

## A MOLECULAR PHYLOGENY OF MALARIAL PARASITES RECOVERED FROM CYTOCHROME *b* GENE SEQUENCES

Susan L. Perkins\* and Jos. J. Schall

Department of Biology, University of Vermont, Burlington, Vermont 05405. e-mail: susan.perkins@colorado.edu

**ABSTRACT:** A phylogeny of haemosporidian parasites (phylum Apicomplexa, family Plasmodiidae) was recovered using mitochondrial cytochrome *b* gene sequences from 52 species in 4 genera (*Plasmodium*, *Hepatocystis*, *Haemoproteus*, and *Leucocytozoon*), including parasite species infecting mammals, birds, and reptiles from over a wide geographic range. *Leucocytozoon* species emerged as an appropriate out-group for the other malarial parasites. Both parsimony and maximum-likelihood analyses produced similar phylogenetic trees. Life-history traits and parasite morphology, traditionally used as taxonomic characters, are largely phylogenetically uninformative. The *Plasmodium* and *Hepatocystis* species of mammalian hosts form 1 well-supported clade, and the *Plasmodium* and *Haemoproteus* species of birds and lizards form a second. Within this second clade, the relationships between taxa are more complex. Although jackknife support is weak, the *Plasmodium* of birds may form 1 clade and the *Haemoproteus* of birds another clade, but the parasites of lizards fall into several clusters, suggesting a more ancient and complex evolutionary history. The parasites currently placed within the genus *Haemoproteus* may not be monophyletic. *Plasmodium falciparum* of humans was not derived from an avian malarial ancestor and, except for its close sister species, *P. reichenowi*, is only distantly related to haemosporidian parasites of all other mammals. *Plasmodium* is paraphyletic with respect to 2 other genera of malarial parasites, *Haemoproteus* and *Hepatocystis*. Explicit hypothesis testing supported these conclusions.

The malarial parasites (phylum Apicomplexa, family Plasmodiidae; Levine, 1988) are taxonomically diverse, cosmopolitan in distribution over all the warm continents, and ecologically successful, exploiting a wide range of vertebrate and invertebrate hosts (Garnham, 1966; Levine, 1988). Approximately 450 described species have been placed in 12 genera, the best known being *Plasmodium* and *Haemoproteus*. Parasitologists have traditionally described species, and classified them into subgenera and genera, on the basis of life-history characteristics, morphological traits, symptomatology expressed in the vertebrate host, and taxon of vertebrate hosts and insect vectors infected (Levine, 1988). The phylogenetic significance of such characters, however, has long been questioned (Manwell, 1936, 1957). For example, morphological traits seen under the light microscope are often distorted by preservation, and life-history traits could evolve convergently on the basis of ecological pressures experienced by the parasites. Manwell (1936) concluded that phylogenetic relationships of malarial parasites will not be revealed by morphology, life-history traits, or host taxa, and predicted that genetic characters would be far more informative.

Investigators have now turned to molecular data, primarily DNA sequences, to recover the evolutionary relationships of the Plasmodiidae (McCutchan et al., 1984, 1996; Waters et al., 1991, 1993; Escalante and Ayala, 1994; Escalante et al., 1995, 1998; Qari et al., 1996; Rathore et al., 2001). These studies lend support to Manwell's prescient views. For example, Escalante et al. (1998) concluded that life-history traits such as periodicity in asexual replication and virulence do not concord with phylogenetic relationships of *Plasmodium* species, and the parasites now placed in other genera (on the basis of life-history traits) appear within the *Plasmodium* clade. Molecular data also reveal cryptic species not detected by the examination of morphology under the light microscope. For example, *P. azurophilum*, a parasite of *Anolis* species of lizards in the Caribbean islands, was found to be 2 (or perhaps 3) morphologically indistinguishable species (Perkins, 2000, 2001).

Here, we present a molecular phylogeny of malarial parasites, based on sequences of the cytochrome *b* gene (1,116 bp, approximately 92% of the gene) from 52 parasite species currently placed in 4 genera, approximately triple the largest number of taxa used in previous molecular studies. These malarial parasites were isolated from reptilian, avian, and mammalian hosts from a very broad geographic range. Previous studies have been hampered by lack of a suitable out-group; here, *Leucocytozoon* is identified as the closest sister out-group. Both parsimony and maximum-likelihood analyses were conducted, as was explicit hypothesis testing using the Shimodaira–Hasegawa (SH) tests (Shimodaira and Hasegawa, 1999), to explore alternative phylogenetic groupings.

### MATERIALS AND METHODS

A list of the parasite species used in this study, their vertebrate hosts, the geographic site of collection, and the collector or data source is provided in Table I. We used data for 9 species of *Plasmodium* infecting lizards from North America, Central America, South America, and Africa; 9 species of *Plasmodium* from birds from southeast Asia, Africa, Japan, Europe, and North America; 5 species of *Plasmodium* from African rodents; the 4 human malarial parasites; 10 *Plasmodium* species from nonhuman primates from both the New and the Old World; and haemosporidian parasites belonging to 3 other named genera, *Haemoproteus* (2 species from lizards [Israel and Pakistan] and 9 from birds [India, North America, and Europe]), 2 *Hepatocystis* species (from an African baboon and a southeast Asian fruit bat), and 2 species of *Leucocytozoon* from birds (North America).

Identification of malarial parasites to species as seen in blood films, especially from poorly known taxa, is challenging. Most samples could be positively identified to species by experienced parasitologists, but approximately one-third of the isolates are indicated only to genus because some uncertainty remained. This conservative approach follows that of Bensch et al. (2000). It is possible that some of these isolates represent new, undescribed species. Also, very small differences between some samples (for example, *P. gallinaceum* from Vietnam and a *Plasmodium* from a bird from Vermont) suggested technical errors (polymerase chain reaction [PCR] copy errors or contamination) or that the isolates represented geographic variation within a single species. Multiple sequencing runs producing the same gene sequence eliminated the possibility of technical errors. Also, other forms generally regarded as separate species (for example *P. vivax* and *P. simium*; Coatney et al., 1971) also were found to be very similar genetically. In any case, the primary goal of this study was to recover the broad phylogeny of the Plasmodiidae rather than to stress the relationships among particular

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\* Present address: Department of Environmental, Population, and Organismal Biology, University of Colorado, Boulder, Colorado 80309.

species; therefore, the inclusion of species identified only to genus does not distract from the major aim of the work.

Cytochrome *b* sequences from the *Plasmodium* species infecting humans, nonhuman primates, the *Hepaticystis* of baboons, 1 *Plasmodium* species of birds (*P. elongatum*), and another apicomplexan parasite, *Theileria annulata*, used as an out-group, were retrieved from GenBank (see Table I for accession numbers). All other sequences are new. For most of these, DNA was extracted from blood samples that had been dried onto Whatman filter paper using the DNeasy Extraction kit (QIAGEN, Valencia, California), following protocols of the manufacturer, except eluting in only 50  $\mu$ l of buffer. Some samples were received as already extracted DNA from S. Bensch (noted in Table I). The cytochrome *b* gene fragment was amplified using a nested PCR design, taking standard precautions to prevent cross-contamination of samples. The PCR reactions were carried out using Ready-to-Go PCR beads (Amersham-Pharmacia, Piscataway, New Jersey) under the following conditions: an outer reaction using primers DW2 (5'-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3') and DW4 (5'-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3' [=AL1356, Escalante et al., 1998]), and 2  $\mu$ l of genomic DNA was subjected to 35 cycles of 94 C for 20 sec, 60 C for 20 sec, and 72 C for 1.5 min. For most species, a 0.5- $\mu$ l aliquot of this product was used as a template for a nested reaction with primers DW1 (5'-TCA ACA ATG ACT TTA TTT GG-3' [Creasey et al., 1993]) and DW6 (5'-GGG AGC TGT AAT CAT AAT GTG-3' [=AL1413, Escalante et al., 1998]) under 40 cycles of 94 C for 20 sec, 50 C for 20 sec, and 72 C for 1 min and then 72 C for 7 min. The samples of avian *Haemoproteus* parasites were reamplified in 2 separate reactions using a 0.5- $\mu$ l aliquot of the product from the first reaction (as described above). One nested reaction was carried out with primers DW1 and HaemoR (5'-CAT ATC CTA AAG GAT TAG AGC TAC CTT GTA A-3') and the other with primers HaemoF (5'-TTA CAA GGT AGC TCT AAT CCT TTA GGA TAT G-3') and DW6. (Primers HaemoF and HaemoR are reverse complements of each other.) All these primers are specific to malarial parasites and do not amplify host DNA or even that of other Apicomplexa such as *Hepatozoon* (S. Perkins, pers. obs.).

PCR products were directly sequenced along both strands using either outer primers DW1 and DW6, and internal primers DW3 (5'-TGC TGT ATC ATA CCC TAA AG-3' [Creasey et al., 1993]) and DW8 (5'-GCA CAA ATC CTT TAG GGT ATG ATA C-3') or for the avian *Haemoproteus* products, using primers DW1 and HaemoR, and HaemoF and DW6. All automated sequencing reactions were carried out using BigDye<sup>®</sup> terminator mix (Applied Biosystems, Foster City, California) and were run on an ABI Prism 377 or on an ABI 3700.

Nucleotide sequences were aligned first using the program CLUSTAL W (Thompson et al., 1994), with minor adjustments made by eye. All phylogenetic analyses were performed using PAUP\* 4.0b6 (Swofford, 1999). Unweighted parsimony using tree-bisection-reconnection (TBR) in a heuristic search was employed with 30 replicates of random addition sequences of taxa. Nodal support was determined using 100 replicates of jackknifing with 37% deletion of characters, emulated "Jac" resampling, and the complete heuristic search with TBR branch-swapping. For maximum-likelihood analyses, 56 models of DNA substitution were tested using the program Modeltest v3.06 (Posada and Crandall, 1998), which suggested a general time reversible model, estimated base frequencies, proportion of invariable sites = 0, and a gamma distribution shape parameter of 1.6368. Preliminary analysis suggested that *T. annulata*, a member of the Piroplasmida, followed different molecular models compared with the other taxa, i.e., incorporating this taxon resulted in unresolved trees. Therefore, *Theileria* was not included in maximum-likelihood analyses.

Several of the conclusions emerging from the phylogenies were tested by constraining the trees to alternative topologies and testing for significantly different tree scores under maximum likelihood. Kishino-Hasegawa (KH) tests (Kishino and Hasegawa, 1989) have typically been used to estimate standard errors and confidence limits of different topologies and to determine if varying topologies are significantly different from one another. But as Goldman et al. (2000) have demonstrated, KH tests are frequently used inappropriately because they were originally devised only for competing hypotheses that were chosen a priori, independent of the data. Here, we follow the recommendations of Goldman et al. (2000) in using the test devised by Shimodaira and Hasegawa (1999), in which the best topology is tested against other competing

topologies. In conducting the SH tests, each hypothesis was evaluated by constraining relevant taxa to fall within clades based on a priori topologies expected under alternative hypotheses. The resulting trees were then compared with the best trees obtained using a resampling estimated log likelihood technique with 1,000 bootstrap replications, as implemented in PAUP\*. The model of evolution used to generate maximum-likelihood scores was obtained using the results of the Modeltest program (described above). The results were subjected to a 1-tailed test for significance.

## RESULTS

The cytochrome *b* sequences were highly A-T rich (mean = 73.36%, excluding *T. annulata*), typical of mitochondrial genes of malarial parasites (McIntosh et al., 1998). This bias, however, was uniformly found across taxa (chi-square test of base homogeneity across taxa = 45.74,  $P = 1.0000$ ). Uncorrected genetic distances ( $P$ ) between pairs of cytochrome *b* sequences of all ingroup taxa ranged from 0.3 to 23%. Such small differences for taxa most likely separated by hundreds of millions of years (for example, there is only a 15% difference for *P. mexicanum*, a parasite of North American lizards and for *P. berghei* of African rodents), might suggest saturation of the gene and a loss of phylogenetic signal. However, a standard test for saturation refuted this possibility (Fig. 1).

Despite the large number of taxa in the study, unweighted parsimony analysis produced just 2 equally parsimonious trees, with 1 of these shown in Figure 2. (The only difference between the 2 topologies was a minor rearrangement within a clade of primate *Plasmodium* species.) Rooting the trees with *T. annulata* supported *L. simondi* and *L. dubreuli* as a close sister out-group to the other malarial parasites. All other species form a well-supported (98% jackknife support) monophyletic clade. Two major clades are contained within this ingroup, the *Plasmodium* and *Hepaticystis* of mammals and the *Plasmodium* and *Haemoproteus* of lizards and birds. Thus, *Plasmodium* is paraphyletic, containing species of 2 other described genera: *Hepaticystis* and *Haemoproteus*. Within the clade of avian and lizard parasites, the *Haemoproteus* species that infect birds and lizards each appear as well-supported clades (jackknife support of 100% and 96%, respectively) but not as 1 monophyletic lineage. The lizard *Plasmodium* species also appear to be divided into 3 groups that are in accordance with their geographic distribution: a clade of North American parasites (*P. floridense*, *P. mexicanum*, and *P. chiricahuae*); a clade of neotropical parasites (*Plasmodium* species from Brazil, *P. azurophilum* in erythrocytes, *P. azurophilum* in leukocytes, and *P. fairchildi*); and an African clade (*P. giganteum* and *P. agamae*). Nodal support is strong only for the neotropical group (98%), but these 9 species still represent only approximately 10% of the described lizard malarial taxa (Schall, 1996).

Maximum-likelihood analysis produced a phylogeny with a very similar topology ( $-\ln$  likelihood = 9696.53, results not shown). SH tests demonstrated that the topologies obtained under the parsimony and maximum-likelihood heuristic searches were not significantly different from each other. Again, the malarial parasites of mammals and those infecting lizards and birds form 2 clusters, and the *Haemoproteus* species infecting birds are monophyletic. The *Plasmodium* of lizards and birds, however, are interspersed within their cluster, again suggesting that lizard and avian *Plasmodium* may not form 2 monophyletic clades.

TABLE I. Parasite taxa used in the study are given in the approximate order as they appear in the phylogeny presented in Figure 2.\*

Parasite	Host	Geographic locale	Source†	GenBank accession no.
<i>Plasmodium</i> of primates				
<i>P. cynomolgi</i>	Old World monkeys	Southeast Asia	P	AF069616
<i>P. falciparum</i>	Humans	Tropical regions	P	AF069605
<i>P. fieldi</i>	Old World monkeys	Malaysia	P	AF069615
<i>P. gonderi</i>	Old World monkeys	Central Africa	P	AF069622
<i>P. hylobati</i>	<i>Hylobati moloch</i>	Malaysia	P	AF069618
<i>P. inui</i>	Old World monkeys	India and Southeast Asia	P	AF069617
<i>P. knowlesi</i>	Old World monkeys	Malaysia	P	AF069621
<i>P. malariae</i>	Humans	Tropical and subtropical	P	AF069624
<i>P. ovale</i>	Humans	Tropics of Africa and Asia	P	AF069625
<i>P. reichenowi</i>	<i>Pan troglodytes</i>	Central Africa	P	AF069610
<i>P. simiovale</i>	Old World monkeys	Sri Lanka	P	AF069614
<i>P. simium</i>	<i>Alouatta fuscus</i>	Brazil	P	AF069620
<i>P. vivax</i>	Humans	Brazil	P	AF069619
<i>Plasmodium</i> sp. (Mandrill)	<i>Mandrillus leucophaeus</i>	Gabon	P	AF069623
<i>Plasmodium</i> of rodents				
<i>P. atheruri</i>	<i>Atherurus africanus</i>	Congo and Cameroon	L	AY099054
<i>P. berghei</i>	<i>Grammomys surdaster</i>	Katanga, Congo	L	AY099049
<i>P. chabaudi</i>	<i>Thamnomys rutilans</i>	Central African Republic	L	AY099050
<i>P. vinckei</i>	<i>G. surdaster</i>	Katanga, Congo	L	AY099052
<i>P. yoelii</i>	<i>T. rutilans</i>	Central African Republic	L	AY099051
<i>Plasmodium</i> of birds				
<i>P. elongatum</i>	<i>Passer domesticus</i>	North America	P	AF069611
<i>P. gallinaceum</i>	<i>Gallus gallus</i>	Vietnam	W1	AY099029
<i>P. relictum</i>	<i>Zenaida macroura</i>	Nebraska	A	AY099032
<i>Plasmodium</i> sp. (bird 1)	<i>Acrocephalus arundinaceus</i>	Kenya	D	AY099041
<i>Plasmodium</i> sp. (bird 2)	<i>Quiscalus quiscula</i>	Vermont	W2	AY099031
<i>Plasmodium</i> sp. (bird 3)	<i>Turdus migratorius</i>	Vermont	W2	AY099033
<i>Plasmodium</i> sp. (bird 4)	<i>Ninox scutulata</i>	Singapore	W3	AY099035
<i>Plasmodium</i> sp. (bird 5)	<i>Larosterna inca</i>	Washington, D.C.	C	AY099036
<i>Plasmodium</i> sp. (bird 6)	<i>Acrocephalus orientalis</i>	Japan	D	AY099044
<i>Plasmodium</i> of lizards				
<i>P. agamae</i>	<i>Agama agama</i>	Ghana	W4	AY099048
<i>P. azurophilum</i> (erythrocytes)	<i>Anolis oculatus</i>	Dominica	W5	AY099055
<i>P. azurophilum</i> (leukocytes)	<i>A. oculatus</i>	Dominica	W5	AY099058
<i>P. chircahuae</i>	<i>Sceloporus jarrovi</i>	Arizona	W6	AY099061
<i>P. fairchildi</i>	<i>Norops cupreus</i>	Costa Rica	W5	AY099056
<i>P. floridense</i>	<i>A. oculatus</i>	Dominica	W5	AY099059
<i>P. gignateum</i>	<i>Agama agama</i>	Ghana	W4	AY099053
<i>P. mexicanum</i>	<i>Sceloporus occidentalis</i>	California	W5	AY099060
<i>Plasmodium</i> sp. (lizard)	<i>Ameiva ameiva</i>	Manaus, Brazil	W7	AY099047
<i>Hepatocystis</i> of mammals				
<i>Hepatocystis</i> sp. (baboon)	<i>Papio nubensis</i>	Ethiopia	P	AF069626
<i>Hepatocystis</i> sp. (bat)	<i>Cynopterus brachyoti</i>	Singapore	W3	AY099030
<i>Haemoproteus</i> of birds				
<i>H. majoris</i>	<i>Parus caeruleus</i>	Sweden	D	AY099045
<i>H. sylvae</i>	<i>Acrocephalus arundinaceus</i>	Sweden	D	AY099040
<i>Haemoproteus</i> sp. (bird 7)	<i>Vireo olivaceus</i>	Vermont	W8	AY099034
<i>Haemoproteus</i> sp. (bird 8)	<i>A. schoenobaeneus</i>	Sweden	D	AY099037
<i>Haemoproteus</i> sp. (bird 9)	<i>Phylloscopus occipitalis</i>	India	D	AY099043
<i>Haemoproteus</i> sp. (bird 10)	<i>A. scirpacues</i>	Spain	D	AY099046
<i>Haemoproteus</i> sp. (bird 11)	<i>Phylloscopus trochilus</i>	Sweden	D	AY099039
<i>Haemoproteus</i> sp. (bird 12)	<i>P. trochilus</i>	Sweden	D	AY099038
<i>Haemoproteus</i> sp. (bird 13)	<i>Phylloscopus humeri</i>	India	D	AY099042
<i>Haemoproteus</i> of lizards				
<i>H. ptyodactylus</i>	<i>Ptyodactylus hasselquistii</i>	Israel	W3	AY099057
<i>H. kopki</i>	<i>Teratoscincus scincus</i>	Pakistan	W9	AY099062

TABLE I. Continued.

Parasite	Host	Geographic locale	Source†	GenBank accession no.
<i>Outgroups</i>				
<i>Leucocytozoon dubreuli</i>	<i>Catharus guttatus</i>	Vermont	W8	AY099063
<i>L. simondi</i>	<i>Anas platyrhynchos</i>	Ontario, Canada	C	AY099064
<i>Theileria annulata</i>			P	M63015

\* Parasites traditionally placed within *Plasmodium* are presented first from mammal, bird, and then from lizard hosts. Following are *Hepatocystis* and *Haemoproteus* of birds, reptiles, and last the parasites of the outgroups, *Leucocytozoon* and *Theileria*. Also given are the vertebrate host, geographic location for the isolate, the collector or the source for the isolate and the GenBank accession number of each sequence. The geographic location for some of the isolates from previously published data has only the general location for those parasites.

† A, ATCC culture; C, captive animal, naturally infected; D, DNA donated by S. Bensch, Lund University, Sweden; L, passed laboratory culture from Richard Carter, University of Edinburgh; P, published sequence; W, wild-caught infected host collected by 1) R. Paul, Institute Pasteur; 2) W. Barnard, Norwich University; 3) I. Paperna, Hebrew University of Jerusalem, Israel; 4) S. Yeboah, University of Cape Coast, Ghana; 5) authors; 6) J. Foubopoulos, Princeton University; 7) C. R. de Silva, University of Sao Paulo, Brazil; 8) S. Hudman, University of Vermont; 9) S. R. Telford Jr, Florida State Museum.

Several other hypotheses were subjected to SH tests. H1: *P. falciparum* and its close sister species *P. reichenowi* of chimpanzees fall within the clade of parasites of mammals and are unrelated to avian-infecting *Plasmodium*. This was tested by constraining both *P. falciparum* and *P. reichenowi* (because separating these sister taxa may artificially bias the results) to fall within the clade of avian malarial parasites. H2: The genus *Plasmodium* is paraphyletic and contains species of 2 other genera, namely *Hepatocystis* and *Haemoproteus*. This was tested by constraining all genera to form separate, monophyletic lineages. H3: *P. falciparum* and *P. reichenowi* are not within the cluster of *Plasmodium* species infecting other primates (the “primate malaria” clade). Testing was done by constraining these 2 species to group with the other primate malarial species, exclusive of the other mammalian parasites (rodent *Plasmodium*). Test results supported the first 2 hypotheses but not the

third (Table II). Although constraining *P. falciparum* and *P. reichenowi* within the clade of other primates, *Plasmodium* species did not result in significantly different trees, and all topologies still place the 2 species on a very long branch relative to the other primate parasites. Furthermore, topologies placing *Hepatocystis* as basal to the other mammalian malarial parasites were not significantly different from those grouping these species with *P. ovale* as shown in Figure 2.

## DISCUSSION

The broad story of the phylogenetic relationships of the malarial parasites presented here is likely to be robust because the study incorporated data from a large number of taxa, identified a close out-group, found concordance of results from parsimony (with only 2 equally parsimonious trees) and maximum-likelihood methods (with different model assumptions), and conducted hypothesis testing to evaluate the major conclusions. Several striking results emerge from the analysis.

First, *Plasmodium* is currently paraphyletic because of the presence of taxa of other named genera within the clade of *Plasmodium* parasites (*Haemoproteus* and *Hepatocystis*). Escalante et al. (1998) first included a species of *Hepatocystis* (the species included here from baboons) in a phylogeny of malarial parasites and found it clustered within the primate *Plasmodium*. Addition here of a second species of *Hepatocystis* from a fruit bat confirms the result of Escalante et al. (1998), but this is a demonstration of paraphyly, and not polyphyly, as those authors proposed. As noted above, however, the placement of the 2 *Hepatocystis* species within the clade with *P. ovale* as shown in Figure 2 is not necessarily resolved. *Hepatocystis* may not fall within the primate *Plasmodium* cluster, but it still is contained within the clade of *Plasmodium* of mammals.

The divergent life-history traits used to place the malarial parasites in different genera are not always informative characters for understanding the evolutionary relationships among these parasites (Escalante et al., 1998). The 2 *Hepatocystis* species, with their enormous asexually replicating cells in the liver, fall on a long branch but are contained within the clade of mammalian *Plasmodium*. The reproductive strategy that defines *Haemoproteus* (casting only gametocytes into blood cells) is found within 2 well-supported clades of avian and lizard parasites that are now called *Haemoproteus*. *Haemoproteus* is,

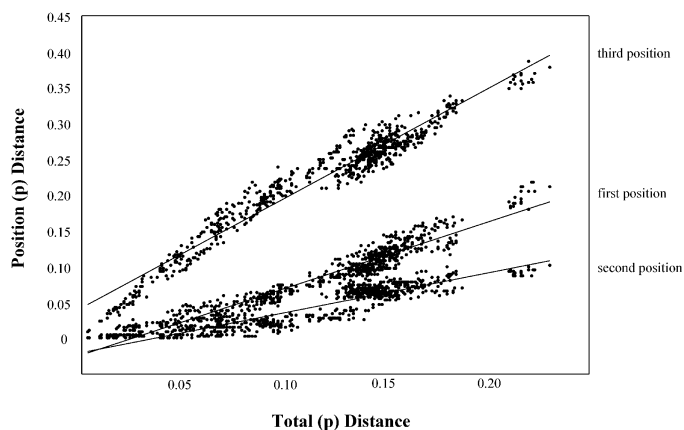


FIGURE 1. Uncorrected pairwise cytochrome *b* distances partitioned by codon position (372 codons analyzed). Each point represents 1 pair of taxa appearing in the phylogeny. One axis presents the overall difference for the genetic sequences for the 2 taxa, and the other axis partitions this genetic distance by the 3 codon positions. As expected, the third position where silent substitutions are most common contributes a greater share of the overall genetic distance. Evidence of saturation of nucleotide changes in a codon position, e.g., third position wobble, minimally requires demonstration of an asymptoting curve function describing pairwise substitutions. All 3 positions exhibit linearly increasing substitutions relative to total divergence; thus, there is no evidence of saturation by excessive overwriting of base changes.

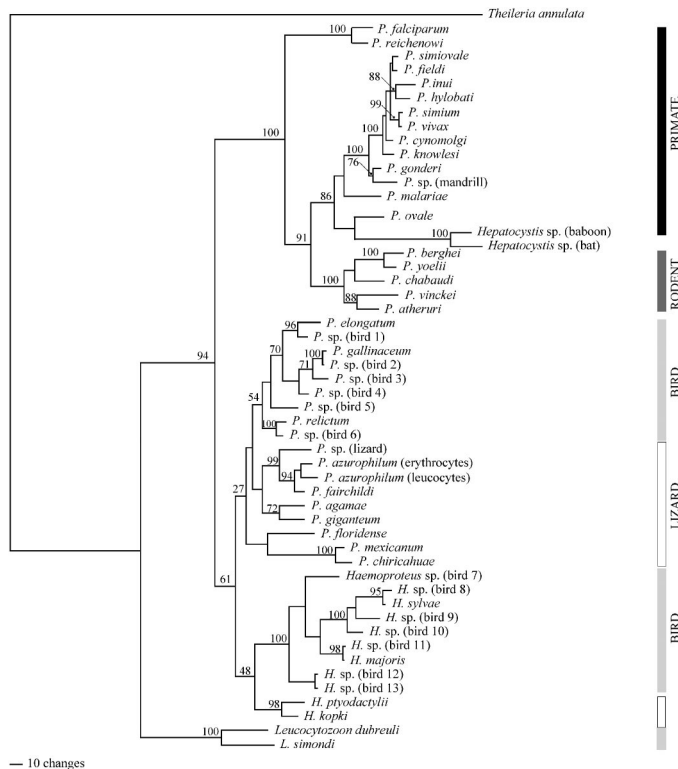


FIGURE 2. Phylogeny of malarial parasites using mitochondrial cytochrome *b* gene. Vertebrate class exploited as the parasite's host is indicated with bars. This phylogeny is 1 of the 2 equally parsimonious trees (2,216 steps) rooted with *T. annulata*. Branch lengths are proportionate to number of nucleotide changes, and the numbers above nodes represent percent jackknife support (if >75% or on a key node) out of 100 replicates of the full heuristic search.

thus, not monophyletic, but both the clusters are included within an overall clade of *Plasmodium* taxa.

Second, several life histories and morphological traits are also not always informative for comparisons among species presently regarded as *Plasmodium*. The number of daughter cells produced in schizogony perhaps can be a hundred in *P. giganteum* of lizards, yet this species is most closely related to *P. agamae*, a species that produces very few daughter cells (Schall and Bromwich, 1994). *Plasmodium azurophilum* appears in the phylogeny twice because it consists of 2, widely distributed monophyletic lineages (2 species) in the *Anolis* liz-

ards of the Caribbean islands (Perkins, 2000, 2001). The apparent ancestor species was a typical *Plasmodium* that infects erythrocytes, whereas 1 daughter species has altered its life history to infect 2 classes of white blood cells, which have very different habitats than that found in erythrocytes. Neither of these species stores hemozoin pigment (the remnant of catabolism of the host hemoglobin by the parasite), and yet they fall among other lizard malarial parasites that do so (Schall, 1996). Likewise, *P. inui*, a primate malarial parasite with quartan periodicity, is not closely related to other quartan parasites with both immunological (Kamboj and Cochrane, 1988) and DNA sequence data (Kissinger et al., 1998). Virulence can also be considered a life-history trait; species causing malaria in lizards with known high versus low virulence do not cluster on the tree (Schall, 2002). Thus, life-history traits and morphological characters in *Plasmodium* must evolve rapidly, and similar traits can be convergent.

Third, the major clades coincide with the taxon of the vertebrate host exploited by the parasites, suggesting that vertebrate host switching is rare and that the establishment of the parasite lineages in different vertebrate host classes had an ancient origin. The rodent *Plasmodium* species are monophyletic and, as discussed above, constraining the tree to force all primate *Plasmodium* species (including *P. falciparum* and *P. reichenowi*) to be monophyletic (hypothesis 3) did not produce a significantly worse tree, thus, a single evolutionary switch into primates cannot be discarded as a possibility. The pattern for lizard versus avian *Plasmodium* is less clear, but the current evidence suggests that these parasites fall into a single clade of *Plasmodium* of birds and 3 clades of *Plasmodium* species of lizards. The pattern of high jackknife support for the mammalian clade coupled with the lower jackknife value for the bird-lizard clade is consistent with the supposition that malarial parasites invaded mammalian hosts at just 1 point in their evolutionary history, whereas lizard parasites (and perhaps those of birds) are a more ancient and diverse lineage (F. Lutzoni, pers. comm.). These results underscore the pressing need to sample and include a far greater proportion of the immense diversity of lizard and avian malarial parasites to uncover the more complete evolutionary history of this family.

Fourth, the results cast light, albeit dimly, on the evolutionary origin of *P. falciparum*, the most virulent human malarial parasite. Malariologists long suspected that *P. falciparum* was recently acquired by humans because high virulence was unexpected for well-adapted parasite-host associations (Boyd, 1949;

TABLE II. Shimodaira-Hasegawa tests (Shimodaira and Hasegawa, 1999) of significance for phylogenies with alternative topologies. *Theileria annulata* was pruned from the parsimony topologies and compared with the constrained topology using maximum likelihood under the model estimated from Modeltest (Posada and Crandall, 1998).

Alternative a priori hypotheses	Tree score (-ln L)	Diff*	P
H <sub>1</sub> : <i>P. falciparum</i> and <i>P. reichenowi</i> belong to the mammal parasite clade	9,708.70		
H <sub>2</sub> : <i>P. falciparum</i> and <i>P. reichenowi</i> belong to the bird parasite clade	9,854.63	145.93	<0.0001
H <sub>1</sub> : <i>Plasmodium</i> is a paraphyletic genus with respect to <i>Haemoproteus</i> and <i>Hepatocystis</i>	9,708.70		
H <sub>2</sub> : <i>Plasmodium</i> is monophyletic	10,081.15	372.45	<0.0001
H <sub>1</sub> : <i>P. falciparum</i> and <i>P. reichenowi</i> fall outside the primate parasite clade	9,708.70		
H <sub>2</sub> : <i>P. falciparum</i> and <i>P. reichenowi</i> belong to the primate parasite clade	9,709.13	0.43	0.956

\* Minimum value for all trees.

Hoeprich, 1989). Several previous molecular systematics studies based on the 18S rRNA genes lent support to this hypothesis when *P. falciparum* clustered with 2 avian malarial parasites, suggesting that *P. falciparum* arose from an avian host, perhaps when birds were first domesticated (Waters et al., 1991, 1993). Escalante and Ayala (1994) included *P. reichenowi* in an analysis and found that these 2 species cluster and may have diverged within the time scale consistent with divergence of the human and pongid lineages (6–10 million yr). Thus, *P. falciparum* did not recently originate from an avian malarial parasite. The *P. falciparum* + *P. reichenowi* pair, however, fell as a sister group to the parasites of birds and lizards. Qari et al. (1996) included even more taxa and concluded that *P. falciparum* is within the clade of primate malarial parasites. Escalante et al. (1995), using sequence data for the circumsporozoite gene, found the *P. falciparum* + *P. reichenowi* pair to be sister to the malarial parasites of other primates and those of rodents; thus, the “primate” malarial parasites appear more closely related to those of rodents than to the *P. falciparum* + *P. reichenowi* pair. The first phylogeny based on the cytochrome *b* gene (Escalante et al., 1998) produced the opposite result, showing *P. falciparum* and *P. reichenowi* as sister to a cluster of *Plasmodium* and *Haemoproteus* infecting birds.

Part of the confusion concerning *P. falciparum* arises from taxon bias, in which previous studies included a preponderance of parasite species from primates, which can lead to spurious clustering of the few dissimilar species when constructing phylogenetic trees, especially if distance measures (neighbor joining) are used (Farris et al., 1996). Also, using a distant out-group will often lose phylogenetic signal (Siddall and Barta, 1992), and midpoint rooting will be confounded by unequal rates of genetic change or uneven taxon sampling. We attempted to correct for these potential pitfalls by including a large number of taxa from all reptiles, birds, and mammals, and from a worldwide distribution, and used a close sister taxon as the out-group (*Leucocytozoon*).

The phylogeny presented here shows that *P. falciparum* and *P. reichenowi* are not related to the malarial parasites of birds and reptiles. But we find that these species exhibit a deep root within the *Plasmodium* species infecting mammals. *Plasmodium falciparum* and *P. reichenowi* have a fairly deep root when compared with other pairs of species that must have been separated for millions of years (within the lizard–bird clade). This result suggests that the immediate ancestor of *P. falciparum* was present at the separation of lineages leading to *Homo* and *Pan*, the chimpanzee, consistent with the conclusions of Escalante and Ayala (1994). The high virulence of *P. falciparum*, therefore, cannot be explained by its recent entry into human hosts but is most likely a result of its overall ecology and transmission biology (Ewald, 1994).

Examination of the phylogeny of malarial parasites offers questions of ecological, physiological, and medical importance. For example, if significant differences in life-history traits among named genera of malarial parasites have little phylogenetic significance, what selective factors have led to the evolution of such divergent ecological strategies? As more extensive sequence data become available for *Plasmodium* and *Haemoproteus*, we anticipate that comparative analyses will reveal important details of how the developmental fate of the parasite cells is controlled. Also, the evolutionary origin of the *P. fal-*

*ciparum* and *P. reichenowi* lineage remains a mystery. Bio-medical researchers have long sought an “animal model” for *P. falciparum* and over the past 80 yr have studied *Plasmodium* infections of lizards, birds, rodents, and monkeys to understand the basic biology of human malaria. Although each of these model systems may have 1 or more biological characteristics in common with *P. falciparum*, none is a close relative. Only *P. reichenowi* of chimpanzees is related to *P. falciparum*, but use of this species as a model has important ethical and logistical shortcomings. Close sister taxa may no longer exist or may await discovery by sampling poorly known species of *Plasmodium* such as those of bats and ungulates. Discovery of other close relatives of *P. falciparum* could open novel windows to the biology of the most important vector-borne human parasite.

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