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Dunthorn, Micah; Katz, Laura A.; Stoeck, Thorsten; and Foissner, Wilhelm, "Congruence and Indifference Between Two Molecular Markers for Understanding Oral Evolution in the Marynidae sensu lato (Ciliophora, Colpodea)" (2012). Biological Sciences: Faculty Publications, Smith College, Northampton, MA.  
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Congruence and indifference between two molecular markers for understanding oral evolution in the Marynidae sensu lato (Ciliophora, Colpodea)

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Received 18 September 2011; received in revised form 16 January 2012; accepted 18 January 2012
Available online 20 February 2012

Abstract

Our understanding of the evolution of oral structures within the Colpodea 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 Colpodea are presented.

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Keywords: Bootstrap; Colpodeans; Mitochondrial SSU-rDNA; Nuclear SSU-rDNA; Node support; Phylogeny

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 LKm 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,
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 area of the cell, a postoral calyx (=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 characters. 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 *Colpodida/Maryna* oral ciliary pattern originates from a cyrtolophosid 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 Colpoda and increased the number of included nucleotide positions, there was still no support in the intervening nodes between *Ilsiella* and *Marynidae/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., 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 (CodonCode 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 v.4.05 (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.
v1.1 (Penn et al. 2010a,b), but found no difference (data not shown). Taxon inclusion for the mtSSU alignment was limited to just the Colpodida and an outgroup (Cyrtolophosis mucicola). Taxon inclusion for the nSSU-rDNA alignment was generated to match the mtSSU-rDNA alignment.

Genealogical analyses

Pairwise distances were calculated as uncorrected "p" distances in PAUP* v4.0b8 (Swofford 2002). For all alignments the GTR-I-Gamma model was the best fitted model selected by AIC as implemented in jModeltest v0.1.1 (Guindon and Gascuel 2003; Posada 2008). ML analyses were carried out in RAxML-HPC v7.2.5 (Stamatakis et al. 2008), with node support from a majority rule consensus tree of 1000 multiparametric bootstrap replicates. BI was carried out using MrBayes v3.2.1 (Huelsenbeck and Ronquist 2003). Posterior probability was estimated using four chains running one million generations sampling every 100 generations. The first 25% of sampled trees were considered burn-in trees and were discarded prior to constructing a 50% majority rule consensus trees. FigTree v1.3.1 (Rambaut 2006) was used for visualization. For the ML bootstraps, we consider values <70 as low, 70–94 as moderate, and ≥95 as high following Hillis and Bull (1993). For the Bayesian posterior probabilities, we consider values <94 as low, and ≥95 as high following Alfaro et al. (2003).

Constrained analyses

Constrained analyses in RAxML were carried out on all three alignments, where the three Marynidae s.l. (Ilsiella palustris, Maryna sp., M. umbrellata) were forced to be monophyletic. All other relationships were unspecified. Resulting constrained topologies were compared to the non-constrained topologies using the S-H test (Shimodaira and Hasegawa 1999) as implemented in PAUP* v4.0b8 (Swofford 2002).

Results

Characterization of the new Ilsiella isolate

Because there was no genomic DNA remaining from the original isolate of Ilsiella palustris used by Dunthorn et al. (2008), here a new isolate was collected so as to obtain mtSSU-rDNA sequences. The previously published nSSU-rDNA (GenBank number EU039901) has a pairwise distance of 0.0095% to the nSSU-rDNA sequence from this new isolate. This value is well within the variation caused by population variation and/or errors introduced during amplification and sequencing reactions. Therefore, the nSSU-rDNA sequence from the new isolate and the original mtSSU-rDNA sequences were concatenated in the final analyses.

Mitochondrial SSU-rDNA tree

The mitochondrial alignment of 830 included characters resulted in identical ML and Bayesian topologies for moderately to highly supported nodes. Here we present the most likely ML tree with node support from both methods (Fig. 1). For non-Marynidae sequences, the mtSSU-rDNA topology is congruent with a previously published tree (Dunthorn et al.

Fig. 1. Mitochondrial SSU-rDNA topology of the Colpodida. 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 for moderately to highly supported nodes. Node support is as follows: ML bootstrap/BI posterior probability. Support <50% is shown as ‘—’.
Fig. 2. Nuclear SSU-rDNA topology of the Colpodida. 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 for moderately to highly supported nodes. Node support is as follows: ML bootstrap/BI posterior probability. Support <50% is shown as ‘–’.

2011) for most nodes. The only substantial difference is the clade formed by Bardeliella and Hausmanniella (along with Ilsiella), but this node is not supported (<50 ML bootstrap/50 Bayesian posterior probability).

The Marynidae s.l. are not monophyletic. The two Maryna sequences (Maryna sp. and M. umbrellata) form a separate clade that has full node support (100/100) and is distinct from the Ilsiella sequence. The two Maryna sequences branch sister to all Colpodida, except Colpoda aspera. Ilsiella nests within the clade formed by Bardeliella and Hausmanniella. Because there are no moderately to well-supported intervening nodes between Ilsiella and Maryna, the mtSSU-rDNA tree provides little confidence in its support for non-monophyly of the Marynidae.

Nuclear SSU-rDNA tree

To evaluate the possibility of low taxon sampling affecting the inferred tree, taxon inclusion for nSSU-rDNA was limited to match the alignment for mtSSU-rDNA. This nuclear alignment of 1676 included characters resulted in identical ML
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 *Haustramanniella* 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 morphological hypotheses of oral evolution 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 (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 Ilisiellidae 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 ilisiellids 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 ilisiellids 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, *Cyrtochloris* and *Bardelebiella*. 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.

Acknowledgements

This work was supported by a postdoctoral fellowship from the Alexander von Humboldt Foundation to M.D., the United States National Science Foundation (grant DEB 0816828) to L.A.K., the Deutsche Forschungsgemeinschaft (grant STO414/3-1) to T.S., and the Austrian Science Fund (FWF), projects P 20360-B17 and P 22846-B17 to W.F.

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