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PHYLOGENY OF NUCLEAR SMALL SUBUNIT rRNA GENES OF HEMOGREGARINES AMPLIFIED WITH SPECIFIC PRIMERS

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ABSTRACT: Hemogregarines, apicomplexan intracellular blood parasites, are cosmopolitan in distribution and infect a broad range of vertebrate and invertebrate hosts. Molecular phylogenetic studies have been hampered by lack of hemogregarine-specific polymerase chain reaction primers that would allow amplification of parasite, but not host, DNA. A novel method for separating parasite and host 18S rRNA genes has been developed, and new primers that are specific for hemogregarine rRNA genes have been designed. These primers were used to obtain sequences from 4 isolates of hemogregarines of lizards from California, the Caribbean island of Grenada, eastern Australia, and Israel. Combining these results with already published sequences, a preliminary phylogeny of hemogregarines and several other apicomplexan taxa has been created. The hemogregarines form a monophyletic group and appear to be more closely related to coccidia than to *Plasmodium* species. The difficulty of using 18S genes that have multiple copies in some apicomplexan parasites was explored for systematic studies.

Hemogregarine parasites (Apicomplexa, Adeleorina, Haemogregarinidae) are intracellular parasites known from all continents except Antarctica (Smith, 1996). These parasites display a complex life cycle, alternating between a vertebrate host (including mammals, birds, fishes, crocodilians, snakes, lizards, and turtles) and a hematophagous invertebrate (ticks, mites, mosquitoes, other arthropods, and leeches) (Smith, 1996; Davies and Johnston, 2000). Some species have evolved a 3-host life cycle requiring carnivory (Smith, 1996). Six genera have been described (*Hepatozoon, Hemogregarina, Desseria, Cyrilia, Karyolysus,* and *Hemolivia*). With over 300 species currently assigned to *Hepatozoon,* Smith and Desser (1997) recommended that this genus should be partitioned further into at least 2 genera, upon a phylogenetic analysis of several *Hepatozoon* species. The great taxonomic diversity, variation in life cycles and hosts, and geographic range of these parasites argues that they are an ancient and successful group.

Traditionally, taxonomic classification of hemogregarines was based on morphological characters (primarily the size and shape of gamont stages found in host erythrocytes), life history and development characteristics, and host taxon (Siddall, 1995; Mathew et al., 2000). Based on morphological data, Barta (1989) concluded that hemogregarines were basal to the piroplasms. Recently, molecular data in the form of DNA sequences have been employed to examine the phylogenetic relationships of these parasites. Lang-Unnasch et al. (1998) included a species of *Hepatozoon* in a phylogenetic study of the rRNA genes of the plastid genome of several apicomplexan taxa. Their results supported a *Plasmodium–Babesia–Hepatozoon* grouping. Carreno et al. (1999) used nuclear small subunit rRNA (SSU rRNA) genes in their examination of the phylogenetic placement of gregarines. Their analyses also included 1 species of *Hepatozoon* that clustered with *Plasmodium knowlesi* and *Plasmodium gallinaceum.* Mathew et al. (2000) conducted a detailed examination of the hemogregarines using morphological, life-cycle, and molecular data. Although these authors used data for 18 species of hemogregarines from all 6 genera, they sequenced the SSU rRNA genes for only 3 *Hepatozoon* species. Their results showed a well-supported grouping of *Hepatozoon, Plasmodium,* and *Cryptosporidium.*

Prior to Mathews et al.'s (2000) study, only a single pair of polymerase chain reaction (PCR) primers to amplify the nuclear SSU rRNA gene for hemogregarines had been described (Wozniak et al., 1994). Subsequent work showed that these primers were not useful for amplifying hemogregarine SSU rRNA genes if using samples from whole blood because they are extremely conservative and amplify the SSU rRNA gene from a wide variety of organisms, including the host (Perkins and Martin, 1999). Many, if not all, *Plasmodium* species have an insertion of ~200 nucleotides in the portion of the SSU rRNA gene amplified by this primer pair, making it possible to separate the lizard and parasite genes based on size (Feldman et al., 1995). However, hemogregarine and host SSU rRNA genes are approximately the same size and thus cannot be separated on an agarose gel (Fig. 1).

A novel screening procedure was used to differentiate the host and parasite genes that were amplified with the conserved primers. Once this was completed, new hemogregarine-specific PCR primers were designed and sequences of an approximately 900-bp fragment of the 18S rRNA gene were obtained from 4 hemogregarine isolates (presumably different species) from lizards from northern California, the Caribbean island of Grenada, eastern Australia, and Israel. A preliminary phylogeny was constructed by combining these data with a published sequence of another hemogregarine, *Hepatozoon canis* (GenBank accession no. AF176835), as well as several other apicomplexan taxa. The results showed that the hemogregarines from 3 continents and the Caribbean form a monophyletic group and that hemogregarines are not closely related to *Plasmodium.*

MATERIALS AND METHODS

Collection of specimens

The hemogregarine parasites used in the present study were all found in lizard hosts. Thin blood smears stained with Giemsa were scanned for 6 min or more at 1,000× to determine infection status. Only lizards that were infected with a single morphotype of hemogregarine and no other hemoparasites, e.g., *Plasmodium,* were used for genetic analysis. Hemogregarine parasites from *Anolis richardi* from Grenada, *Anolis roquet* from Martinique, *Sceloporus occidentalis* from Mendocino County, California, *Eulamprus quoyii* from Queensland, Australia, and *Agama stellio* from Israel were used for subsequent genetic analysis. Only 1 hemogregarine was identified to species (*Hemolivia mariae* from Israel; Smallridge and Paperna, 1997). In light of not wishing to exer-

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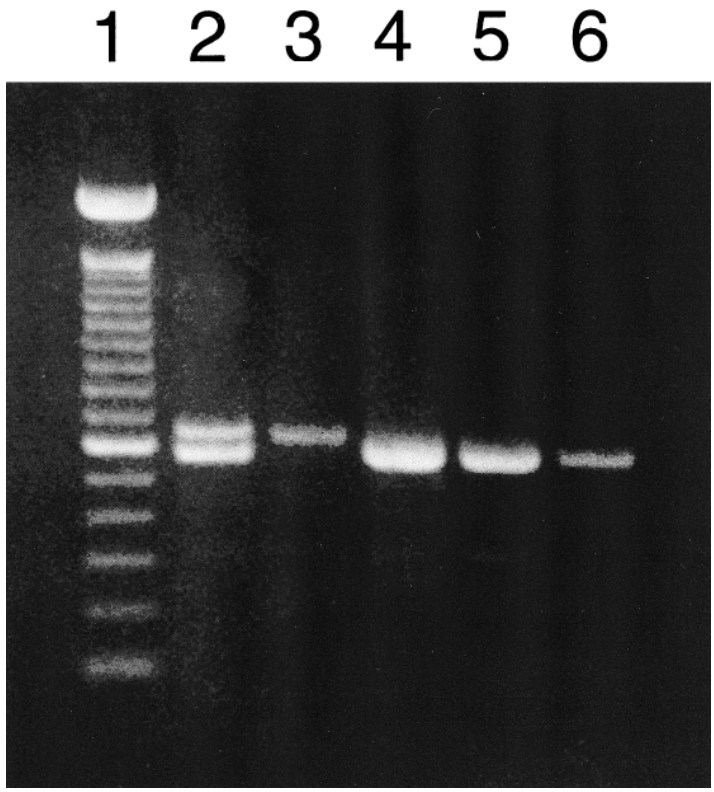

FIGURE 1. Agarose gel showing PCR products amplified with conserved primers 18AP853.F and 18AP1488.R. Lane 1, 100-bp ladder; lane 2, lizard infected with *Plasmodium*; lane 3, cloned *Plasmodium falciparum*; lane 4, uninfected lizard; lanes 5 and 6, lizards infected with hemogregarines. Note that the lizard infected with *Plasmodium* shows 2 bands; the larger product is from the parasite, and the smaller product is from the host. Lizards infected with hemogregarines show only 1 band as host and parasite genes are similar in size.

cise the practice of naming species based on gamont stages alone, the remaining morphotypes were not given species names.

Design of hemogregarine-specific primers

Total DNA was extracted from dried blood dots on filter paper using proteinase K digestion in 100 μl extraction buffer (0.01 M Tris, 0.01 M Na_2EDTA, 0.1 M NaCl, plus 20 g SDS/L, pH adjusted to 8.0), followed by separation of proteins with 7.5 M ammonium acetate, precipitation of DNA with cold isopropynol, and washing the pellet with 70% ethanol.

PCRs were performed to amplify the SSU rRNA gene both of the host and of the hemogregarine parasites from Martinique and Australia using the primers 18AP853.F (5′-GAGTAAATTAGAGTGTTCAAAGCA-3′) and 18AP1488.R (5′-CGGAATTAACCAGACAAATC-3′) designed by Wozniak et al. (1994) and Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, New Jersey). Reactions were subjected to 35 cycles of 94 C for 1 min, 54 C for 1 min, and 72 C for 1 min.

To separate hemogregarine SSU rRNA gene products from lizard SSU rRNA gene products, amplified fragments were cloned into pSTBlue-1 vectors using the Perfectly Blunt Cloning Kit (Novagen, Madison, Wisconsin). A random sample of 50 colonies was picked from each plate, and each colony was resuspended in 50 μl of water. Cells were heat shocked for 5 min at 95 C to release the plasmid, and colonies were screened for the presence of the correct insert with PCR using 5 μl of the lysate and the original primers. The reactions were heated at 94 C for 2 min and then subjected to 35 cycles of 94 C for 1 min, 52 C for 1 min, and 72 C for 1.5 min, with an additional extension period of 4 min at 72 C. The DNA of *Plasmodium* species is generally AT-rich (Weber, 1987; Musto et al., 1999), so based on a presumption that hemogregarine sequences might also be AT-rich, it was predicted that a restriction enzyme lacking these bases in its recognition sequence would cleave the host-derived PCR product more often than the parasite-derived DNA. The enzyme *Hae*III (GGCC) was chosen and a sequence of a lizard 18S gene (Perkins and Martin, 1999) was examined to confirm that this 4 base sequence was present in the host.

Once isolates were scored as likely either to contain hemogregarine or lizard 18S DNA, PCR products were sequenced using Big Dye terminator mix (Applied Biosystems, Foster City, California) and run on an ABI Prism automated sequencer (Vermont Cancer Center, Burlington, Vermont). A BLAST Search (National Center for Biotechnology Information, Bethesda, Maryland) was done on the sequences to confirm our identification of predicted host and parasite products. New PCR primers specific for the hemogregarine DNA were designed (see Results) by comparing aligned sequences from hemogregarine and lizard PCR products, as well as published SSU rRNA sequences for 4 other apicomplexan parasites, i.e., *Plasmodium falciparum*, *Toxoplasma gondii*, *Babesia bovis*, and *Lankesterella minima* (GenBank accession numbers listed below). That is, new primers were sought that would amplify the 18S genes from only hemogregarines and not those of any other organisms, including hosts and malarial parasites.

Phylogenetic analyses of apicomplexan 18S gene sequences

DNA sequences from *H. mariae* from *A. stellio* and the 3 unidentified hemogregarines from Grenada, Australia, and California were obtained by amplification with the new hemogregarine-specific primers. PCR products were cloned using the Perfectly Blunt cloning kit (Novagen, Madison, Wisconsin), plasmid DNA was extracted using UltraClean plasmid Miniprep kit (Doc Frugal Scientific, San Diego, California), and the insert was sequenced in both directions. For each of the hemogregarine isolates, 3 cloned 18S rRNA PCR products were sequenced. In addition to the sequences obtained from the 3 taxa above, another hemogregarine sequence, *H. canis* (AF176835), as well as 17 other apicomplexan sequences, were obtained from GenBank (*Cryptosporidium parvum* [L25642]; *B. bovis* [L19077]; *Eimeria falciformis* [AF080614]; *Gregarina caledia* [L31799]; *L. minima* [AF080611]; *Plasmodium vivax* asexually expressed gene [U93233]; *P. vivax* spo-

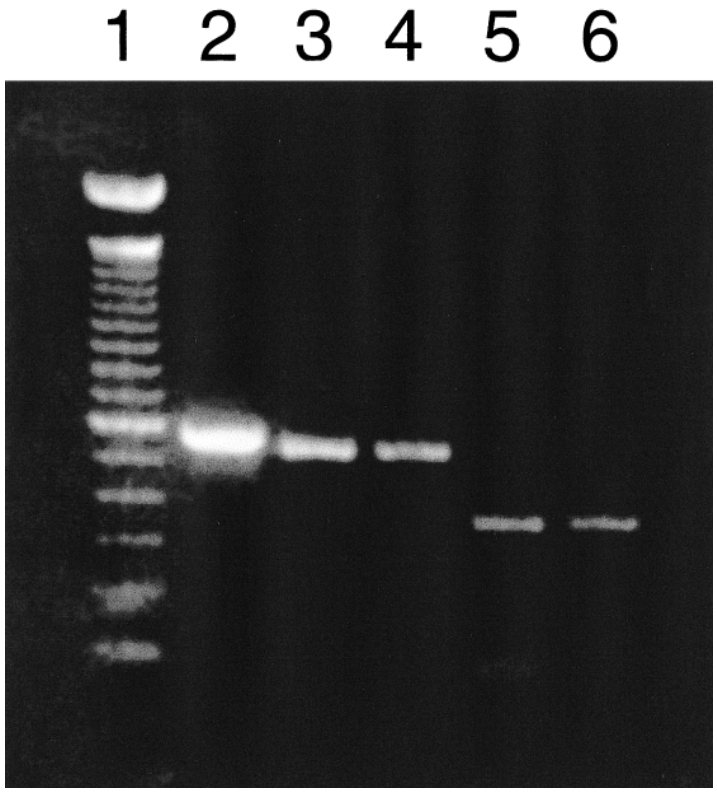

FIGURE 2. Agarose gel showing cloned and reamplified PCR products after restriction digest with *Hae*III. Lane 1, 100-bp ladder; lane 2, undigested PCR product; lanes 3 and 4 were suspected to be parasite; lanes 5 and 6 were suspected to be host.

rozooite-expressed gene [U93234]; *P. vivax* oocyst-expressed gene [U93235]; *P. falciparum* asexually expressed gene [PFARGEA]; *P. falciparum* sporozooite-expressed gene [PFARGEB]; *Plasmodium berghei* asexually expressed gene [AJ243513]; *P. gallinaceum* asexually expressed gene [M61723]; *Sarcocystis tenella* [L24383]; *Theileria parva* [L02366]; *T. gondii* [L24381]; and *Perkinsus atlanticus* [U07697] that was used as the outgroup taxon [Siddall et al., 1997]).

Published sequences were truncated to the length of those obtained in this study, and all were aligned using Clustal W (Thompson et al., 1994) with minor adjustments made by eye. Phylogenetic relationships of the hemogregarines and of other apicomplexan parasites were assessed using PAUP* 4.0b4 (Swofford, 1999) using both parsimony and maximum likelihood methods. Unweighted parsimony using branch breaking as a heuristic search strategy was employed with 10 replicates of random addition sequences of taxa. For the maximum likelihood analyses, a set of 40 evolutionary models was tested on the neighbor-joining tree obtained from an initial search using the program ModelTest (Posada and Crandall, 1998). The simplest, best model (via log likelihood ratio tests) was then chosen for the maximum likelihood algorithm. This model consisted of a 6-step (general time reversible) matrix, proportion of invariable sites = 0, and gamma shape parameter of 0.6006. Reliability of each clade was assessed using bootstrapping (Felsenstein, 1985) with 1,000 replicates for the parsimony-based trees and 100 replicates of the maximum likelihood heuristic search.

RESULTS

Design of hemogregarine-specific primers

Amplification with the conserved primers of Wozniak et al. (1994) produced PCR products of equal size for lizard host and hemogregarine parasites (Fig. 1, lanes 5 and 6). When these products were cloned, reamplified, and digested with *Hae*III, 2 banding patterns resulted (Fig. 2). One banding pattern appeared to be uncut, whereas the other had at least 1 restriction site. Upon sequencing the products and performing a BLAST search, it was clear that the uncut 18S gene was very similar to published apicomplexan sequences, e.g., *Isospora belli* (GenBank accession no. AF106935; 92% identity), and was thus not a host-derived product. The sequence of the cut product was similar to published 18S gene sequences for vertebrates, e.g., *Alligator mississippiensis* (GenBank accession no. AF173605; 99% identity) and was thus concluded to be from the lizard host.

A forward primer specific for hemogregarines (HEMO1: 5′TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG-3′) and a reverse primer specific to apicomplexan parasites (HEMO2: 5′CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC3′) were designed. These primers were tested by PCR to confirm that they would not amplify DNA of the host nor of 2 malarial parasites, *P. falciparum* and *Plasmodium mexicanum* (Fig. 3).

Phylogenetic analyses

The 3 sequences for each of 3 hemogregarine morphotypes were identical (California and Australia and *H. mariae*); however, a 26-base insertion was found in 1 of the cloned PCR products for the isolate from Grenada. The overall sequence divergence between pairs of morphotypes ranged from 0.45% to 1.39%.

The cladograms obtained using the 18S sequences from hemogregarine isolates and the published sequences from several other apicomplexan taxa are shown in Figures 4 and 5. The hemogregarines form a monophyletic group that has high boot-

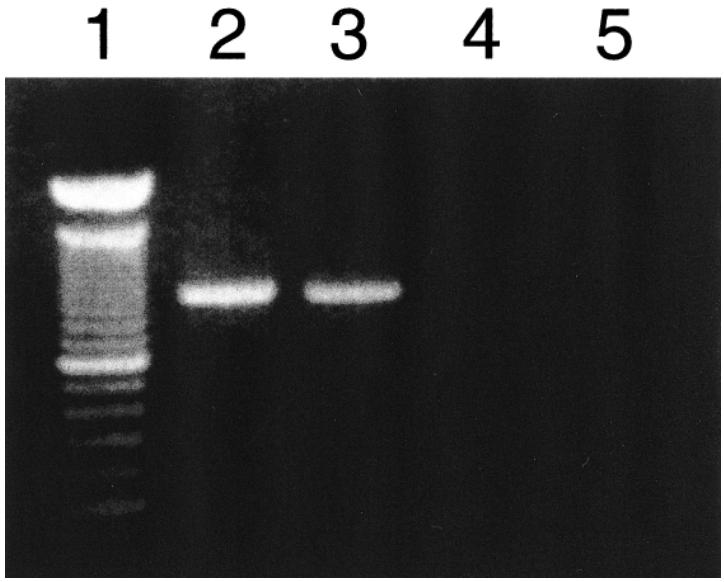

FIGURE 3. Agarose gel showing PCR products amplified with new, hemogregarine-specific primers, HEMO1 and HEMO2. Lane 1, 100-bp ladder; lanes 2 and 3, lizards infected with hemogregarines; lane 4, lizard infected with *Plasmodium mexicanum*; lane 5, cloned *Plasmodium falciparum.* Note: there was no amplification for the lizard infected with malaria, thus, also no amplification of host 18S genes.

strap support (98% for parsimony and 88% for maximum likelihood). Both tree-building methods yielded similar results, with 5 major groups of apicomplexan parasites that conform to Levine's (1988) classification: gregarines (*Gregarina*); hemogregarines (*Hepatozoon, Hemolivia,* plus 3 unidentified hemogregarines); coccidia (*Sarcocystis, Toxoplasma, Lankesterella,* and *Eimeria*); hemosporinids (*Plasmodium* spp.); and piroplasms (*Theiieria* and *Babesia*). The conserved sequences for hemogregarines from 3 continents contrast strongly with the divergence for species of *Plasmodium,* and for different 18S copies within a single species of *Plasmodium* (Figs. 4, 5).

DISCUSSION

As shown by Perkins and Martin (1999), Wozniak et al.'s (1994) primers are not specific for hemogregarines and will also amplify the SSU rRNA genes of the vertebrate host if DNA is extracted from blood. Although Mathew et al. (2000) used the conserved primers, they extracted the parasite DNA from oocysts taken from infected tick vectors, thus presumably there was little or no contamination with host. However, for general diagnostic purposes, such as might be employed by zoo or wildlife management personnel, the primers designed in this study would seem to be more appropriate. PCR using our HEMO1 and HEMO2 primers and genomic DNA extracted from whole blood of the vertebrate host should allow the rapid diagnosis of hemogregarine infections. Infections with *Plasmodium* species may be detected with the nested primer design described in Perkins et al. (1998). Here a protocol was described for the design of specific 18S primers for parasites that are difficult to extract pure from the host such as blood parasites from hosts with nucleated erythrocytes. Although a restriction enzyme was chosen based on an assumption of an AT-rich apicomplexan genome compared to that of the vertebrate, the hemogregarine gene fragment proved actually to show no such bias ($\chi^2 = 0.036$, $P > 0.995$). Thus, the successful choice of this restriction enzyme was merely fortuitous. However, by picking the enzyme after comparison with a vertebrate 18S sequence, the chance of choosing a useful enzyme was greatly improved.

Preliminary phylogenies obtained in the present study are in general agreement with the 5 major groupings of Apicomplexa proposed by Levine (1988). The only anomaly in these trees is the placement of *Cryptosporidium.* The tree constructed with unweighted parsimony placed *Cryptosporidium* as basal to the eimeriids, piroplasms, and hemogregarines, but with only weak bootstrap support (<5%). The maximum likelihood tree grouped *Cryptosporidium* with *Gregarina,* but again, there was no support for this node. Carreno et al.'s (1999) results also placed *Cryptosporidium* with the gregarines, though their bootstrap support was only 64%.

Use of the 18S rRNA gene for phylogenetic studies of apicomplexan parasites is confounded by 2 characteristics of this gene. First, as with all 18S genes, numerous insertions/deletions may prevent accurate alignment. Morrison and Ellis (1997) demonstrated that the recovery of phylogenetic relationships of Apicomplexa was sensitive to the alignment of nucleotide sequences, though this appears to be less of a problem when armed with a very large taxonomic sample (Barta et al., 2001). Second, many apicomplexan parasites reveal an unusual organization of their 18S rRNA genes. All species of *Plasmodium* examined so far (including rodent, avian, primate, and human malarial parasites) have multiple (4–8) copies of nuclear rRNA genes scattered among many chromosomes that are expressed at different times during the life cycle (Waters, 1994). Some genes are transcribed when the parasites asexually divide in the blood, others are expressed in the sporozooites, and some (but not all) *Plasmodium* species have a third set of genes that are transcribed only during the oocyst stage (Li et al., 1997; Spaendonk et al., 2000). Several other taxa also have been found to have this unusual rDNA organization. For example, *T. parva* has 2 rDNA loci (Kibe et al., 1994), the lowest copy number

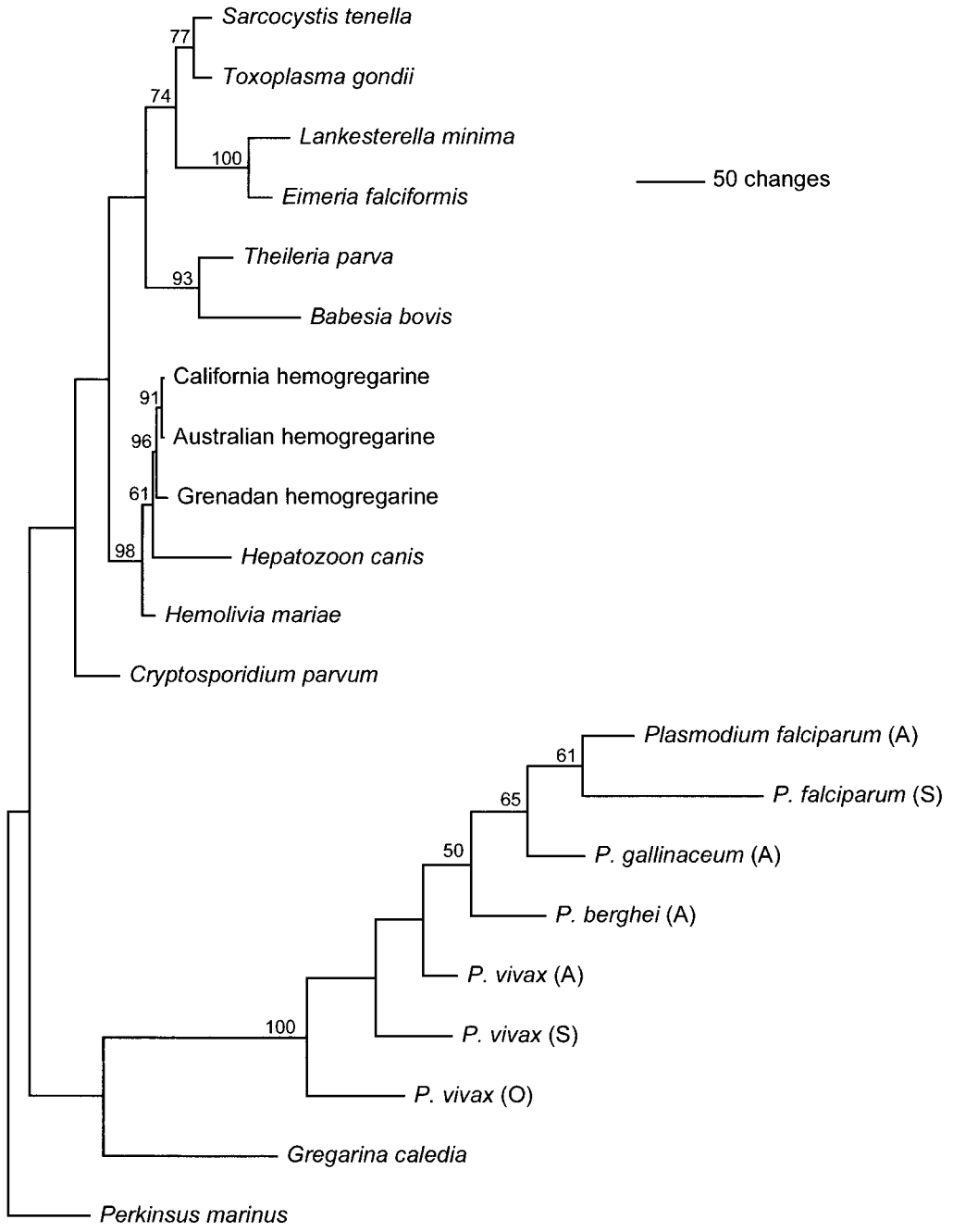

FIGURE 4. Single most parsimonious tree obtained using heuristic search of 1,224 aligned bp of the 18S rRNA gene of 3 unidentified species of hemogregarines and other apicomplexan parasites, rooted with *Perkinsus marinus.* The stage at which the 18S gene is expressed in the *Plasmodium* species is indicated as follows: A = asexually expressed; S = sporozooite expressed; O = oocyst expressed. The length of the tree is 1,598 steps with a consistency index of 0.685 and a retention index of 0.730. Numbers above nodes represent percent bootstrap support out of 1,000 replicates.

ever observed in eukaryotes. *Cryptosporidium* parasites have a simple life cycle, completed within a single host species, and have 5 distinct rDNA loci (Le Blanq et al., 1997); however, all genes appear to be expressed at all stages of the life cycle (Widmer et al., 1999). Interestingly, *T. gondii* appears to have a more typical eukaryotic rDNA organization with 110 homogenous copies arranged in head-to-tail arrays (Gagnon et al., 1996).

Figures 4 and 5 show the substantial difference among the 18S copies even within species of *Plasmodium.* Adding these sequences to the phylogenic data matrix demonstrates a potentially serious problem with sequencing a single 18S sequence from different taxa; that is, paralagous genes may be combined into 1 analysis. Only minor sequence divergence within the cloned PCR products of the rRNA gene of 1 hemogregarine, the isolate from the Grenadan *Anolis,* were found, and it is possible that this was a reflection of the presence of multiple copies of rRNA genes in these parasites. As hemogregarines

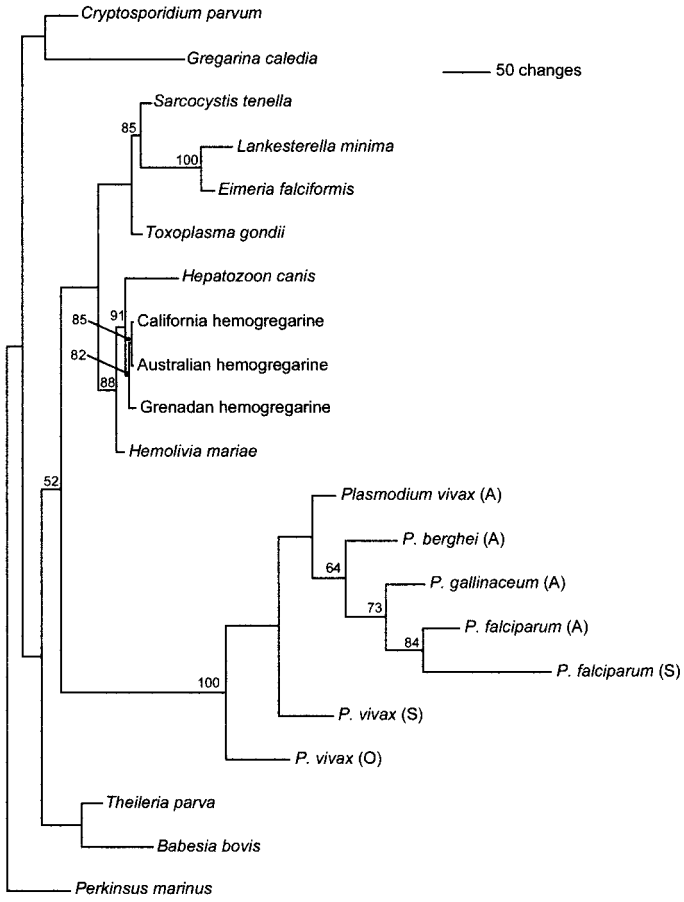

FIGURE 5. Maximum likelihood phylogeny of apicomplexan parasites constructed with a GTR model of substitution, proportion of invariable sites = 0, and a gamma shape parameter of 0.6006 rooted with *Perkinsus marinus.* The stage at which the 18S gene is expressed in the *Plasmodium* species is indicated as follows: A = asexually expressed; S = sporozooite expressed; O = oocyst expressed. The log likelihood score of the phylogeny is −8,634.95. Numbers above nodes represent percent bootstrap support out of 100 replicates.

have a life cycle more similar to *Plasmodium* (they also are vectorborne), we predict that they might also have developmentally regulated rRNA genes that are similar to those in *Plasmodium.* However, the variation observed in the limited number of cloned SSU rRNA PCR products sequenced for the hemogregarines was much smaller than the sequence divergence in *Plasmodium* (Rogers et al., 1995). It is possibly that the variation instead was caused by mixed-species infection as was suggested by Lang-Unnasch et al. (1998). Larger-scale sequencing of cloned PCR products should be attempted before this is concluded. The similar sequences that were determined for the hemogregarine isolates from 3 continents suggests either that these parasites have only 1 18S rRNA copy, or that clones were fortuitously chosen that carried homologous gene copies for all isolates.

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