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Peer reviewed

African apes as reservoirs of *Plasmodium falciparum* and the origin and diversification of the *Laverania* subgenus

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Contributed by Francisco J. Ayala, April 21, 2010 (sent for review February 26, 2010)

We investigated two mitochondrial genes (*cytb* and *cox1*), one plasmid gene (*tufA*), and one nuclear gene (*ldh*) in blood samples from 12 chimpanzees and two gorillas from Cameroon and one lemur from Madagascar. One gorilla sample is related to *Plasmodium falciparum*, thus confirming the recently reported presence in gorillas of this parasite. The second gorilla sample is more similar to the recently defined *Plasmodium gaboni* than to the *P. falciparum*–*Plasmodium reichenowi* clade, but distinct from both. Two chimpanzee samples are *P. falciparum*. A third sample is *P. reichenowi* and two others are *P. gaboni*. The other chimpanzee samples are different from those in the ape clade: two are *Plasmodium ovale*, and one is *Plasmodium malariae*. That is, we have found three human *Plasmodium* parasites in chimpanzees. Four chimpanzee samples were mixed: one species was *P. reichenowi*; the other species was *P. gaboni* in three samples and *P. ovale* in the fourth sample. The lemur sample, provisionally named *Plasmodium malagasi*, is a sister lineage to the large cluster of primate parasites that does not include *P. falciparum* or ape parasites, suggesting that the *falciparum* + ape parasite cluster (*Laverania* clade) may have evolved from a parasite present in hosts not ancestral to the primates. If malignant malaria were eradicated from human populations, chimpanzees, in addition to gorillas, might serve as a reservoir for *P. falciparum*.

ape malaria | human malaria | *Plasmodium malagasi* | *Plasmodium* phylogeny

There is a revolution afoot concerning our understanding of human malaria. It was shown in 1994/1995 that the closest relative of *Plasmodium falciparum*, the agent of malignant malaria was *Plasmodium reichenowi*, a chimpanzee parasite, the only ape malaria parasite that had been molecularly characterized (1–4). The close phylogenetic relationship between *P. falciparum* and *Plasmodium reichenowi*, their distinctness from the three other known human malaria parasites (*Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*), as well as from other primate parasites, and their remoteness from bird or lizard parasites, was soon confirmed by other studies (5–7). It was assumed, as a working hypothesis, that *P. falciparum* and *P. reichenowi* had evolved from a common ancestor parasite, independently in their respective hosts, humans and chimpanzees, as these two lineages gradually diverged from one another over the last 5–7 million years—the cospeciation hypothesis. Two alternative hypotheses—either (i) a human origin (*P. reichenowi* evolved from an introduction of *P. falciparum* into chimpanzee hosts) or (ii) a chimpanzee origin (*P. falciparum* evolved from the introduction of *P. reichenowi* into the human lineage)—could not be tested

against each other or against the cospeciation hypothesis, because only one *P. reichenowi* isolate was available, which had been isolated from a captive chimpanzee.

It was soon demonstrated by Rich et al. (8), Rich and Ayala (9), and Ayala and Rich (10) that *P. falciparum* has very low levels of neutral genetic polymorphism, a result that was subsequently confirmed by other investigators (11–15). The scarcity of neutral polymorphism was explained as the result of a recent world expansion of *P. falciparum*—the Malaria's Eve hypothesis (8)—which was estimated to have happened starting a few thousand years ago, rather than millions of years ago, as expected according to the cospeciation hypothesis. Martin et al. (16) suggested a mechanism compatible with a relatively recent origin of *P. falciparum* from *P. reichenowi* based on the differential expression of host sialic acid (Sia) ligands that populate the surface of erythrocytes and other cells, and on differences in Sia-binding preferences among parasite receptors. A mutation in the human lineage inactivated the *CMAH* gene rendering human ancestors unable to generate the Sia Neu5Gc from its precursor Neu5Ac, thus making humans resistant to *P. reichenowi*. A later mutation in the dominant invasion receptor *EBA175* provided the *P. falciparum* lineage with preference for the overabundant Neu5Ac precursor, accounting for its extreme human pathogenicity (16, 17).

Rich et al. (18) have recently analyzed, in eight new isolates of *P. reichenowi*, gene fragments from the three principal genomes of *Plasmodium* parasites, mitochondrial (*cytB*), apicoplast (*clpC*), and nuclear (*18S rDNA*). The isolates were obtained from blood samples of wild and wild-born captive chimpanzees in Cameroon and Côte d'Ivoire. The genetic analysis shows that *P. reichenowi* is a geographically widespread and genetically very diverse chimpanzee parasite. The genetic lineage comprising the totality of global *falciparum* is fully included within the much broader genetic diversity of *P. reichenowi*. The genetic analysis supports the hypothesis proposed by the authors that all extant populations of *P. falciparum* originated from *P. reichenowi*, likely by a single host transfer, which may have occurred as early as 2–3 million years ago or as recently as 10,000 years ago (18). This hypothesis is consis-

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The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. HM000105 and HM000122).

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tent with the hypothesis proposed by Martin et al. (16) and Varki and Gagneux (17) of the two successive mutational events, first in our human ancestors and then in the parasite, which account for the extreme pathogenicity of *P. falciparum*.

Other recent developments in the malaria story include the discovery of new *Plasmodium* species parasitic to apes, and the presence in chimpanzees and gorillas of *Plasmodium* parasites previously thought to be human specific. Moreover, a cercopithecoid parasite, *Plasmodium knowlesi*, has now been found in humans (19–22).

Duval et al. (23) screened DNA isolated from blood samples in captivity from 113 chimpanzees and 17 gorillas from Cameroon, initially kept as pets for variable time periods and then brought to local zoos or sanctuaries or confiscated by the Ministry of Environment and Forestry. Two chimpanzees from the subspecies *Pan troglodytes troglodytes* presented with single infections of *Plasmodium ovale*, molecularly characterized by means of partial fragments from three genes, the mitochondrial genes *cytochrome b* and *cytochrome c oxidase* and the nuclear gene *lactate dehydrogenase*.

Hayakawa et al. (24, 25) screened the blood of 60 captive chimpanzees, imported to Japan 30 years earlier, and obtained two isolates of *Plasmodium malariae*, molecularly characterized by a nearly complete sequence of the nuclear *small subunit rRNA* and the complete mitochondrial genome. Indigenous malaria disappeared in Japan more than 50 years ago. Thus, the two chimpanzees most likely were infected in Africa and the *P. malariae* parasites brought into Japan from Africa. One chimpanzee was a male from Sierra Leone, assumed to have been born in 1978, and exported to Japan in April 1980. The other chimpanzee is a female, assumed to have been born in 1976, and exported to Japan in March 1977 from an unrecorded African country of origin. Both chimpanzees have been asymptomatic to the present.

As pointed out above, *P. reichenowi* was identified in the 1990s as the sister lineage of *P. falciparum* (1–3) and had remained since then, until very recently, as the only known ape parasite that had been molecularly characterized. Ollomo et al. (26) obtained blood samples from 17 chimpanzees kept as pets by villagers, collected from different parts of Gabon. Two chimpanzees were infected by a new species named by the authors *Plasmodium gaboni*. Analysis of its complete mitochondrial genome showed that the new species is much more closely related to *P. falciparum* and *P. reichenowi* than to any other known *Plasmodium* species. Indeed, the three species make up a distinctive clade, considerably separated from the clade that includes all other known primate parasites, with *P. gaboni* about equally different from *P. falciparum* as from *P. reichenowi*. The time of divergence of *P. gaboni* from the clade making up *P. falciparum* and *P. reichenowi*, assuming that the divergence of the later two species occurred 4–7 million years ago, was estimated at $\sim 21 \pm 9$ Mya, a time congruent with the radiation of the hominoids, suggesting that the *Plasmodium* lineage encompassing the three species may have been present in early hominoids and may have diversified simultaneously with their hosts (26).

The recent discovery that *Plasmodium* can be genotyped from urine, saliva, and fecal primate samples (5, 27, 28) has opened up considerably the opportunities to explore the presence of *Plasmodium* in apes, as well as in other primates and in all other sorts of animals, by noninvasive methods. Prugnolle et al. (29) have analyzed partial *Cytb* sequences (704 nucleotides) from five chimpanzee and seven gorilla DNA samples, obtained from fecal samples from wild chimpanzees ($n = 125$) and gorillas ($n = 84$) from Cameroon, plus three blood samples from captive wild-born gorillas from Gabon. The chimpanzee isolates clustered with either *P. reichenowi* or *P. gaboni*, congruent with previous observations. The gorilla isolates, however, clustered into three different groups. Two isolates identify new lineages and potentially new species. One lineage, labeled *Plasmodium GorA*, was a sister lineage to *P. gaboni*; the second lineage, *Plasmodium GorB*, clustered

with the clade made up of *P. reichenowi* plus *P. falciparum*. Surprisingly, three gorilla samples, two fecal Cameroon samples and one blood sample from a captive gorilla from Gabon, turned out to be *P. falciparum*. The presence of *P. falciparum* in nonhuman primates had not been previously reported.

Also recently, Krief et al. (30) found four sequences related to *P. falciparum* and two related to *P. malariae* in blood samples from bonobos (*Pan paniscus*). In chimpanzees (*Pan troglodytes*), the investigators found, in addition to *P. reichenowi*, two sequences related to *P. vivax*, as well as two newly named species, *Plasmodium billbrayi* (four sequences) and *Plasmodium billcollinsi* (three sequences), which form a monophyletic clade with the previously discovered *P. gaboni* (see below).

Results

We investigated two mitochondrial genes (*cytB* and *cox1*), one plastid gene (*tufA*) and one nuclear gene (*ldh*) in blood samples positive for *Plasmodium* from 12 chimpanzees and two gorillas from Cameroon and one lemur from Madagascar. One gorilla sample (GGcam10) belongs within the *P. falciparum* cluster, thus confirming the presence of *P. falciparum*-related parasites in gorillas, as reported by Prugnolle et al. (29). The second gorilla sample (GGcam13) appears to be closest to *P. gaboni*, although statistically not closer to *gaboni* than to the *falciparum*–*reichenowi* clade. The chimpanzee samples proved to be extremely diversified. Two samples (CPZcam46 and CPZcam137) cluster with *P. falciparum*, suggesting that this parasite is present not only in gorillas, as already shown by Prugnolle et al. (29) and as observed by us, and in bonobos (30), but also in chimpanzees. A third chimpanzee sample (CPZcam61) appears to be *P. reichenowi*, and two others (CPZcam80 and CPZcam155) associate with *P. gaboni*. The other chimpanzee samples are very different from those in the ape cluster. Two samples (CPZcam89 and CPZcam91) are *P. ovale*; these are the same samples already reported (23). Another sample (CPZcam83) is similar to *P. malariae*. That is, at least three human *Plasmodium* parasite species have also been found in chimpanzees. Four chimpanzee samples manifested mixed infections: three samples (CPZcam72, CPZcam86, and CPZcam103) had mixed infections of *P. reichenowi* and *P. gaboni*, and one sample (CPZcam63) of *P. reichenowi* and *P. ovale*.

The lemur *Plasmodium* sample appears in our tree as a sister lineage to a large cluster of cercopithecoid and hominid primates, which does not include *falciparum* or the ape parasites, thus suggesting that this cluster must have originated from a parasite present in hosts that were not ancestral to the primates at large.

We screened a total of 113 chimpanzee, 17 gorilla, and 55 lemur DNA samples for *Plasmodium* parasite infection by PCR using two mitochondrial partial genes, *cytochrome b* (*cyt b*) and *cytochrome c oxidase 1* (*cox1*). Two additional partial genes were sequenced for the purpose of confirming certain results, the nuclear gene *lactate dehydrogenase* (*ldh*) and the apicoplast elongator gene *tufA*. Twelve chimpanzees (7 *Pan t. troglodytes* and 5 *Pan t. vellerosus*) and two gorillas (*Gorilla gorilla*) from Cameroon and one lemur (*Propithecus verreauxi*) from Madagascar were infected with *Plasmodium* parasites (Table 1). As shown in Table 1, four chimpanzees had mixed infections consisting of *P. reichenowi* and either *P. gaboni* (three cases) or *P. ovale* (one case). The mixed-infection sequences were not used in the phylogenetic reconstruction.

Figure 1 shows the phylogeny of the remaining newly obtained sequences (eight from chimpanzees, two from gorillas, and one from a lemur) together with relevant sequences from other *Plasmodium* species, listed in Table 2. The phylogenetic analysis was carried out using a concatenate sequence of 1672 bp, consisting of partial *cyt b* (708 bp) and *cox1* (964 bp) genes. Maximum likelihood and Bayesian methods were used. Identical topologies were obtained by both methods. Bayesian posterior probabilities and bootstrap values are both given in Fig. 1. The

Table 1. Gorilla, chimpanzee, and lemur samples analyzed for *cytb* and *cox1* partial gene sequences: Sample code, host species, sample collection date, gender, age, *Plasmodium* strain, and GenBank accession number

Sample code	Host	Sample collection date	Gender, age	<i>Plasmodium</i> strain	GenBank accession no.	
					<i>cyt b</i>	<i>cox1</i>
GGcam10	<i>Gorilla gorilla</i>	March 2000	M, <6 mo	<i>P. falciparum</i>	HM000105	HM000114
GGcam13	<i>Gorilla gorilla</i>	April 1999	M, 1 y	<i>P. GorB</i>	HM000106	HM000115
CPZcam46	<i>Pan t. vellerosus</i>	February 2002	M, 3 y	<i>P. falciparum</i>	HM000107	HM000116
CPZcam61	<i>Pan t. troglodytes</i>	February 1999	F, NA	<i>P. reichenowi</i>	HM000108	HM000117
CPZcam63	<i>Pan t. vellerosus</i>	September 1998	M, adult	<i>P. reichenowi/P. ovale*</i>	AJ251941/ FJ409564	AJ251941/ FJ409568
CPZcam72	<i>Pan t. troglodytes</i>	February 2000	F, 2 y	<i>P. reichenowi/P. gaboni*</i>	AJ251941/ FJ895307	AJ251941/ FJ895307
CPZcam80	<i>Pan t. troglodytes</i>	July 2001	M, 1 y	<i>P. gaboni</i>	HM000109	HM000118
CPZcam83	<i>Pan t. vellerosus</i>	April 1999	F, juvenile	<i>P. malariae</i>	HM000110	HM000119
CPZcam86	<i>Pan t. vellerosus</i>	February 2000	M, 1 y	<i>P. reichenowi/P. gaboni*</i>	AJ251941/ FJ895307	AJ251941/ FJ895307
CPZcam89	<i>Pan t. troglodytes</i>	February 2000	F, juvenile	<i>P. ovale</i>	FJ409565	FJ409569
CPZcam91	<i>Pan t. troglodytes</i>	February 2001	M, adult	<i>P. ovale</i>	FJ409564	FJ409568
CPZcam103	<i>Pan t. vellerosus</i>	April 2000	M, 1 y	<i>P. reichenowi/P. gaboni*</i>	AJ251941/ FJ895307	AJ251941/ FJ895307
CPZcam137	<i>Pan t. troglodytes</i>	February 2003	F, NA	<i>P. falciparum</i>	HM000111	HM000120
CPZcam155	<i>Pan t. troglodytes</i>	March 2004	M, 1.5 y	<i>P. gaboni</i>	HM000112	HM000121
Lemur	<i>Propithecus verreauxi</i>	November 2002	M, adult	<i>Plasmodium malagasi</i>	HM000113	HM000122

M, male; F, female; NA, not available.
*Mixed infections.

topology of the reference sequences (Table 2) is consistent with previously published phylogenies (5, 24).

Five chimpanzees, CPZcam61 plus the four chimpanzees with mixed infection (CPZcam63, CPZcam72, CPZcam86, and CPZcam103), were infected with *P. reichenowi*, the parasite previously considered chimpanzee specific but recently shown to infect gorillas as well (29). Five chimpanzees are infected with *P. gaboni*, the recently identified chimpanzee parasite (26, 29): CPZcam80, CPZcam155, and the doubly infected CPZcam72, CPZcam86, and CPZcam103. One gorilla, GGcam13, is infected with a strain identical to the recently discovered gorilla parasite named *P. GorB* by Prugnolle et al. (29).

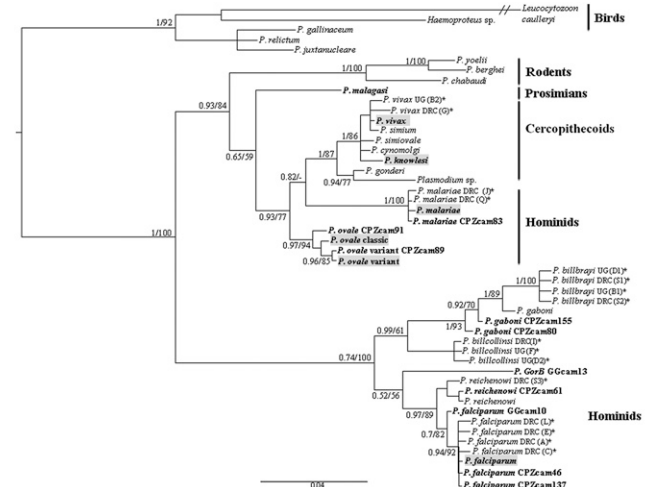


Fig. 1. Phylogeny of *Plasmodium* species inferred from *cyt b* and *cox1* nucleotide sequences. Values are Bayesian posterior probabilities (left of slash) and bootstrap percentages obtained by maximum likelihood (right of slash). Values less than 0.55 or 55% are not shown. CPZcam, chimpanzee from Cameroon; GGcam, gorilla from Cameroon. Boldface type indicates species and strains discovered in this work; gray overlay represents human parasites. *Sequences from Krief et al. (30). DRC, Democratic Republic of Congo; UC, Uganda. *P. simium* is a parasite of New World monkeys that is genetically identical to *P. vivax*. Hosts are shown on the right. *P. knowlesi* is a simian parasite recently found in humans.

Six chimpanzees and one gorilla were infected with parasites previously thought to be human specific. One gorilla sample (GGcam10) belongs within the *P. falciparum* cluster, thus confirming the presence of *P. falciparum*-related parasites in gorillas, as reported by Prugnolle et al. (29). However we found two chimpanzees, CPZcam46 and CPZcam137, that were also infected with *P. falciparum*, and a chimpanzee, CPZcam83, infected with *P. malariae*. Krief et al. (30) found four bonobos also infected with *P. falciparum* and one bonobo infected with *P. malariae*. In addition, three chimpanzees are shown in Table 1 to be infected with *P. ovale*; one of the chimpanzees, CPZcam89, in a mixed infection (with *P. reichenowi*) and two more, CPZcam89 and CPZcam91, previously reported by Duval et al. (23). Of these two chimpanzees, CPZcam 89 was infected with “classic” *P. ovale*. The parasites from CPZcam91 are more similar to the “variant” *P. ovale* strain, although somewhat different from it, which might suggest the presence of a distinct strain of *P. ovale* (23).

The identification of strains GGcam10, CPZcam46, and CPZcam137 as *P. falciparum*-related parasites was confirmed by two additional partial gene sequences obtained from GenBank (Table 2): nuclear *ldh* (368 bp) and plasmid *tufA* (677 bp). The identification of CPZcam61 as *P. reichenowi* was confirmed by the *ldh* partial sequence (no *tufA* sequence of *P. reichenowi* was available from GenBank). The monophyly of the set of strains made up of the three *P. falciparum* and one *P. reichenowi* strains plus the *P. gaboni* CPZcam155 and CPZcam80, plus the *P. GorB* GGcam13 was confirmed by both *ldh* and *tufA* (as well as *cyt b* and *cox1*). The monophyly has 100% bootstrap support and 1.00 Bayesian posterior probability (Fig. 1). All *tufA* sequences of this monophyletic group (*Laverania* clade, discussed below) show a three-nucleotide insertion when compared with the *tufA* sequences of other *Plasmodium* parasites from GenBank. These three nucleotides coding for either a proline (CCT) or a serine (TCT) at position 263 in the protein sequence are specific to the *Laverania* parasites.

Of the 55 Madagascar lemur samples examined, one *Plasmodium* strain was isolated from *Propithecus verreauxi*. We provisionally name this strain *Plasmodium malagasi*. This parasite forms a monophyletic clade with the clade that includes all Cercopithecoid parasites plus *P. malariae* and *P. ovale* (Fig. 1). Notably, this larger monophyletic clade does not include the clade encompassing *P. falciparum*, *P. reichenowi*, *P. gaboni*, *P. GorA*, *P. GorB*, and the newly named (30) *P. billbrayi* and *P. billcollinsi* (29) (Fig. 1). Thus, the latter clade (*Laverania*, discussed below) evolved from

Table 2. *Cytb* and *cox1* DNA GenBank sequences used in the phylogeny presented in Fig. 1, with their natural hosts and accession numbers

<i>Plasmodium</i> parasites species	Natural hosts	GenBank accession no.	
		<i>cyt b</i>	<i>cox1</i>
<i>P. falciparum</i>	<i>Homo sapiens</i>	M76611	M76611
<i>P. falciparum</i> DRC (A)*	<i>Pan paniscus</i>	GQ355474	GQ355474
<i>P. falciparum</i> DRC (C)*	<i>Pan paniscus</i>	GQ355473	GQ355473
<i>P. falciparum</i> DRC (E)*	<i>Pan paniscus</i>	GQ355472	GQ355472
<i>P. falciparum</i> DRC (L)*	<i>Pan paniscus</i>	GQ355475	GQ355475
<i>P. vivax</i>	<i>Homo sapiens</i>	AY598139	AY598139
<i>P. vivax</i> DRC (G)*	<i>Pan t. troglodytes</i>	GQ355480	GQ355480
<i>P. vivax</i> UG (B2)*	<i>Pan t. schweinfurthii</i>	GQ355481	GQ355481
<i>P. ovale</i> classical	<i>Homo sapiens</i>	FJ409567	FJ409571
<i>P. ovale</i> variant	<i>Homo sapiens</i>	FJ409566	FJ409570
<i>P. malariae</i>	<i>Homo sapiens</i>	AB489194	AB489194
<i>P. malariae</i> DRC (J)*	<i>Pan paniscus</i>	GQ345486	GQ355486
<i>P. malariae</i> DRC (Q)*	<i>Pan paniscus</i>	GQ355485	GQ355485
<i>P. reichenowi</i>	<i>Pan t. troglodytes</i>	AJ251941	AJ251941
<i>P. reichenowi</i> DRC (S3)*	<i>Pan t. troglodytes</i>	GQ355476	GQ355476
<i>P. gaboni</i>	<i>Pan t. troglodytes</i>	FJ895307	FJ895307
<i>P. gaboni</i> DRC (S1)*	<i>Pan t. troglodytes</i>	GQ355468	GQ355468
<i>P. gaboni</i> DRC (S2)*	<i>Pan t. troglodytes</i>	GQ355469	GQ355469
<i>P. gaboni</i> UG (B1)*	<i>Pan t. schweinfurthii</i>	GQ355470	GQ355470
<i>P. gaboni</i> UG (D1)*	<i>Pan t. schweinfurthii</i>	GQ355471	GQ355471
<i>P. gaboni</i> DRC (I)*	<i>Pan t. troglodytes</i>	GQ355479	GQ355479
<i>P. gaboni</i> UG (D2)*	<i>Pan t. schweinfurthii</i>	GQ355477	GQ355477
<i>P. gaboni</i> UG (F)*	<i>Pan t. schweinfurthii</i>	GQ355478	GQ355478
<i>P. knowlesi</i>	Old World monkeys [†]	AY598141	AY598141
<i>P. simiovale</i>	Old World monkeys	AY800109	AY800109
<i>P. cynomolgi</i>	Old World monkeys	AY800108	AY800108
<i>P. gonderi</i>	Old World monkeys	AY800111	AY800111
<i>P. simium</i>	New World monkeys	AY800110	AY800110
<i>Plasmodium</i> sp.	Old World monkeys	AY800112	AY800112
<i>P. yoelii</i>	<i>Thamnomys rutilans</i>	M29000	M29000
<i>P. berghei</i>	<i>Grammomys surdaster</i>	AF014115	AF014115
<i>P. chabaudi</i>	<i>Thamnomys rutilans</i>	AF014116	AF014116
<i>P. gallinaceum</i>	<i>Gallus gallus</i>	AB250690	AB250690
<i>P. relictum</i>	Birds	AY099032	EU254593
<i>P. juxtannucleare</i>	<i>Gallus gallus</i>	AB250415	AB250415
<i>Haemoproteus</i> sp.	<i>Lichenostomus frenatus</i>	AY733087	AY733087
<i>Leucocytozoon caulleryi</i>	Birds	AB302215	AB302215

*Sequences from Krief et al. (30).

[†]*P. knowlesi* has recently been found also in human hosts (19–22).

parasites acquired by host transfer from an evolutionary lineage that had diverged from the primate lineage before the divergence between lemurs (prosimians) and primates, which occurred before the Cretaceous/Tertiary boundary, ~75–80 Mya (31, 32).

Discussion

Apes as Reservoirs for Human Malaria Parasites. Prugnolle et al. (29) identified *P. falciparum* in fecal samples from two wild gorillas in Cameroon (two distant localities) and in one blood sample from a captive gorilla from Gabon, and Krief et al. (30) found *P. falciparum* in blood samples from bonobos. The likely success of the campaign to eradicate malaria is hardly in sight. Were it to succeed, one consideration to keep in mind, as pointed out by Prugnolle et al. (29), is that gorillas might serve as a reservoir for malignant malaria and the possibility that humans might acquire *P. falciparum* by host transfer from gorillas, or from bonobos (30). We confirm the presence of *P. falciparum*-related parasites in gorillas, sample GGcam10 (Table 1 and Fig. 1). Moreover, we have found *P. falciparum* in blood samples from two chimpanzees belonging to two different subspecies, *Pan troglodytes vellerosus* (CPZcam46) and *P. troglodytes* (CPZcam137) (Table 1 and Fig. 1).

The possibility that African apes might serve as a reservoir for malignant malaria is thereby much increased.

We have found the human parasite *P. ovale* in samples from two chimpanzees (CPZcam89 and CPZcam91) of the subspecies *P. t. troglodytes* and in one sample (CPZcam63) from *P. t. vellerosus*. Krief et al. (30) found the human parasite *P. malariae* in two blood samples from bonobos, and we have found it in one sample (CPZcam83) from *P. t. vellerosus* chimpanzees. These chimpanzees were held in captivity. Increased contact with humans, relative to wild chimpanzees, may have facilitated cross-species malaria transmission. There is no information on the capacity of the human *Plasmodium* parasites to produce viable gametocytes in chimpanzees that would be required for transmission by mosquitoes from chimpanzees or bonobos to humans or to other chimpanzees, gorillas, or different primate species.

The increased contact of humans with apes in the wild raises the question whether likely transmissions of *P. falciparum* (and other human *Plasmodium* parasites) may endanger the survival of the ape species in the wild. There are no definitive answers to this question. Experimental infections with human blood infected with *P. falciparum* fail to induce malaria in chimpanzees (reviewed in

ref. 33). Nonsplenectomized chimpanzees experimentally infected with *P. falciparum* developed a low-grade parasitemia (up to 1,000/mm³) and maintained the infection without evidence of eliminating the parasites (34). Even after splenectomy to increase parasite survival, experimentally infected chimpanzees did not develop a parasitemia equivalent to that observed in humans (33, 34).

The strong human specificity of *P. falciparum* may be due to species-specific erythrocyte recognition profiles (16, 17). The mutational loss of the common primate Sia Neu5Gc may have protected our human ancestors from *P. reichenowi*. However, the major merozoite-binding protein (the erythrocyte-binding antigen-175, EBA-175) would have consequently evolved in *P. falciparum* to take advantage of the accumulated excess on human erythrocytes of the Neu5Gc precursor, the Sia Neu5Ac. These evolutionary changes would account for the reduced virulence of *P. falciparum* in apes and the presumed similar reduced virulence of *P. reichenowi* in humans. These two-way attenuations of virulence are consistent with the specific binding activities demonstrated by Martin et al. (16). The enhanced specificity of *falciparum* EBA-175 for the human Sia Neu5Ac (rather than for the ape Sia Neu5Gc) indicates that *P. falciparum* evolved in association with the hominid lineage, rather than in chimpanzees, bonobos, or gorillas. The presence of *P. falciparum* in these apes would therefore have resulted from host transfer from humans to apes. The older age and much greater genetic diversity of *P. reichenowi* compared with *P. falciparum* (18) also support the evolution of *P. falciparum* in the human lineage from ancestral *P. reichenowi* acquired in turn by host transfer from chimpanzees (18).

The Laverania Clade. *Plasmodium schwetzi* was originally described by Reichenow in 1920 in the blood of apes in Cameroon (33). Experimental infections by *P. schwetzi* in humans have also been reported (35). In 1970, Contacos asserted its potential as a zoonosis in Africa (36). *P. schwetzi* is morphologically similar to the two human pathogens, *P. vivax* and *P. ovale*. Arguments have been advanced in favor of one or the other of these species as the most closely related to *P. schwetzi* (33). No isolate of this parasite is available from which molecular sequences could be obtained to decide its phylogenetic position.

The only known genetic relative of *P. malariae* is *Plasmodium brasilianum* (37), a parasite of several species of New World monkeys. The two species are indistinguishable at the molecular level, so that a very recent host transfer between humans and monkeys must have occurred (1, 3, 38). *Plasmodium rodhaini* was reported in 1920 from blood smears of chimpanzees and gorillas in Cameroon. It was morphologically described as being similar to the human parasite *P. malariae* (33). Transmission studies with quartan parasites isolated from chimpanzees have shown that *P. rodhaini* is actually *P. malariae* (39).

Plasmodium falciparum was discovered by Alphonse Laveran in 1880; *P. falciparum* has been classified with its sister species, *P. reichenowi*, a malaria parasite of chimpanzees, into the subgenus *Laverania* because of specific characters such as aspects of life cycle, high antigenic polymorphism, crescent-shaped gametocytes, and long gametocytogenesis duration as compared with that of other human malaria species placed in the subgenus *Plasmodium* (33, 40). Our results show that *P. falciparum* and *P. reichenowi* belong to the same clade as the recently discovered *P. gaboni*, *P. GorA*, and *P. GorB* (29), and *P. billbrayi* and *P. billcollinsi* (30). The set of these seven monophyletic species may appropriately be included in the *Laverania* subgenus, and be referred to as the *Laverania* clade (41).

The evolutionary origin of the *Laverania* clade predates the divergence of the prosimian *P. malagasi* from the Cercopithecoidea clade, which may have occurred before 75–80 Mya. Currently, there seems to be no evidence of the *Plasmodium* parasites from which the *Laverania* clade evolved, or the nature of their hosts, or if and when a host transfer may have occurred from some remote ancestor to a more recent ancestor of the *Plasmodium Laverania*

clade. What we know is that such a host transfer must have occurred before ~10 Mya, the time of divergence of the gorilla lineage from the human-plus-chimpanzee lineage. Be that as it may, our results confirm that (i) as asserted by Rich et al. (18), Ollomo et al. (26), Prugnolle et al. (29), and Krief et al. (30), the molecular diversity of the *Laverania* clade is much greater than previously thought, and (ii) chimpanzees, bonobos, and gorillas are a potential reservoir of *P. falciparum*, as well as of *P. malariae* and *P. ovale*.

Materials and Methods

Most of the chimpanzees and gorillas used in the study originated from different areas of Cameroon, and were initially kept as pets for different periods of time and then brought to local primate facilities or confiscated by the Ministry of Environment and Forestry. These animals had blood samples extracted for virological studies at the Virology Unit of Centre Pasteur du Cameroon (42, 43). A DNA bank was established between 1998 and 2004.

In total, we tested 130 DNA samples from great apes for *Plasmodium* infection, using primarily *cytochrome b* (*cyt b*) molecular tools (44); the apes included 105 chimpanzees from four subspecies (60 *Pan t. troglodytes*, 39 *P. t. vellerosus*, three *P. t. schweinfurthii*, and three *P. t. verus*), eight chimpanzees of undetermined subspecies, and 17 gorillas (*Gorilla gorilla*).

A total of 55 lemurs belonging to six genera (*Hapalemur*, *Eulemur*, *Indri*, *Avahi*, *Varecia*, and *Propithecus*) were caught between 1996 and 2002 in different areas of Madagascar. These animals were sampled for cytogenetic and molecular taxonomic studies by the Institut d'Embryologie of the University Louis Pasteur in France (45). Under an agreement with the Institut Pasteur de Madagascar, the University Louis Pasteur of Strasbourg provided blood samples from these different lemurs to the Institut Pasteur de Madagascar, where they were frozen and stored. DNA was obtained from lemur blood samples using a phenol/chloroform extraction technique and then screened for *Plasmodium* infection using *cyt b* molecular tools (44).

Isolated parasites from African great apes (chimpanzees and gorillas) and lemurs were molecularly characterized with the mitochondrial genes *cytochrome b* (*cyt b*) and *cytochrome c oxidase 1* (*cox1*). The gene *cox1* has been selected for biodiversity identification within the international barcoding program (46). Like *cyt b* gene, *cox1* is a conserved gene and is useful for resolving phylogenetic relationships between parasite species that diverged over tens to hundreds of millions of years ago (47). In addition, we used the nuclear gene *lactate dehydrogenase* (*ldh*) gene and the *tufA* apicoplast elongation factor for additional characterization of parasites isolated from chimpanzees, gorillas and lemurs. All primers used are specific of Haemosporidia parasites and do not amplify other Apicomplexa parasites or host DNA. All PCR products were sequenced by Macrogen in Korea.

Primers and PCR conditions used to amplify the *cytb*, *cox1*, *ldh* and *tufA* genes were as follows. *Cytb* gene fragments of 708 bp were amplified using published primers (44). The PCR and nested PCR reactions were carried out in a final volume of 25 μ L under the following conditions: 2.5 μ L of each primer, 2 mM of each dNTP, 2.5 U of Taq polymerase (Solis), 2 mM MgCl₂, 5 min at 94 °C, 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C for 40 cycles, and a final 10-min extension at 72 °C. *Cox1* gene fragments of 964 bp were obtained with the following primers: *cox1a*: 5'-CGCCTGACATGGATGGATAATC -3' and *cox1b*: 5'-CCATTTAAAGCGTCTGGATAATC -3', and nested PCR primers: *cox1c*: 5'-GATTAACCGCTGTCTGGGACTG -3' and *cox1d*: 5'-CGTCTAGGCATTACATTAATCC -3'. The PCR and nested PCR reactions were carried out as for *cytb*, except 30 s at 94 °C, 30 s at 53 °C for PCR, and 30 s at 58 °C for nested PCR, and 2 min at 72 °C for 40 cycles and a final 10-min extension at 72 °C. *Cox1c* and *cox1d* were used for sequencing. We amplified *ldh* gene fragments of 350 bp with two primers for PCR: *Ldh1* (5'-GGNTCDGGHATGATHGGAGG-3') and *Ldh2* (5'-GC-CATTTTCRATRDGACAGC-3'), and two for nested PCR: *Ldh7* (5'-TGTDATGGC-WTAYTCVAATTGYMARGT-3') and *Ldh8* (5'-CCATYTRTTNCCATGWGCWSCD-ACA-3'). PCR and nested PCR were carried out as for *cytb*, except 10 pmol/ μ L of each primer; 0.2 mM of each dNTP, 2.5 mM MgCl₂, 2.5 μ L PCR buffer 10 \times , and 2 μ L of DNA, heating for 5 min at 94 °C, 30 s at 94 °C, 30 s at 53 °C, and 30 s at 52 °C for nested PCR, and 1 min at 72 °C for 40 cycles and a final 10-min extension at 72 °C. *Ldh7* and *Ldh8* were used for sequencing. 677 bp of the *tufA* gene were amplified using two primers for PCR: *TufA1* (5'-GGGCATGTASATCATGGGA-AAAC-3') and *TufA2* (5'-CCTGCTCTATWGTTTTWCCT-3'), and two for nested PCR reactions: *TufA3* (5'-GGAGCTACAAATGGATRAGC-3') and *TufA4* (5'-GGTTTATGACGACCHCCYTC-3'). PCR and nested PCR were as for *ldh*, except 1 min at 53 °C for PCR and 90 s at 72 °C for 40 cycles and a final extension phase for 10 min at 72 °C. *TufA3* and *TufA4* were used for sequencing. All primers used are specific for Haemosporidia parasites and do not amplify other Apicomplexa parasites or host DNA. All PCR products were sequenced by Macrogen in Korea.

The *cytb*, *cox1*, *ldh*, and *tufA* sequences were checked using chromatograms and CLUSTAL W alignment to ensure that no position was ambiguous (48). Mixed infections were discarded from the phylogenetic study. Phylogenetic analyses were based on the use of 708 bp *cyt b* and 964 bp *cox1* concatenated sequences. Reference sequences without ambiguous positions for either *cyt b* or *cox1* were obtained from GenBank (Table 2).

We performed a statistical analysis, based on the method of Xia and Xie (49), to ascertain whether the number of substitutions was saturated. In this method, both transitions and transversions are plotted against evolutionary distances calculated with the JC69 model. The relative rates at which transitions and transversions saturated at the third position were compared by counting substitutions in all pairwise comparisons between sequences. The analysis showed that the third base was saturated, and this base was therefore discarded for subsequent phylogenetic analyses.

The most appropriate nucleotide substitution model was identified, based on hierarchical likelihood ratio tests and the Akaike information criterion, using PHYML (50) and Garli (51). Model selection was also performed using MrBayes by computing the Bayes factor. The General Time Reversible model

incorporating a proportion of invariant sites and a gamma correction for variable sites (52) was favored by the hierarchical likelihood ratio tests, Akaike information criterion, and Bayes factor. Maximum likelihood analysis was carried out with PHYML, with clade support evaluated by nonparametric bootstrapping (1,000 replicates). Bayesian analysis was performed with MrBayes (53), using two runs of 1 million generations sampled every 100 generations. Convergence was determined using the standard deviation of the split frequencies, and runs were terminated when a value of less than 0.01 was reached. The burn-in phase was defined as the first 250,000 generations. Partitioning the alignment using independent models for each of the two genes did not improve the likelihood estimates in MrBayes.

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