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,c], Benjamin Ollomo[d], Francisco J. Ayala[e,2], Céline Arnathau[a], Lucie Etienne[b], Eitel Mpoudi-Ngole[c], Dieudonné Nkoghe[d], Eric Leroy[d,f], Eric Delaporte[b], Martine Peeters[b], and François Renaud[a,2]

[a]Laboratoire Génétique et Evolution des Maladies Infectieuses, Unité Mixte de Recherche 2724, Centre National de la Recherche Scientifique–Institut de Recherche pour le Développement, Institut de Recherche pour le Développement Montpellier, 34394 Montpellier Cedex 5, France; [b]Unité Mixte de Recherche 145, Virus de l'Immunodéficience Humaine/ Syndrome de l'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; [e]Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525; and [f]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.**

cytochrome b gene | ecology | evolution | host specificity | infectious diseases

Plasmodium falciparum is the agent of malignant malaria, one of the worst scourges of mankind, with 2 million infant deaths and ~500 million clinical cases per year, 85% in sub-Saharan Africa. *Plasmodium reichenowi*, a chimpanzee parasite, was the only known closely related sister lineage (Fig. 1*A*), of which only one single exemplar was known (1, 2). It was assumed that the two parasites had evolved along with their hosts since the separation of the two lineages, ~7 million years ago (the cospeciation hypothesis). The existence of only one *P. reichenowi* strain did not allow testing two alternative hypotheses: whether *P. reichenowi* was ancestral to *P. falciparum* or vice versa (1, 3). Recent genetic analysis of eight previously undescribed isolates of *P. reichenowi* from wild-born chimpanzees in Cameroon and Ivory Coast has shown that *P. reichenowi* is a geographically widespread and genetically diverse chimpanzee parasite and that *P. falciparum* is fully included within the much broader genetic diversity of *P. reichenowi* (4). Phylogenetic analysis indicates that all extant *P. falciparum* populations originated from *P. reichenowi*, likely by a single transfer from chimpanzees. Also recently, a unique closely related species, named *Plasmodium gaboni*, was discovered in two wild-born chimpanzees kept as pets in remote villages of Gabon (5), suggesting the possible existence of additional *Plasmodium* species infecting chimpanzees.

Although a number of *Plasmodium* species parasitic to apes and other primates have been identified, no primates have been studied systematically in their natural habitat owing to the difficulty of collecting blood samples. The recent discovery that *Plasmodium* can be genotyped from urine or saliva in humans (6, 7) and that fecal primate samples have allowed researchers to identify the ancestors of the HIV-1 epidemic in chimpanzees and gorillas (8, 9) moved us to explore whether similar noninvasive approaches could be used to investigate the diversity of malaria parasites in wild populations of apes (chimpanzees and gorillas) in Africa.

This is, indeed, the case. Thus, we have discovered (*i*) two unique gorilla parasites closely related to *P. falciparum* and (*ii*) that *P. falciparum* is not an exclusively human parasite, but can also infect gorillas.

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

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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. For a list of accession numbers, see Table S2.

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

[2]To whom correspondence may be addressed. E-mail: prugnoll@mpl.ird.fr, frrenaud@mpl.ird.fr, or fjayala@uci.edu.

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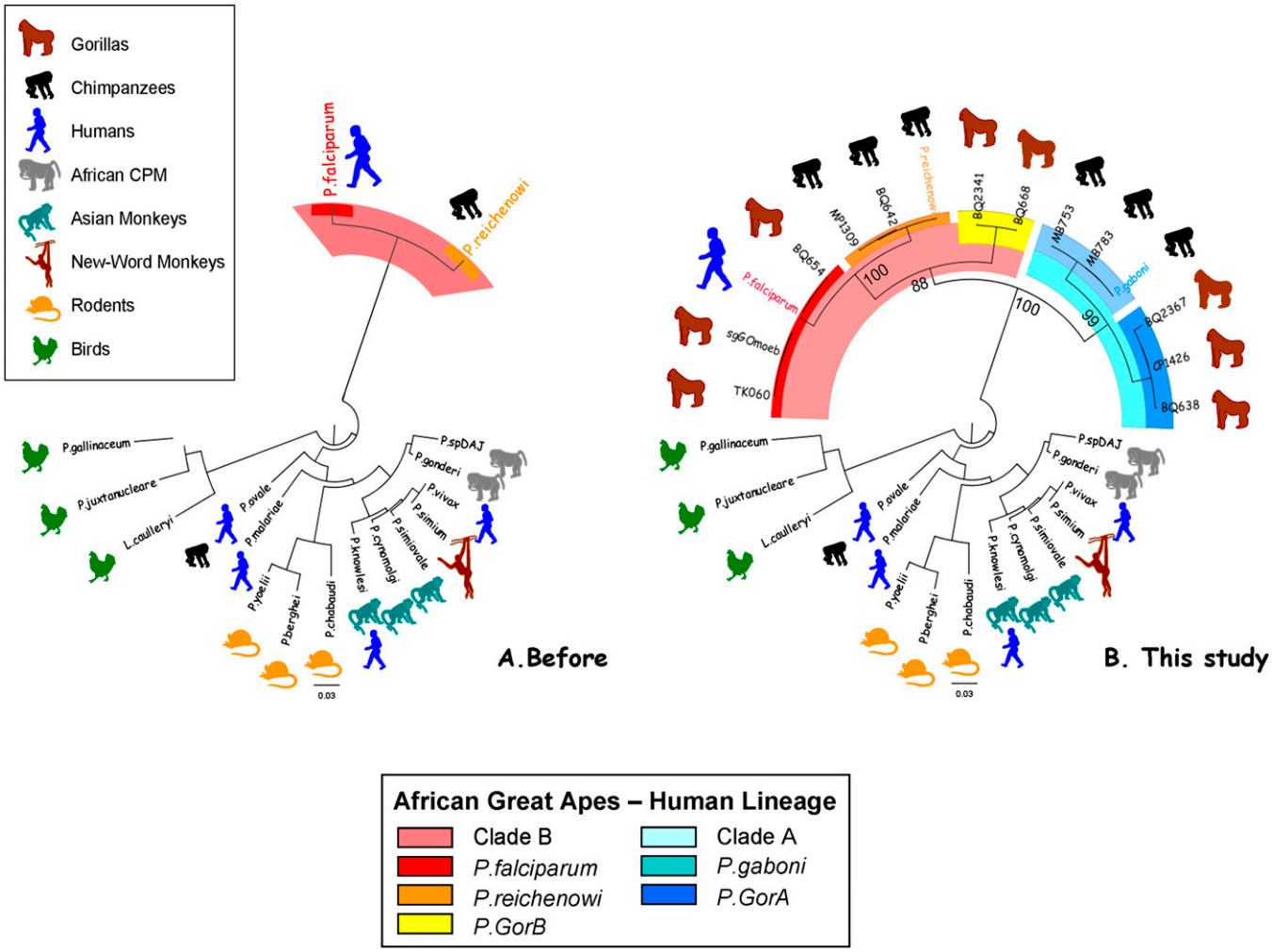

Fig. 1. Phylogenetic relationships among *Plasmodium* species. (*A*) Before: *Cytochrome b* phylogeny based on gene sequences available before 2009. Only two species, *P. falciparum*, parasitic to humans, and *P. reichenowi*, parasitic to chimpanzees, were known within the clade that includes humans 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 phylograms 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.

ods). It may be noted, at the outset, that all nucleotide sequences in our study represented in Fig. 1*B* 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*B*). 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 troglodytes* 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. 1*B* 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. 1*B*). 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 GorB*. The second lineage (referred as *Plasmodium GorA*) 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

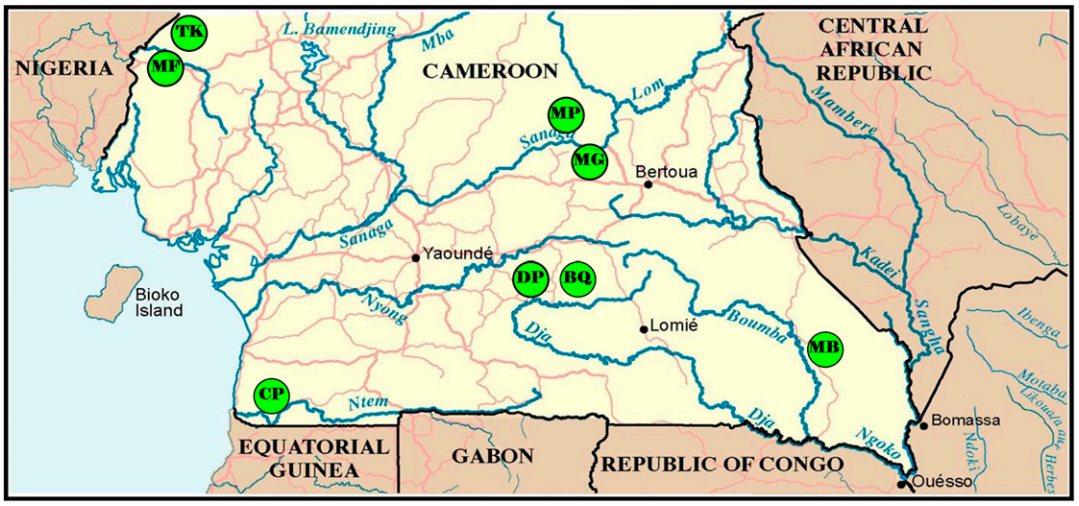

Fig. 2. Location of the wild chimpanzee and gorilla populations in Cameroon. The Sanaga River defines the boundary between *P. t. troglodytes* in the south and *P. t. vellerosus* in the north. *G. g. gorilla* is found in the south of Cameroon and *G. g. diehli* in the northwest of Cameroon at the border with Nigeria. BQ, CP, DP, MB, MF, MG, MP, TK: Sites of chimpanzee and gorilla feces collection (more details in Table S1).

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 km^2). 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. reichenow, Plasmodium rodhaini*, and *Plasmodium schwetzi*, 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. schwetzi* are unlikely to correspond to the two newly identified gorilla lineages, because they are morphologically related to *Plasmodium vivax* and *Plasmodium malariae*, two human parasites, which, like *Plasmodium ovale*, another human parasite, belong to very distant evolutionary lineages (Fig. 1 *A* 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. 1*B*) 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 human-specific 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 (EBA 175) of *P. falciparum*, would have evolved to take advantage of the accumulated excess on human erythrocytes of the Neu5Gc precursor, the Sia *N*-acetylneuraminic 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

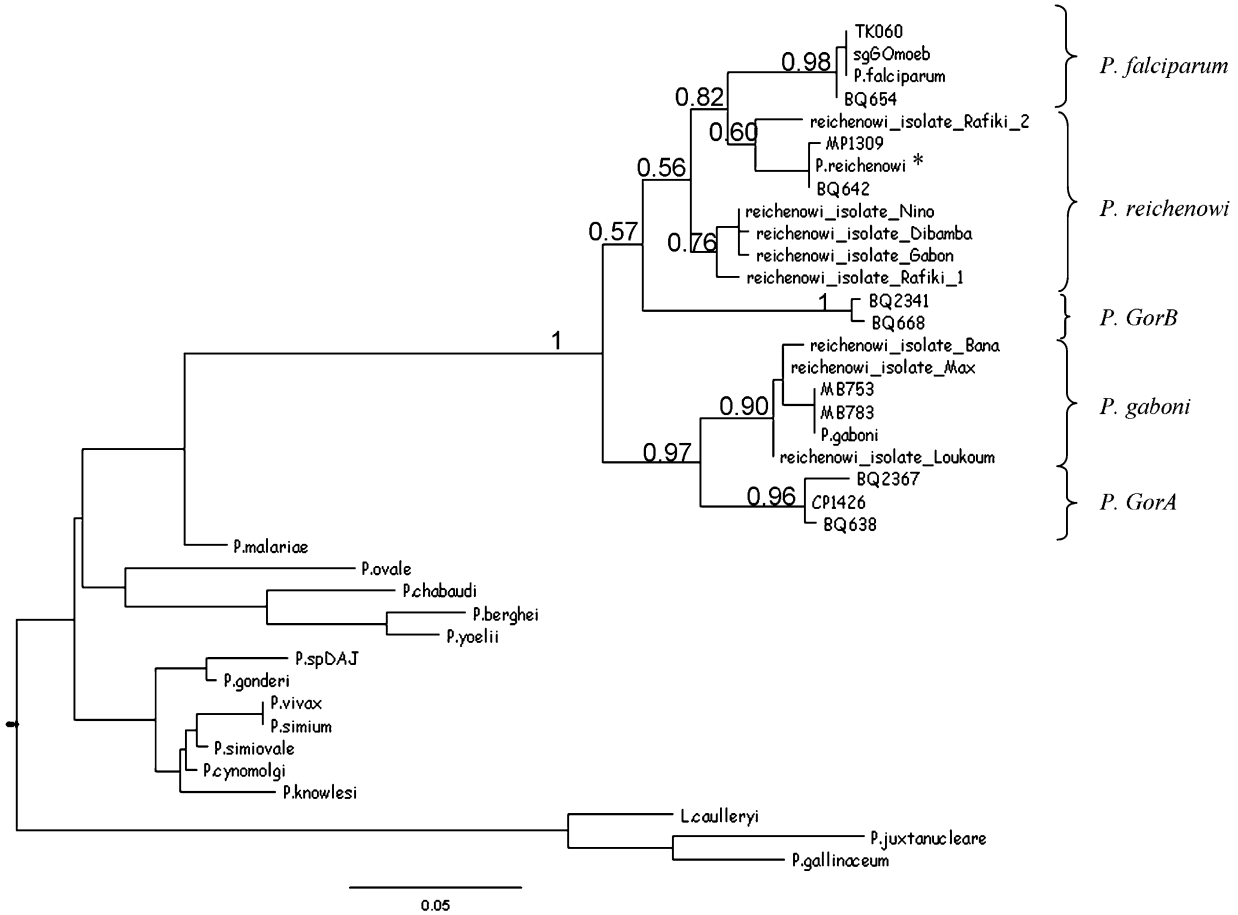

Fig. 3. Phylogeny of *Plasmodium* species including our samples and those from Rich et al. (4), i.e., "reichenowi_isolate." This phylogram was constructed using maximum likelihood from partial *Cyt b* sequence data (i.e., 350 nucleotides shared between the two studies). Bootstrap values were obtained using 100 iterations. (Scale bar, 0.05 substitutions per site.) *P. reichenowi** corresponds to the reference GenBank sequence (Table S2).

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 malignant malaria and the possibility that humans might acquire *P. falciparum* by host transfer from gorillas. Our observations do not determine whether the three gorilla carriers of *P. falciparum* acquired the parasite from humans (or from other primates), nor do we know the pathogenicity of these infections in gorillas. The three infected gorillas come from distant localities, which makes it unlikely that they are accidental or recent infections. Independent foci of *P. falciparum* may be active in natural populations of gorillas in Central Africa. It should be noted, in addition, that *P. malariae*, a human parasite considerably less widespread and malignant than *P. falciparum*, has recently been found in two captive asymptomatic chimpanzees that were exported to Japan ~30 years ago (19). The authors propose that the *P. malariae* isolates were exported from Africa to Japan with their hosts (19).

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 Lambaréné, 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 template in a 25-μL reaction volume, containing 2.5 μL of 10× buffer, 1.25 mM $MgCl_2$, 250 μM of each dNTP, 37.5 pmol of each primer (Pfcyb1 and Pfcytb2), 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: (*i*) All DNA extractions were done in a laboratory where no work is conducted on *Plasmodium* to prevent contamination of the native DNA [Unité 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 Evolution des Maladies Infectieuses (GEMI, IRD, Montpellier, France). The amplifications were done on Mastercycler Eppendorf Thermocyclers. 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 (Laboratory Diversité et Génome des Plantes Cultivées, IRD, Montpellier, France). For each newly extracted sample, four PCR replicates were done 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. (4) 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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