African great apes are natural hosts of multiple related malaria species, including *Plasmodium falciparum*

Franck Prugnolle\(^a,1,2\), Patrick Durand\(^a,1\), Cécile Neel\(^b,3\), Benjamin Ollomo\(^d\), Francisco J. Ayala\(^a,2\), Céline Arnaud\(^a\), Lucie Etienne\(^b\), Eitel Mpoudi-Ngole\(^c\), Dieudonné Nkoghe\(^b\), Eric Leroy\(^d,1\), Eric Delaporte\(^b\), Martine Peeters\(^b\), and François Renaud\(^a,2\)

\(^a\)Laboratoire Génétique et Évolution des Maladies Infectieuses, Unité Mixte de Recherche 3724, Centre National de la Recherche Scientifique–Institut de Recherche pour le Développement, Montpellier, 34394 Montpellier Cedex 5, France; \(^b\)Unité Mixte de Recherche 145, Virus de l’Immunodéficience Humaine/Syndrome d’Immunodéficience Acquise et Maladies Associées, Institut de Recherche pour le Développement and University of Montpellier 1, 34394 Montpellier, France; \(^c\)Institut de recherches Médicales et d’études des Plantes Médicinales Prévention du Sida au Cameroun Centre de Recherche Médicale, BP 906, Yaoundé, Cameroon; \(^d\)Unité des Maladies Virales Émergentes, Centre International de Recherches Médicales de Franceville, BP 769, Franceville, Gabon; \(^1\)Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2325; and \(^2\)Emergence des Pathologies Virales, Unité Mixte de Recherche 190, Institut de Recherche pour le Développement–Université de la Méditerranée, 13005 Marseille, France

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*Plasmodium reichenowi*, a chimpanzee parasite, was until very recently the only known close relative of *Plasmodium falciparum*, the most virulent agent of human malaria. Recently, *Plasmodium gaboni*, another closely related chimpanzee parasite, was discovered, suggesting that the diversity of *Plasmodium* circulating in great apes in Africa might have been underestimated. It was also recently shown that *P. reichenowi* is a geographically widespread and genetically diverse chimpanzee parasite and that the world diversity of *P. falciparum* is fully included within the much broader genetic diversity of *P. reichenowi*. The evidence indicates that all extant populations of *P. falciparum* originated from *P. reichenowi*, likely by a single transfer from chimpanzees. In this work, we have studied the diversity of *Plasmodium* species infecting chimpanzees and gorillas in Central Africa (Cameroon and Gabon) from both wild-living and captive animals. The studies in wild apes used noninvasive sampling methods. We confirm the presence of *P. reichenowi* and *P. gaboni* in wild chimpanzees. Moreover, our results reveal the existence of an unexpected genetic diversity of *Plasmodium* lineages circulating in gorillas. We show that gorillas are naturally infected by two related lineages of parasites that have not been described previously, herein referred to as *Plasmodium GorA* and *P. GorB*, but also by *P. falciparum*, a species previously considered as strictly human specific. The continuously increasing contacts between humans and primate populations raise concerns about further reciprocal host transfers of these pathogens.

**Results and Discussion**

In the present study we analyzed fecal samples from wild chimpanzee (*n* = 125) and gorilla (*n* = 84) populations from eight distant localities in Cameroon (Fig. 2 and Materials and Methods) and 3 blood samples from captive wild-born gorillas from Gabon. Using a *Plasmodium*-specific PCR assay, based on the mitochondrial *Cytochrome b* (*Cyt b*) gene, known to be suitable for exploring phylogenetic relationships in *Plasmodium* and related genera (10–12), 22 chimpanzee samples and 18 gorilla samples (including 1 blood sample) were detected positive by PCR for *Plasmodium* (Tables S1 and S2). Unambiguous and good quality partial *Cyt b* sequences (704 nucleotides) were obtained for 5 chimpanzee and 7 gorilla samples. Failure to obtain good sequences from the remaining samples was mainly due to insufficient amplified DNA for sequencing (due to scarce *Plasmodium* DNA within the DNA extracted from feces).

Fig. 1B displays the phylogenetic relationships of the newly identified *Plasmodium* strains infecting the two host primates, obtained by maximum-likelihood methods (Materials and Methods).


The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database. For a list of accession numbers, see Table S2.

1F.P. and P.D. contributed equally to this work.

2To whom correspondence may be addressed. E-mail: prugnolle@iml.fr, fnrenea@iml.fr, or fjayala@uci.edu.

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ods. It may be noted, at the outset, that all nucleotide sequences in our study represented in Fig. 1 translated well into proteins, without any stop codons in the partial Cyt b gene sequence.

As recently observed (4), there is a much greater genetic diversity in chimpanzee parasites than among the numerous strains representing the global populations of human P. falciparum (Fig. S1). We have now identified in southeast Cameroon two unique chimpanzee isolates (MB753 and MB783), genetically very similar to the previous chimpanzee isolates from Gabon, named P. gaboni (5) (Fig. 1). A supplementary phylogenetic analysis (based on partial Cyt b sequences of 350 nucleotides in length, Fig. 3) including the Cyt b sequences recently published by Rich et al. (4) also shows that three of their isolates collected in Cameroon and Ivory coast (Bana, Max, and Loukoum) belong to the P. gaboni lineage, as previously suggested (4). Thus, P. gaboni is widespread, present in at least three different countries (Gabon, Cameroon, and Ivory Coast), and it infects at least two different chimpanzee subspecies (Pan troglodytes vellerosus in Gabon and Cameroon and Pan troglodytes verus in Ivory Coast).

We have also identified two unique Plasmodium specimens from Cameroon (MP1309 and BQ642) that cluster with the reference sequence of P. reichenowi (Figs. 1B and 3). One specimen is from the MP locality (Fig. 2), north of the Sanaga river, a region home to the Pan troglodytes vellerosus chimpanzee subspecies; the other specimen is from the BQ site, north of the Dja Reserve (south-central Cameroon), which is home to the P. t. troglodytes subspecies. P. reichenowi is, therefore, geographically widespread (Cameroon and Ivory Coast) and infects three chimpanzee subspecies (P. t. troglodytes, P. t. vellerosus, and P. t. verus).

In gorillas, we have identified two unique and previously unknown lineages of parasites (Fig. 1B). One lineage, which is closely related to, but statistically distinct (bootstrap value = 88) from P. reichenowi and P. falciparum, includes two specimens (BQ2341 and BQ668) from the BQ locality. We hereafter refer to this lineage as Plasmodium GorA. The second lineage (referred as Plasmodium GorB) is closer to, but statistically different from P. gaboni (bootstrap value = 99). It includes three isolates (two from the BQ locality, BQ2367 and BQ638) and one from CP (CP1426), in the Campo Ma’an reserve in southwest Cameroon (Fig. 2). Surprisingly, we also identified P. falciparum in fecal samples from two gorillas in Cameroon and in one blood sample from a captive gorilla from Gabon. These three P. falciparum positive samples were collected in three different geographic localities and from two different gorilla subspecies: The TK site in

A. Before

B. This study

**Fig. 1.** Phylogenetic relationships among Plasmodium species. (A) Before: Cytochrome b phylogeny based on gene sequences available before 2009. Only two species, P. falciparum, parasitic to humans, and P. reichenowi, parasitic to chimpanzees, were known within the clade that includes humans and the great apes (above). (B) This study: Phylogeny that includes the sequences obtained in our study. Three additional species are shown: P. gaboni, which infects chimpanzees (5), and P. GorA and P. GorB, which infect gorillas. Moreover, P. falciparum is shown to infect gorillas in addition to humans. The phylogenograms were constructed using a maximum-likelihood method from partial Cyt b sequence data (704 nucleotides). Bootstrap values are shown for the nodes inside the African great apes/human clade. (Scale bar, 0.03 substitutions per site.) cpm: Cheek-Push monkeys.
western Cameroon is home to the highly endangered cross-river gorilla (Gorilla gorilla dielhi) and the samples collected just above the Dja Reserve (BQ) in Cameroon and Gabon were from western lowland gorillas (Gorilla gorilla gorilla). In summary, we have identified Plasmodium infections in five of the eight localities studied in Cameroon. At least five different Plasmodium lineages (including *P. falciparum*, *P. reichenowi*, *P. gaboni*, *P. GorA*, and *P. GorB*) circulate among apes in Cameroon; four of them occur in BQ, within a small geographic area (of ~15 km²). The frequency of positive fecal samples (22 of 125 for chimpanzees and 18 of 84 for gorillas) is high, which demonstrates the high prevalence of Plasmodium infections in apes in their natural habitat, as well as the suitability of this noninvasive method to study Plasmodium infection in wild animals.

Coatney et al. (13) and Garnham (14) reported several forms of Plasmodium infecting gorillas, including *P. reichenowi*, *Plasmodium rodhaini*, and *Plasmodium schwetzii*, although none of them was genetically characterized. It is possible that some of the parasites previously identified as *P. reichenowi* may have been *P. GorB* or *P. GorA* parasites, given their genetic proximity to *P. reichenowi*. However, *P. rodhaini* and *P. schwetzii* are unlikely to correspond to the two newly identified gorilla lineages, because they are morphologically related to *Plasmodium vivax* and *Plasmodium ovale*, two human parasites, which, like *Plasmodium ovale*, another human parasite, belong to very distant evolutionary lineages (Fig. 1A and B; refs. 1 and 3–5).

Are *P. GorA* and *P. GorB* lineages two unique species of *Plasmodium*? Three broad classes of criteria can be used to define species for sexually reproducing organisms: the similarity species concept, which is based on phenotypic features; the biological or reproductive species concept, which focuses on the ability of sexually reproducing organisms to cross and produce fertile offspring; and the phylogenetic or lineage-based species concept, which emphasizes shared evolutionary history of individuals (15, 16). Species of malaria have traditionally been described using the similarity species concept based primarily on differences in morphology, life-history traits, species of hosts infected, and/or symptoms of infection (13, 14). Whereas this approach has been useful, it has now proved to be unreliable for several reasons. First, parasite morphology may be altered during blood smear preparation (which may result in artifactual differences in the appearance of the parasite under the light microscope, ref. 16) and, second, morphology and life history traits may vary depending on the host species in which the parasites find themselves (17, 18). The similarity species concept may, therefore, lead to the splitting of true species on the basis of phenotypic differences, but also to the lumping of taxa that show morphological similarities (16). Species should ideally be identified using all three criteria listed above, but in practice this is often not easily feasible. In the present case, our results clearly indicate that *P. GorA* and *P. GorB* constitute two distinct monophyletic lineages, which could justify the definition of two unique species. However, we submit that it is prudent to gather additional information about these lineages before identifying them as different species. Perhaps, at a minimum, it will be appropriate to obtain their complete mitochondrial sequences, as was previously done for *P. gaboni* (5), before deciding to define it as a separate species.

The evolutionary origin of the ape–human clades A and B (Fig. 1B) remains to be determined, but it is apparent that the origin of *P. falciparum* is within clade B, which includes *P. reichenowi* and *P. GorB*. Our results corroborate the recent proposal (4) that *P. falciparum* likely originated from *P. reichenowi* following a host transfer (Fig. 3).

It has been assumed that *P. falciparum* is strictly a humanspecific pathogen. First, experimental infections with human blood infected with *P. falciparum* fail to induce malaria in chimpanzees (review in ref. 13). Even after splenectomy to increase parasite survival, experimentally infected chimpanzees do not develop a parasitemia equivalent to that observed in humans (13, 19). Second, captive chimpanzees in Gabon do not get infected with *P. falciparum* despite high rates of infection among their keepers and being exposed to the same vector mosquitoes (20). Finally, Martin, Varki, and colleagues (21, 22) have shown that the strong human specificity of *P. falciparum* may be linked to species-specific erythrocyte recognition profiles. The human-specific loss of the common primate Sia N-glycolylneuraminic acid (Neu5Gc) would have protected our human ancestors from *P. reichenowi*. Consequently, however, the major merozoite-binding protein, the erythrocyte-binding antigen-175 (EBAd) of *P. falciparum*, would have evolved to take advantage of the accumulated excess on human erythrocytes of the Neu5Gc precursor, the Sia N-acetyleneuraminic acid (Neu5Ac).

Our observations challenge the assumed human specificity of *P. falciparum*. We have detected *P. falciparum* in three gorillas from three different localities. This might be because the gorilla...
erythrocyte has both the Neu5Ac and the Neu5Gc forms of sialic acids (23), including the Neu5Ac form that the *P. falciparum* parasite prefers to bind to (21). However, the density of Neu5Ac may be much lower in gorilla cells (23), which could account for the rare observation of *P. falciparum* in gorilla samples. In any case, our results raise the distinct possibility that gorillas may be a reservoir for the very virulent parasite, *P. falciparum*. The amplification and characterization of *P. falciparum* from the blood and/or the feces of the three gorillas were carried out under very stringent conditions to exclude the possibility that the *P. falciparum* isolates were due to contamination with human strains. DNA extraction, amplification, and sequencing were repeated several times for each sample. One repetition was performed independently as a “blind test” in a *Plasmodium*-free laboratory (additional details in Materials and Methods).

The likely success of the campaigns to eradicate malaria is hardly in sight. One consideration to keep in mind as the campaigns proceed is that gorillas (and perhaps other primates) may serve as a reservoir for the very virulent parasite, *P. falciparum*. The amplification and characterization of *P. falciparum* from the blood and/or the feces of the three gorillas were carried out under very stringent conditions to exclude the possibility that the *P. falciparum* isolates were due to contamination with human strains. DNA extraction, amplification, and sequencing were repeated several times for each sample. One repetition was performed independently as a “blind test” in a *Plasmodium*-free laboratory (additional details in Materials and Methods).

In conclusion, molecular tools and noninvasive sampling strategies have allowed us to discover a great diversity of *Plasmodium* species circulating in wild apes from Central Africa and the fragility of species barriers against the transmission of vector-borne pathogens, at least in primates. Our results enlarge the diversity of species within the clade that previously included only two species, *P. falciparum* and *P. reichenowi*, with the addition of two unique *Plasmodium* lineages parasitic to gorillas and the confirmation of our recently discovered chimpanzee parasite, *P. gaboni* (5). The continuously increasing contact between humans and primate populations, mostly due to logging and deforestation, increases the possibility of transmission of new pathogens from primates to humans and from humans to primates, including the endangered great apes.

**Materials and Methods**

**Origin of Samples and Blood and Feces Collection.** We analyzed blood and fecal samples collected from chimpanzees and gorillas in Gabon and Cameroon. In Cameroon, only fecal samples from wild chimpanzees and gorillas were analyzed (Fig. 3). The fecal samples were obtained from existing banks of specimens previously collected for molecular epidemiological studies of SIVcpz and SIVgor (9, 24). For chimpanzees, fecal samples were analyzed from two subspecies, *P. t. troglodytes* (*n* = 90) and *P. t. vellerosus* (*n* = 35) (Table S1). For gorillas, we analyzed populations of the cross-river subspecies *G. g. diehli*...
(northwest of Cameroon, near the Nigeria border, TK in Fig. 2) (n = 12) and the western lowland subspecies G. g. gorilla (other localities in Cameroon) (n = 72) (Table S1). Samples were preserved in RNA later, shipped, and processed as described (8). All fecal samples were collected in the wild, and the species and subspecies (for chimpanzees only) were confirmed by mitochondrial DNA analysis as reported (8). In Gabon, blood aliquots from three gorillas (G. g. gorilla) were collected. The samples were collected from wild-born animals kept in a sanctuary on island Evaro, near Lambarené, Moyen-Ogooué. This investigation was approved by the Government of the Republic of Gabon and by the Animal Life Administration of Libreville, Gabon (no. CITES 00956). All animal work was conducted according to relevant national and international guidelines. Blood samples were collected in 7-mL EDTA vacutainers from gorillas under ketamine anesthesia. Clots and plasma were obtained by centrifugation and stored at −20 °C until they were transported to the Centre International de Recherches Médicales de Franceville (CIRMF), Gabon, where they were stored at −80 °C until they were processed for testing.

**DNA Extraction, PCR Conditions, and Sequencing.** Total DNA (Plasmodium and host) was isolated and purified from blood using the DNeasy blood kit (Qiagen) according to the manufacturer’s instructions. Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) as described (8).

The Plasmodium Cytochrome b (Cyt b) gene was amplified using published primers (12, 25, 26). A nested PCR was performed using an MJ Research PTC100 thermal cycler or Mastercycler Eppendorf thermocycler. For the first round of amplifications, we used 4 μL of DNA template in a 25-μL reaction volume, containing: 12.5 μL of Mix PCR (Qiagen), 2.5 μL solution Q (Qiagen), and 2 pmol of each primer (DW2 and DW4). Cycling conditions were 3 min at 94 °C, 20 s at 94 °C, 20 s at 60 °C, 90 s at 72 °C (35 cycles), and 10 min at 72 °C. For the second round of amplification, we used 1 μL of the first PCR product in a 25-μL reaction containing 2.5 μL of 10× buffer, 1.25 mM MgCl₂, 250 μM of each dNTP, 37.5 pmol of each primer (PrfC1 and PrfCt), and 0.5 unit Taq DNA polymerase (Invitrogen). Cycling conditions for the second round were 5 min at 95 °C, 30 s at 94 °C, 30 s at 50 °C, 90 s at 72 °C (35 cycles), and 10 min at 72 °C.

The amplified products (5 μL) were run on 1.5% agarose gels in TAE buffer. The PCR amplified products were used as templates for sequencing. DNA sequencing was performed by CoGenics Genome Express.

For fecal samples positive for the same *Plasmodium* species/genetic entities and collected in the same site, we used microsatellite markers specific for chimpanzees and gorillas to determine if they originated from the same host individual or different ones (8).

**Prevention of PCR Contaminations and Confirmation of Results in a "Plasmodium Free" Laboratory.** To avoid PCR contaminations, we followed strict rules for DNA extraction and PCR: (1) All DNA extractions were done in a laboratory where no work is conducted on *Plasmodium* to prevent contamination of the native DNA (Union Mixte de Recherche 145, Laboratory SIV/HIV et Maladies Associées, Institut de Recherche pour le Développement (IRD), Montpellier, France). Extractions were made in a P3 laboratory, under a laminar flow safety cabinet reserved for the treatment of primate samples (human samples are never manipulated under this hood). (ii) For the first set of amplifications, we did all PCRs in the laboratory Génétique et Évolution des Maladies Infectieuses (GEMI, IRD, Montpellier, France). The amplifications were done on Mastercycler Eppendorf Thermocycler. In all steps of PCR preparation we used specially assigned lab coats, gloves, and filter tips, as well as DNA–RNase-free water (Qiagen). All PCR mixes were prepared in a single room under a safety hood with UV light, using pipettes dedicated to the preparation of the PCR mix. DNA templates were deposited in a second room. PCR cycles on thermocyclers were performed in a third room. Electrophoreses of the PCR products were realized in a fourth room. (iii) For all samples positive for *P. falciparum* or any other *Plasmodium*, DNA extractions, PCR, and sequencing were repeated. PCRs were done in a Plasmodium-free laboratory working on rice (Laboratoire Diversité et Génomique des Plantes Cultivées, IRD, Montpellier, France). For each newly extracted sample, four PCR replicates were done in the same day on a PTC100 MJ Research Thermocycler. A master mix was prepared in a hood with laminar flow using a new set of micropipettes, new aerosol-resistant pipette tips, new PCR reagents, new primers, and new DNA–RNase-free water.

For this second set of amplifications, all manipulations were carried out blindly (all tubes were initially randomized on the plate by a third person). Six DNA templates negative to *Plasmodium* were also randomly distributed among the samples to be amplified. Negative controls with water instead of DNA were tested for each PCR.

In all cases (first and second sets of amplification), we never detected any band in the negative template controls.

**Alignment and Phylogenetic Analyses.** For phylogenetic analyses we used, in addition to our sequences, 18 previously published Cyt b sequences from different *Plasmodium* species (Cyt b sequence length: 704 nucleotides). Hosts and GenBank accession numbers for these taxa are given in Table S2. An additional set of phylogenetic analyses was performed using the same 18 previously published sequences, but also adding sequences recently published by Rich et al. (4). Because our Cyt b sequences and those from Rich et al. only partially overlapped, the final length of the sequences used for this latter analysis was shorter (i.e., 350 nucleotides). The multiple alignment of all partial Cyt b sequences was conducted using ClustalW (v 1.8.1 in BioEdit v.7.0.9.0. software) (27).

Maximum-likelihood (ML) tree construction was based on the Cyt b sequences. The best-fitting ML model under the Akaike information criterion was general time reversible plus Gamma distribution (GTR + Γ) for nucleotides as identified by ModelTest (28). The highest-likelihood DNA trees and corresponding bootstrap support values were obtained by PhyML (freely available at the ATGC bioinformatics platform http://www.atgc-montpellier.fr), using nearest neighbor interchange plus subtree pruning regrafting (NNI + SPR) branch swapping and 100 bootstrap replicates (29).

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