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

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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 fukayoi, 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; Kuvardina et al. 2002; 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, Clpton, 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; Noreén, Moestrup, and Rehnstrom-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).

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 (Noreén 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 García, 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 proprorocentri 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 fukayoi 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 algae—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 Diabosquella and an undescribed red plankemidal parasite superficially similar to the rhizarian Paradinium have identified some Group I algae 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 algae are parasites of other planktonic hosts, such as radiolarians (Dolven et al. 2007), Group II algae, 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 algae, syndineans (i.e.
Group II alveolates), and dinokaryotes, collectively called “dino-
flagellates” (Diez et al. 2001; Dolven et al. 2007; Groisillier et al. 
2006; Lefranc et al. 2005; Lepère, Domaizon, and Debroas 2008; 
Lopez-García et al. 2001; Moon-van der Staay et al. 2001; More-
ira and López-García 2002; Takishita et al. 2007; Worden 2006). 
Molecular phylogenetic analysis of P. prorocentri—a taxon pos-
sessing 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 con-
ditions 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) amplifi-
cation, and sequencing. Forty closed sporangia containing zoos-
pores 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 sub-
unit (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 eukaryo-
tic 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 Corpora-
tion, Carlsbad, CA). One clone was completely sequenced with 
ABI big-dye reaction mix using both vector primers and two in-
ternal 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, re-
sulting 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 substi-
tutions were performed on both alignments; this model was se-
lected with MODELTEST version 3.06 (Posada and Crandall 
1998). All gaps were excluded from the alignments before phylo-
genetic 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: \( \alpha = 0.431 \), \( i = 0.143 \); 68-taxon alignment with 1,269 sites: \( \alpha = 0.412 \), \( i = 0.152 \)). 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 es-
timated from each dataset. Maximum likelihood bootstrap ana-
lyses 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+In-
variable sites model and four Monte-Carlo–Markov chains (de-
fault 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 trans-
mision 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 forma-
tion 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 Hop-
penrath 2008), the reniform zooids of P. prorocentri had the fol-
lowing morphological characteristics: (1) a posterior refractile 
body, (2) a long anterior (transverse) flagellum with a hetero-
morphic pair of central microtubules in the anterior axoneme, (3) 
a short posterior flagellum, (4) a reduced pseudonucleon, (5) mi-
cronemes, (6) oblong microbodies, (7) bipartite trichocysts, (8) a 
syndinean-like nucleus (i.e. a nucleus with a conspicuous nucle-
olus and condensed chromatid 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 poste-
rior 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 con-
sisting 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 poste-
rior 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 primar-
ily free-living dinoflagellates (Fig. 11). 

Phylogenetic analyses of the 68-taxon alignment, which ex-
cluded 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 per-
kinsid clade was unchanged (i.e. 63% ML bootstrap value/1.0 
Bayesian posterior probability). The relationships between P. proro-
centri, 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 Hop-
penrath 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 syn-
dineans (e.g. nuclear morphology and bipartite trichocysts) (Table 1). The presence of clustered, ovoid, membrane-bound “micro-
bodies” is a novel feature of P. prorocentri that has not been re-
ported 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.).

<table>
<thead>
<tr>
<th></th>
<th>Syndineans</th>
<th>Perkinsus</th>
<th>Parvi. infectans</th>
<th>Parvi. prorocentri</th>
<th>Parvi sinerae</th>
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<tr>
<td>Reniform zoospores</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Alveoli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
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<tr>
<td>Anterior (transverse) flagellum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
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<tr>
<td>Refractile body</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mito with tubular cristae</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
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<tr>
<td>Micronemes</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>?</td>
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<tr>
<td>Oblong microbodies</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Nucleus syndinean-like</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Bipartite trichocysts</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Reduced pseudoconoid</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Short posterior flagellum</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Heteromorphic pair of central Microtubules in anterior axoneme</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Golgi with six cisternae</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Germ tube</td>
<td></td>
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<td>+</td>
<td>+</td>
<td>?</td>
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<tr>
<td>Ovoid &quot;microbodies&quot;</td>
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<td>+</td>
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+, present; -, absent; (+), not demonstrated yet, but extremely likely; ?, not known.
Although *P. prorocentri* differs significantly from *P. infectans* in many respects, we conservatively decided to describe it as a 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* possess a sporangium with a germ tube(s) like those found in *Perkinsus* species and *Parvilucifera* (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 colpodelids, such as two heterodynamic flagella, an open-sided conoid or pseudoconoid, rhoptry-like vesicles, micronemes, and myotoxins-based feeding (Leander and Keeling 2003, 2004). Along the apicomplexan lineage, the flagella of colpodelid-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.
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LITERATURE CITED


