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Molecular Phylogeny of *Parvilucifera prorocentri* (Alveolata, Myzozoa): Insights into Perkinsid Character Evolution

MONA HOPPENRATH[1] and BRIAN S. LEANDER

Departments of Botany and Zoology, University of British Columbia, Vancouver, Canada BC V6T 1Z4

ABSTRACT. Perkinsids and colpodellids are lineages that diverged near the origins of dinoflagellates and apicomplexans, respectively, and provide compelling insights into the earliest stages of alveolate evolution. Perkinsids, including *Perkinsus* and *Parvilucifera*, are intracellular parasites of animals and dinoflagellates and possess traits also known in syndineans, dinokaryotes (mainly free living dinoflagellates), and colpodellids. An improved understanding of perkinsid biodiversity and phylogeny is expected to shed considerable light on the evolutionary origins of syndineans and dinokaryotes as well as the cellular identities of environmental sequences derived from marine and freshwater habitats. Accordingly, the small subunit (SSU) rDNA sequence from *Parvilucifera prorocentri*, a tube-forming intracellular parasite of the marine benthic dinoflagellate *Prorocentrum fukuyoi*, was determined. Molecular phylogenetic analyses demonstrated, with very high statistical support, that *P. prorocentri* branched as a sister lineage to a divergent clade consisting of *Parvilucifera infectans* and *Parvilucifera sinerae*. The entire *Parvilucifera* clade was nested within a more inclusive and modestly supported clade consisting of *Perkinsus* and several environmental sequences. Because *P. prorocentri* possessed a novel combination of ultrastructural features known in *Perkinsus, Parvilucifera*, and/or syndineans (i.e. germ tubes, trichocysts, and a syndinean-like nucleus), establishing the molecular phylogenetic position of this species enabled us to build a more comprehensive framework for understanding the earliest stages in the evolution of myzozoans.

Key Words. Alveolates, dinoflagellates, parasite, Perkinsozoa, *Perkinsus*, SSU rDNA, *Syndinium*.

ALVEOLATES are monophyletic and consist of three extremely diverse and morphologically distinctive subgroups: ciliates, dinoflagellates, and apicomplexans (e.g. Cavalier-Smith 1993; Ellis, Morrison, and Jeffries 1998; Fensome et al. 1993; Katz 2001; Leander and Keeling 2003, 2004; Patterson 1999; Schlegel and Eisler 1996; Siddall et al. 1997; Taylor 1987). Molecular phylogenetic analyses of several different markers have consistently shown that dinoflagellates and apicomplexans—collectively known as the Myzozoa—are more closely related to each other than to ciliates (e.g. Cavalier-Smith 1993; Cavalier-Smith and Chao 2004; Fast et al. 2002; Gajadhar et al. 1991; Leander and Keeling 2003). *Parvilucifera*, *Perkinsus*, *Chromera*, and colpodellids are significant from an evolutionary perspective because these lineages do not fall neatly within any of the three main subgroups and have retained several characteristics inferred to be ancestral for the Myzozoa and perhaps the Alveolata as a whole (Cavalier-Smith and Chao 2004; Kuvardina et al. 2002; Leander and Keeling 2003, 2004; Leander, Clopton, and Keeling 2003; Moore et al. 2008). Perkinsids, including *Perkinsus* and *Parvilucifera*, are a group of intracellular parasites that form a relatively close (and modestly supported) sister lineage to generally free-living dinoflagellates (e.g. Leander and Keeling 2003; Norén, Moestrup, and Rehnstam-Holm 1999; Saldarriaga et al. 2003; Siddall et al. 1997). Molecular phylogenetic analyses and comparative morphology indicate that free-living colpodellids (e.g. *Colpodella*) and *Chromera* form the nearest sister lineages to the obligately parasitic apicomplexans (Kuvardina et al. 2002; Leander and Keeling 2003; Leander et al. 2003; Moore et al. 2008). The suite of traits shared by perkinsids and colpodellids provides a compelling example of morphostasis within alveolates and sheds considerable light onto the mosaic of traits present in the most recent common ancestor of apicomplexans and dinoflagellates (Kuvardina et al. 2002; Leander and Keeling 2003; Leander et al. 2003; Siddall et al. 2001).

Corresponding Author: Mona Hoppenrath, Departments of Botany and Zoology, University of British Columbia, Vancouver, Canada BC V6T 1Z4—Telephone number: +04421 9475 116; FAX number: +04421 9475 111; e-mail: mhoppenrath@senckenberg.de

[1]Present address: Forschungsinstitut Senckenberg, Deutsches Zentrum für Marine Biodiversitätsforschung, Südstrand 44, D-26382 Wilhelmshaven, Germany.

Parvilucifera Norén and Moestrup is a genus of marine intracellular parasites of dinoflagellates; the type species *Parvilucifera infectans* has been characterized at both the ultrastructural and molecular phylogenetic levels (Norén et al. 1999). For nearly a decade, this was the only known species in the genus. However, a second species, namely *Parvilucifera sinerae* Figueroa and Garcés, was discovered most recently and investigated with an emphasis on seasonal occurrence, infection rates, host specificity, and life cycle (Figueroa et al. 2008). Both of these *Parvilucifera* species infect planktonic dinoflagellates, especially toxic species, and comparative morphology and molecular phylogenetic analyses have shown that they are very closely related (Figueroa et al. 2008). In contrast, a third species named *Parvilucifera prorocentri* Leander and Hoppenrath was characterized at the ultrastructural level and was shown to possess a novel combination of features also known in either perkinsids or syndineans (Leander and Hoppenrath 2008). This species is morphologically divergent from the other two species in the genus and infects the marine, benthic, non-toxic dinoflagellate *Prorocentrum fukuyoi* Murray and Nagahama (Leander and Hoppenrath 2008).

Marine environmental sequencing surveys have demonstrated at least two large and diverse alveolate groups—sometimes referred to as Group I and Group II alveolates—that show close phylogenetic affinity to perkinsids, syndineans, and dinokaryotes (Diez, Pedros-Alio, and Massana 2001; Dolven et al. 2007; Groisillier et al. 2006; Lopez-Garcia et al. 2001; Moon-van der Staay, De Wachter, and Vaulot 2001; Moreira and López-García 2002; Takishita et al. 2007; Worden 2006). Molecular phylogenetic analyses of *Duboscquella* and an undescribed red plasmodial parasite superficially similar to the rhizarian *Paradinium* have identified some Group I alveolates as parasites of tintinnid ciliates and planktonic copepods (Harada, Ohtsuka, and Horiguchi 2007; Skovgaard and Daugbjerg 2008). Moreover, molecular phylogenetic evidence has demonstrated that at least some of these alveolates are parasites of other planktonic hosts, such as radiolarians (Dolven et al. 2007). Group II alveolates, on the other hand, have been identified as syndineans sensu stricto, a group consisting of several different genera of marine parasites (Coats 1999; Moon-van der Staay et al. 2001; Moreira and López-García 2002; Saldarriaga et al. 2004; Skovgaard et al. 2005). Perkinsids have been reported in both marine and freshwater environments and tend to branch as the nearest sister group to a weakly supported clade consisting of Group I alveolates, syndineans (i.e.

Group II alveolates), and dinokaryotes, collectively called ''dinoflagellates'' (Diez et al. 2001; Dolven et al. 2007; Groisillier et al. 2006; Lefranc et al. 2005; Lepère, Domaizon, and Debroas 2008; Lopez-Garcia et al. 2001; Moon-van der Staay et al. 2001; Moreira and López-García 2002; Takishita et al. 2007; Worden 2006). Molecular phylogenetic analysis of *P. prorocentri*—a taxon possessing a novel combination of features described in *Perkinsus*, *Parvilucifera*, and/or syndineans (Leander and Hoppenrath 2008)—enabled us to refine the hypothetical framework required for understanding character evolution during the earliest stages of perkinsid and dinoflagellate evolution.

MATERIALS AND METHODS

Sample collection. Sand samples containing *P. fukuyoi* were collected with a spoon during low tide at Centennial Beach, Boundary Bay, BC, Canada on August 28, 2007. Dinoflagellate extraction was as described in Leander and Hoppenrath (2008). Raw cultures of *P. fukuyoi* (in f/2-medium, Guillard and Ryther 1962) were maintained at room temperature and natural light conditions in the laboratory. It took about 6 wk from sample collection and extraction until the parasitic infection was evident in the raw culture. The cell density of *P. fukuyoi* increased visibly during the period before the infection became evident.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing. Forty closed sporangia containing zoospores with the host cell already disintegrated were manually isolated and washed twice in f/2-medium. Genomic DNA was extracted from the cells using the MasterPure complete DNA and RNA purification Kit (Epicentre, Madison, WI). The small subunit (SSU) rDNA sequence was PCR-amplified using puReTaq ready-to-go PCR beads (GE Healthcare, Quebec, Canada), with an error rate of 1 per 20,000–40,000 bases, and universal eukaryotic primers as reported previously (Leander et al. 2003). The PCR products of expected size were gel isolated and cloned into pCR2.1 vector using a TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA). One clone was completely sequenced with ABI big-dye reaction mix using both vector primers and two internal primers oriented in both directions (GenBank accession code FJ424512).

Alignment and phylogenetic analysis. The new SSU rDNA sequence was aligned with 69 other alveolate sequences using MacClade 4 (Maddison and Maddison 2000), forming a 70-taxon alignment. We also analyzed a similar alignment that excluded the highly divergent sequences from *P. infectans* and *P. sinerae*, resulting in a 68-taxon alignment. These alignments are available on request. Maximum likelihood (ML) and Bayesian methods using the General Time Reversible (GTR) model of nucleotide substitutions were performed on both alignments; this model was selected with MODELTEST version 3.06 (Posada and Crandall 1998). All gaps were excluded from the alignments before phylogenetic analysis. The alpha shape parameters were estimated from the data using GTR, a gamma distribution with invariable sites and eight rate categories (70-taxon alignment with 1,265 sites: $\propto = 0.413$, $i = 0.143$; 68-taxon alignment with 1,269 sites: $\propto = 0.412$, $i = 0.152$). The ML trees were analyzed using the parameters listed above and were constructed with PhyML (Guindon and Gascuel 2003; Guindon et al. 2005). Maximum likelihood bootstrap analyses were performed on both alignments with PhyML on 500 re-sampled datasets using an HKY+Gamma+invariable sites model, with the alpha shape parameter, proportion of invariable sites, and transition/transversion ratio estimated from each dataset. Maximum likelihood bootstrap analyses were done using the HKY substitution model (rather than GTR) in order to reduce the computational burden required. We also examined both SSU rDNA datasets with Bayesian analysis using the program MrBayes 3.0 (Huelsenbeck and Ronquist 2001). The program was set to operate with a GTR+Gamma+Invariable sites model and four Monte-Carlo–Markov chains (default temperature = 0.2). A total of 2,000,000 generations was calculated with trees sampled every 100 generations and with a before burn-in of 200,000 generations (i.e. 2,000 sampled trees were discarded; burn-in was checked manually). A majority rule consensus tree was constructed from 18,000 post-burn-in trees, and the posterior probabilities correspond to the frequency at which a given node is found in these trees.

Light microscopy, scanning electron microscopy, and transmission electron microscopy. Procedures for these methods were described in Leander and Hoppenrath (2008).

RESULTS

The sporangia of *P. prorocentri* grew in the host cytoplasm until they occupied nearly the complete host cell and a thick sporangium wall had formed (Fig. 1, 7). During zoospore formation a prominent closed germ tube or discharge tube developed (Fig. 2–4). Eventually the germ tube opened (Fig. 5, 6) and the zoospores were released. As shown previously (Leander and Hoppenrath 2008), the reniform zooids of *P. prorocentri* had the following morphological characteristics: (1) a posterior refractile body, (2) a long anterior (transverse) flagellum with a heteromorphic pair of central microtubules in the anterior axoneme, (3) a short posterior flagellum, (4) a reduced pseudoconoid, (5) micronemes, (6) oblong microbodies, (7) bipartite trichocysts, (8) a syndinean-like nucleus (i.e. a nucleus with a conspicuous nucleolus and condensed chromatin distributed beneath the nuclear envelope), and (9) a golgi apparatus with six cisternae (see also Fig. 7–10) (Table 1).

Phylogenetic analyses of the 70-taxon alignment demonstrated that the SSU rDNA sequence of *P. prorocentri* branched with very strong support (i.e. 99% ML bootstrap value/1.0 Bayesian posterior probability) as the sister lineage to the clade containing *P. infectans* and *P. sinerae* ($-\ln L = 14{,}262.88307$) (Fig. 11). The *Parvilucifera* clade clustered within a more inclusive clade consisting of three *Perkinsus* species and a clade consisting of three environmental sequences (Fig. 11). The entire perkinsid clade was modestly supported (60% ML bootstrap value/1.0 Bayesian posterior probability) and formed a weakly supported sister lineage to a much larger dinoflagellate clade, consisting of the parasitic Group I and Group II alveolates (i.e. syndineans) and the primarily free-living dinokaryotes (Fig. 11).

Phylogenetic analyses of the 68-taxon alignment, which excluded the highly divergent sequences from *P. infectans* and *P. sinerae*, produced similar results ($-\ln L = 13744.67909$). The internal branching order and relative statistical support for the perkinsid clade was unchanged (i.e. 63% ML bootstrap value/1.0 Bayesian posterior probability). The relationships between *P. prorocentri*, *Perkinsus*, and the environmental sequences remained unresolved within the perkinsid clade.

DISCUSSION

The phylogenetic analyses of the SSU rDNA sequence from *P. prorocentri* supported the earlier systematic placement of this species based on ultrastructural comparisons (Leander and Hoppenrath 2008). This species is most similar to *P. infectans*, but it also shares features that are exclusive to *Perkinsus* (e.g. germ tubes) and other character combinations that are exclusive to syndineans (e.g. nuclear morphology and bipartite trichocysts) (Table 1). The presence of clustered, ovoid, membrane-bound ''microbodies'' is a novel feature of *P. prorocentri* that has not been reported in any other myzozoan lineage (Leander and Hoppenrath

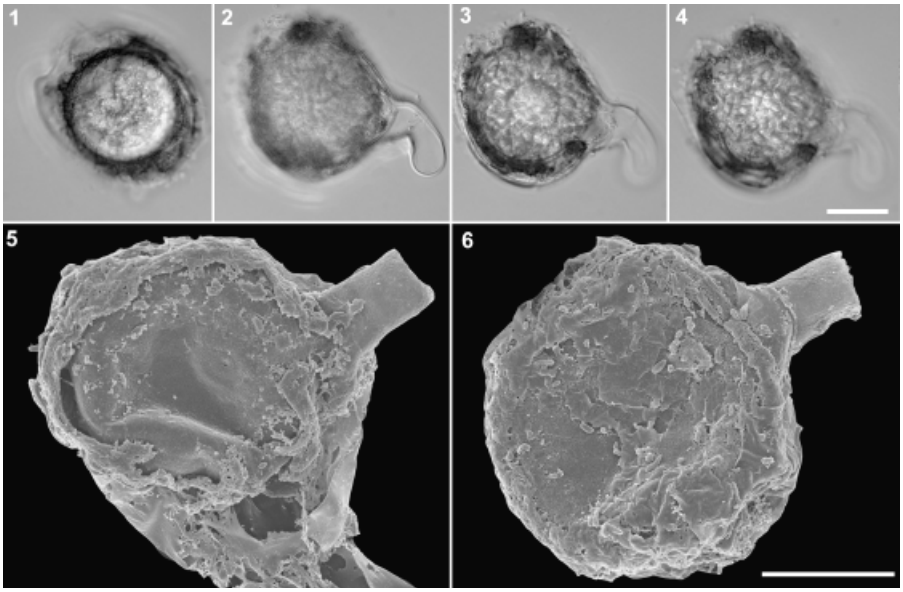


Fig. **1–6.** Differential interference contrast (DIC) light micrographs and scanning electron micrographs (SEM) of the sporangia of *Parvilucifera prorocentri*. **1**. A DIC micrograph showing a large growing undifferentiated sporangium occupying nearly the whole host cell. **2–4**. A focal series of DIC micrographs through a host cell with a sporangium undergoing early stages of zoospore differentiation. At this stage, the sporangium consists of an undifferentiated central mass that is surrounded by a more granulated cytoplasm. A germ tube is already developed but is still closed. Figure 1–4 are at the same scale (scale bar = 10 μm). **5–6**. A late stage of sporangium development, the open germ tube, the sporangium wall and remnants of the host theca. Figure 5, 6 are SEMs at the same scale (scale bar = 10 μm).

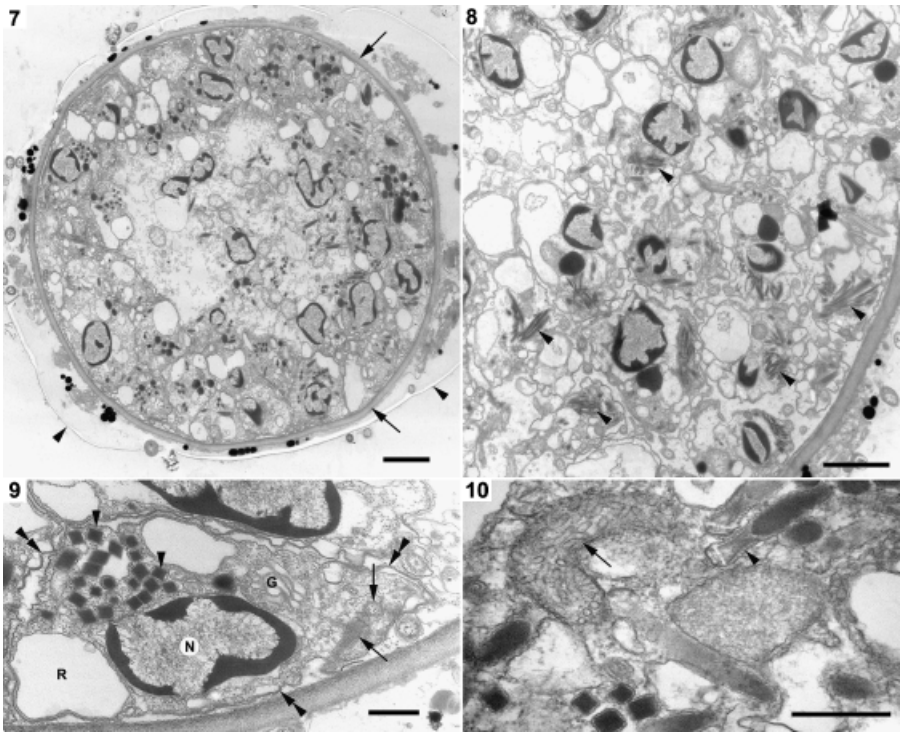
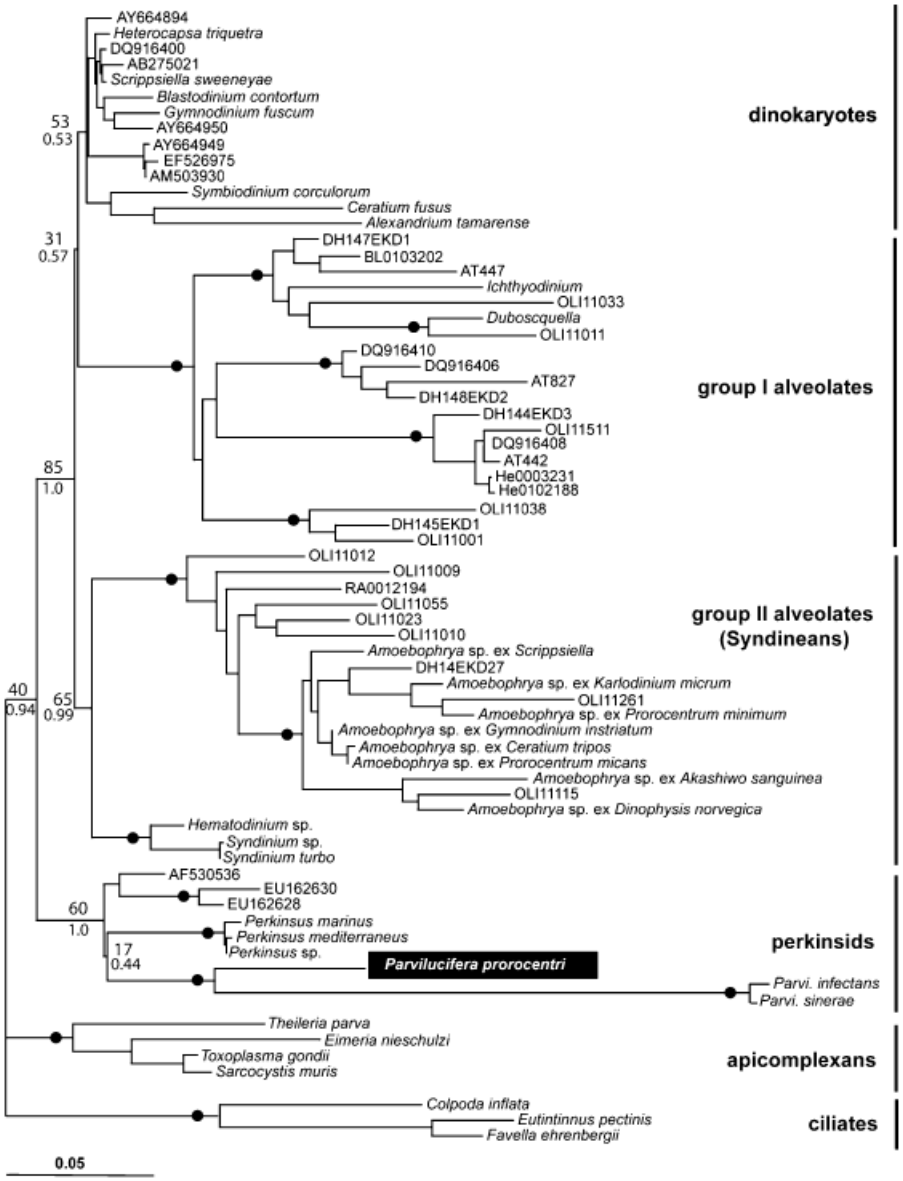


Fig. **7–10.** Transmission electron micrographs (TEM) of *Parvilucifera prorocentri*. **7**. An almost fully developed sporangium showing the robust sporangium wall (arrows) and remnants of the host cell (arrowheads) (scale bar = 2 μm). **8**. The sporangium showing the early stages of zoospore differentiation; arrowheads indicate the batteries of trichocysts associated with the apical complex in each zoospore (scale bar = 2 μm). **9**. The main components in an individual zoospore: plasma membrane (double arrowheads), nucleus (N), refractile body (R), flagellar apparatus consisting of two basal bodies (arrows), Golgi body, and a battery of trichocysts (arrowheads) associated with the apical complex (scale bar = 0.5 μm). **10**. A zoospore showing a mitochondrion with tubular cristae (arrow) and a bipartite trichocyst (arrowhead) (scale bar = 0.5 μm).

Table **1.** Morphological characteristics of syndineans, *Perkinsus*, and *Parvilucifera (Parvi.)*.

	Syndineans	*Perkinsus*	*Parvi. infectans*	*Parvi. prorocentri*	*Parvi sinerae*
Reniform zoospores	+	+	+	+	+
Alveoli	+	+	+	+	(+)
Anterior (transverse) flagellum	+	+	+	+	+
Refractile body	+	+	+	+	+
Mito with tubular cristae	+	+	+	+	(+)
Micronemes	+	+	+	+	?
Oblong microbodies	+	+	+	+	?
Nucleus syndinean-like	+	−	−	+	?
Bipartite trichocysts	+	−	−	+	?
Reduced pseudoconoid	−	−	+	+	?
Short posterior flagellum	−	−	+	+	+
Heteromorphic pair of central Microtubules in anterior axoneme	−	−	+	+	?
Golgi with six cisternae	−	−	+	+	?
Germ tube	−	+	−	+	−
Ovoid ''microbodies''	−	−	−	+	?

+, present; −, absent; (+), not demonstrated yet, but extremely likely; ?, not known.



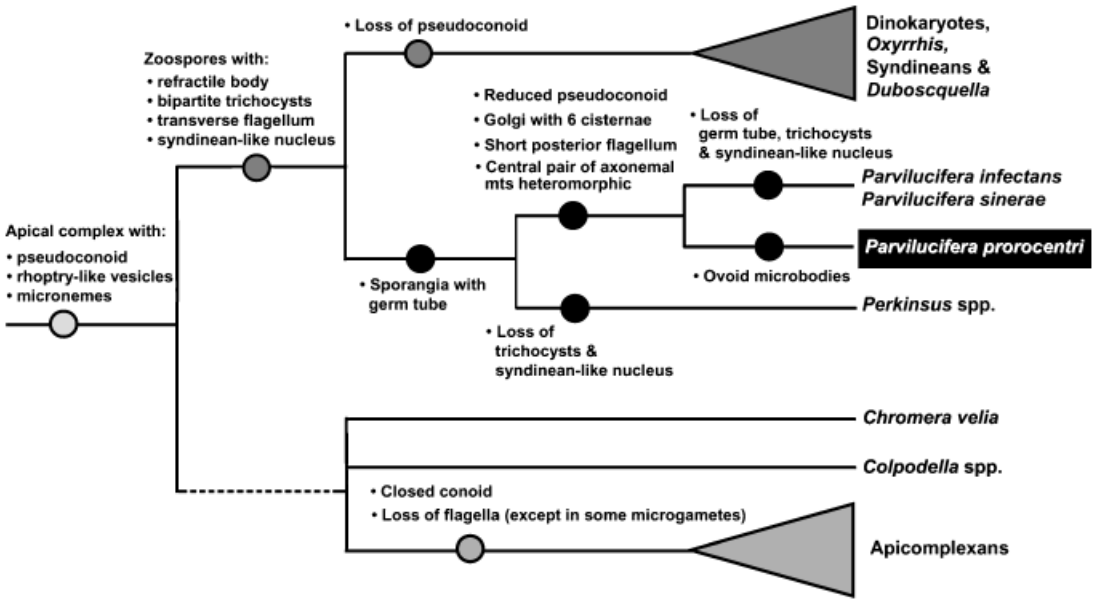


Fig. **12.** A framework for inferring morphological character evolution in dinoflagellates and perkinsids. Colored circles denote positions of relevant character states as constrained by parsimony. Triangles indicate species radiations; mts, microtubules; dashed line, significant phylogenetic uncertainty.

2008). Although *P. prorocentri* differs significantly from *P. infectans* in many respects, we conservatively decided to describe it as new species in the genus *Parvilucifera* rather than erecting a new genus (Leander and Hoppenrath 2008). A subsequent study described a new species, called *P. sinerae*, which is morphologically and phylogenetically very similar to the type species *P. infectans* (Figueroa et al. 2008). All three described *Parvilucifera* species form a strongly supported clade using SSU rDNA sequences. However, the phylogenetic distances between *P. prorocentri*, the *Perkinsus* clade, and the clade consisting of *P. infectans* and *P. sinerae* suggests that *P. prorocentri* warrants classification within a different genus altogether. This approach would also be consistent with morphological disparities between the species as well as differences in the type of hosts; for instance, *P. infectans* and *P. sinerae* mainly infect planktonic *Alexandrium* species and *P. prorocentri* infects a benthic *Prorocentrum* species (Figueroa et al. 2008; Leander and Hoppenrath 2008; Norén et al. 1999). Nonetheless, we are still hesitant to erect a new genus for *P. prorocentri* because knowledge of perkinsid biodiversity is so poor, and although ultrastructural data from the zoospores of *P. sinerae* is still missing, *P. prorocentri* and *P. infectans* share several ultrastructural features in common (e.g. a reduced pseudoconoid, Golgi bodies with six cisternae, a short posterior flagellum, and a heteromorphic pair of central microtubules in the anterior axoneme) (Table 1).

The relationships between the three perkinsid clades—*Perkinsus*, *Parvilucifera*, and the group of environmental sequences—as well as the relationship between perkinsids and dinoflagellates (i.e. the large clade containing dinokaryotes and syndineans) were not convincingly resolved in our molecular phylogenetic analyses of SSU rDNA (see ''Results,'' also Figueroa et al. 2008). Nonetheless, we were able to refine the hypothetical framework necessary for understanding character evolution during the earliest stages of perkinsid and dinoflagellate evolution by integrating the novel combination of ultrastructural features in *P. prorocentri* with the strongly supported relationships between this species, other *Parvilucifera* species, and *Perkinsus* (Fig. 12).

Strong evidence suggests that the most recent common ancestor of apicomplexans and dinoflagellates—the ancestral myzozoan—possessed the character states shared by perkinsids and colpodellids, such as two heterodynamic flagella, an open-sided conoid or pseudoconoid, rhoptry-like vesicles, micronemes, and myzocytosis-based feeding (Leander and Keeling 2003, 2004). Along the apicomplexan lineage, the flagella of colpodellid-like ancestors were lost, except in some microgametes, and the ancestral open-sided conoid became closed in association with obligate parasitism. Perkinsids, on the other hand, diversified along the lineage leading to dinoflagellates (i.e. Group I alveolates, syndineans, and dinokaryotes) (Leander and Keeling 2004; Saldarriaga et al. 2003). The most recent common ancestor of perkinsids and dinoflagellates is inferred to have had zoospores with a refractile body, bipartite trichocysts, a transverse (anterior) flagellum, and a syndinean-like nucleus; the most recent ancestor of perkinsids possessed a sporangium with a germ tube(s) like those found in *Perkinsus* species and *P. prorocentri* (Fig. 12).

Our phylogenetic mapping analyses suggest that several of these features were independently lost within the perkinsids and before the dinoflagellate radiation. For instance, the pseudoconoid was lost in the most recent ancestor of dinoflagellates, and the germ tube was lost in the clade consisting of *P. infectans* and *P. sinerae* (Fig. 12). Moreover, bipartite trichocysts and the syndinean-like nucleus were both independently lost in the *Perkinsus* clade and in the clade consisting of *P. infectans* and *P. sinerae* (Fig. 12). Altogether, understanding the molecular phylogenetic position and unique combination of ultrastructural features in *P. prorocentri* has demonstrated that inferences about the earliest stages of alveolate evolution will continue to be refined as we learn more about perkinsid biodiversity.

Fig. **11.** Gamma-corrected maximum likelihood (ML) tree ($-\ln L = 14{,}262.88307$, $\alpha = 0.413$, number of rate categories = 8) inferred using the GTR+gamma+invariable sites model of substitution on an alignment of 70 small subunit (SSU) rDNA gene sequences and 1,265 sites. Upper numbers at the branches denote ML bootstrap percentages using a HKY+gamma+invariable sites model. The lower numbers refer to Bayesian posterior probabilities—GTR+gamma+invariable sites model. Black dots indicate ML bootstrap values and Bayesian posterior probabilities over 95% and 1.0%, respectively.

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