Big Bang in the Evolution of Extant Malaria Parasites

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Malaria parasites (genus *Plasmodium*) infect all classes of terrestrial vertebrates and display host specificity in their infections. It is therefore assumed that malaria parasites coevolved intimately with their hosts. Here, we propose a novel scenario of malaria parasite–host coevolution. A phylogenetic tree constructed using the malaria parasite mitochondrial genome reveals that the extant primate, rodent, bird, and reptile parasite lineages rapidly diverged from a common ancestor during an evolutionary short time period. This rapid diversification occurred long after the establishment of the primate, rodent, bird, and reptile host lineages, which implies that host-switch events contributed to the rapid diversification of extant malaria parasite lineages. Interestingly, the rapid diversification coincides with the radiation of the mammalian genera, suggesting that adaptive radiation to new mammalian hosts triggered the rapid diversification of extant malaria parasite lineages.

Introduction

Coevolution between parasites and their hosts is a widely recognized phenomenon in many parasite–host systems and constitutes an essential component of evolution and the diversity of life. Malaria parasites, the genus *Plasmodium*, cause malaria, one of the major infectious diseases prevalent in most tropical and subtropical areas of the world, and are found from all classes of terrestrial vertebrates (mammals, birds, and reptiles) (Levine 1988). Each malaria parasite species generally exhibits a restricted host range, for example, primate parasites infect only primates and cannot infect other mammals, birds, or reptiles (Levine 1988; Coatney et al. 2003). It is therefore assumed that malaria parasites have coevolved along with their hosts over long time periods. Previous studies have examined the evolutionary history of malaria parasites in order to investigate parasite–host coevolution (Waters et al. 1991; Escalante and Ayala 1994; Escalante et al. 1995, 1998; Martinsen et al. 2008). However, the assumption of parasite–host coevolution has not been fully addressed because of the difficulty in assigning a timescale to parasite evolution due to the lack of a fossil record of parasites. Here, we employ a unique parasite–host phylogenetic comparison to obtain a reliable timescale for evolution without a fossil record of parasites by detecting parasite–host codivergence points for calibration. Thus, we are able to construct a reliable evolutionary history of extant malaria parasites and propose a novel scenario of malaria parasite–host coevolution.

Materials and Methods

DNA Samples

Monkey blood infected with *Plasmodium fieldi* (N-3 strain), *Plasmodium inui* (IM-Perak), *Plasmodium hylobati* (WAK), *Plasmodium cynomolgi* (langur), *Plasmodium simiovale*, and *Plasmodium gonderi*, respectively, was obtained from the American Type Culture Collection. Reptile blood infected with *Plasmodium mexicanum* was generous gift from Joseph J. Schall (University of Vermont, VT). Genomic DNA of these primate *Plasmodium* species and *P. mexicanum* (a reptile malaria parasite) was extracted by using QIAamp DNA mini kit (Qiagen, Hilden, Germany). Genomic DNAs of *Plasmodium malariae* (Uganda I) and *Plasmodium ovale* (Nigeria II) were obtained from the Centers for Disease Control and Prevention (United States). *Plasmodium coatneyi* genomic DNA was kindly provided by Satoru Kawai (Dokkyo University School of Medicine, Tochigi, Japan).

Mitochondrial Genome Sequences

The whole mitochondrial genome was amplified by genomic polymerase chain reaction (PCR). To obtain whole mitochondrial genome sequences from *P. hylobati*, *P. fieldi*, *P. inui*, *P. coatneyi*, *P. cynomolgi*, and *P. simiovale*, six PCR primers (PvmtF5488, PvmtR3088, PvmtF2959, PvmtR0, PvmtF4978, and PvmtR1169) were designed based on the mitochondrial genome sequence of *Plasmodium vivax* (GenBank accession number NC_007243). PCR reactions were performed with 4 pmol of each primer and 1 μl of extracted genomic DNA solution in a total volume of 20 μl containing 400 μM dNTPs and 1 unit of LA-Taq DNA polymerase (Takara, Otsu, Shiga, Japan) in PCR buffer containing 2.5 mM $MgCl_2$. GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) was used to generate the following conditions: denaturation at 93 °C for 1 min followed by 40 cycles of 93 °C for 20 s, 62 °C for 5 min, and extension at 72 °C for 10 min.

Genomic PCR of *P. malariae* and *P. ovale* was performed using two primers (MOcytbF1 and MOcytbR1) that were designed on the basis of partial sequences of their respective cytochrome *b* genes (GenBank accession numbers AF069624 and AB182496). PCR using these primers was performed under the following conditions: denaturation at 93 °C for 1 min followed by 40 cycles of 93 °C for 20 s, 62 °C for 7 min, and extension at 72 °C for 10 min. For complete coverage of the entire mitochondrial genome with PCR products, two further primers, PvmtF2959 and PvmtR0, were also used in the genomic PCR.

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Table 1
Parasite Species Used in This Study

Species	Natural host	Accession number
Plasmodium falciparum	Human	AY282930
Plasmodium vivax	Human	NC_007243
Plasmodium malariae	Human	AB354570 (this study)
Plasmodium ovale	Human	AB354571 (this study)
Plasmodium reichenowi	Chimpanzee	NC_002235
Plasmodium hylobati	Gibbon	AB354573 (this study)
Plasmodium cynomolgi	Asian OWMs	AB434919 (this study)
Plasmodium simiovale	Asian OWMs	AB434920 (this study)
Plasmodium fieldi	Asian OWMs	AB354574 (this study)
Plasmodium inui	Asian OWMs	AB354572 (this study)
Plasmodium fragile	Asian OWMs	AY722799
Plasmodium coatneyi	Asian OWMs	AB354575 (this study)
Plasmodium knowlesi	Asian OWMs	NC_007232
Plasmodium gonderi	African OWMs	AB434918 (this study)
Plasmodium yoelii	Rodents	M29000
Plasmodium chabaudi	Rodents	AF014116
Plasmodium relictum	Birds	AY733090
Plasmodium gallinaceum	Birds	AB250690
Plasmodium juxtanucleare	Birds	AB250415
Plasmodium mexicanum	Lizards	AB375765 (this study)
Plasmodium floridense	Lizards	NC_009961
Theileria parva	Cattle	Z23263

As for genomic PCR of *P. gonderi*, four primers (PgeneralF2s, PgeneralR2, PgeneralF3s, and PgeneralR1s) were designed. PCR using these primers was performed under the following conditions: denaturation at 93 °C for 1 min followed by 40 cycles of 93 °C for 20 s, 60 °C for 1 min, 72 °C for 3 or 5 min, and extension at 72 °C for 10 min.

To amplify the whole mitochondrial genome from *P. mexicanum*, four PCR primers (PmemtF2, PmemtF3, PmemtR2, and PmemtR3) were designed based on the partial sequence of *P. mexicanum* cytochrome *b* gene (GenBank accession number AY099060). The PCR conditions were as follows: denaturation at 93 °C for 1 min followed by 40 cycles of 93 °C for 20 s; 60 °C for 1 min; 72 °C for 1 or 7 min; and extension at 72 °C for 10 min. The primer sequences used in this study are given in supplementary table 1 (Supplementary Material online).

The PCR products were purified using the QIAquick PCR purification Kit (Qiagen) and directly sequenced on an ABI 3130 genetic analyzer (Applied Biosystems). The GenBank accession numbers of sequences determined in this study are given in table 1.

The complete or partial sequences of the mitochondrial genomes of *Plasmodium falciparum*, *P. vivax*, *Plasmodium reichenowi*, *Plasmodium fragile*, *Plasmodium knowlesi*, *Plasmodium yoelii*, *Plasmodium chabaudi*, *Plasmodium relictum*, *Plasmodium gallinaceum*, *Plasmodium juxtanucleare*, *Plasmodium floridense*, and *Theileria parva* were obtained from the NCBI Web site (http://www.ncbi.nlm.nih.gov/). The GenBank accession numbers of sequences used in our analyses are given in table 1.

Sequence Analysis

DNASIS software (Hitachi, Tokyo, Japan) was used to assemble sequences. There are three protein-coding genes on the *Plasmodium* mitochondrial genome: cytochrome *c* oxidase III, cytochrome *c* oxidase I, and cytochrome *b* genes (Aldritt et al. 1989; Vaidya et al. 1989). The entire mitochondrial genome is regarded as a single genetic unit in tracing evolutionary history because there is no evidence of recombination and positive selection (Joy et al. 2003). Thus, the amino acid sequences of the three gene products were concatenated and then used for our analyses. The lengths of concatenated amino acid sequences used were 1100–1167 amino acids (for the details, see supplementary table 2, Supplementary Material online). Phylogenetic tree construction and relative rate tests (Tajima 1993) were performed using MEGA2 software (Kumar et al. 2001). Branch length tests were performed using LINTREE program (Takezaki et al. 1995). Phylogenetic trees were constructed using Neighbor–Joining (Saitou and Nei 1987) and UPGMA methods with gamma distances. The gamma shape parameter (α) was estimated using the PAML program (Yang 1997).

Results and Discussion

Phylogenetic Relationship among *Plasmodium* Species

We used the mitochondrial genome of *Plasmodium* species in this study. It contains three protein-coding genes involved in respiration: cytochrome *c* oxidase subunits I and III and cytochrome *b* genes (Aldritt et al. 1989; Vaidya et al. 1989). The mitochondrial genome undergoes no recombination and is free from positive selection pressure (Joy et al. 2003). It has previously been reported that the differences in G + C content between sequences can bias the estimation of tree topology due to codon usage difference (Sueoka 1961; Crozier RH and Crozier YC 1993). However, because the G + C content is similar among the mitochondrial genomes of all *Plasmodium* species used in this study (see supplementary table 2, Supplementary Material online), the possibility of such a topological bias needs not be considered here. Taken together, the mitochondrial genome is considered as an ideal target for phylogenetic analysis. A phylogenetic tree of 21 *Plasmodium* species including primate, rodent, bird, and reptile malaria parasites was constructed using the amino acid sequences of all three mitochondrial protein-coding genes (fig. 1). This phylogenetic tree demonstrates that the extant malaria parasite species can be divided into four major lineages based on host category: 1) *P. malariae*/*P. ovale*/*P. hylobati*/Old World monkey (OWM) parasites (primate parasite group 1), 2) rodent parasites, 3) *P. falciparum*/*P. reichenowi* (primate parasite group 2), and 4) bird/reptile parasites (fig. 1). The bird/reptile malaria parasite lineage (*P. relictum*, *P. gallinaceum*, *P. juxtanucleare*, *P. mexicanum*, and *P. floridense*) diverges from the other lineages at the root of the tree. The primate parasite group 2 lineage branches off from the primate parasite group 1 lineage/rodent parasite lineage, followed by the divergence of primate parasite group 1 lineage from the rodent parasites (*P. yoelii* and *P. chabaudi*). The primate parasite group 1 lineage consists of two sublineages: two human malaria parasites, *P. malariae* and *P. ovale*, compose one sublineage (fig. 1) and another sublineage consists of one human malaria parasite (*P. vivax*), a gibbon malaria parasite (*P. hylobati*), and

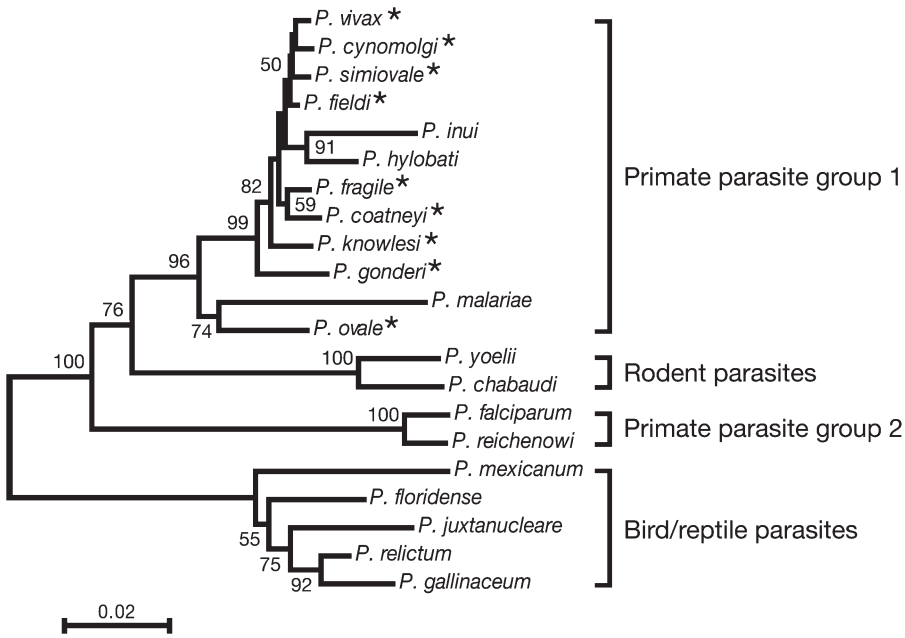

FIG. 1.—The phylogenetic relationships between primate, rodent, and bird malaria parasites. Neighbor–Joining tree of amino acid sequences of three mitochondrial genes was constructed with gamma distance ($\alpha = 0.3$). Numbers on the phylogenetic tree represent bootstrap values based on 1000 replications. The *Plasmodium* species used here can be divided into two groups depending on mutation rate: groups A and B. Species with stars are members of group A and the rest of the species belong to group B.

the OWM malaria parasites (*P. gonderi*, *P. cynomolgi*, *P. simiovale*, *P. fieldi*, *P. inui*, *P. fragile*, *P. coatneyi*, and *P. knowlesi*). Within the sublineage containing OWM parasites, an African OWM malaria parasite, *P. gonderi*, is the earliest to diverge from the others, showing separate Asian and African clades of OWM malaria parasites. The overall phylogenetic relationship shown in figure 1 is generally consistent with those previously obtained from cytochrome *b*, small subunit ribosomal RNA (SSU rRNA), and circumsporozoite protein genes (CSP) (Escalante and Ayala 1994; Escalante et al. 1995, 1998).

Recently, these separate clades of OWM malaria parasites have been contradicted based on the genomic deletion (~100 bp) shared between *P. gonderi*, *P. simiovale*, and *P. cynomolgi* (Roy and Irimia 2008). However, the complete sequences of these three malaria parasites that we obtained in this study have no such genomic deletion. The reported "deletion" probably corresponds to a short region of sequence encompassing the priming sites that were originally used to obtain the near-complete mitochondrial genome sequences by the original sequence depositors (Mu et al. 2005). We therefore conclude that separate Asian and African clades of OWM parasites are well supported in our analysis.

Comparison of Evolution between Malaria Parasites and Their Hosts

A comparison of the phylogenetic tree of malaria parasites with that of their hosts reveals two topological matches (fig. 2). One match lies between *P. gonderi*–Asian OWM malaria parasites and African OWMs–Asian OWMs (either macaque or colobine monkeys) (fig. 2*B*). Another match is seen between the *P. falciparum*–*P. reichenowi* and human–chimpanzee relationships (fig. 2*C*). It is therefore assumed that parasite–host codivergence occurred between OWM malaria parasites and OWMs and between *P. falciparum*/*P. reichenowi* and humans/chimpanzees.

One possible mechanism for parasite–host codivergence is through geographical isolation of two populations of host species which consequently undergo divergence. This would also lead to the geographical isolation of the parasites of the two host populations, resulting in parasite species divergence. Geographical isolation is evidently considered as the evolutionary driving force in the Asian OWMs–African OWMs divergence (Stewart and Disotell 1998) but not in the human–chimpanzee divergence (contradiction between Coppens [1994] and McBrearty and Jablonski [2005]). It has been reported that both OWM malaria parasites and OWMs originated in Africa (Escalante et al. 1998; Stewart and Disotell 1998). Colobine and macaque monkeys migrated into the Eurasian continent from Africa 10 MYA and 6 MYA, respectively (Stewart and Disotell 1998), and became the ancestors of extant Asian OWM species. This evolutionary history means that the divergence between African OWMs and Asian OWMs was initiated by geographical isolation and that the ancestors of Asian OWM malaria parasites migrated into Eurasia along with their hosts. Asian OWM malaria parasite species infect both colobine and macaque monkeys (Coatney et al. 2003). It is, therefore, highly likely that the divergence between *P. gonderi* and Asian OWM malaria parasites was caused by intercontinental allopatric speciation along with their hosts either 6 or 10 MYA.

Divergence Times among Malaria Parasite Lineages

To obtain a reliable estimate of the divergence time of *Plasmodium* lineages, a molecular clock must be validated

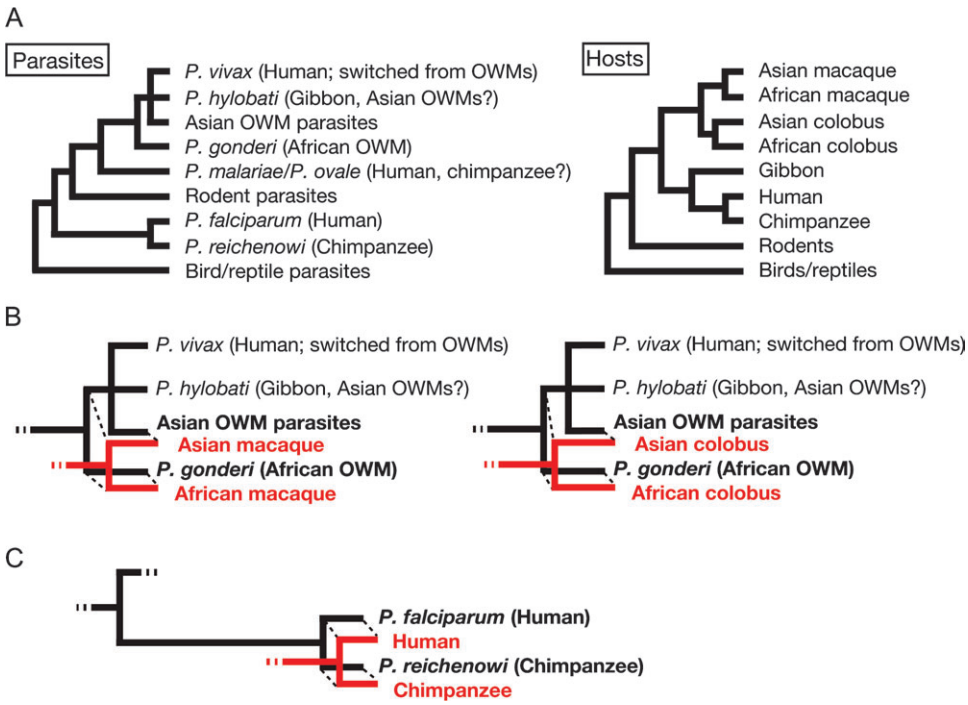

FIG. 2.—Comparison of phylogenetic trees between parasites and hosts. (*A*) Parasite tree and host tree. The phylogenetic tree shown in figure 1 is simplified here and shown as the parasite tree. Host category is given in parentheses on the parasite tree on the basis of Coatney et al. (2003). Note that *Plasmodium vivax* is considered to have become a human parasite by a host-switch event from an OWM parasite (Escalante et al. 2005; Mu et al. 2005). Also, *Plasmodium hylobati* is most closely related to *Plasmodium inui*, an Asian OWM parasite (see fig. 1), and can be maintained in Asian OWMs (Coatney et al. 2003). Hence, *P. vivax* and *P. hylobati* are evolutionarily regarded as members of Asian OWM parasites. Because *Plasmodium malariae* and *Plasmodium ovale* are possibly found from chimpanzees, *Plasmodium falciparum* is the sole parasite that infects only humans (Coatney et al. 2003). The phylogenetic relationships between primates, rodents, and birds are well characterized by both molecular and fossil record data (Kumar and Hedges 1998; Stewart and Disotell 1998; Bromham et al. 1999; Nei et al. 2001) and are summarized in the host tree. In this host tree, two OWMs, that is, macaque and colobine monkeys, are subdivided into African and Asian groups. (*B*) Topological matches between OWM malaria parasites and their hosts. (*C*) A topological match between *P. falciparum–Plasmodium reichenowi* and human–chimpanzee.

for the *Plasmodium* mitochondrial genome. We examined evolutionary rate constancy by performing relative rate tests (Tajima 1993) and branch length tests (Takezaki et al. 1995). The relative rate tests revealed consistent rates of amino acid substitution in *P. vivax*, *P. cynomolgi*, *P. simiovale*, *P. fieldi*, *P. fragile*, *P. coatneyi*, *P. knowlesi*, *P. gonderi*, and *P. ovale* (members of group A in fig. 1). *Plasmodium malariae*, *P. inui* and *P. hylobati* exhibit discrepancies in substitution rate compared with other OWM malaria parasites, *P. vivax* and *P. ovale* in relative rate tests. However, *P. malariae*, *P inui*, and *P. hylobati* show rate constancy with *P. yoelii*, *P. chabaudi*, *P. falciparum*, *P. reichenowi*, *P. relictum*, *P. gallinaceum*, *P. juxtanucleare*, *P. mexicanum*, and *P. floridense* in the branch length test using *T. parva*, a close relative of the malaria parasites, as an outgroup (group B in fig. 1). The *Plasmodium* species examined here can, therefore, be divided into two groups defined in terms of their substitution rates: group A and group B. Because a different molecular clock exists for each group, we constructed a UPGMA tree independently for each group in order to calculate divergence times (fig. 3). Such independent construction does not significantly affect the tree topology (see figs. 1 and 3).

One reliable calibration point for malaria parasite evolution is the divergence time between *P. gonderi* and Asian OWM malaria parasites. As mentioned above, *P. gonderi*–Asian OWM parasites divergence occurred either 6 or 10 MYA. Because *P. gonderi* and most Asian OWM malaria parasites belong to group A, we initially calculated the divergence times for this group by examining the genetic distances between species. The average pairwise genetic distance (d_b distance) between *P. gonderi* and Asian OWM malaria parasites is 0.0251 ± 0.0051 and that between *P. ovale* and OWM malaria parasites is 0.0488 ± 0.0079 (table 2). Assuming that the d_b distance between *P. gonderi* and Asian OWM malaria parasites corresponds to either 6 or 10 Myr, the divergence time between *P. ovale* and OWM malaria parasites is either 12 or 19 Myr (see table 2 and fig. 3*A*). The divergence time between *P. ovale* and OWM malaria parasites in group A is identical to that between *P. malariae* and *P. inui*/*P. hylobati* in group B (see fig. 1). To calculate the divergence times for group B, we therefore used 12 or 19 Myr for the divergence time between *P. malariae* and *P. inui*/*P. hylobati* (corresponding genetic distance of 0.0765 ± 0.0096; see table 2) as the calibration point. The d_b distance between *P. malariae*/*P. inui*/*P. hylobati* and rodent malaria parasites (*P. chabaudi* and *P. yoelii*), which is 0.1033 ± 0.0103, yields a divergence time of 16.2 ± 1.6 Myr or 25.7 ± 2.6 Myr (table 2 and fig. 3*B*). The d_b distances between *P. falciparum*/*P. reichenowi* and *P. malariae*/*P. inui*/*P. hylobati*/rodent malaria species (0.1280 ± 0.0115) and between bird/reptile

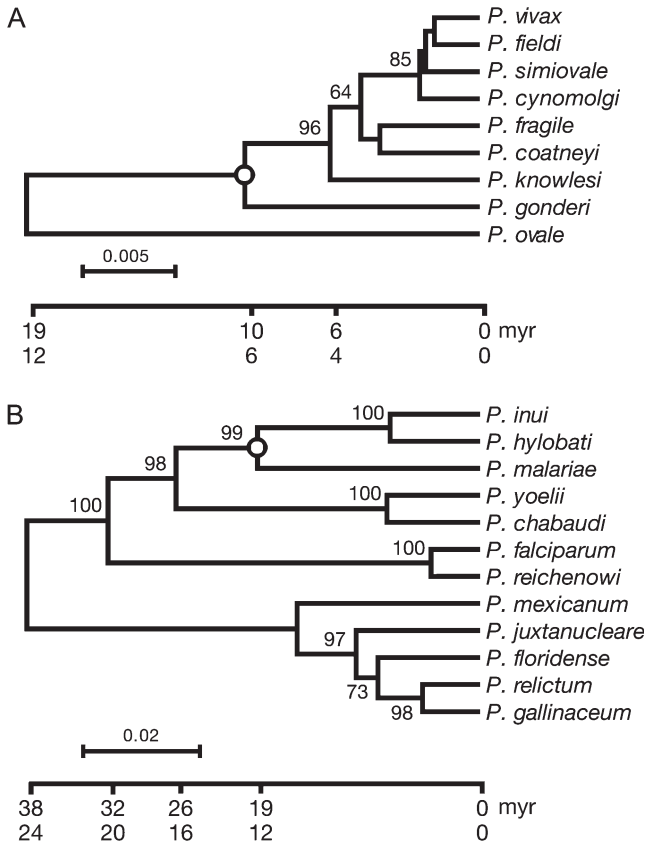

FIG. 3.—Separate tree construction for two malaria parasite groups and the divergence times among malaria parasite lineages. A UPGMA tree was separately constructed for groups A and B because of a difference in mutation rates between the two groups. Numbers on the phylogenetic tree represent bootstrap values based on 1000 replications. The time frame for parasite evolution is given under the trees. The timing calculation was performed by assuming that the divergence time between *Plasmodium gonderi* and Asian malaria parasites is 6 or 10 Myr. The open circle indicates the calibration time point used for the timing calculation. (*A*) The UPGMA tree ($\alpha = 0.1$) of group A. (*B*) The UPGMA tree ($\alpha = 0.3$) of group B.

malaria parasites and others (0.1545 ± 0.0128) gives 20.1 ± 1.8 Myr or 31.8 ± 2.9 Myr and 24.2 ± 2.0 Myr or 38.4 ± 3.2 Myr, respectively (table 2 and fig. 3*B*).

The four major malaria parasite lineages, primate parasite group 1, primate parasite group 2, rodent parasites, and bird/reptile malaria parasites, diverged rapidly during the early phase of the evolution of the extant malaria parasite lineages (incipient rapid diversification; see fig. 4). The estimated timing of this incipient rapid diversification is either 16–24 MYA or 26–38 MYA. Surprisingly, this timing is much later than the divergence times of their hosts (75–310 MYA; 75 Myr of primate–rodent divergence; 310 Myr of mammal–bird divergence; Kumar and Hedges 1998; Bromham et al. 1999; Nei et al. 2001; Waterston et al. 2002; fig. 4). This large discrepancy in divergence times between parasites and hosts indicates that the incipient rapid diversification was not caused by codivergence along with hosts. Host-switch events, therefore, must have contributed to the rapid divergence between the four major parasite lineages.

Table 2
Divergence Timings among Malaria Parasite Lineages

Group	Divergence[a]	Genetic distance[b]	Timing (Myr)	
A	*Plasmodium gonderi* and Asian OWM parasites	0.0251 ± 0.0051	10[c]	6[c]
	Plasmodium ovale and others	0.0488 ± 0.0079	19.4 ± 3.1	11.7 ± 1.9
	Plasmodium knowlesi and other Asian OWM parasites	0.0159 ± 0.0035	6.3 ± 1.4	3.8 ± 0.8
B	*Plasmodium malariae* and *Plasmodium hylobati*/*Plasmodium inui*	0.0765 ± 0.0096	19[c]	12[c]
	Bird/reptile lineage and others	0.1545 ± 0.0128	38.4 ± 3.2	24.2 ± 2.0
	Plasmodium falciparum/*Plasmodium reichenowi* lineage and Rodent parasites/*P. malariae*/*P. hylobati*/*P. inui*	0.1280 ± 0.0115	31.8 ± 2.9	20.1 ± 1.8
	Rodent lineage and *P. malariae*/*P. hylobati*/*P. inui*	0.1033 ± 0.0103	25.7 ± 2.6	16.2 ± 1.6
	P. falciparum and *P. reichenowi*	0.0160 ± 0.0038	4.0 ± 0.9	2.5 ± 0.6
	Plasmodium yoelii and *Plasmodium chabaudi*	0.0311 ± 0.0056	7.7 ± 1.4	4.9 ± 0.9

[a] Three incipient divergences producing four major lineages are underlined.
[b] Gamma distance.
[c] Calibration time point of each group.

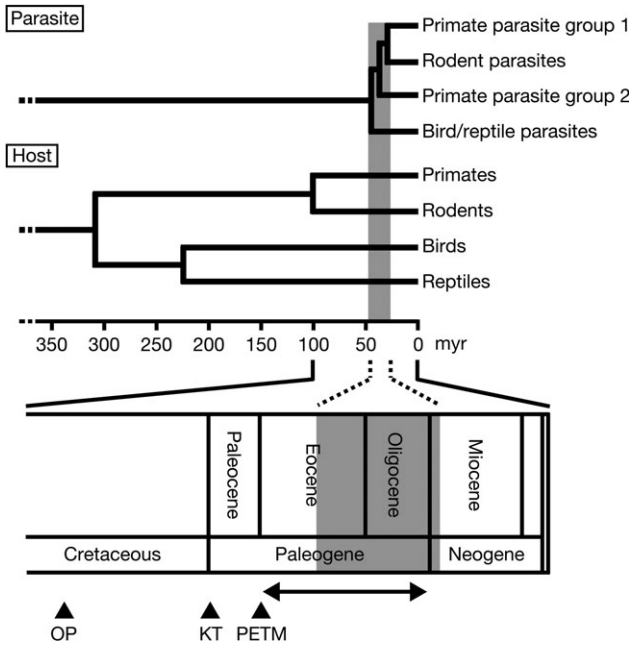

FIG. 4.—Evolution of malaria parasite lineages and their host lineages. Parasite divergence times based on a calibration point of 10 Myr are employed here (see main text for details). Divergence times between host lineages are based on Kumar and Hedges (1998), Bromham et al. (1999), Nei et al. (2001), and Waterston et al. (2002). The shaded areas represent the period of the incipient rapid diversification of extant malaria parasite lineages. The double-headed arrow indicates the period of rapid radiation of mammalian genera (Bininda-Emonds et al. 2007). OP, origin of placental superorders and orders (~93 Myr; Bininda-Emonds et al. 2007); KT, Cretaceous/Tertiary boundary (extinction of dinosaurs) (65 Myr; Bininda-Emonds et al. 2007); PETM, Paleocene–Eocene thermal maximum (~55 Myr; Gingerich 2006).

The use of 6 or 10 Myr as a calibration point, that is, the divergence time of the *P. gonderi* and the Asian OWM malaria parasite species, gives 2.5 ± 0.6 Myr or 4.0 ± 0.9 Myr for the *P. falciparum–P. reichenowi* divergence time, respectively (table 2). Because the latter value (4.0 ± 0.9 Myr) is closer to the accepted human–chimpanzee divergence time (5–7 Myr; Haile-Selassie 2001; Brunet et al. 2002), the calibration time of 10 Myr is more likely considering the parasite–host topological match between the *P. falciparum–P. reichenowi* and human–chimpanzee relationships (fig. 2*C*). Also, the use of 10 Myr is supported by the fact that the diversification timing of Asian OWM malaria parasites (6.3 ± 1.4 Myr; see fig. 3*A* and table 2) is consistent with the time at which colobine fossils become abundant in Southern Asia (7 MYA; Stewart and Disotell 1998).

The previous estimates of parasite divergence times based on the phylogenetic trees of the SSU rRNA and CSP genes are much older than our estimates (Escalante and Ayala 1994; Escalante et al. 1995). For example, the divergence time of OWM parasites/rodent parasites/*P. malariae* and *P. falciparum*/*P. reichenowi* is 165 Myr, whereas it is 32 or 20 Myr in this study. However, these older estimates have suffered from weaknesses in methodology that were previously accepted for compelling reasons: these are 1) the application of an evolutionary rate obtained from prokaryotic endosymbionts of aphids (1% or 2% per 50 Myr in the SSU rRNA gene) to malaria parasites and 2) the unexamined adoption of the same mutation rate in the gene to all *Plasmodium* lineages. In contrast, our estimation of divergence times is free from these methodological weaknesses.

We compared amino acid substitution rates between mitochondrial genes and the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, a housekeeping gene of nuclear genome. The substitution rates in mitochondrial genes from both groups A and B are comparable to that of the G3PDH gene (see supplementary table 3, Supplementary Material online). It seems unlikely, therefore, that the mitochondrial genes show exceptionally high or low rates of amino acid substitution compared with housekeeping genes of nuclear genome.

A Possible Scenario of Malaria Parasite–Host Coevolution

Our estimation of parasite divergence times has led us to conclude that the incipient rapid diversification began around the latter half of Eocene epoch (fig. 4). A dramatic radiation of mammalian genera occurred at the Eocene epoch (Bininda-Emonds et al. 2007), resulting in an abundance of new potential host species (see fig. 4). This acceleration of mammalian diversification continued into the Oligocene epoch (Bininda-Emonds et al. 2007) and coincides with the incipient rapid diversification of extant malaria parasite lineages (see fig. 4). Thus, the incipient rapid diversification of extant malaria parasite lineages could have been a consequence of the adaptive radiation to new mammalian hosts via host-switch events. Because the mammalian malaria parasite and bird/reptile malaria parasite lineages diverged at the root of the phylogenetic tree (see figs. 1, 3*B*, and 4), the common ancestor of the extant malaria parasites is most likely to have been a bird or reptile malaria parasite.

This study demonstrates that host-switch events principally drove the evolution of the extant *Plasmodium* species and highlights the parasite's ability to switch between host species, which has been previously underestimated, as an important adaptive trait of the parasite.

Supplementary Materials

Supplementary tables 1–3 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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