Gregarina niphandrodes may Lack Both a Plastid Genome and Organelle

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ABSTRACT. Gregarines are early diverging apicomplexans that appear to be closely related to Cryptosporidium. Most apicomplexans, including Plasmodium, Toxoplasma, and Eimeria, possess both plastids and corresponding plastid genomes. Cryptosporidium lacks both the organelle and the genome. To investigate the evolutionary history of plastids in the Apicomplexa, we tried to determine whether gregarines possess a plastid and/or its genome. We used PCR and dot-blot hybridization to determine whether the gregarine Gregarina niphandrodes possesses a plastid genome. We used an inhibitor of plastid function for any reduction in gregarine infection, and transmission electron microscopy to search for plastid ultrastructure. Despite an extensive search, an organelle of the appropriate ultrastructure in transmission electron microscopy, was not observed. Triclosan, an inhibitor of the plastid-specific enoyl-acyl carrier reductase enzyme, did not reduce host infection by G. niphandrodes. Plastid-specific primers produced amplicons with the DNA of Babesia equi, Plasmodium falciparum, and Toxoplasma gondii as templates, but not with G. niphandrodes DNA. Plastid-specific DNA probes, which hybridized to Babesia equi, failed to hybridize to G. niphandrodes DNA. This evidence indicates that G. niphandrodes is not likely to possess either a plastid organelle or its genome. This raises the possibility that the plastid was lost in the Apicomplexan following the divergence of gregarines and Cryptosporidium.

Key Words. Apicomplexa, apicoplast, fatty acid synthesis, gregarine.

THE phylum Apicomplexa consists of unicellular parasites that infect a wide variety of hosts. Apicomplexans that cause diseases in humans or livestock, such as coccidiosis, babesiosis, toxoplasmosis, and malaria, are well studied. Gregarines are apicomplexans that infect invertebrates and have primarily a monoxenous life cycle. They are considered to represent an early diverging apicomplexan lineage, thus making them a key group for questions regarding apicomplexan evolution. Phylogenetic analyses of the small subunit (SSU) ribosomal RNA (rRNA) gene suggest the gregarines are a sister group to Cryptosporidium, and represent an early divergence within the phylum Apicomplexa (Carreno, Martin, and Barta 1999; Leander, Clopton, and Keeling 2003). Life-cycle characteristics shared between gregarines and Cryptosporidium also suggest that these two groups are sisters (Hijjawi et al. 2002; Rosales et al. 2005).

Apicoplasts are non-photosynthetic plastids found in a number of apicomplexans (Lang-Unnasch et al. 1998). Discovery of the ~35–40 kb plastid genome was a surprise as apicomplexans are non-photosynthetic. However, the presence of plastid DNA indicated that they had a photosynthetic ancestor (Cai et al. 2004; Kohler et al. 1997; McFadden and van Dooren 2004). To date, four species of apicomplexans have had their entire plastid DNA sequenced: Plasmodium falciparum (Wilson et al. 1996), Theilea parva (Gardner et al. 2005), Toxoplasma gondii, and Eimeria tenella (Cai et al. 2004). The genes encoded by the small apicoplast genome constitute only a small fraction of the gene products in the apicoplast. Owing to the extensive gene transfer from the plastid to the nucleus most of the plastid-localized proteins are encoded in the nuclear DNA (Waller et al. 1998).

Cryptosporidium is the only member of the phylum Apicomplexa in which a plastid has been sought but not found. Analysis of the complete genomes of C. parvum and C. hominis identified neither a plastid genome nor genes with putative plastid-targeting sequences (Abrahamsen et al. 2004; Xu et al. 2004). Primers designed to amplify plastid-encoded sequences failed to produce products with Cryptosporidium DNA, yet did produce products using T. gondii and B. bovis DNA as templates. Likewise, apicomplexan plastid probes failed to hybridize to C. parvum DNA, yet hybridized to DNA from T. gondii, P. falciparum, and E. bovis (Zhu, Marchewka, and Keithly 2000a). Plastid-like structures have not been revealed through microscopy of Cryptosporidium (Riordan et al. 2003). Cyanobacterial-like genes have been discovered in Cryptosporidium; however, they lack plastid-targeting sequences (Huang et al. 2004). Together this evidence indicates that Cryptosporidium does not have a plastid genome or the organelle.

A common pathway among plastid bearing organisms is the Type II fatty acid synthesis (FAS II) pathway. This pathway is associated with both plants (Harwood 1996b) and apicomplexan plastids (Waller et al. 1998) and is derived from the plastid’s ancestral endosymbiont. Inhibitors to this pathway have been shown to stunt P. falciparum growth and survival (Surolia and Surolia 2001). Cryptosporidium lacks the FAS II pathway and compounds that inhibit this pathway have no effect on Cryptosporidium (Zhu et al. 2000b), providing further supporting evidence that Cryptosporidium lacks a plastid.

The lack of a plastid in Cryptosporidium raises the question of when this organelle was introduced or lost in the apicomplexan lineage. That is, did the lineage leading to apicomplexans have plastids and subsequently the branch leading to Cryptosporidium lose them? Alternatively, did the incorporation of this organelle occur in the apicomplexan lineage after the branch leading to Cryptosporidium? Whether gregarines have a plastid is key to answering these questions.

MATERIALS AND METHODS

Collection of gametocysts from Gregarina niphandrodes. Briefly, gametocysts from G. niphandrodes were collected from the frass of adult Tenebrio molitor, separated on a step sucrose gradient, manually collected, extensively washed in sterile distilled water, and stored in ethanol at ~20 °C (Omoto et al. 2004). Gametocysts collected in the triclosan drug study were not dehydrated in ethanol so that they could be returned to their respective host populations.

Extraction of DNA from gametocysts of Gregarina niphandrodes. Ethanol-preserved gametocysts from G. niphandrodes were hydrated in sterile distilled water. The samples were frozen for 20 min at ~80 °C and incubated at 50 °C for 15 min in lysis buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 1 mM EDTA, pH 8, 1% [w/v] SDS, 0.2% [v/v] DTT). DNA was extracted using standard phenol–chloroform–isoamyl extraction and ethanol precipitation. The final pellet was suspended in sterile distilled water.

PCR. Six pairs of degenerate plastid-specific primers were used to amplify highly conserved regions of the plastid genome (Table 1) (Zhu et al. 2000a). To account for mismatched bases between template DNA and primers a 5 mM magnesium
concentration and annealing temperature of 48°C were used. DNA from *B. equi*, *P. falciparum*, and *T. gondii* was used for plastid positive controls. Primers designed to amplify the nuclear large subunit (LSU) rRNA gene from *G. niphandrodes* were used for the positive control for *G. niphandrodes* DNA (Table 1). Polymerase chain reaction products were separated by electrophoresis using 1% (w/v) Tris–Acetate–EDTA agarose.

*Babesia equi* products (*L*, Fig. 1) were excised from the gel, re-amplified, and sequenced. DNA sequencing was carried out by the DyeDeoxy terminator cycle protocol with synthetic primers synthesized by Invitrogen (Carlsbad, CA). Sequencing reactions were analyzed on an Applied Biosystems 377 DNA Sequencer at the Washington State University Laboratory of Bioanalysis and Biotechnology.

### Table 1. Polymerase chain reaction primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Genes</th>
<th>5' Primer</th>
<th>R Primer</th>
<th>3' Primer</th>
<th>B</th>
<th>G</th>
<th>Reference</th>
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<tr>
<td>1</td>
<td>Plastid LSU rRNA</td>
<td>CTGAATCATCTTAGTACTAAAG</td>
<td>A(A/G)TGAGCT(T/A)TACGCACTCTTT</td>
<td>+</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Plastid LSU rRNA</td>
<td>ATT(T/G)TCAA(A/G)AGGGACACGCC</td>
<td>TTACACCTTTG(A/G)TGC(G/A)GTTC</td>
<td>+</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Plastid LSU rRNA</td>
<td>GCGAAATTCCTACTGCCGGTTAACTCC</td>
<td>TTT(C/T)(C/T)(G/A/T)TCCCCGTCTACT</td>
<td>+</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Plastid SSU rRNA</td>
<td>AGAGTTTGATCATTTGC</td>
<td>TACCTGTGACGACCTT</td>
<td>+</td>
<td>−</td>
<td>1</td>
<td></td>
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<tr>
<td>5</td>
<td>Plastid SSU rRNA</td>
<td>AGGATTAGATACCTGTTAG</td>
<td>TACCTTGTTACGACTT</td>
<td>+</td>
<td>−</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Plastid tufA-rRNA</td>
<td>CATGT(A/T)GATCATGG(A/T)AAAAC</td>
<td>GGTAGAAGCAATGGATTGAAG</td>
<td>+</td>
<td>−</td>
<td>1</td>
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<td>7</td>
<td>Nuclear LSU rRNA</td>
<td>TCTCGAGGGAACCTTGGAGG</td>
<td>GATAGAGAACTGATGTTAG</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td></td>
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Amplions produced (+) or not produced (−) with *Babesia equi* DNA; G, *Gregarina niphandrodes* DNA; LSU, large subunit; SSU, small subunit.

**Triclosan/bran preparation.** Triclosan (Irgasan™, Sigma-Aldrich), was dissolved in acetone and applied to wheat bran at two concentrations: 4 mg/g of bran and 0.4 mg/g bran. The bran with beetles was covered with an unbleached paper towel. Four milliliters of water were dropped on the paper towel daily. Every 7 days the numbers of beetles were counted and gametocysts were collected from each group as described above and replaced in the container. The bran was replaced every 7 days with triclosan–acetate–treated bran for each group’s respective treatment. This was continued for 42 days.

**Thin layer chromatography (TLC).** To determine the level of triclosan in the intestinal environment of the gregarines, TLC was used. After 1 wk, five beetles each from the 0.4 mg/g and the 4.0 mg/g groups were dissected and the intestines were frozen at −20°C for 20 min in 1.5 ml microfuge tubes. The tissue was homogenized and the triclosan extracted with a 1:1 (v/v) mixture of methanol:chloroform. An intestine from a beetle in the acetone group was used as a negative control. For a triclosan positive control, an intestine from a beetle unexposed to triclosan was used, but spiked with 1.5 μg of triclosan prior to extraction. The samples were analyzed on Silica Gel 60F254 TLC sheet (EM Separations) with chloroform as solvent and visualized under short wavelength UV illumination.

**Determination of triclosan concentration in beetle intestine.** The intestines of five beetles were placed in a graduated 1.5 ml microfuge tube and centrifuged for 1 min at 13,000 g to estimate the intestine volume. Five intestines occupied approximately 130 μl. To estimate the quantity of triclosan in the intestine, a triclosan standard from 0.2 to 1.6 μg TLC was made. This standard was then visually compared with the TLC from each triclosan-exposed beetle. The quantity of triclosan found in the intestines of drugged beetles divided by the intestinal volume provided an estimated concentration of triclosan in the intestines. Our procedure for volume estimate will err on the high end, and thus lower the estimate for the triclosan concentration.

**Determination of gregarine infection.** The level of gregarine infection was assessed by counting gametocysts weekly. As beetles naturally died over time, the ratio of gametocysts per beetle was calculated. The natural log of this ratio was used as the response variable in our analysis. We performed a repeated measures analysis of variance (ANOVA) with time (the seven time points) and treatment (control/acetone, 4.0 mg/g, 0.4 mg/g) as our factors. The log-transformed response satisfied the assumptions of normality and homogeneity of variances.

At the end of the experimental treatment, five beetles from each group were selected at random for dissection. The dissected intestines were placed in insect Ringer’s (120 mM NaCl, 1.2 mM
KCl, 1.4 mM CaCl₂), and the trophozoites were counted under a dissecting microscope. Trophozoite counts were log-transformed as above and analyzed using a one-way ANOVA to assess the differences between the three treatments.

**Transmission electron microscopy.** Trophozoites of *G. niphandrosides* were dissected from *T. molitor* in insect Ringer’s solution and fixed overnight at 4°C in 2% (w/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Samples were rinsed three times in 0.1 M cacodylate buffer before a 1 h postfix with 2% (w/v) osmium tetroxide in 0.1 M cacodylate buffer. They were rinsed three times in distilled water and stained in 1% (w/v) tannic acid for 1 h. For potassium permanganate fixation, trophozoites were fixed overnight with 1% (w/v) potassium permanganate in 0.1 M veronal acetate buffer. The trophozoites were then washed three times in the buffer. All the specimens were dehydrated in an acetone series and embedded in Spurr’s resin. Ultrathin sections were cut with a glass knife on a Leica Reichert microtome and placed on formvar-coated copper grids. Sections were stained with uranyl acetate and Sato’s lead. Sam-

**Results**

**PCR analysis of DNA from Gregarina niphandrosides.** Polymerase chain reaction was performed to investigate if *G. niphandrosides* possesses DNA sequences similar to those found in other apicomplexan plastids. Six plastid-specific primer pairs designed for highly conserved apicoplast sequences. These sequences had highly significant matches to apicoplast genome sequences: for example, the sequence of the product in lane 4 matched *B. bigemina* apicoplast small subunit ribosomal RNA gene (Accession number: AF409698) with an e-value of 1e⁻²⁰. Thus, the plastid-specific primers amplified plastid sequences of *B. equi* DNA.

We also used primer pairs 1 and 5 with *T. gondii* and *P. falci-

**Triclosan drug studies.** Intestines from five beetles each from low and high triclosan treatments (0.4 mg/g bran and 4 mg/g bran) and acetone control, and an unexposed beetle but spiked with 1.5 μg of triclosan were extracted and analyzed. Triclosan was not detectable in the intestine of a beetle unexposed to the triclosan (Fig. 2). The beetle exposed to 0.4 mg/g triclosan bran had ~ 0.3 μg triclosan. The beetles exposed to 40 mg/g triclosan bran had ~ 1.5 μg triclosan. Using ~ 20 μl as the volume of an intestine, the triclosan concentration is estimated to be ~ 15,000 ng/ml at the lower dose of triclosan and ~ 75,000 ng/ml in the high triclosan treatment.

To determine the effect of triclosan exposure on gregarine infesta-

After the final collection of gametocysts at week 7, five beetles from each group were dissected to determine the degree of trophozoite infection. The huge variance between the numbers of trophozoites between individuals is evident in the large standard deviation for the average number of trophozoites: control (18.4 ± 26.5), 0.4 mg/g (33.3 ± 42.3), and 4.0 mg/g (44.5 ± 56.0). The higher number of trophozoites in both triclosan treatments compared with the control and the higher number of trophozoites in the higher concentration of triclosan was slightly significant (F = 4.53; P = 0.017). Thus, triclosan, a specific inhibitor of conditions, as we did not try to optimize the conditions to produce a single amplicon band.

The *B. equi* products (*, Fig. 1) were re-amplified and sequenced to determine whether the products were indeed apicoplast sequences. These sequences had highly significant matches to apicoplast genome sequences: for example, the sequence of the product in lane 4 matched *B. bigemina* apicoplast small subunit ribosomal RNA gene (Accession number: AF409698) with an e-value of 1e⁻²⁰. Thus, the plastid-specific primers amplified plastid sequences of *B. equi* DNA.

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To determine the effect of triclosan exposure on gregarine infestation of beetles, gametocysts released in the frass of the beetles were counted weekly for 7 wk. The results indicated no treatment effect (F = 0.15; P = 0.860) with no effect of time and no treatment interaction (F = 0.86; P = 0.589).

After the final collection of gametocysts at week 7, five beetles from each group were dissected to determine the degree of trophozoite infection. The huge variance between the numbers of trophozoites between individuals is evident in the large standard deviation for the average number of trophozoites: control (18.4 ± 26.5), 0.4 mg/g (33.3 ± 42.3), and 4.0 mg/g (44.5 ± 56.0). The higher number of trophozoites in both triclosan treatments compared with the control and the higher number of trophozoites in the higher concentration of triclosan was slightly significant (F = 4.53; P = 0.017). Thus, triclosan, a specific inhibitor of
FAS II pathway, if anything, increased rather than decreased gregarine infestation.

Electron microscopy. Transmission electron microscopy was used to search for plastid-like organelles. Both fixative treatments revealed mitochondrion-like structures (Fig. 3, 5, 6). Despite extensive searches through multiple sections from 10 different trophozoites, no multi-membranous organelle characteristic of plastids was observed. Other membranous organelles, such as epicytic folds and Golgi (Fig. 4) were visible. While potassium permanganate fixation is poor overall, it is useful for revealing membranes, making it an ideal method to reveal multi-membranous organelles, like the plastid. Using this fixation, mitochondria were clearly seen scattered throughout the cytoplasm (Fig. 5). At higher magnification, the details of the mitochondria, their cristae and the dual membranes, are visible (Fig. 6). Various examples of cytoplasm are visible in Fig. 7–10, no structures were observed with features reminiscent of plastids in any sections.

Dot-blot hybridization. Hybridizations were also used to investigate if G. niphandrodes possesses plastid DNA. A mixture of LSU and SSU plastid sequences from B. equi plastid DNA (total of ~4 kb) was used to probe for plastid DNA. The hybridizations were performed at a lower stringency to allow for cross species hybridization. The plastid probes hybridized to total DNA from B. equi. However, there was no hybridization signal with G. niphandrodes DNA (Fig. 11A). The nuclear LSU probe from G. niphandrodes hybridized to both the G. niphandrodes and B. equi DNA (Fig. 11B).

DISCUSSION

We used PCR, an inhibitor specific to a plastid pathway, thin-section electron microscopy, and dot-blot hybridization to investigate whether G. niphandrodes has a plastid. None of the four approaches provided any evidence to suggest that G. niphandrodes possesses a plastid.

No amplicons were obtained with plastid-specific primers using G. niphandrodes DNA as a template. This DNA produced amplicons with primers to nuclear LSU and to a nuclear protein-encoding gene, RPB1, clearly demonstrating that the DNA was of sufficient quality to amplify sequences. The plastid-specific primers have been shown to amplify DNA from numerous apicomplexan species, as well as chloroplast DNA (Zhu et al. 2000a), and we produced amplicons using B. equi, T. gondii and P. falciparum templates as positive controls. Thus, if G. niphandrodes possesses a plastid genome, our results indicate that its sequence must be significantly divergent from those of other organisms harboring plastids. Obornik et al. (2002) also tried to determine whether the gregarine Gregarina garnhami possessed a plastid genome using a plastid-specific SSU rRNA PCR primers but they also could not produce a product.
We investigated whether triclosan reduced the level of *G. niphandrodes* infection in *T. molitor*. We initially tested a range of triclosan concentrations to determine the maximum concentration that did not cause significant beetle mortality over a 1-wk period (data not shown). We then used triclosan at that concentration, 4 mg/g bran, and also at 10-fold lower concentration.

The FASII pathway is found in all organisms harboring plastids (Harwood 1996; Ryall, Harper, and Keeling 2003; Waller et al. 1998). Within this pathway, ENR is inhibited by the compound triclosan in bacteria (Escalada et al. 2005) and in other apicomplexans that contain plastids (McLeod et al. 2001; Surolia and Surolia 2001). *Cryptosporidium* synthesizes fatty acids with a giant multienzymatic Type I FAS (Zhu 2004) and not by the FASII (Abrahamsen et al. 2004; Xu et al. 2004; Zhu et al. 2000a). Consequently, it is unaffected by inhibitors of the FASII pathway (Zhu 2004). If *G. niphandrodes* contained a plastid, we expected triclosan treatment to inhibit the FASII and significantly decrease the number of gametocysts and trophozoites. There was no statistically significant reduction in gametocyst production with triclosan treatment. Unexpectedly, there were higher numbers of trophozoites in the triclosan treatments compared with controls, though it was only slightly statistically significant. Thus, triclosan clearly does not decrease the level of gregarine infection in *T. molitor* adults. The higher trophozoite numbers in triclosan-treated beetles and higher trophozoite numbers in the higher triclosan treatment may be explained by triclosan inhibition of the bacterial flora within the intestine. The bacteria may act as competitors to gregarine growth and/or survival. Hence, inhibiting the bacteria with triclosan may indirectly benefit gregarines and lead to higher survival.

Triclosan inhibits ENR through the binding of specific amino acids; bacteria (*E. coli*), plants (*B. napus*), and *P. falciparum* all share identical amino acid sequence at this binding site, indicating a conserved target for triclosan in a wide range of organisms (McLeod et al. 2001). Previous studies on apicomplexans used direct application of triclosan on the parasites to inhibit growth.

Fig. 7–10. Transmission electron micrographs (TEM) of epicytic folds (7, 8) and cytoplasm (7, 9, 10) of *Gregarina niphandrodes* trophozoites. 7. Epicytic folds and mitochondria are visible in *G. niphandrodes*. Scale bar = 200 nm. 8. TEM of epicytic folds in *G. niphandrodes*. Scale bar = 500 nm. 9. TEM of *G. niphandrodes* cytoplasm showing large vacuoles. Scale bar = 200 nm. 10. TEM of typical cytoplasm and *G. niphandrodes* trophozoite. Scale bar = 200 nm.
and survival. However, we cannot grow gregarines in the absence of the host. Therefore, we grew the hosts in triclosan to determine its effect upon G. niphandrodes. We estimated the concentration of triclosan in the intestine to be \( \sim 15,000 \, \text{ng/ml} \) at the lower exposure concentration of triclosan. Previous studies showed that direct exposure of 150 ng/ml for *P. falciparum* and 62 ng/ml for *T. gondii* inhibited their growth (McLeod et al. 2001). Thus, our gregarines were estimated to be exposed to at least 100 times the concentration of triclosan shown to be inhibitory in previous studies.

Triclosan clearly did not decrease gregarine infestation. Indeed, perhaps the increased gregarine infestation strongly indicates that *G. niphandrodes* lacks this common plastid localized enzyme. *Gregarina niphandrodes* may obtain fatty acids from its host, *T. molitor*, making this pathway unessential. However, in that case, it suggests that it may also not require a plastid. This triclosan study suggests that it may also not require a plastid. This triclosan study is the first to probe the fatty acid metabolism of gregarines, suggesting *G. niphandrodes*, like *Cryptosporidium*, may lack the FASII pathway.

The apicoplast is a multimembranous organelle. Two to four membranes have been observed in the Apicomplexa (Hopkins et al. 1999; Kohler et al. 1997; McFadden et al. 1996). The observation of three or four membranes led to the hypothesis that the apicoplast arose through secondary endosymbiosis of an alga. However, studies of the *T. gondii* apicoplast have revealed two membranes that undergo complex in-foldings, in effect revealing two to four membrane layers in thin section. Such a structure suggests an alternative evolutionary pathway for the plastid in *T. gondii* as a primary endosymbiosis of a cyanobacteria (Kohler 2005). We looked for a multimembranous organelle characteristic of plastid ultrastructure in TEM. No organelle with this ultrastructure was observed. Common membranous structures were seen, such as mitochondria and Golgi. The application of permanganate fixation, which is particularly useful for revealing multi-membrane structures, revealed the dual membrane mitochondria, but also failed to reveal plastid-like organelles.

Dot-blot hybridization also failed to demonstrate a plastid genome in *G. niphandrodes*. The experiment was performed using probes that encompass significant portion of highly conserved plastid sequences at lower stringency to allow for hybridization of mismatched bases between the probe DNA of *B. equi* and *G. niphandrodes*. The apicoplast probe bound to *B. equi* DNA but not to *G. niphandrodes* DNA. This provides further support that *G. niphandrodes* lacks the homologues of SSU and LSU apicoplast sequences. A nuclear LSU *G. niphandrodes* probe was used as a positive control. This hybridization was performed at a low stringency to replicate the conditions of the apicoplast probe hybridizations. The nuclear LSU probe bound to both *G. niphandrodes* and *B. equi* DNA.

Collectively these four studies provide strong evidence that *G. niphandrodes* may not possess either a plastid organelle or a plastid genome. The shared ancestry of apicomplexan plastids with dinoflagellate plastids implies that the common ancestor of the Apicomplexa, and thus the ancestor of gregarines and *Cryptosporidium* harbored a plastid. Studies of the nuclear-encoded plastid targeted glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene indicate a common origin of the apicomplexan plastids and the dinoflagellate plastids and its cyanobacterial/algae origin (Fast et al. 2001). Consistent with this hypothesis, analysis of the genome of *C. parvum* revealed several genes of cyanobacterial/algae origin (Huang et al. 2004). The close relationship between gregarines and *Cryptosporidium* within the Apicomplexa and the possible absence of a plastid in both suggest that the plastid was lost following the divergence of *Cryptosporidium* and gregarines from the other apicomplexan species harboring a plastid.

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**LITERATURE CITED**


