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Authors: SIDDALL, MARK E., REECE, KIMBERLY S., NERAD, TOM A., and BURRESON, EUGENE M.

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Molecular Determination of the Phylogenetic Position of a Species in the Genus *Colpodella* (Alveolata)

MARK E. SIDDALL,[1] KIMBERLY S. REECE,[2] TOM A. NERAD,[3] AND EUGENE M. BURRESON[4]

ABSTRACT

The phylogenetic position of a species of the free-living protist genus *Colpodella* is assessed using its SSU rDNA gene sequence in the context of a wide array of other alveolate taxa. Phylogenetic analyses indicates that this species is not related to species of *Bodo* as has previously been suggested. However, SSU rDNA data alone are insufficient to provide a wholly stable hypothesis of relationships for *Colpodella* sp. among the various alveolate phyla. Much of this instability can be attributed to alignment procedure sensitivity. Analyses of SSU rDNA and actin gene sequences in combination provide strong support for a species of *Colpodella* as sister group to the ciliates, irrespective of alignment procedure. Moreover, use of SSU rDNA data alone strongly support a recent common ancestry of the genera *Perkinsus* and *Parvilucifera* with the dinoflagellates, and not with the apicomplexans. These findings, and the nearly identical ultrastructural characteristics of *Colpodella, Perkinsus,* and *Parvilucifera* species, suggest that this morphology is the ancestral condition for all of Alveolata.

INTRODUCTION

It is now well understood that protistan phyla Apicomplexa, Ciliophora, and Dinozoa share a recent common ancestor and form the ''super-phylum'' Alveolata (Gajadhar et al., 1991). Each of these three phyla share only a few common features such as a second inner membrane system (alveoli) and micro-

[1] Assistant Curator, Division of Invertebrate Zoology, American Museum of Natural History.
[2] Virginia Institute of Marine Science, Gloucester Point, Virginia.
[3] American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia.
[4] Virginia Institute of Marine Science, Gloucester Point, Virginia.

pores (Siddall et al., 1997) but otherwise they have unique characteristics. For example, dinoflagellates once thought to be ancestral to all eukaryotes (Loeblich, 1976), have uniquely condensed chromosomes. Ciliates stand apart not only with uniquely arranged cortical ciliature but also by having two nuclei. The apicomplexans, so named for their distinctive apical organelles (Levine, 1988), also exhibit gliding motility unlike any other group of organisms. In light of this uniqueness, the ancestral conditions for the alveolates are not obvious.

Perkinsus marinus was thought to be related to apicomplexans when it was discovered to possess structures reminiscent of a conoid and micronemes (Perkins, 1976; Levine, 1978). Levine (1988), though he held that this oyster parasite may be ancestral to the group, added that this was ''conjectural and never will be proven. There are no fossils.'' However, several molecular phylogenetic analyses clearly have placed *Perkinsus* species with the dinoflagellates (Siddall et al., 1995, 1997; Reece et al., 1997). The recent discovery and characterization of a *Perkinsus*-like protist, *Parvilucifera infectans,* have complicated this picture (Norén et al., 1999; Erard-Le Denn et al., 2000). Like *Perkinsus* species, *P. infectans* is a parasite, but one of toxic dinoflagellates not of metazoans. Its ultrastructural characteristics are markedly similar to those seen in species of *Perkinsus.* A phylogenetic analysis of SSU rDNA strongly supports the monophyly of these two genera, but as the sister group to Apicomplexa, not to Dinoflagellata (Norén et al., 1999).

Less well studied are species of the genus *Colpodella,* which except for their free-living habits, also are nearly indistinguishable from *Perkinsus* species. Species of *Colpodella* (prev. *Spiromonas*) are predators of other free-living protists (Brugerolle and Mignot, 1979; Myl'nikov, 1991; Simpson and Patterson, 1996). With a reinforced rostrum, they penetrate through the cell membrane of their protistan prey and consume the cytoplasmic contents or ingest whole cells (Brugerolle and Mignot, 1979). The rostrum of *Colpodella* species is identical in structure and function to the so-called ''conoid'' of zoospores of *Perkinsus* and *Parvilucifera* species and each of these taxa have elongate extrusomes that are hypothesized to be homologous with apicomplexan rhoptries. Previously, however, molecular data have not been available for any species in the genus *Colpodella.*

MATERIALS AND METHODS

An undescribed species of *Colpodella* found feeding on *Bodo caudatus* was isolated from brown woodland soil in Gambrill State Park, Maryland, in 1993. This species was maintained in vitro at the American Type Culture Collection [ATCC 50594] with its naturally occurring bodonid prey. Frozen 500 μl samples were thawed, and 50 μl 2M NaCl in 1500 μl cold ethanol was added to precipitate any DNA released from ruptured cells prior to DNA isolation. After centrifugation and washing with 70% ethanol, the pellet was resuspended in lysis buffer and digested with proteinase-K at 55°C. DNA isolation then followed standard phenol-chloroform extraction procedures.

Amplification of the small subunit ribosomal RNA gene used combinations of primers 5′-AACCTGGTTGATCCTGCCAGT-3′ and 5′-TGATCCTTCCGCAGGTTCACCT-3′ (primers ''A'' and ''B'' respectively) and internal primers (primer ''L'': 5′-CCAACTACGAGCTTTTTAACTG-3′, primer ''C'': 5′-CGGTAATTCCAGCTCCAATAG-3′, primer ''Y'': 5′-CAGACAAATCGCTCCACCAAC-3′, primer ''O'': 5′-AAGGGCACCACCAGGAGTGGAG-3′). Reaction mixtures were heated to 94°C for four minutes and then cycled 35 times at 94°C (20 s), 47°C (20 s), 68°C (105 s) with a final extension at 70°C (seven minutes). ''Universal'' actin gene primers ''480'': 5′-AA(TC)GGIGA(AG)AA(AG)ATGACICA-(AG)AT(TCA)ATGTT-3′ and ''483'': 5′-CCAIACI(CG)(AT)(AG)TA(CT)TTIC-(GT)(CT)TCIGGIGG-3′ designed by G. Warr (Medical University of South Carolina) and M. Wilson (Mississippi State Medical Center) for amplification of vertebrate actin genes were used to amplify a central coding region of the actin genes corresponding to residues 127 through 333 in vertebrate actin protein sequences. Five to 10 ng of genomic DNA was amplified in 50 μl reactions using the BRL PCR Reagent System (Life Technologies, Gai-

thersburg, Maryland). Reagent concentrations were as follows: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmoles of each primer, 0.2 mM each of dATP, dGTP, dCTP, dTTP, 20% (v/v) BSA, and 1.25 units of Taq DNA polymerase. Following an initial denaturation of four minutes at 95°C, reactions were subjected to 40 cycles of one minute at 95°C, one minute at 45°C and three minutes at 65°C. A final extension of five minutes at 65°C was added to each amplification reaction. Amplification products were electrophoresed in 1.5% low-melting-point agarose and removed with the QiaQuick Gel-Extraction protocol (QiaGen), or were cloned into the plasmid vector pCR2.1 (TA Cloning kit, InVitrogen) following the manufacturer's protocol. The ligated plasmids were transformed into *E. coli* DH5α cells and recombinants were selected for sequence analysis. Some sequencing reactions employed Big-Dye (Applied Biosystems) according to the manufacturer's instructions and were electrophoresed in 4.5% polyacrylamide on an ABI 377 automated sequencer. Chromatograms from fragments sequenced in both directions were reconciled in Sequence Navigator. For the cloned amplification products, both strands of the plasmid inserts were sequenced in a single tube using the ThermoSequenase kit (Amersham) and fluorescently labeled (IRD700 and IRD800, LI COR) M13 forward and reverse primers in simultaneous bidirectional sequencing (SBS). The labeled DNA fragments were separated on 4% Long Ranger acrylamide (Baker) gels and detected by infrared lasers in a LI COR automated sequencer (model 4200L–2).

Because of the contaminating prey item (*Bodo caudatus*) DNA, multiple clonal isolates of amplification products were sequenced to ensure that *Colpodella* sp. sequences were obtained. An appropriately structured suite of taxa was obtained for phylogenetic analyses of SSU rDNA alone including 18 dinoflagellates [AF022153, AF022154, AF022156, AF022191, AF022194, AF022197, AF022199, AF022200, AF022201, AF022202, AF052190, AF077055, AF080096, AF080097, L13717, L13719, M14649, SNU52357], two species of *Perkinsus* [L07375, U07701], 15 apicomplexans [M97703, AF006470, AF009244, AF060975, AF080611, AF080612, AF111183, L02366, L19078, L19080, L24383, NSPRG18S, U40264, U67117, U94787], 18 ciliates [X56171, AF164136, U97111, U17354, U57769, U57770, U57771, X03948, X03772, SPU97112, ECU57765, EMU57766, ECU5776, FVU97110, OCU17355, AHU51554, CHU97109], and *Parvilucifera* [AF133909] as well as seven outgroup taxa [X89518, M87327, M32705, J01353, U83128, M97959, M32704]. *Bodo caudatus* [X53910] was included in preliminary alignments to assess the identity of clones. Analyses employing combined SSU rDNA *and* actin gene sequences were conducted with the same taxonomic composition as above and coding actin as missing for those taxa for which it is not available. Combined SSU rDNA and actin analyses also were conducted with a different taxonomic composition using only those taxa for which both genes were available. The latter analyses include SSU rDNA and actin for two slime molds [X00134, X13160; X03282, M21500], four fungi [X04971, Z75578, Z75307, X89518; X16377, V01288, U17498, U78026], a chlorophyte [M32703; D50839], *Acanthamoeba* [U07413; V00002], a rhodophyte [Z14140; U03677], four stramenopiles [AB011423, M32705, X53229, X54265; U11697, X59937, X59936, M59715], a haptophyte [M87327; S64188], two dinoflagellates, [M14649, L13719; U84290, U84289], four ciliates [X03948, AF164136, X56165, X03772; X05195, J01163, AF043608, J04533], two apicomplexans [U12138, AF108864; U10429, M86241], and *Perkinsus* [L07375; U84287].

Alignment was explored using phenetic CLUSTAL alignments, and parsimonious sequence alignment with MALIGN (Wheeler and Gladstein, 1996a) including and excluding regions of highly variable alignment; in addition parameter sensitivity was assessed with optimization alignment using POY (Wheeler and Gladstein, 1996b). Alignment parameters varied for 18S rDNA sequences was the cost-ratio of insertion-deletion events to base substitutions (1, 2, 3, 4 and 5). The most parsimonious solution, or that found to be optimal under likelihood with the no common mechanism model (Tuffley and Steel, 1997), was determined with Nona (Goloboff,

1998) using the Ratchet option with 10 replicates of 50 iterations. Levels of support for clades examined included Bremer support values (Bremer, 1988) obtained with Nona (Goloboff, 1998), parsimony jackknife (Farris, et al., 1996) values using 100 replicates with branch breaking, and 10 random addition sequences in Xac (Farris, 1998).

RESULTS

Analysis of SSU rDNA aligned with CLUSTAL or MALIGN for the preliminary assessment including *Bodo* species yielded one optimal tree. This tree placed *Bodo caudatus* with the other sequence available for this species, *Perkinsus* species with the dinoflagellates, and *Colpodella* sp. as sister to the Ciliophora which together were hypothesized to be the sister clade to the apicomplexans. More than 20 extra steps were required to lose the *Bodo* clade. Monophyly of *Perkinsus* spp. and dinoflagellates had a Bremer support of 17 but nonmonophyly of *Colpodella* and Ciliophora could be found in a tree only two steps longer. Analysis of these same data, aligned with CLUSTAL in the absence of the distantly related *Bodo* species, resulted in two optimal trees disagreeing only on relationships within the dinoflagellates. These trees indicated monophyly of the genus *Perkinsus* and the dinoflagellates but placed *Colpodella* sp. as sister to the apicomplexans. Analyses of parameter sensitivity with optimization alignment when substitutions and indels had equal costs indicated an apicomplexan relationship for *Colpodella* sp. but failed to find monophyly of any alveolate phyla. Optimization alignment with double or higher costs for indels all supported *Colpodella* as sister to the Ciliophora.

Realignment with MALIGN (using a 2:1 indel:substitution cost) yielded six trees of length 7136 with *Colpodella* as sister to the Apicomplexa but in which *Perkinsus* species and *Parvilucifera infectans* did not group either with dinoflagellates or with apicomplexans. The relationship between *Colpodella* and Apicomplexa could be refuted with two extra steps. Parsimony jackknife values of whole SSU rDNA sequences are illustrated for higher taxonomic groups in the consensus of these trees in figure 1A. There was strong support for each of the three recognized alveolate phyla and for monophyly of the genera *Parvilucifera* and *Perkinsus.* Exclusion of highly variable alignment regions (fig. 1B) yielded two equally parsimonious trees of length 4022. Again *Colpodella* grouped with the Apicomplexa; however, this clade could be refuted with only two additional steps. Although monophyly of *Perkinsus* and *Parvilucifera* species with the Dinozoa was indicated with these truncated data, neither this nor the affiliation of *Colpodella* were found with parsimony jackknifing.

Because these analyses of protists necessarily entail the use of highly divergent outgroup taxa (specifically a combination of fungal and algal taxa), the preceding analyses were conducted again in the absence of the outgroup taxa. Analyses both with and without variable aligned regions did not group *Colpodella* with the apicomplexans but rather suggested a closer relationship to the ciliates (fig. 1 C,D). Nonetheless, the most parsimonious solution grouping *Colpodella* with Ciliophora using all aligned sites (5873 steps) or using only more conservative sites (3338 steps) each could be refuted with only three additional steps.

Combined analyses using 63 eukaryotic taxa and including actin for 17 taxa for which this gene is available, yielded three equally parsimonious trees of length 8385 for the analysis using all 18S rDNA sites (fig. 2A). An additional 12 steps were required to group *Colpodella* with the Apicomplexa and 37 steps were required to group *Colpodella* with its look-alikes, species of *Perkinsus* and *Parvilucifera.* Using only conservative 18S rDNA sites and actin combined (fig. 2B) minimum length trees (5287 steps) were 13 steps shorter than those grouping *Colpodella* with the Apicomplexa and 30 steps shorter than those grouping *Colpodella* with species of *Perkinsus* and *Parvilucifera.*

Using only 24 taxa for which both 18S rDNA and actin sequences are currently available, phenetically aligned data (length = 7243) placed *Colpodella* and *Perkinsus* species as sister taxa to Ciliophora, and Dinoflagellata, respectively. Identical results (fig 2C) were found with the phylogenetically aligned data (length = 7176). The latter result was strongly supported by parsimony

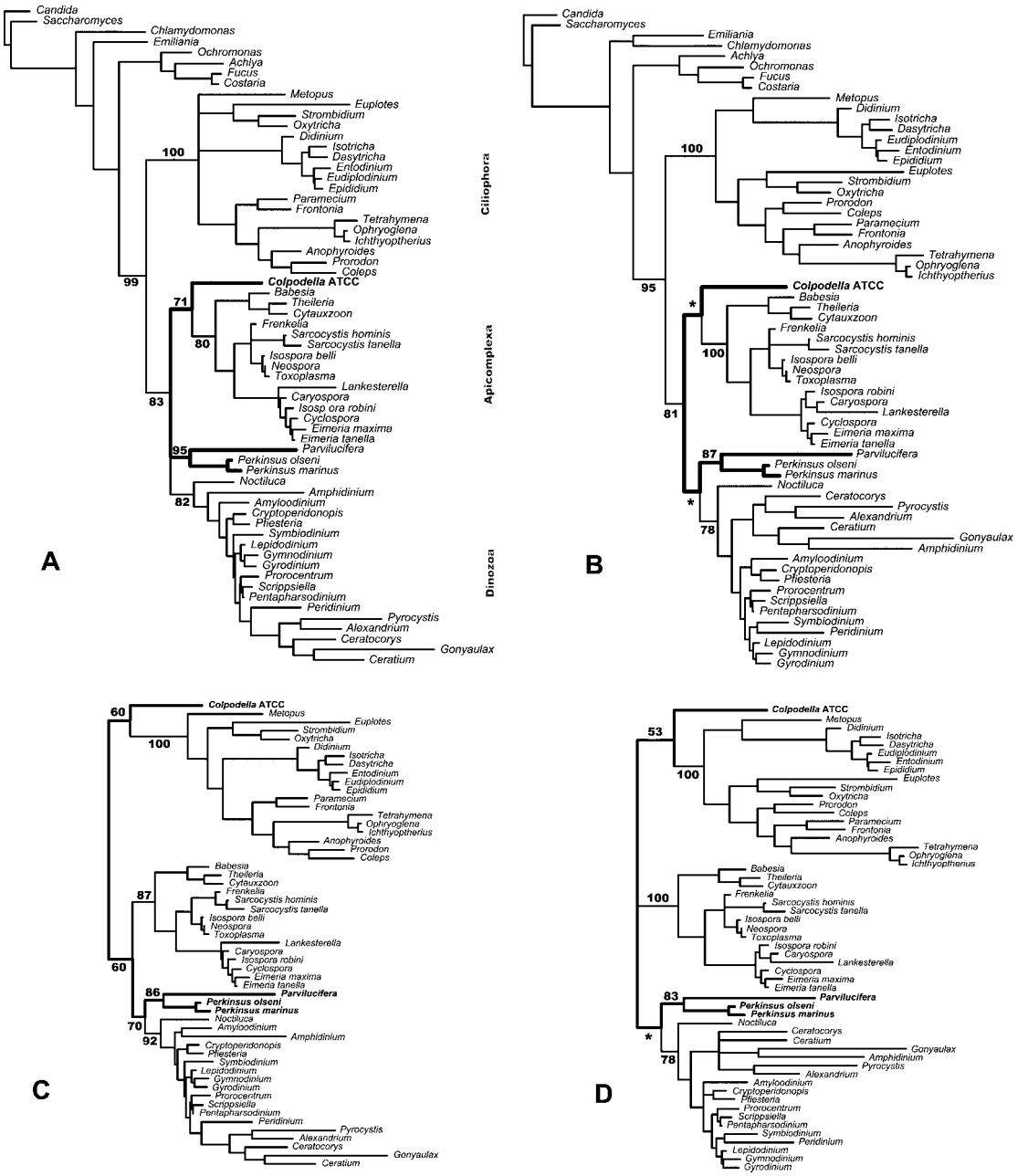

Fig. 1. Consenses of optimal trees found from parsimony analysis of SSU rDNA aligned with MALIGN using all aligned sites **(A),** only conservative sites **(B),** and excluding outgroup taxa for all sites **(C)** and conservative sites only **(D).** Branches are drawn proportional to amount of change. Values at internodes for groups of interest are parsimony jackknife support indices (asterisk indicates not supported with this method). Thickened branches indicate implied retention of the *Colpodella*/*Perkinsus* morphology.

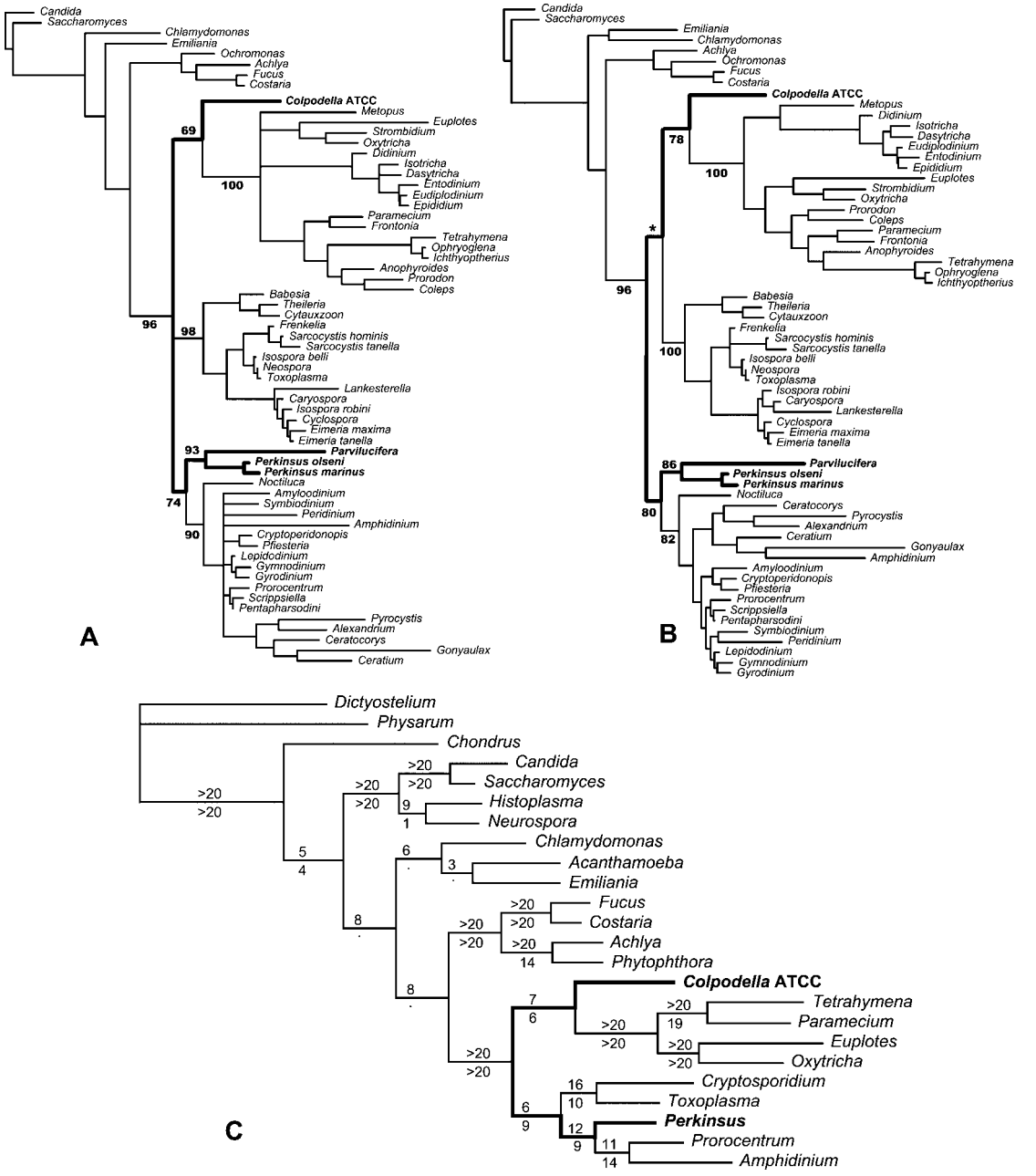

Fig. 2. Consenses of optimal trees found from parsimony analysis of combined 18S rDNA and actin nucleotide sequences (SSU rDNA data were only available for 24 taxa) for all available sites **(A)** and for conservative SSU rDNA sites **(B)**; values at internodes are Bremer support indices. Optimal tree **(C)** found for combined analyses using only those taxa for which both genes are available; values at internodes are Bremer support indices for the 18S rDNA data (above nodes) and for the actin data (below nodes). Thickened branches indicate implied retention of the *Colpodella*/*Perkinsus* morphology.

jackknifing and 38 to 40 extra steps were required to make the genera *Colpodella* and *Perkinsus* monophyletic. Topological results for these taxa did not change when gapped sites were excluded from these data sets.

DISCUSSION

On the whole, the molecular evidence points to this species of *Colpodella* as sister to the ciliates. Species of *Colpodella*, previously included in the genus *Spiromonas*, had been thought to be related to *Bodo* spp. on the basis of light microscopy alone. Electron microscopy, however, provided ample evidence that these predators are not closely related to kinetoplastids (Brugerolle and Mignot, 1979; Myl'nikov, 1991; Simpson and Patterson, 1996). The ultrastructural findings of Brugerolle and Mignot (1979), for example, led them to speculate on a close affinity for *Colpodella perforans* with the dinoflagellates or the apicomplexans. Inclusion of the bodonid prey item and another species of *Bodo* in preliminary analyses confirms the distinct isolation of the two co-occurring sequences and clearly separates *Colpodella* from the kinetoplastids. Small subunit ribosomal sequences remain the tool of choice for protozoologists. Although the use of 18S rDNA data alone corroborated the expected alveolate affinity for *Colpodella* sp., comparison of phenetic and cladistic alignment regimes and a variety of additional parameters suggests that this gene alone is insufficient to resolve higher-level relationships in the group. Even with a broad taxonomic composition it is highly sensitive to alignment optimality criterion, to alignment parameters within an optimality criterion, and to the choice of taxa used to root the ingroup.

It is surprising that *Colpodella* does not group with *Perkinsus* or *Parvilucifera* species in any of the analyses. *Colpodella* species are morphologically indistinguishable from *Perkinsus* species and are quite similar developmentally. Simpson and Patterson (1996) created a new apicomplexan family Colpodellidae for those predatory, non-endoparasitic flagellates that otherwise look like *Perkinsus* species. They argued that ''any attempt to define the Apicomplexa to include only endoparasites . . . founders on the absence of any unambiguous synapomorphies'' since they considered micropores and the apical complex to be necessary and sufficient conditions for inclusion of *Perkinsus* or *Colpodella* species in the phylum Apicomplexa. However, Siddall et al. (1997) noted that micropores are exhibited by all alveolate taxa and that the anterior structures in *Perkinsus* species are no more like an apical complex than they are like feeding peduncles in some dinoflagellates. The combined analyses with two genes, and even some of the results found from the use of only SSU rDNA, indicate that this species of *Colpodella* is sister to the ciliates. To the best of our knowledge, no such arrangement previously has been suggested for these free-living flagellates. Simpson and Patterson retained the genus *Perkinsus* for the parasitic taxa.

That neither *Colpodella* nor *Perkinsus* species grouped with apicomplexans, is not entirely surprising. Lipscomb et al. (1998) attributed the notion of a recent ancestry for *Perkinsus* and the dinoflagellates to Levine (1985). However, the hypothesis that *Perkinsus* species are more closely related to the dinoflagellates than to the apicomplexans originated with Vivier (1982) who argued rather forcefully against the prevailing views stating ''j'estime que *Dermocystidium* (=*Perkinsus*) et *Spiromonas* ont des characters incontestables de Mastigophora et sans doubte peuvent-ils être rapproches des Dinoflagelles.'' In contrast, Levine (1985) clearly drew Perkinsasida as sister to the euapicomplexans and later articulated his belief that *Perkinsus marinus* ''is an apicomplexan'' (Levine, 1988). Vivier's (1982) position repeatedly has been vindicated with respect to *Perkinsus* species. Morphological data, SSU rDNA and actin sequences, separately and in combination, consistently have supported a more recent common ancestry between *Perkinsus* species and the Dinozoa (Siddall et al., 1995, 1997; Reece et al., 1997; Litaker et al., 1999; de la Herran et al., 2000). That relationship is again corroborated by the analyses conducted here, despite of recent suggestions that the inclusion of *Parvilucifera infectans* might alter this arrangement (Norén et al., 1999). These phylogenetic conclusions have received additional

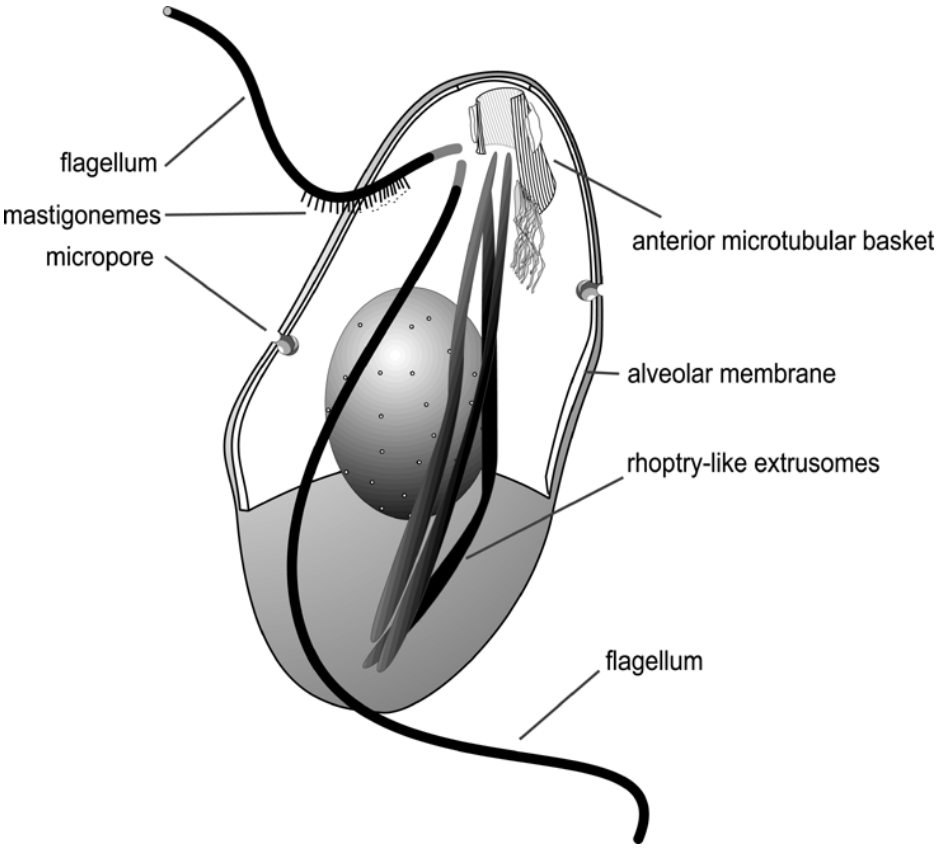

Fig. 3. Illustration of the morphology of *Colpodella, Perkinsus,* and *Parvilucifera* which is indicated to be the plesiomorphic condition for the Alveolata. Thickened branches indicate implied retention of the *Colpodella*/*Perkinsus* morphology.

corroboration. Polyclonal antibodies thought to have binding specificities limited to *Perkinsus* spp. (Dungan and Roberson, 1993) now are known to share epitopes with free-living and parasitic dinoflagellates (Chris Dungan, personal commun.). Vivier's (1982) belief in a close association of *Colpodella* and *Perkinsus* species, however, cannot be supported.

One necessary implication of these flagellates not grouping together, in spite of their ultrastructural identity, is that this same morphology must have been retained from their common ancestor. That is, for this morphology to be found in the sister of the ciliates and in the sister to the dinoflagellates simultaneously, it must have been retained from the ancestor of all Alveolata. This morphology (fig. 3) then, appears to be the original condition for each of the various alveolate groups. It long has been suggested that a *Perkinsus*-like organism gave rise to the apicomplexans. That hypothesis would appear to be correct with the important caveat that *Perkinsus* species do not themselves share a recent common ancestor with Apicomplexa. There are several other species of *Colpodella* for which there are sufficient morphological data to include them in this genus (Simpson and Patterson, 1996), however, in light of the implication that this morphology is entirely plesiomorphic, there is no particular reason to expect all of these to form a monophyletic group. If the phylum Perkinsozoa is to be recognized (Norén et al., 1999) for the sister group of the Dinoflagellata, yet another new phylum would be required for this and any other species of Colpodella grouping sister to

the Ciliophora, but yielding two distinct phyla for morphologically identical taxa. Alternatively, *Perkinsus* and *Parvilucifera* could be included in Dinoflagellata and *Colpodella* left without phylum rank within the Alveolata.

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