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Evolution

The evolution of primate malaria parasites based on the gene encoding cytochrome *b* from the linear mitochondrial genome

(*Plasmodium*/Apicomplexa)

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ABSTRACT **We report a phylogenetic analysis of primate malaria parasites based on the gene encoding the cytochrome *b* protein from the mitochondrial genome. We have studied 17 species of *Plasmodium*, including 14 parasitic in primates. In our analysis, four species were used for rooting the *Plasmodium* phylogenetic tree: two from closely related genera (*Hepatocystis* sp. and *Haemoproteus columbae)* and two other Apicomplexa (*Toxoplasma gondii* and *Theileria parva*). We found that primate malaria parasites form a monophyletic group, with the only exception being the *Plasmodium falciparum–Plasmodium reichenowi* lineage. Phylogenetic analyses that include two species of non-*Plasmodium* Haemosporina suggest that the genus *Plasmodium* is polyphyletic. We conclude that the biologic traits, such as periodicity and the capacity to relapse, have limited value for assessing the phylogenetic relationships among *Plasmodium* species. For instance, we found no evidence that would link virulence with the age of the host–parasite association. Our studies also reveal that the primate malaria parasites originated in Africa, which contradicts the presently held opinion of Southeast Asia as their center of origin. We propose that the radiation of Asian monkey parasites is a recent event where several life history traits, like differences in periodicity, appeared *de novo*.**

Malaria is one of the leading causes of infant mortality in the tropics. Between 200 and 500 million people in the world are infected every year with malaria (1, 2). Malaria parasites are classified into the genus *Plasmodium*, family Plasmodiidae, phylum Apicomplexa (3). The phylogenetic relationships among *Plasmodium* species have been the focus of attention in recent years, especially the phylogenetic relationships discerning the origin of human malaria parasites (4–11).

Classical parasitologic studies have led to the conclusion that the genus is polyphyletic. *Plasmodium* species are considered related to other genera of haemosporidian parasitic in the same class of vertebrate hosts. Specifically, the primate malarial parasites and *Hepatocystis* are considered a monophyletic group, and the avian malaria parasites appeared as sister taxa of *Leucocytozoon* and *Haemoproteus* (12–15).

Molecular systematic studies provide an independent source of evidence for elucidating the evolution of malaria parasites. So far, two genes have been used in molecular phylogenetic studies of *Plasmodium*: the 18S small subunit rRNA (18S SSU rRNA) expressed in the asexual stage and the circumsporozoite protein (CSP) gene. The phylogenetic trees obtained with both of these genes are congruent; however, no appropriate outgroup was included (6, 7). The complex pattern of gene expression from 18S SSU rRNA raised concern about the phylogenetic trees derived from these genes (16). Partial gene conversion occurs among nonhomologous copies of the 18S SSU rRNA genes that are expressed during different parasite stages. This partial gene conversion among nonhomologous genes can generate misleading phylogenetic results (11, 17). The CSP, a polymorphic and immunogenic surface protein expressed at the sporozoite stage, is considered to be under selective pressure for accumulating polymorphism (18). In addition, the tandem repeat motifs do not allow the alignment of the full-length *CSP* gene (8). Because of the limitations of the genes used for molecular phylogenetic studies so far, we have studied the *cytochrome b* gene from the mitochondrial genome.

Our phylogenetic analysis included 17 species of *Plasmodium*. We found that primate malaria parasites form a monophyletic group with the exception of the *Plasmodium falciparum* and *Plasmodium reichenowi* lineage. The results derived by rooting the *Plasmodium* phylogenetic tree using non-*Plasmodium* Haemosporidia reveal that the genus is polyphyletic. We also find that biologic traits, such as periodicity and virulence, have limited value for inferring phylogenetic relationships in *Plasmodium*.

MATERIALS AND METHODS

We studied the gene encoding cytochrome *b* from the 17 species of *Plasmodium* listed in Table 1. Information about their host, geographic range, and major biologic characteristics (periodicity and the capacity of relapse) is also provided. Additional details can be obtained elsewhere (19–22). We included in our study a new species isolated from a drill, *Mandrillus leucophaeus*, in Gabon. In addition, we included data from *Plasmodium hylobati*, one of the four species parasitic in gibbons, and two monkey malaria parasites, *Plasmodium inui* and *Plasmodium fieldi*. These two monkey parasites are considered similar to the human parasites *Plasmodium malariae* and *Plasmodium ovale* based on the characteristics of the disease (19). We obtained five sequences of *P. falciparum*, one from a field isolate from western Kenya and four laboratory adapted strains: "Santa Lucia" from Central America, "3D7" from West Africa, "Malaysian-4" from Malaysia, and one from China. The *Plasmodium yoelii* sequence was obtained from the literature (23).

The cytochrome *b* sequences were amplified by PCR using three sets of primers: pair 1, AL 1355: CAU-CAU-CAU-CAU-TTA-TGG-AAT-TAI-GGI-TTC-CTT-TTA-GGA with AL 1356: CUA-CUA-CUA-CUA-ATG-TTT-GCT-TGG-GAG-CTG-TAA-TCA-TAA-TGT-G; pair 2, AL 1412: CAU-CAU-CAU-CAU-GGA-ATT-AIG-GIT-TIC-TGA-TTT-TAG-G

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Abbreviations: CSP, circumsporozoite protein; GG, Galtier and Gouy distance; ML, maximum likelihood; My, million years; NJ, neighbor joining; SSU, small subunit.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF069605–AF069626).

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Table 1. *Plasmodium* species with their host, geographic range, and two life history traits: Periodicity and their capacity for relapse

Species	Natural hosts	Geographic range	Periodicity	Relapse
P. falciparum	*Homo sapiens*	Tropical regions	Tertian	No
P. vivax	*H. sapiens*	Tropical and subtropical	Tertian	Yes
P. malariae	*H. sapiens*	Tropical and subtropical	Quartan	No
P. ovale	*H. sapiens*	Tropics of Africa and Asia	Tertian	Yes
P. reichenowi	*Pan troglodytes*	Central Africa	Tertian	No
P. hylobati	*Hylobati moloch*	Malaysia	Tertian	No
Plasmodium cynomolgi	Old world monkeys	Southeast Asia	Tertian	Yes
P. fieldi	Old world monkeys	Malaysia	Tertian	Yes
P. inui	Old world monkeys	India and Southeast Asia	Quartan	No
P. knowlesi	Old world monkeys	Malaysia	Quotidian	No
Plasmodium simiovale	Old world monkeys	Sri Lanka	Tertian	Yes
Plasmodium gonderi	Old world monkeys	Central Africa	Tertian	No
Plasmodium sp.	*Mandrillus leucophaeus*	Gabon	*	*
Plasmodium simium	*Alouatta fuscus*	Brazil	Tertian	*
P. yoelii	*Thamnomys rutilans*	Central Africa	24h	*
P. gallinaceum	*Gallus gallus*	Asia	36h	NA
P. elongatum	*Passer domesticus*	North America	24h	NA

*, Insufficient information. NA, not applicable.

with AL 1413: CUA-CUA-CUA-CUA-GGG-AGC-TGT-AAT-CAT-AAT-GTG; pair 3, AL 1510: CAU-CAU-CAU-CAU-TGG-AAT-TAG-GGG-TTC-CT with AL 1511: CUA-CUA-CUA-CUA-TTG-GGA-GCT-GTA-ATC-ATA-AT. "I" codes for Inosine. All primers contained ends for using the CloneAmp system of GIBCO for cloning. The amplification conditions were as follows: first, 1 min at 94°C, followed by 30 cycles with 0.5 min of denaturation at 94°C, annealing at 40°C for 0.5 min, and elongation at 72°C for 1.5 min. After 30 cycles, a final elongation step at 72°C for 3 min was carried out. The amplified product of 1038 bp (346 residues of the 377 that are part of this protein) was purified, cloned, and sequenced. Both strands were sequenced from at least two clones. The sequences were obtained using an automated sequencer ABI Prism 377 from Perkin–Elmer. The alignment was performed using ClustalW version 1.7 (24) with manual editing for reestablishing the reading frame.

We used the neighbor joining (NJ) method (25) with the Galtier and Gouy (GG) correction (26) in our phylogenetic analyses. This model corrects for unequal base composition among the sequences. The GG correction seems appropriate to use in plasmodial phylogenies because of the observed high content of A + T in the *cytochrome b* genes (27). We also estimated phylogenetic trees using maximum likelihood (ML) methods (28) as implemented in the algorithm DNAML of the PHYLIP package (29). The ML analyses were performed assuming different transition to transversion ratios: 1, 2, and 5.

We performed three phylogenetic analyses. First, NJ and ML trees were estimated for all *Plasmodium* species. The root of the tree was estimated using the algorithm DNAMLK of the PHYLIP package, which assumes a molecular clock. We tested this assumption by comparing the trees obtained from the DNAML and the DNAMLK algorithms using the likelihood ratio test. Second, we rooted the *Plasmodium* tree by using two closely related outgroups: *Haemoproteus columbae* and *Hepatocystis* sp. The isolate from *H. columbae* was obtained from *Columba livia* in Venezuela whereas the isolate of *Hepatocystis* sp. was obtained from *Papio nubensis* in Ethiopia. Both NJ and ML trees were estimated on this new alignment. Finally, nucleotide sequences were translated into amino acids. Protein base alignments were performed for each of two additional Apicomplexa parasites that were used as outgroups: *Toxoplasma gondii* (GenBank accession no. AF023246 submitted by D. C. McFaden and J. C. Boothroyd, Stanford University, CA) and *Theileria parva* (30). These Apicomplexa parasites are considered distantly related to *Plasmodium* (7, 31). A phylogeny was estimated by NJ on distances calculated with the Tajima and Nei model (32).

Tree reliability for the NJ was assessed by the bootstrap method (33) with 1000 replications. We use a 95% value as statistically significant (34); however, values above 70% are reported because bootstrap may be a conservative estimate for the reliability of a clade (35). For the purposes of this study, we consider the nodes with bootstrap support between 70 and 95% as tentative associations that require additional data. All NJ analyses were performed using the program TreeconW (36). In the case of the ML trees, alternative topologies were compared using the Kishino and Hasegawa test (37) and the ML test ratio.

RESULTS

The gene encoding cytochrome *b* of *Plasmodium* has a high A + T content in all of the lineages studied, which is in agreement with previous reports (23, 27, 38). The average A + T content is 73.35%, with a 95% confidence interval around the mean of 72.98–73.73%, calculated using only one sequence from *P. falciparum*. The average A + T content at first, second, and third codon positions are 66.82% (confidence interval, 66.21–67.43%), 64.47% (confidence interval, 64.23–64.71%), and 88.53% (confidence interval, 87.90–89.16%), respectively. Most of the substitutions were synonymous. The ratio of synonymous versus nonsynonymous substitutions is approximately 6.5 as calculated according to the method of Nei and Gojobori (39) using all 17 species of *Plasmodium* (data not shown).

The five sequences obtained from *P. falciparum* isolates are almost identical (average of 0.001 using all pairwise comparisons), even though the isolates included are from diverse geographic origins (Malaysia, China, Central America, and Africa). The NJ tree estimated from the GG distances is depicted in Fig. 1. The four human parasites do not form a monophyletic group as previous studies reported (4, 7), which is in agreement with traditional parasitologic studies (12, 19). The group of primate malaria parasites, excluding *P. falciparum* and *P. reichenowi*, appears as a monophyletic group with a bootstrap support of 90%. The *P. falciparum*/*P. reichenowi* lineage shares a common ancestor with avian malaria parasites. Parasites from Southeast Asian monkeys form a monophyletic group with *Plasmodium simium* from South American monkeys, *P. hylobati* from gibbons, and the human parasite *Plasmodium vivax*. The lack of informative sites does not allow a clear resolution of their phylogenetic relationships. African

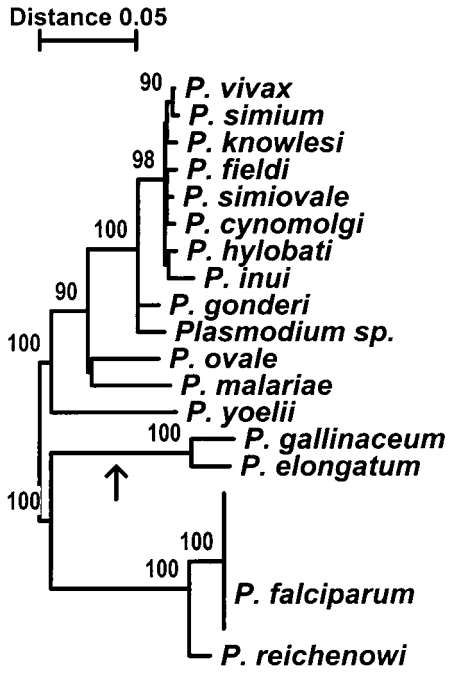

FIG. 1. Phylogenetic relationship among the 17 *Plasmodium* species inferred from the gene encoding cytochrome *b*. The tree was estimated using the NJ method (25). The GG distance matrix is provided in Table 1. Bootstrap values are provided as percents over 1000 replications. The tree ↑ shows the location of the root as estimated by the DNAMLK algorithm from PHYLIP, which assumes a molecular clock.

monkey parasites form a monophyletic group with the primate parasites from Southeast Asia and *P. vivax*. This clade is also supported by an insertion of one amino acid, isoleucine, at residue 31 (based on the *P. falciparum* sequence).

P. inui, a quartan parasite, is related to other macaque parasites and does not form a monophyletic group with *P. malariae*. ML analyses lead to similar results. The root obtained using the program DNAMLK is shown by an arrow. The root separates the avian parasites from the rest of the *Plasmodium* species; however, the likelihood ratio test rejected the assumption of a molecular clock for this dataset. Therefore, the location of the root estimated by this procedure is questionable.

Fig. 2 shows the NJ tree including the two closely related outgroups, *Hepatocystis* sp. and *H. columbae*. *H. columbae* appears closely related to the avian malaria parasites. *Hepatocystis* appears as a sister taxa of *P. ovale*. Given the long branch of *Hepatocystis* sp., and the poor bootstrap support of the deep branches of the tree, additional analyses were performed (40). NJ trees, estimated using protein sequences and only transversions (data not shown), does not elucidate the position of *Hepatocystis* in the tree. ML methods do not differentiate the *Plasmodium* tree rooted by *Hepatocystis* in the *P. ovale* branch from the tree where primate malaria parasites form a monophyletic group.

Fig. 3 shows an NJ tree using Tajima and Nei (32) distance based on proteins, including the sequence of *T. gondii*. This distant-related parasite rooted the Haemosporidia tree, separating the avian from the mammalian parasites. The same result was obtained with *T. parva* (data not shown).

DISCUSSION

The A + T content of the *Plasmodium cytochrome b* gene is comparable to the homologous gene in other eukaryotic organisms (41). Most of the substitutions observed are synonymous, suggesting that the protein is not under selective pressure for accumulating polymorphism. This is a common feature in the *cytochrome b* gene that has made it a valuable tool for phylogenetic studies (42). The fact that the cytochrome *b* protein is universally found in the mitochondrial genome

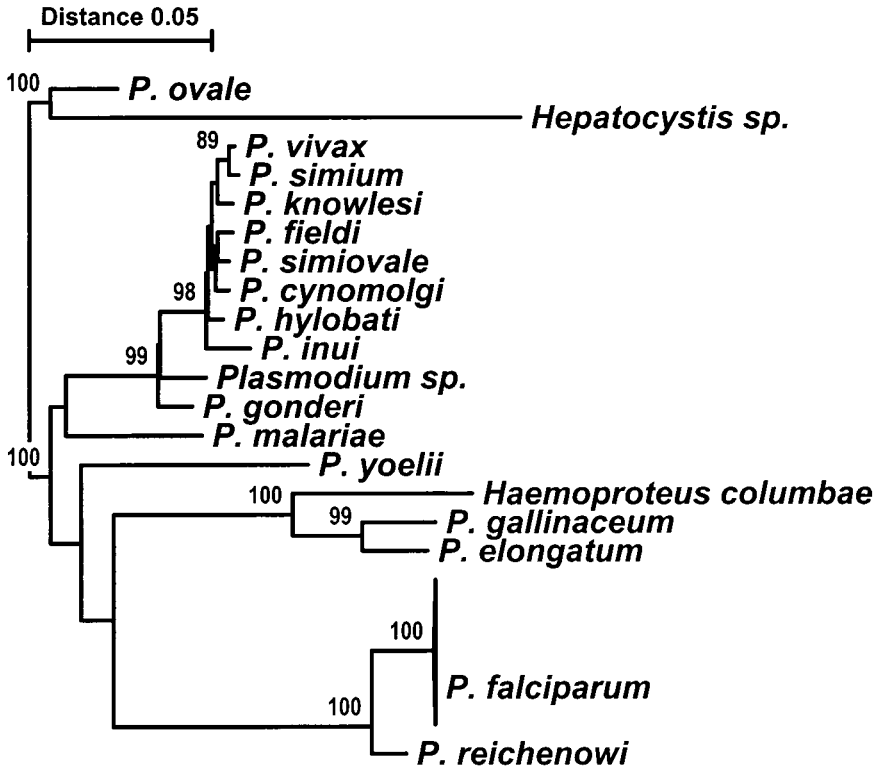

FIG. 2. Phylogenetic tree of the 17 *Plasmodium* species and the two Haemosporina outgroups. The tree was estimated using the NJ method (25) with the GG distance (26). Bootstrap values are provided as percents over 1000 replications.

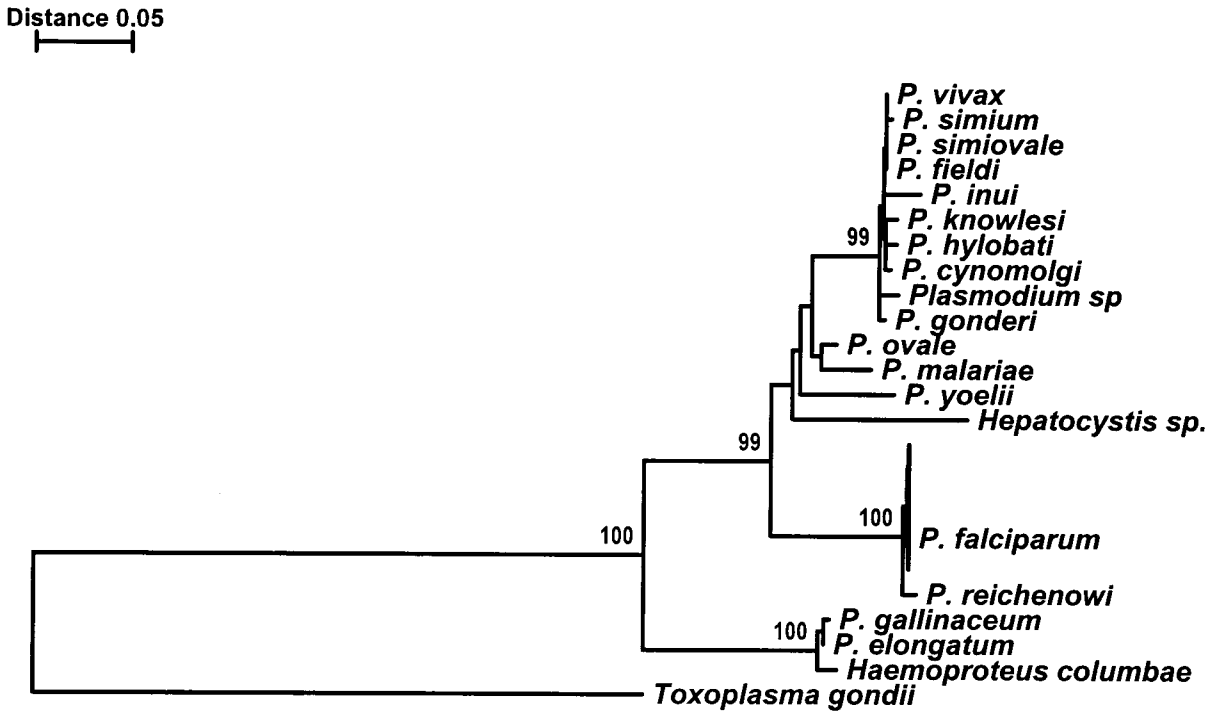

FIG. 3. Phylogenetic tree of the 19 Haemosporina species using *T. gondii* as an outgroup. The tree was estimated using the NJ method (25). The distance matrix was calculated on proteins using the TN distance (32). Bootstrap values are provided as percents over 1000 replications.

makes it an interesting target for phylogenetic studies in *Plasmodium* spp. and other Apicomplexa.

The genus *Plasmodium* was considered polyphyletic in classical parasitologic studies (12–15); however, the molecular phylogenetic studies conducted so far were not able to address this issue. Three observations derived from the cytochrome *b* phylogeny suggest that the genus *Plasmodium* is polyphyletic. First, *Hepatocystis* appears sharing a common ancestor closer to primate malaria parasites than to avian parasites. Second, we found that *H. columbae* is closely related to the two avian malaria parasites. Finally, the position of the *T. gondii* and *T. parva* roots supports the hypothesis that avian parasites evolved separately from mammalian parasites. The clarification of the taxonomic status of *Plasmodium* requires the study of other species of Haemosporina (the genera *Hepatocystis*, *Haemoproteus*, and *Leucocytozoon* among others) and lizard malaria parasites (43). Other genes evolving at a faster rate may also elucidate some of the branches that are not clearly solved with cytochrome *b*.

Our data indicate that *P. falciparum*/*P. reichenowi* lineage shares a common ancestor with avian parasites (4, 7, 10). More interestingly, our results support a host switch from mammals to birds when the criteria of phylogenetic optimization are used, as was previously pointed out on a more limited dataset (5). An alternate hypothesis of a host switch from birds to mammals has also been proposed (4). We agree that a host switch from mammals to birds is a more parsimonious explanation for characters that separate *Plasmodium* parasitic in mammals from those found in birds (44). It is also consistent with the CSP data (10). In this protein, an amino terminal, hepatocyte-binding domain, termed as R1 region, would need to appear *de novo* in *P. falciparum*/*P. reichenowi* if this lineage originated by a host switch from birds to mammals. It is important to notice that only two species of avian malarial parasites are considered in this study; therefore, the inclusion of more species may change the current scenario.

As far as the origin of primate malaria parasites is concerned, the commonly held opinion is that the primate malaria parasites originated in Southeast Asia, with the only exception being *P. falciparum* (45, 46). This hypothesis was made based on the relative abundance of primate malaria parasites in Southeast Asia in comparison to Africa (19, 45). We propose an African origin for the primate malaria parasites. This proposal is supported by two observations: (*i*) the very close relationship among all of the species of primate malaria parasites studied from Southeast Asia, and (*ii*) the position of the African primate malaria parasites in the phylogenetic tree. It is important to note that based on the evolutionary history of the host, an African origin of several lineages of primate malaria parasites was previously proposed (12).

The radiation of the primate malaria parasites in Southeast Asia can be explained by the association of suitable vectors with a highly successful host population (19, 21). The anopheline vectors incriminated in monkey malaria transmission are in the Leucosphyrus group (20). This group has a broad host range that also includes humans and orangutans (45, 47), suggesting that they may be responsible in malaria parasite host switches. The other aspect of the Asian primate malaria parasite radiation is the availability of several species of vertebrate host. Most of the species from Southeast Asia included in our study are macaque parasites, which are a very successful group of primates as evidenced by their geographic distribution and population sizes (48). The radiation of Asian macaques is a recent event that took place around 2.1–2.5 million years (My) ago as estimated by data from mitochondrial DNA (49) and fossil evidence (50). This time frame for the radiation of the vertebrate host is consistent with the data obtained for the parasites.

In terms of the origin of human malaria, we provide further evidence that *P. falciparum* is closely related to *P. reichenowi*, a chimpanzee parasite. We propose a coevolution and a host switch scenario as two hypotheses regarding the origin of *P. falciparum*. The first implies that the parasite followed the separation of *Homo* and *Pan*. The average distance between *P. reichenowi* and *P. falciparum* using cytochrome *b* is approximately 1.9 times greater than that observed among Asian monkey parasites. If we accept that the radiation of Asian monkey malaria parasites took place with the radiation of their

host, approximately 2.1–2.5 My ago (49), the separation of *P. falciparum* and *P. reichenowi* could have taken place between 4 and 4.75 My ago. This time frame is consistent with the time of divergence of *Homo* and *Pan*, which is estimated between 4 and 5 My ago (51). It is also consistent with previous estimates for the separation of *P. falciparum* and *P. reichenowi* based on the 18S SSU rRNA genes (7). However, we need more data from other genetic markers to obtain a robust estimate of the time of divergence (52). A particular problem in the study of the evolutionary rates of genes in parasites is the use of the host for obtaining the time points needed in their estimation. In this case, the circularity in the analysis needs to be avoided by excluding the *Homo*/*Pan* divergence time as a point of calibration. Data from several *Plasmodium* species are required so that other events can be used for estimating the rates. This adds the problem of estimating an overall rate for several *Plasmodium* lineages, a problem that is evidenced in this dataset where the assumption of constant rate of evolution was rejected.

The second hypothesis, the host switch scenario, implies that *P. falciparum* originated from a nonhuman host, and the most likely species will be *P. reichenowi* or a *P. reichenowi*-like parasite. This hypothesis implies that the lack of genetic diversity at genes not involved in the host–parasite interaction is evidence of a bottle neck produced by the colonization of a new host (53). This is consistent with the observation of host switches in primate malaria parasites and with the fact that several primate species live in sympatry (54).

At this time we are not favoring one over the other hypothesis. Our capacity for addressing the *P. falciparum* origin is limited by the dynamic of the human populations (52, 55, 56) and the availability of information from only one isolate of *P. reichenowi*. It may be possible that a few lineages of *P. falciparum* followed the human population expansion out of Africa, producing the bottleneck effect observed today, even when the parasites coevolved with the hominoids.

It is generally accepted that *P. vivax* originated in Asia (12, 19, 46). This Asian origin has been controversial because of the high frequency of the resistant genotype for *P. vivax* of the Duffy blood group in African human populations (57). The finding that *P. vivax*-like parasites evolved in Africa may explain why the Duffy polymorphism has been maintained in African primates (58). However, the observation of *P. vivax*-like parasites in Africa does not reject the origin of *P. vivax* as *Homo* parasite in Asia. The lineage of *P. vivax* could be introduced in Asia by any of the primates that also had their origin in Africa (59, 60), making possible the host switch from nonhuman primates to humans in Asia. Our data and the fact that fossils of *Homo erectus* have been found in Asia (61) indicate that the origin of *P. vivax* is part of the parasite speciation process that took place in the region in the last 2–3 My.

Previous studies that used CSP phylogenies could not rule out the origin of *P. vivax* from *P. simium*, a South American primate parasite (8). The CSP phylogeny lacked resolution in several important branches and it did not include parasites from African monkeys. If we apply the criteria of phylogenetic optimization, the direction of the *P. simium*/*P. vivax* switch will be from a Catarrhini (old world primates) to a Platyrrhini primate (new world primates); therefore, the origin of *P. simium* from *P. vivax* is the most parsimonious hypothesis.

The disease characteristics, such as periodicity and virulence, have been used in drawing evolutionary inferences in malaria parasites (46, 62–64). Our study indicates that disease traits have no value in establishing phylogenetic relationships. For instance, *P. inui* and *P. malariae* do not form a monophyletic group, demonstrating that periodicity is convergent in the evolution of the genus. Additional evidence can be found by comparing the primate parasite *Plasmodium knowlesi* with several species parasitic to birds and rodents, which also have a 24-h erythrocytic cycle (65, 66). Another example is the capacity of relapse (Table 1). The parasites that share this characteristic are not a monophyletic group. A similar comment can be made regarding virulence, a major characteristic that has been used as evidence of the recent origin of *P. falciparum* as a human parasite (46, 62–64).

There is no evidence in malaria that indicates an evolutionary trend toward reducing virulence as a result of the age of the host–parasite relationship. It is known that the virulence of parasite strains of rodent malaria change with few passages (67). No evidence relating virulence with the age of the host–parasite relationship has been found in the lizard parasites (68). There is evidence of host switches between human and new world monkeys parasites, *P. simium*/*P. vivax* and *P. malariae*/*Plasmodium brazilianum* (8, 11, 44); however, no detectable evidence has been found suggesting an association between virulence and the duration of the host–parasite relationship (20). In experimental infections, *P. malariae* shows higher parasitemia in humans than in monkeys, whereas *P. brazilianum* exhibits higher parasitemia in its natural host than in humans (20); a pattern that is not consistent with a trend toward reducing virulence with the time of the host–parasite association. In this study, the distance of *P. falciparum* from *P. reichenowi* (average 0.025) is twice the distance between *P. vivax* and monkey parasites (average 0.011). These differences in genetic distances and the assumption that *P. vivax* originated from Asian monkeys suggest that *P. falciparum* could be the older parasite in hominoids when compared with *P. vivax*. This hypothesis is contrary to past predictions based on virulence (46, 63, 64). Virulence is the result of complex genetic and ecological interactions between the host and the parasite. It has been the case in organisms as diverse as fig wasp and viruses (69–72), and malaria parasites do not appear to be an exception. Malaria pathogenesis has been related with the transmission dynamics and genetic factors in both host and parasite populations (70, 73–75).

In conclusion, we have found evidence suggesting that the genus *Plasmodium* is a polyphyletic group, as the traditional parasitologists had concluded several decades ago. We provide evidence in support of an African origin for primate malaria parasites. We conclude that the radiation of primate *Plasmodium* species in Southeast Asia is a recent event, with several different parasite life histories appearing *de novo*, as evidenced by periodicity and the capacity to relapse. Our results do not support the commonly held notion that virulence is an indicator of the duration of the host–parasite association in malarial parasites. Taken together, this study demonstrates that ''disease traits'' have very limited value for making phylogenetic inferences in malaria parasites.

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