been used to construct radically novel hypotheses of sister-group relationships. However, the low number of morphological characters in ciliates, at least

in relation to plants and animals, can cause difficulty when assessing support for differing hypotheses (e.g. Dunthorn and Katz 2008). Aggravating the problem is that some characters may not be independent from each other, such as apical oral structures and enantiotropic (right-angle) division. Assessments of morphological hypotheses, then, often have to rely on phylogenetic trees inferred from molecules.

Molecular studies have also provided insights into ciliate evolution including: removing the Spirotrichea from the Heterotrichea (Baroin-Tourancheau et al. 1992; Hirt et al. 1995), breaking up of the Cyrtolophosida in the Colpodea (Dunthorn et al. 2008, 2011), and recognizing the Armorphorea as a class (Lynn, 2003). Hence, there is indeed a role for molecules in ciliate systematics for testing morphological hypotheses, as well as instigating novel views.

The power of molecules, though, only comes when we have confidence in their inferred trees; i.e., when node support is moderate to high from ML bootstraps and BI posterior probability, when independent loci result in similar topologies, and when constrained analyses can significantly reject one hypothesis over another. Mitochondrial and nuclear SSU-rDNA analyses that have assessed the morphologically based Marynidae s.l. only partially fulfill these criteria with the current taxon sampling. While the inferred mitochondrial (Fig. 1), nuclear (Fig. 2), and concatenated (Fig. 3) trees here, and nuclear trees elsewhere (Bourland et al. 2011; Foissner et al. 2011), are congruent in showing *Ilsiella* and *Maryna* as non-sister taxa within the Colpodida, we do not have confidence because the intervening nodes have mostly low to no support. Our confidence is bolstered only in that the multiple markers yield similar insights of non-monophyly, and the constrained analyses significantly reject monophyly.

Beyond the Marynidae s.l. not being monophyletic, the topologies of the inferred trees are indifferent to what exactly is the relationship is between *Ilsiella* and *Maryna/Pseudomaryna* with the current taxon sampling. The low node support in each gene tree, and the shifting positions of these taxa within the Colpodida, provide no information on the true branching order of taxa; i.e., in the mtSSU-rDNA tree (Fig. 1) *Maryna* branches first, while in the nSSU-rDNA tree (Fig. 2) and concatenated (Fig. 3) trees *Ilsiella* branches first.

Oral evolution within the Colpodida

Given this lack of molecular support – from mitochondrial and nuclear SSU-rDNA – alternative, and equally valid, hypotheses of oral evolution within the Colpodida should be considered. Generally, the morphological interpretation of the molecular Colpodean trees shows a basic problem: below what is classified at the order level, the taxa are usually weakly supported, and appear influenced by the number and kind of species included, the alignment, and the tree algorithm. Typical examples are the recent trees of Bourland et al. (2011) and Foissner et al. (2011).

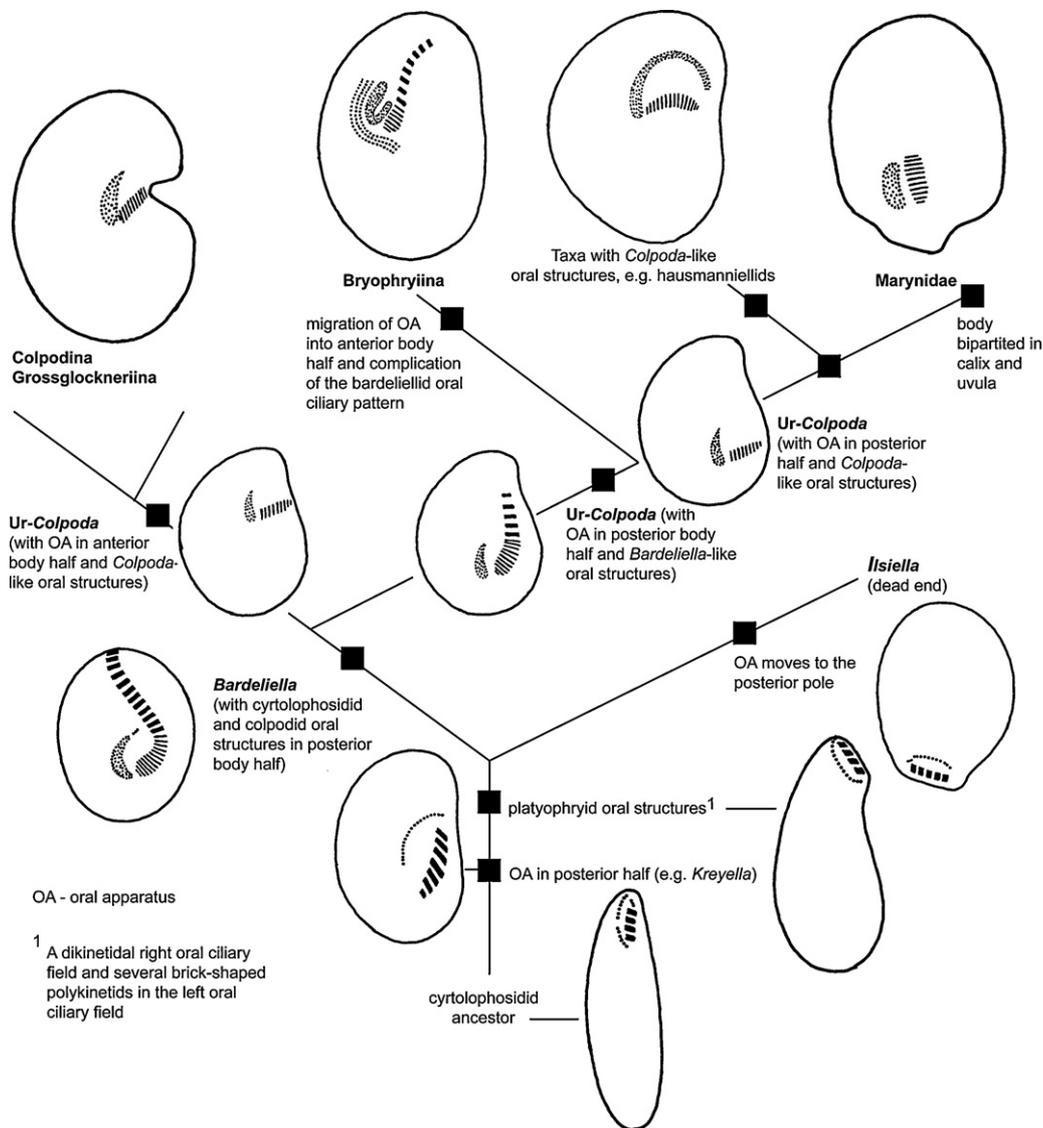


Fig. 4. Development of oral features in the order Colpodida, using evolutionary systematics, as explained by Foissner et al. (2011). This scenario is part of a larger one because *Colpoda*-like oral structures occur also in several other small clades, e.g., *Colpoda steinii* and *Bromeliothrix metopoides* (Foissner et al., 2011). See Foissner (1993) and Foissner et al. (2011) for details of characters and the suborders Colpodina and Grossglockneriina.

Based on a new sequence each from *Bryophrya* and *Maryna* and five *Colpoda* species from GenBank, Bourland et al. (2011) suggest that the posterior position of the oral apparatus evolved convergently in the families Ilsiellidae and Marynidae s.str. Further, they suggest *Bardeliella* as the most basal colpodid, which originated from the cyrtolophosidids and directly developed to *Bryophrya* and *Colpoda*; i.e., they consider the bryophryids as ancestors of the colpodas s.str. While we agree that *Bardeliella* is the earliest diverging Colpodida, and the posterior location of the oral apparatus may have developed convergently in the ilsliellids and marynids (Foissner et al. 2011), we strongly doubt the bryophryids represent the morphological state of the last common ancestor of the Colpodas s.str. Further, we assume that the ilsliellids are a dead end because

additional genera that could belong to this group have been not described.

The tree of Foissner et al. (2011), which includes 12 *Colpoda* species, shows small and large *Colpoda* clades distributed over the entire Colpodida tree. For instance, there is a clade with *Colpoda steinii* and *Bromeliothrix metopoides*, although *C. steinii* is morphologically much more similar to *C. aspera* than to *Bromeliothrix*. The same applies for the *C. aspera/Hausmanniella* clade and the *C. maupasi/C. augustini* clade, which are far away from the *Colpoda* s.str. clade. Thus, Foissner et al. (2011) suggest a rapid basal radiation of the genus *Colpoda*, where the *Colpoda* stem species remained largely unchanged and repeatedly produced new taxa. This hypothesis explains the jumping appearance of clades with *Colpoda* species throughout the Colpodida tree and requires

a new hypothesis on the origin of the *Colpoda/Maryna* oral apparatus (Fig. 4). The *Colpoda* stem species (“Ur-*Colpoda*”) should have been a small, bacterivorous ciliate, as are the last common ancestors, *Cyrtolophosis* and *Bardeliella*. Further, it should have had an oral apparatus similar to that of present-day colpodas s.str. These features are retained by several extant species, e.g., *C. aspera* and *C. ecaudata*.

To sum up, Bourland et al.’s (2011) hypothesis was reasonable with the data available at that time, but it cannot accommodate the new molecular data from Foissner et al. (2011) and here. Finally, we emphasize that our phylogeny should be considered as only one of several possibilities. Very likely, the marynid phylogeny will become better resolved when more sequences from additional taxa become available.

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