# *Plasmodium falciparum*-like parasites infecting wild apes in southern Cameroon do not represent a recurrent source of human malaria

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**Wild-living chimpanzees and gorillas harbor a multitude of *Plasmodium* species, including six of the subgenus *Laverania*, one of which served as the progenitor of *Plasmodium falciparum*. Despite the magnitude of this reservoir, it is unknown whether apes represent a source of human infections. Here, we used *Plasmodium* species-specific PCR, single-genome amplification, and 454 sequencing to screen humans from remote areas of southern Cameroon for ape *Laverania* infections. Among 1,402 blood samples, we found 1,000 to be *Plasmodium* mitochondrial DNA (mtDNA) positive, all of which contained human parasites as determined by sequencing and/or restriction enzyme digestion. To exclude low-abundance infections, we subjected 514 of these samples to 454 sequencing, targeting a region of the mtDNA genome that distinguishes ape from human *Laverania* species. Using algorithms specifically developed to differentiate rare *Plasmodium* variants from 454-sequencing error, we identified single and mixed-species infections with *P. falciparum*, *Plasmodium malariae*, and/or *Plasmodium ovale*. However, none of the human samples contained ape *Laverania* parasites, including the gorilla precursor of *P. falciparum*. To characterize further the diversity of *P. falciparum* in Cameroon, we used single-genome amplification to amplify 3.4-kb mtDNA fragments from 229 infected humans. Phylogenetic analysis identified 62 new variants, all of which clustered with extant *P. falciparum*, providing further evidence that *P. falciparum* emerged following a single gorilla-to-human transmission. Thus, unlike *Plasmodium knowlesi*-infected macaques in southeast Asia, African apes harboring *Laverania* parasites do not seem to serve as a recurrent source of human malaria, a finding of import to ongoing control and eradication measures.**

diagnostic *Laverania* PCR | great apes | nextgen sequencing | *Plasmodium* coinfections | *Plasmodium* diversity

Malaria is one of the most devastating infectious diseases of humans worldwide, with hundreds of millions of cases of clinical illness and over 650,000 deaths occurring annually (1). Given this enormous health burden, efforts to control and potentially eradicate this disease have become an urgent public health priority (2, 3). Effective control and elimination measures require a clear understanding of parasite, vector, human, and environmental factors that sustain malaria transmission. This includes a systematic evaluation of potential zoonotic reservoirs and the risk that they may pose for humans. Recently, close genetic relatives of the human malaria parasites *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium vivax* have been identified in wild-living apes in sub-Saharan Africa (4–8). These parasites were tentatively classified on the basis of their sequence relationships into a number of different species, six of which were closely related to human *P. falciparum* and placed into a separate *Plasmodium* subgenus, termed *Laverania* (4, 7, 9, 10). Of these six *Laverania* species, *Plasmodium reichenowi*, *Plasmodium gaboni*, and *Plasmodium billcollinsi* were identified only in chimpanzees, whereas *Plasmodium adleri*, *Plasmodium blacklocki*, and *Plasmodium praefalciparum* were only found in gorillas. Moreover, *P. praefalciparum* was shown to be the immediate precursor of human *P. falciparum* (4). Because candidate *Anopheles* vectors have been identified that may transmit both ape and human parasites (11), the fact that a large fraction of wild-living apes is endemically *Plasmodium* infected has raised concerns that they might represent a source of recurring human infections (4, 5, 9, 12, 13).

In this study, we tested humans who live in remote rural areas of southern Cameroon for ape *Plasmodium* zoonoses. We specifically screened for *Laverania* infections, because these are the most abundant and widespread in resident ape populations, and because one of them, *P. praefalciparum*, has crossed the species barrier from gorillas to humans already once (4). Moreover, ape *Laverania* parasites have been studied extensively at the molecular level, with numerous mitochondrial, apicoplast, and nuclear sequences available for analyses. To detect zoonotic infections, we (*i*) developed a *Plasmodium* species-specific diagnostic PCR, (*ii*) used ultra-deep sequencing to search for low-abundance ape parasites in mixed *Plasmodium* species infections, and (*iii*) used single-genome amplification (SGA) to characterize the genetic diversity of human *P. falciparum* in southern Cameroon. Our study systematically searched for *Plasmodium* zoonoses in west central Africa, thus providing insight into the host range of human and great ape parasites.

## Results

**Genetic Analysis of Human *Plasmodium* Infections in Rural Cameroon.** Cameroon is an area of high malaria endemicity, with nearly 100% of clinical cases believed to be caused by *P. falciparum* (1).

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The authors declare no conflict of interest.

Data deposition: All newly derived *Plasmodium* sequences have been deposited in the GenBank database (accession nos. KC175306–KC175322 and KC203521–KC203587). The 454 pyrosequencing read data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA), www.ncbi.nlm.nih.gov/sra (accession no. SRP019191).

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However, few of these infections have been molecularly characterized and the extent of parasite diversity, both at the intraspecies and interspecies level, is largely unknown. Studying the epidemiology and natural history of HIV type 1 (HIV-1) infections in Cameroon, we previously collected large numbers of buffy coat samples, which represent thin layers of leukocytes on the surface of sedimented erythrocytes (14). These samples also contain *Plasmodium* DNA, because parasite-infected red blood cells concentrate immediately below the buffy coat layer and are thus harvested together with the leukocytes (15). To characterize the *Plasmodium* species that commonly infect humans in rural Cameroon, we selected samples from 318 inhabitants of seven remote villages (Fig. 1 and *SI Appendix*, Table S1). These study sites were chosen because of the high *Laverania* prevalence rates in chimpanzee and gorilla populations in adjacent forest regions (Fig. 1*A*). All sampled individuals lived in close proximity to ape habitats (Fig. 1*B*) and included forest dwellers, hunters, members of local pygmy tribes, and individuals who worked at logging concessions. Because many of these subjects spent numerous hours and days in the forest, we reasoned that at least some of them were exposed to ape *Plasmodium*-infected *Anopheles* mosquitoes. To examine whether such exposures had resulted in parasite transmission, we screened buffy coat DNA for ape parasites by diagnostic PCR. Using primers previously shown to amplify ape *Laverania* parasites with high sensitivity and specificity (4), we targeted a 939-bp region spanning most of the cytochrome *b* (*cytb*) gene of the *Plasmodium* mitochondrial DNA (mtDNA) genome (Fig. 2). This analysis identified 194 of the 318 blood samples to be PCR positive (61%), all of which contained human parasites as determined by direct sequencing: 181 samples contained *P. falciparum*, 12 samples contained *P. ovale*, and 1 sample contained *P. malariae* as the predominant *Plasmodium* species (*SI Appendix*, Table S1). From this experiment, we concluded that zoonotic *Laverania* infections, if they indeed occurred, were rare and unlikely to represent monoinfections.


**Fig. 1.** Screening of humans in rural Cameroon for zoonotic *Plasmodium* infections. (*A*) Location of human study sites (red stars). Eight rural villages were selected for molecular epidemiological studies because of their proximity to wild-living chimpanzee (yellow circles) and gorilla (yellow hexagons) populations known to harbor *Laverania* infections at high prevalence rates. Previously estimated infection rates (4) are shown for the most proximal field sites (denoted by a two-letter code). Country borders, major rivers, and the capital city of Yaoundé (red triangle) are also shown. A red star with asterisk highlights the location of five closely spaced villages (Mboumo, Eboumetoum, Aviation, Nkonzu, and Kompia). (*B*) View of one rural village, depicting the close proximity of human residences and sleeping quarters to the surrounding forest.

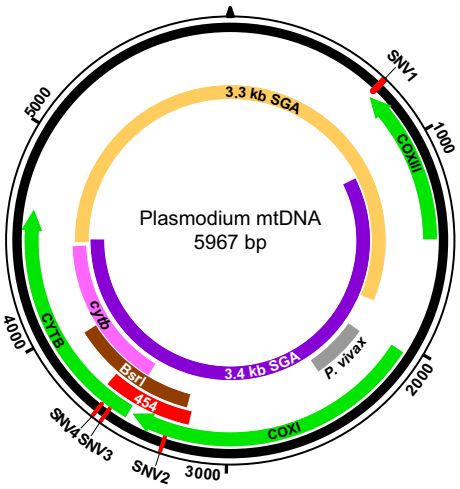


**Fig. 2.** Schematic representation of the *Plasmodium* mitochondrial genome. DNA fragments amplified for diagnostic purposes (*cytb*, BsrI, *P. vivax*), 454 sequencing (454), and SGA (mtDNA-3.3 kb; mtDNA-3.4 kb) are shown in relation to cytochrome *b* (*cytb*), cytochrome *c* oxidase subunit I (*coxI*), and cytochrome *c* oxidase subunit III (*coxIII*) coding regions, respectively. The positions of four SNVs (SNV1–SNV4), which distinguish human *P. falciparum* from ape *Laverania* parasites, are shown in red.

**Diagnostic PCR Capable of Differentiating Human and Ape *Laverania* Species.** To screen a larger number of individuals, we developed a *Plasmodium* species-specific PCR. Aligning several hundred ape and human *Plasmodium* mitochondrial genomes, we had previously noted four single-nucleotide variants (SNVs) that distinguished all known *P. falciparum* strains from the six ape *Laverania* species (4). One of these (SNV4) comprised a BsrI restriction enzyme site (ACTGGN) that was present in 134 of 135 ape *Laverania* sequences, but was absent from all of 859 human *Plasmodium* sequences in the database (Fig. 2). To determine whether PCR amplification followed by BsrI cleavage could be used to screen human blood samples for ape *Laverania* infections, we designed primers for a ~700-bp DNA fragment that spanned the diagnostic SNV4 site (Figs. 2 and 3). Testing these primers on fecal samples from *Plasmodium*-infected apes, we obtained PCR products that were cleaved by BsrI and yielded the expected fragments for the respective ape *Plasmodium* species (Fig. 3 *A* and *B*). In contrast, amplicons from human *P. falciparum*, *P. malariae*, and *P. vivax* reference strains were not cleaved by BsrI, and although *P. ovale* amplicons were cleaved once, the resulting fragments were readily distinguishable from those of the ape parasites. BsrI cleavage products were also visible in mixtures of human and ape parasite DNAs, including in preparations that contained *P. falciparum* at a 10-fold excess. These data indicated that PCR amplification, followed by BsrI cleavage, represented a viable screening approach for zoonotic *Laverania* infections, even when ape and human parasites were present in mixed-species infections.

Using this *Plasmodium* species-specific PCR assay, we screened 1,165 buffy coat samples from villagers native to southeastern Cameroon (Fig. 1 and *SI Appendix*, Fig. S1). For control, we also analyzed 85 samples from HIV-1–infected individuals in the capital city Yaoundé. Testing a total of 1,250 samples, we amplified BsrI-specific fragments from 872 of them (*SI Appendix*, Table S1), three of which were cleaved by BsrI (Fig. 3*C*). Two of these samples (KI051 and EC1592) yielded PCR cleavage products consistent with *P. ovale* infection, which was confirmed by sequence analysis. The third sample (EC1041), from a child in

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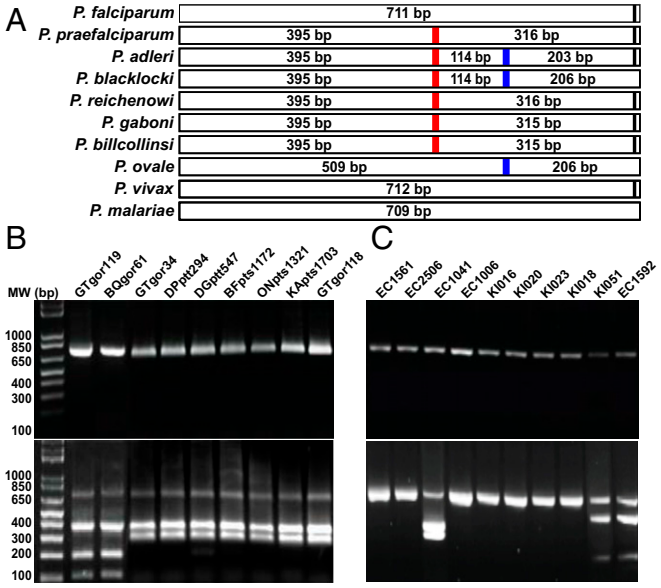


**Fig. 3.** A *Plasmodium* species-specific PCR assay capable of differentiating human and ape *Laverania* parasites. (*A*) Predicted BsrI cleavage products for different *Plasmodium* species infecting humans and apes. A red vertical line highlights a BsrI site unique to all ape *Laverania* parasites. A second BsrI site found only in *P. adleri*, *P. blacklocki*, and *P. ovale* is highlighted in blue. (*B*) Diagnostic PCR of ape *Laverania* infections. *Laverania*-positive ape fecal samples were PCR positive (*Upper*) and yielded appropriately sized fragments upon BsrI cleavage (*Lower*). (*C*) Diagnostic PCR of human *Plasmodium* infections. *Plasmodium*-positive human samples yielded amplicons of predicted size (*Upper*), with BsrI cleavage products observed for three (*Lower*).

Mboumo, yielded the ape-specific PCR cleavage products of 395 and 316 bp, respectively (Fig. 3*C*), suggestive of ape *Laverania* infection. However, sequence analysis of the BsrI fragment failed to confirm this diagnosis, identifying instead a *P. falciparum* variant that exhibited a single point mutation at the SNV4 site. This was confirmed by sequencing the entire mitochondrial genome of this variant (amplified as two partially overlapping 3.4- and 3.3-kb fragments; Fig. 2), which contained the SNV4 point mutation, but lacked additional ape *Plasmodium*-specific signatures. Thus, the BsrI diagnostic PCR had uncovered a rare *P. falciparum* variant whose mitochondrial sequence was identical to that of other *P. falciparum* strains, except for a single (ape-like) back mutation at the SNV4 site.

**Molecular Characterization of Human *Plasmodium* Infections by 454 Deep Sequencing.** The *cytb* and BsrI PCR results indicated that the vast majority of villagers in rural Cameroon harbored human *Plasmodium* parasites, with *P. falciparum* representing the predominant species. We thus reasoned that ape *Laverania* parasites—if they were transmitted to humans—would likely replicate less efficiently and represent only a minor fraction of the total parasite burden within an infected individual. To increase the likelihood of detecting such variants, we selected an ultra–deep-sequencing approach, which generates tens of thousands of sequences of the same genetic locus and can thus detect low-abundance variants with great sensitivity (16–18). Specifically, we used the 454 GS FLX Titanium chemistry to sequence a 405-bp fragment of the *Plasmodium* mtDNA genome that included three of the four diagnostic SNVs (Fig. 2) and thus differentiated even the closest human and ape parasites (*SI Appendix*, Fig. S2).

To explore the utility of the 454-sequencing approach, we initially pooled amplicons from 77 *Plasmodium* positive human buffy coat samples, which yielded 465,391 high-quality reads. Each read was classified by determining its minimum edit distance to a large set of *Plasmodium* reference sequences (*SI Appendix*, Table S2). This approach identified 458,676 reads (98.56%) to represent *P. falciparum*, 76 reads (0.02%) to represent *P. ovale*, and 6,266 reads (1.35%) to represent *P. malariae* (*SI Appendix*, Fig. S3 *A*–*C*), the classification of which was confirmed by phylogenetic analyses (*SI Appendix*, Fig. S4). One single read was classified as *P. praefalciparum* (*SI Appendix*, Fig. S3*D*); however, closer inspection of its sequence revealed multiple indels that caused inactivating frameshift mutations as well as a substitution that was not found in any other *P. praefalciparum* strain. Moreover, this read differed from the closest *P. praefalciparum* reference by five mutations, but from the closest *P. falciparum* reference by six mutations, and contained only two of the three ape-specific SNVs. We thus concluded that this read was erroneously classified as *P. praefalciparum* due to PCR and/or 454 process errors, and that there was no evidence of ape *Laverania* infection in any of the 77 sequenced individuals (*SI Appendix*, *Results and Analysis*).

**Identification of *Plasmodium* Multispecies Infections by 454 Deep Sequencing.** To extend the search for zoonotic *Laverania* infections, we selected an additional 437 samples for 454 sequencing (*SI Appendix*, Fig. S1) but improved the methodology. First, we inserted a 12-mer barcode into the sequencing primer to permit the computational sorting of individual samples (19). Second, we reversed the sequencing direction to increase the number of reads that covered at least two diagnostic SNVs (*SI Appendix*, Fig. S2). Third, we amplified samples using the lowest possible number of cycles to reduce PCR-introduced errors (*SI Appendix*, Table S3). Finally, we included cloned fragments (3.4 kb) of the *P. falciparum*, *P. malariae*, and *P. ovale* mitochondrial genome as controls, which allowed us to perform a formal error calculation for each pyrosequencing run (*SI Appendix*, *Results and Analysis*). The resulting pyrosequencing reads were sorted by sample and analyzed.

The identification of rare ape *Plasmodium* parasites necessitated a method that could differentiate true sequence changes from 454-sequencing error. We thus used a maximum-likelihood based approach to determine which and how many different *Plasmodium* species were present in each barcoded human sample (*SI Appendix*, *Results and Analysis*). For each sample, we generated pairwise alignments of all reads with all *Plasmodium* reference sequences and then used a model of empirically determined 454-sequencing error (*SI Appendix*, Table S4) to calculate the probability that a read was derived from a particular reference. Using this approach, we determined the *Plasmodium* species composition in all barcoded human samples. Of 437 samples, 349 contained only *P. falciparum*, one contained only *P. malariae*, and 4 contained only *P. ovale* sequences (Table 1). A further 61 samples contained both *P. falciparum* and *P. malariae*, 13 samples contained both *P. falciparum* and *P. ovale*, and 9 samples contained all three species (Table 1). Importantly, none of the human blood samples contained any of the six ape *Laverania* species, including *P. praefalciparum*. Moreover, none of the samples contained *P. vivax* sequences.

To be certain that our inability to find ape *Plasmodium* zoonoses was not due to technical limitations, we used the identical 454 methodology to deep sequence *Plasmodium* parasites from fecal samples of infected apes. Analysis of 37,644 filtered reads from two western lowland gorillas (*Gorilla gorilla gorilla*), three central chimpanzees (*Pan troglodytes troglodytes*), and one eastern chimpanzee (*Pan troglodytes schweinfurthii*) confirmed the presence of all six ape *Laverania* species as well as *P. vivax*-like parasites (*SI Appendix*, Fig. S5). We also characterized the proportion of humans who harbored multiple *P. falciparum* variants (*SI Appendix*, *Results and Analysis*). Multiple variant infections were detected in 10% of all subjects, with a maximum of four variants per person (*SI Appendix*, Table S5 and Fig. S6). Importantly, minor variants could be identified at levels as low as 0.006% of the total parasite burden, thus providing direct evidence that our deep-sequencing approach was capable of identifying very low-abundance *Plasmodium* variants.

**Table 1. Species composition of human *Plasmodium* infections in Cameroon as determined by 454 sequencing**

| Identified species* | No. of samples |
|---|---|
| *P. falciparum* | 349 |
| *P. falciparum* and *P. malariae* | 61 |
| *P. falciparum* and *P. ovale* | 13 |
| *P. falciparum*, *P. malariae*, and *P. ovale* | 9 |
| *P. ovale* | 4 |
| *P. malariae* | 1 |
| Total | 437 |

*See *SI Appendix*, Fig. S6, for a breakdown of the parasite composition of individual samples.

**Genetic Diversity of *P. falciparum* in Rural Cameroon.** Although there are over a hundred near–full-length *P. falciparum* mitochondrial DNA sequences in the database, little is known about the extent of genetic diversity of this parasite in central Africa. In particular, there are no molecularly characterized human strains from areas where wild-living apes are endemically infected with *Laverania* parasites. To characterize the *P. falciparum* variants prevalent in rural Cameroon, we selected *Plasmodium*-positive samples from seven different locations (*SI Appendix*, Table S1) and subjected them to SGA, targeting the region of the mitochondrial genome (3.4 kb) known to exhibit the greatest diversity between ape and human *Laverania* lineages (Fig. 2). We selected SGA rather than conventional PCR, because this method eliminates Taq polymerase-induced recombination as well as nucleotide misincorporations in finished sequences, and thus ensures an accurate representation of parasite variants as they exist in vivo (4, 20). Sequencing between one and eight SGA amplicons per sample, we generated a total of 684 half-genome mtDNA sequences. Phylogenetic analyses revealed that these represented 69 unique *P. falciparum* haplotypes, 62 of which had not previously been reported. Despite this diversity, all variants grouped with previously identified *P. falciparum* sequences, forming a single well-supported clade within the radiation of *P. praefalciparum* from gorilla (Fig. 4). This was the case even after inclusion of a *P. falciparum* variant (EC1041; Fig. 3) that contained one of the three ape-specific SNVs at the BsrI cleavage site (Fig. 4). These results failed to uncover additional cross-species transmissions, including human-to-ape transfers, and thus confirmed that extant *P. falciparum* emerged in humans following a single introduction of a gorilla parasite.

**Absence of *P. vivax* in Humans from Southern Cameroon.** Although the great majority of individuals in Cameroon are Duffy negative (21), it has been proposed that *P. vivax* persists in west central human populations at a very low frequency (22). Because deep sequencing failed to identify evidence of *P. vivax* infection in 514 individuals, we considered the possibility that the 454 primers were less efficient in amplifying *P. vivax* compared with the other *Plasmodium* species. We thus designed *P. vivax*-specific primers in the mtDNA *cox1* gene (Fig. 2) and used these to screen an additional 558 human samples (*SI Appendix*, Fig. S1). Although 47 samples yielded a visible amplification product (*SI Appendix*, Table S6), none of these represented *P. vivax* or *P. vivax*-like infections as determined by sequence analysis of the corresponding amplicon. Instead, 37 of the PCR-positive samples contained *P. malariae*, whereas the remaining 10 contained *P. ovale*. Moreover, sequence analysis of the Duffy promoter region from 90 human samples confirmed a Duffy-negative phenotype in all of them (*SI Appendix*, Table S6). Thus, using both conventional PCR and 454-sequencing approaches, we found no evidence of *P. vivax* infections in individuals living in rural Cameroon.

## Discussion

Chimpanzees and gorillas harbor at least 10 different *Plasmodium* species, including six of the subgenus *Laverania* that are closely related to *P. falciparum* (4–8). The discovery of this previously unrecognized reservoir has prompted concerns that wild-living apes might constitute a source of recurrent human infection (4, 5, 9, 11–13). In this study, we set out to examine this possibility for several reasons. First, the ape reservoir is substantial, both in terms of geographic distribution and complexity of *Plasmodium* species. Second, both western gorillas and chimpanzees are infected at high endemicity throughout their habitat. Third, *Plasmodium* zoonoses can have significant public health impact. A case in point is *Plasmodium knowlesi*, a macaque parasite that has been shown to cause hundreds of cases of human malaria every year (23–25). Finally, *Plasmodium* zoonoses have been misdiagnosed in the past: *P. knowlesi* was initially mistaken for *P. malariae*, ultimately requiring the development of molecular tools to facilitate its detection (24). Given that malaria infections in central Africa are rarely genetically characterized, we considered the possibility that ape *Plasmodium* zoonoses might have also been overlooked. To test this, we developed diagnostic PCR assays and next-generation sequencing approaches that permitted the detection of rare *Plasmodium* variants, even when they occurred in the context of mixed-species infection with *P. falciparum*, which is known to reach very high blood titers. Using these approaches to test 1,400 blood samples from individuals native to rural Cameroon, we failed to detect previously unknown human *Plasmodium* infections (*SI Appendix*, Table S1). There was no evidence for zoonotic infection with any of the six ape-specific *Laverania* species or non-*Laverania* parasites identified only in wild apes (4). Instead, we detected *P. falciparum*, *P. ovale*, and *P. malariae* in a large fraction of individuals, both as monospecies and mixed-species infections (Table 1 and *SI Appendix*, Fig. S6). From these data, we conclude that ape *Laverania* zoonoses can be ruled out as an ongoing threat to public health in this region of west central Africa.

Although we failed to find ape *Laverania* infections in humans, our data cannot exclude the possibility of very rare transmission events (4). Depending on the host, parasite, and/or vector properties required for successful transmission, a much larger number of individuals from geographically more diverse regions will have to be screened to exclude rare spillovers. Because the *Anopheles* species that transmit *Plasmodium* parasites among wild apes are only beginning to be characterized (11), it is conceivable that the ecology, distribution, and feeding preferences of these vectors play a much greater role in determining the likelihood of zoonotic transmission than the mere geographic proximity of human habitations to infected ape populations. Nonetheless, it seems unlikely that the absence of zoonotic *Laverania* infections in rural Cameroon is solely due to a lack of human exposure. This is because even among endemically infected chimpanzees and gorillas, there is no evidence that *Laverania* parasites cross between the two ape species (4). This remarkable host specificity suggests a restriction at the parasite–host interface, which is supported by comparisons of *P. falciparum* and *P. reichenowi* gene sequences. It is known that the genes involved in erythrocyte invasion are evolving rapidly between *Laverania* parasites (26). Moreover, erythrocyte invasion of *P. falciparum* is absolutely dependent on the interaction of its PfRh5 ligand with the human Ok blood group antigen basigin (27). Human, chimpanzee, and gorilla homologs of basigin are highly divergent, suggesting that ape *Laverania* species have to overcome significant adaptive hurdles before they can spread efficiently in a different host.

Given these restrictions, the question arises how the gorilla precursor of *P. falciparum* managed to colonize humans. One possibility is that *P. praefalciparum* underwent a very specific mutation in a host-compatibility factor that changed its host preference from gorilla to human. Because *P. falciparum* emerged only once (Fig. 4), this mutation must have been difficult to generate and/or must have arisen under exceedingly favorable transmission conditions. Another possibility is that the immediate precursor of *P. falciparum* was the product of a rare recombination event. Regardless of the circumstances, it seems

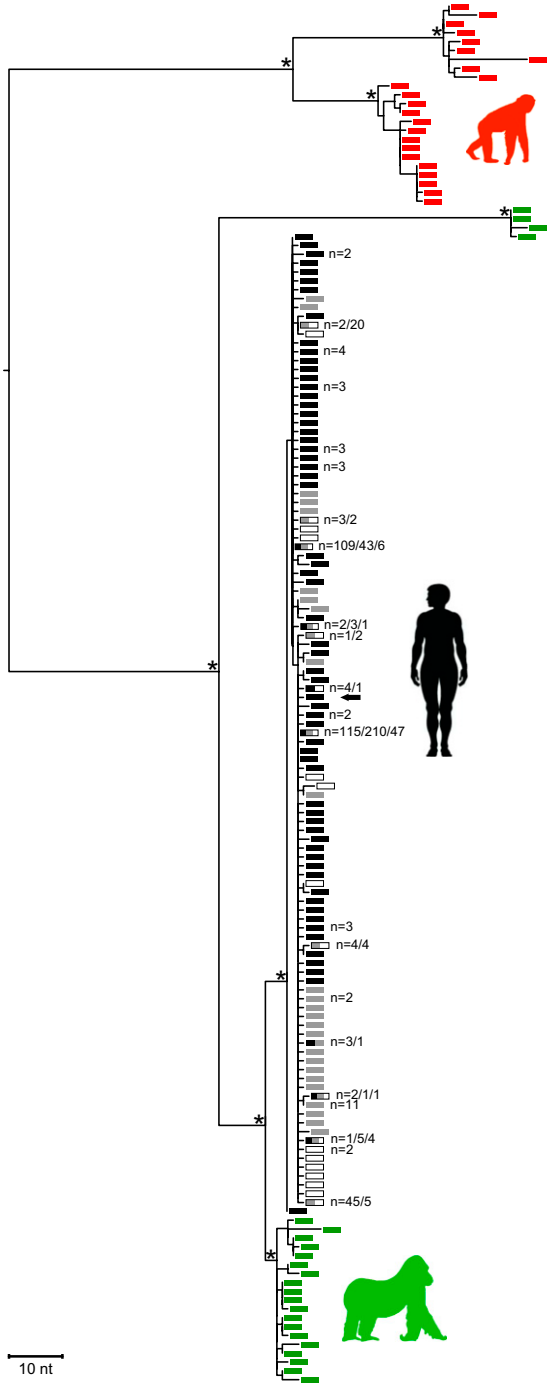


**Fig. 4.** Phylogeny of *P. falciparum* strains from rural Cameroon. Newly derived *P. falciparum* sequences from humans living in Cameroon (black) are shown in relation to *P. falciparum* sequences from GenBank (white) and the Sanger Institute (gray), as well as to *P. praefalciparum* and *P. reichenowi* sequences from gorillas (green) and chimpanzees (red), respectively. The tree includes 684 new SGA-derived 3.4-kb mitochondrial sequences from 229 infected humans, including one that contained a back mutation at the ape-specific SNV4 site (highlighted by arrow; Fig. 3*C*). Numbers at tips indicate the number of times that a sequence was found in Cameroon (black), the Sanger dataset (gray), or GenBank (white; for sequences present in multiple datasets, numbers are listed in sequence). The tree was inferred using maximum-likelihood methods. The asterisks indicate posterior probabilities above 0.9. (The scale bar represents 10-nt substitutions.)

clear that the generation of an ape *Laverania* strain that is capable of spreading in humans is an extremely rare event, which may explain why we failed to detect such variants.

In addition to *Laverania* species, wild-living chimpanzees and gorillas also harbor *P. vivax*, *P. malariae*, and *P. ovale*-like infections. Because very few of these ape parasites have been molecularly characterized, it remains unknown whether they represent members of the same or different *Plasmodium* species as their human counterparts. Transmission studies conducted nearly a hundred years ago demonstrated that non-*Laverania* species cross between apes and humans more readily than *Laverania* species (28–32). Two species, *Plasmodium schwetzi* and *Plasmodium rodhaini*, have been experimentally transmitted to humans in the past (29, 30, 32). Because neither species has been molecularly characterized, it is unknown whether they represent the *P. vivax*, *P. malariae*, and/or *P. ovale*-like infections that have more recently been identified in wild apes. It will thus be important to characterize more of these ape parasites to understand their evolutionary history and characterize their zoonotic potential.

The genetic diversity of *P. falciparum* in central Africa is largely unknown because only very few strains from this geographic region have been molecularly characterized. It has thus been argued that this lack of sequence information has confounded previous evolutionary analyses and led to erroneous conclusions concerning the origin of *P. falciparum* (33). In particular, it has been proposed that *P. praefalciparum* is more likely the result of a human-to-gorilla transmissions of *P. falciparum* than the other way around (33). To examine this possibility, we amplified mitochondrial half-genome sequences from 229 *P. falciparum*-positive samples collected in remote rural areas of Cameroon. We found that *P. falciparum* strains from rural Cameroon were indeed genetically more diverse than previously appreciated. Analyzing 684 single template-derived sequences, we identified 69 unique *P. falciparum* variants, 62 of which had not previously been reported. However, none of these variants changed previous conclusions concerning the evolutionary history of *P. falciparum*. Phylogenetic analysis revealed that all newly characterized variants grouped with previously reported *P. falciparum* strains, forming a well-supported clade within the *P. praefalciparum* radiation (Fig. 4). An additional 354 sequences of cosmopolitan *P. falciparum* strains from the Sanger Institute supported this conclusion (34), indicating that all human *P. falciparum* sequences coalesced to a single common ancestor. Thus, the addition of over 1,000 new *P. falciparum* sequences, including over 600 from individuals living near wild-ape populations, confirmed that *P. falciparum* is of gorilla origin and emerged in humans following a single cross-species transmission event (4).

*P. vivax* is extremely rare in humans in west and central Africa due to the near fixation of the Duffy-negative phenotype, which confers resistance to this parasite (21). Nonetheless, *P. vivax* seems to be able to cause clinical infection of Duffy-negative individuals in Madagascar (35) and *P. vivax*-specific antibodies have been reported in 13% of humans living in Pointe-Noire, a city in the Republic of Congo (36). To examine whether *P. vivax* is maintained in west central African human populations, we screened nearly 700 blood samples from Cameroonian villagers for *P. vivax* mitochondrial sequences (*SI Appendix*, Fig. S1). Using both PCR and 454-sequencing approaches, we failed to identify *P. vivax* infection in inhabitants from seven different rural villages. Finally, all human samples tested were Duffy negative, suggesting that the fraction of Duffy-positive individuals in rural areas of west central Africa is exceedingly low. These data differ from results of others who have reported the presence of *P. vivax* in Equatorial Guinea and Angola. In these studies, the *P. vivax*-infected individuals were either Duffy positive (37) or diagnosed solely based on *P. vivax*-specific PCR products without sequence verification (38). Given the frequency of off-target amplification even with *P. vivax*-specific primers (*SI Appendix*, Table S6), any *P. vivax* diagnosis in central Africa should be confirmed by sequence analysis. Until this is done, the presence of *P. vivax* in rural west central Africa remains questionable.

## Methods

**Sample Collections.** Human buffy coat samples ($n = 1,402$) were selected from anonymized sample collections previously obtained for molecular epidemiological studies of HIV-1 in Cameroon (14). Fecal samples from wild-living apes known to contain *Laverania* and non-*Laverania* parasites served as positive controls (4). The study was reviewed and approved by the Institutional Review Board of the University of Pennsylvania.

**Detection of *Plasmodium* Infections.** Human buffy coat samples were screened for *Plasmodium* sequences using a diagnostic (*cytb*) PCR (4) followed by direct amplicon sequencing. Samples were also screened using a newly developed *Plasmodium* species-specific (BsrI) PCR assay (*SI Appendix, Materials and Methods*, and Fig. S1).

**Pyrosequencing.** A 405-bp fragment of the mitochondrial genome spanning three SNVs unique to ape *Laverania* parasites (Fig. 2) was amplified and sequenced on a Genome Sequencer FLX Titanium Series (Roche) (*SI Appendix, Materials and Methods*).

**SGA and Sequencing.** To derive *Plasmodium* mitochondrial sequences devoid of PCR-induced substitutions and/or recombination, a 3.4-kb fragment was amplified and sequenced from 229 *cytb* PCR-positive human samples as described (4). DNA was endpoint diluted such that single templates were amplified, thus excluding PCR-induced substitution and recombination errors (*SI Appendix, Materials and Methods*).

**Phylogenetic Analyses.** SGA-derived 3.4-kb mitochondrial sequences were aligned with human and simian reference sequences. Trees were inferred using maximum-likelihood and Bayesian methods (*SI Appendix, Materials and Methods*).

**Detection of *P. vivax* Infection.** Human samples were screened for *P. vivax* infections by nested PCR. Primers were specifically designed to avoid off-target amplification of *P. falciparum* or other *Laverania* species, and were shown to amplify ape *P. vivax*-like parasites as well as human *P. vivax* with high sensitivity and specificity (*SI Appendix, Materials and Methods*).

**Duffy Phenotype.** Buffy coat DNA samples were used to amplify the Duffy promoter region by nested PCR analysis. The Duffy phenotype was determined by direct amplicon sequencing (*SI Appendix, Materials and Methods*).

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