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Origin of the human malaria parasite *Plasmodium falciparum* in gorillas

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Author Information. SGA-derived *Plasmodium* nucleotide sequences have been deposited in NCBI GenBank under accession numbers *cytB*: HM234976-HM235117; *ldh*: HM235118-HM235143; *clpC*: HM235144-HM235170; mtDNA-3.3kb: HM235171-HM235268; mtDNA-3.4kb: HM235269-HM235404 (also see Supplementary Table 6).

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Abstract

Plasmodium falciparum is the most prevalent and lethal of the malaria parasites infecting humans, yet the origin and evolutionary history of this important pathogen remain controversial. Here, we developed a novel polymerase chain reaction based single genome amplification strategy to identify and characterize *Plasmodium spp*. DNA sequences in fecal samples of wild-living apes. Among nearly 3,000 specimens collected from field sites throughout central Africa, we found *Plasmodium* infection in chimpanzees (*Pan troglodytes*) and western gorillas (*Gorilla gorilla*), but not in eastern gorillas (*Gorilla beringei*) or bonobos (*Pan paniscus*). Ape plasmodial infections were highly prevalent, widely distributed, and almost always comprised of mixed parasite species. Analysis of more than 1,100 mitochondrial, apicoplast and nuclear gene sequences from chimpanzees and gorillas revealed that 99% grouped within one of six host-specific lineages representing distinct *Plasmodium* species within the subgenus *Laverania*. One of these from western gorillas was comprised of parasites that were nearly identical to *P. falciparum*. In phylogenetic analyses of full-length mitochondrial sequences, human *P. falciparum* formed a monophyletic lineage within the gorilla parasite radiation. These findings indicate that *P. falciparum* is of gorilla and not of chimpanzee, bonobo or ancient human origin.

Malaria is a blood infection caused by mosquito (*Anopheles spp.*) borne apicomplexan parasites of the genus *Plasmodium*[1-3]. Of five *Plasmodium* species known to infect humans, *P. falciparum* causes by far the greatest morbidity and mortality, with several hundred million cases of clinical malaria and more than one million deaths occurring annually[1,2]. While much progress has been made in the treatment and prevention of *P. falciparum*[4], the origin and natural reservoir(s) of this and related plasmodial pathogens remain controversial. Until recently, the closest known relative of *P. falciparum* was a chimpanzee parasite, *P. reichenowi*, which was assumed to have diverged from its human counterpart at the same time as the ancestors of chimpanzees and humans, more than 5 Myr ago[5-8]. Within the past year, other closely related *Plasmodium* strains were detected in chimpanzees (*Pan troglodytes*), western gorillas (*Gorilla gorilla*), and bonobos (*Pan paniscus*), raising the possibility that *P. falciparum* in humans could have arisen as a consequence of cross-species transmission from one or more of these apes[9-12]. However, all of these studies were limited by an analysis of only few apes, many of which were captive and living in close proximity to humans. In addition, all prior studies employed non-limiting dilution polymerase chain reaction (PCR) amplification methods that were prone to generate artifactual mosaic sequences by recombination between genetically distinct templates. Here, we used conventional and single template PCR amplification methods to screen and analyze wild-living chimpanzee, bonobo and gorilla populations across sub-Saharan Africa for *P. falciparum* related parasites.

Prevalence of ape *Plasmodium* infections

To determine the geographic distribution, species association and prevalence of ape *Plasmodium spp.* infections, we adapted a previously described PCR based diagnostic method[10] to amplify a 956 bp fragment of *Plasmodium* cytochrome B (*cytB*) sequences from fecal DNA (Supplementary Fig. 1a). Ape fecal samples were selected from existing

specimen banks that we had collected earlier for molecular epidemiological studies of simian retrovirus infections[13-16]. Except for 28 samples from one habituated gorilla community at the DS field site (Fig. 1), all other specimens were derived from nonhabituated apes living in remote forest areas (Supplementary Table 1). Chimpanzee (n=1,827), gorilla (n=805) and bonobo (n=107) samples were subjected to diagnostic PCR, and all amplification products were sequenced to confirm *Plasmodium* infection. In addition, a subset of samples (n=1,027), including all specimens from eastern gorillas and bonobos, was subjected to microsatellite analysis of host genomic DNA[14-16] to determine the number of individuals tested at particular field sites (Supplementary Table 1). Microsatellite analysis also provided quantitative estimates of specimen integrity (Supplementary Table 2) and redundant sampling (Supplementary Table 3), thereby allowing us to determine the sensitivity of the non-invasive diagnostic test by identifying the proportion of PCR positive specimens from infected apes who were sampled more than once. From 32 such individuals, we estimated the test sensitivity to be 57% (Supplementary Table 4) and calculated the prevalence of ape infection at each field site (Supplementary Table 1). The results revealed widespread *Plasmodium* infection in chimpanzees and western gorillas, but not in eastern gorillas or bonobos.

Ape malaria parasites were detected at 32 of 45 chimpanzee collection sites, and at 17 of 20 western gorilla collection sites (Fig. 1), including every site where at least 10 individuals were estimated to have been sampled. *Plasmodium* infection was endemic in Nigeria-Cameroon (*P. t. ellioti*), central *(P. t. troglodytes*) and eastern (*P. t. schweinfurthii*) chimpanzees as well as in western lowland gorillas (*G. g. gorilla*), with estimated prevalence rates ranging from 32% to 48% (Table 1). The true infection rates are likely to be higher still, since *Plasmodium* detection in fecal samples can be expected to be less sensitive than detection in blood, as is the case for urine and saliva samples[17]. Although wild-living western chimpanzees (*P. t. verus*) and Cross River gorillas (*G. g. diehli*) were not tested in this study, these two subspecies have previously been shown to harbor *Plasmodium* parasites in the wild[9,10]. Based on these data, it is clear that chimpanzees and western gorillas represent substantial *Plasmodium* reservoirs. Surprisingly, we did not find this to be true for eastern gorillas or bonobos. Screening 71 and 58 members of these two species at multiple field sites, we failed to detect *Plasmodium* infection in any of them (Supplementary Table 1). These findings suggest that malaria parasites are rare or absent in some wild-living ape communities, possibly reflecting regional, ecological or seasonal differences in the distribution and/or host specificities of the transmitting mosquito vector(s). Additional field studies are needed to determine whether eastern gorillas and bonobos are infected by *Plasmodium* parasites at other locations or if they harbor divergent parasites not detected by current diagnostic assays.

SGA analysis of ape *Plasmodium* species

To examine the evolutionary relationships of the newly identified *Plasmodium* parasites, we constructed phylogenetic trees for a subset of the diagnostic *cytB* sequences. This analysis showed that all sequences, except for one *P. ovale*-like strain, fell into one large monophyletic clade that also included *P. reichenowi* and *P. falciparum* (Supplementary Fig. 2). Parasites related to *P. reichenowi* and *P. falciparum* have previously been classified into a subgenus, termed *Laverania*, to distinguish them from more divergent *Plasmodium* species[18]. Our results thus indicated that parasites from this subgenus were common and widespread among wild ape populations. However, the topology of the *Laverania* clade was highly unusual, characterized by only few discrete clades and multiple sequences with very short branches attached to internal branches. Moreover, repeated PCR analysis of the same fecal samples yielded sequences that clustered variably in different parts of the tree (Supplementary Fig. 2). These findings indicated simultaneous infection with genetically

diverse *Plasmodium* parasites and suggested that conventional (bulk) PCR amplification had generated *in vitro* recombinants. To examine this possibility, we re-analyzed the same *Plasmodium* positive fecal samples by single genome amplification (SGA), a molecular strategy that has been used extensively to characterize the genetic identity and quasispecies complexity of human and simian immunodeficiency viruses (HIV/SIV)[19-23]. Fecal DNA was diluted so that fewer than 30% of all PCR reactions yielded an amplification product, which ensured amplification of single *Plasmodium* templates in most reactions[19-23]. All amplicons were sequenced directly and sequences containing mixed bases indicative of more than one amplified template were discarded. Using this approach to characterize the genetic complexity of malaria parasites in fecal samples, we could eliminate both *Taq* polymerase-induced recombination (template switching) and nucleotide misincorporations in finished sequences, thereby ensuring an accurate representation of plasmodial variants as they existed *in vivo*[21-23].

Fig. 2 depicts the phylogenetic relationships of a subset of SGA derived mitochondrial *cytB* sequences (the entire set of 697 sequences is analyzed in Supplementary Fig. 3). As in the corresponding tree of bulk PCR-derived sequences (Supplementary Fig. 2), all SGA derived sequences, except for seven *P. ovale, P. vivax* and *P. malariae*-like strains, grouped within the *Laverania* radiation. However, unlike in the bulk PCR tree, *Laverania* sequences in the SGA tree clustered in a strictly host species-specific manner, forming three chimpanzee (C1-C3) and three gorilla (G1-G3) specific clades, each supported by high bootstrap values. Interestingly, this host specificity did not extend to the subspecies level, since *P. t. ellioti, P. t. troglodytes* and *P. t. schweinfurthii* derived sequences were interspersed; however, *cytB* sequences from *P. t. schweinfurthii* segregated into distinct subclades within two of the three chimpanzee lineages (C1, C2), suggesting a phylogeographic distribution of certain *Plasmodium* variants (Supplementary Figs. 3a and b). None of 363 chimpanzee derived *Plasmodium cytB* sequences was closely related to human *P. falciparum*. Instead, all human sequences grouped within a single clade of parasites (G1) that infected western gorillas at numerous sites in Cameroon (LB, BB, CP, NK, BQ, DD, MM, LM), the Central African Republic (DS, ND) and the Republic of Congo (GT) (Fig. 2 and Supplementary Fig. 3d). A notable finding of the SGA analysis, which was obscured by bulk PCR analysis, was that most apes were co-infected with parasites representing multiple different plasmodial lineages, including variants from (i) the same *Laverania* clade, (ii) different *Laverania* clades, or (iii) *Laverania* and non-*Laverania* clades (Supplementary Table 5). Of 65 chimpanzee and 53 gorilla samples characterized, 48 (74%) and 37 (70%), respectively, harbored more than one genetically distinct parasite strain, and 36 (55%) and 23 (43%) contained members of two or more major *Plasmodium* clades (Supplementary Fig. 3). Given this high frequency of co-infection with divergent parasites, conventional recombination-prone PCR approaches are not appropriate for generating ape *Plasmodium* sequences for phylogenetic analysis. Moreover, previously reported ape *Plasmodium* sequences[9-12] must be interpreted with caution since they were subject to these same confounding factors.

To test the robustness of the phylogenetic relationships depicted in Fig. 2, we used SGA to amplify additional genomic regions from *cytB* positive fecal samples, targeting loci in the mitochondrial, apicoplast and nuclear *Plasmodium* genomes. These regions included 390 bp of the caseinolytic protease C (*clpC*) gene (n=126), 772 bp of the lactase dehydrogenase (*ldh*) gene (n=46), and 3.4 kb (n=165) and 3.3 kb (n=127) fragments that together spanned the entire mitochondrial genome (Supplementary Fig. 1a). Phylogenetic analyses of each of these genomic loci revealed very similar topologies. In trees of *clpC* (Supplementary Fig. 4), *ldh* (Supplementary Fig. 5) and mitochondrial sequences (Supplementary Figs. 6 and 7), *Laverania* sequences formed the same number of chimpanzee (C1-C3) and gorilla (G1-G3) specific clades, albeit with some variations in the relationships among these lineages. Importantly, there was no evidence of recombination between chimpanzee and gorilla

specific parasites, although many of them infected apes at the same field sites. This suggested that *Laverania* parasites are largely host specific (recombination between parasites infecting the same host species could not be assessed because of mixed *Plasmodium* infections). These findings, together with the extent of genetic diversity that distinguishes the various clades, argue strongly for the existence of six distinct *Plasmodium* species within the *Laverania* subgenus (Supplementary Figs. 3-8). Formal classification of these lineages must await additional taxonomic evaluation.

Origin of human *P. falciparum*

The new SGA-derived ape *Plasmodium* sequences call for a reassessment of the origin of human *P. falciparum*. Among over 600 sequences derived from ape samples spanning most of central Africa, we failed to find a single chimpanzee parasite that was sufficiently closely related to *P. falciparum* to represent a progenitor (Fig. 2 and Supplementary Figs. 3-8). Thus, *P. reichenowi*, as well as other chimpanzee *Plasmodium* species, can be excluded as precursors of *P. falciparum*. Instead, all new phylogenetic evidence points to a western gorilla origin of human *P. falciparum* (Fig. 2 and Supplementary Figs. 3-8). To investigate how often gorilla parasites might have colonized humans, we constructed phylogenetic trees from complete mitochondrial genome equivalents of the closest *Plasmodium* relatives of human *P. falciparum* (Fig. 3). In a tree of concatenated CytB, CoxI and CoxIII protein sequences (980 amino acids), all available human *P. falciparum* sequences (n=105) coalesced to a single common ancestor nested within the G1 clade of gorilla parasites (Fig. 3a). Nucleotide sequences from the remaining (non-coding) portions of the mitochondrial genome yielded a very similar topology, again showing that human *P. falciparum* formed a monophyletic lineage within the gorilla *P. falciparum* radiation (Fig. 3b). These findings, together with the observation that human parasites exhibit substantially less sequence diversity than the various ape *Plasmodium* species, including the closest gorilla relatives (Table 2), provide compelling evidence for a gorilla origin of human *P. falciparum*. Moreover, the monophyly of the human parasite sequences (Fig. 3) may indicate that all extant human strains evolved from a single gorilla-to-human cross-species transmission event. Notably, four recently reported *Plasmodium* sequences from captive bonobos[11] also clustered closely with *P. falciparum*. However, unlike the gorilla sequences, the bonobo sequences were interspersed with the human sequences (Fig. 3). This finding, together with the fact that the bonobo parasites encoded dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) drug resistance mutations prevalent in the local human population[11], suggests that the bonobos became infected with human parasites while housed in an urban sanctuary. In fact, the topologies in Fig. 3 are consistent with more than one human-to-bonobo transmission, although some (or all) of the substitutions that distinguish bonobo and human sequences could represent PCR misincorporations since they were not generated by SGA methods[11].

Discussion

Using single template amplification strategies and a much larger collection of ape specimens than previously analyzed, we show here that wild-living chimpanzees and western gorillas are naturally infected with at least nine *Plasmodium* species. Among more than 1,100 SGA derived mitochondrial, apicoplast and nuclear gene sequences from 80 chimpanzee and 55 gorilla samples, we found a total of nine sequences that were related to *P. malariae*, *P. ovale* or *P. vivax* (Supplementary Table 5). All others grouped within one of six chimpanzee- or gorilla-specific lineages representing distinct *Plasmodium* species, three of which had not previously been described. Importantly, all currently available human *P. falciparum* sequences comprised a single lineage nested within the G1 clade of gorilla parasites. This finding indicates that human *P. falciparum* is of gorilla origin, and not of chimpanzee[9,10,12],

bonobo[11] or ancient human[5] origin, and that all known human strains may have resulted from a single cross-species transmission event. What is still unclear is when gorilla *P. falciparum* entered the human population and whether present day ape populations represent a source for recurring human infection. It has been suggested that the limited levels of genetic diversity seen at many loci in human *P. falciparum* reflect a relatively recent selective sweep[8]. Our data suggest that this bottleneck or "Eve event" was instead the consequence of cross-species transmission of a gorilla parasite. It is difficult to time this event without reliable dates to calibrate the *Plasmodium* phylogenetic trees. Previous estimates of dates in the evolution of *Plasmodium* have relied largely on the belief that *P. falciparum* and *P. reichenowi* diverged at the same time as the ancestors of humans and chimpanzees[6-8,24], an assumption that is now groundless. Others have proposed a much shorter time scale coincident with the emergence of agricultural societies in sub-Saharan Africa, the incomplete penetration of protective human gene polymorphisms (e.g., hemoglobin C) that are selected by *P. falciparum* infection, or the speciation of African mosquito vectors[3,25]. Complete sequence analysis of members of the ape *Plasmodium* species identified here may help to resolve this conundrum. The second question, whether additional cross-species transmissions of *Laverania* parasites have given rise to human infections, is more immediately approachable. An alignment of over 100 ape *Plasmodium* mitochondrial genome sequences reveals ape-specific single nucleotide polymorphisms (Supplementary Fig. 1b), which can now be used to screen plasmodial sequences from humans living in close proximity to wild gorillas and chimpanzees. Such studies can inform malaria eradication efforts about potential zoonotic *Plasmodium* reservoirs and provide insights into adaptive changes that might be required for ape *Plasmodium* infection of humans[26].

METHODS SUMMARY

For more detailed methods see Supplementary Methods.

Ape samples

Fecal samples from wild-living chimpanzees, gorillas and bonobos were selected from existing specimens banks[13-16] based on their geographic location, available host genetic information, and species and subspecies origin (Supplementary Table 1).

Conventional PCR

Plasmodium mitochondrial, apicoplast and nuclear gene sequences were amplified as described[9,10,27,28], but using modified primers and PCR conditions suitable for fecal DNA. Bulk PCR positive fecal samples were subsequently subjected to SGA analysis.

Single genome amplification

Single genome amplification of *Plasmodium* sequences from fecal DNA was performed as described[21,22]. All amplicons were sequenced directly, and sequences containing double peaks were discarded.

Prevalence estimations

The prevalence rates of *Plasmodium* infection were estimated based on the proportion of PCR positive fecal samples, correcting for specimen degradation, repeat sampling, and the sensitivity of the diagnostic test.

Phylogenetic analysis

Phylogenetic trees of newly derived ape *Plasmodium* mitochondrial, apicoplast and nuclear gene sequences were inferred by Bayesian[29] and maximum likelihood methods[30] (GenBank accession numbers are listed in Supplementary Table 6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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MATERIALS AND METHODS

Ape fecal samples

To screen wild ape populations for *Plasmodium* infection, we selected 2,739 fecal samples from an existing bank of chimpanzee (*P. troglodytes*), western gorilla (*G. gorilla*), eastern gorilla (*G. beringei*), and bonobo (*Pan paniscus*) specimens previously collected for molecular epidemiological studies of simian immunodeficiency virus (SIVcpz and SIVgor)[14-16,31] and simian foamy virus (SFVcpz)[13]. All of these specimens, except for 28 samples from a group of habituated western gorillas (Makumba group) at the DS field site, were collected from non-habituated apes living in remote forest areas. Fecal samples were first subjected to host mitochondrial DNA analysis to determine their species and subspecies origin[13-16,31]. A subset was then selected for host microsatellite analysis to determine the number of individuals at particular field sites (Supplementary Table 1). These included 198 chimpanzee samples from the GT field site, 189 eastern gorilla samples from the KE, LU and OP field sites, and 119 bonobo samples from the LK and KR field sites (Supplementary Table 2). For estimates of sample degradation (Supplementary Table 2) and oversampling (Supplementary Table 3), we also included microsatellite results that we had obtained earlier for specimens collected from central chimpanzees[14-16] and western gorillas[15] in Cameroon, as well as from eastern chimpanzees[31] in the Democratic Republic of Congo.

Microsatellite analyses

Fecal DNA was extracted[14] and used to amplify four (GT) or eight polymorphic microsatellite loci (KE, LU, OP, LK, KR) as described[14,15]. Amplification products were analyzed on an automated sequencer (Applied Biosystems) and sized using GeneMapper 4.0 (Applied Biosystems). For individual identification, samples were first grouped by field site and mitochondrial DNA haplotype. Within each haplotype, samples were then grouped by microsatellite genotypes, but allowing for one allelic mismatch between samples. Specimens

were classified as degraded if they failed to amplify two or more (GT), or three or more (all other sites) microsatellite loci. Samples with evidence of DNA admixture (multiple peaks for the same locus) were discarded.

Amplification of ape *Plasmodium* sequences by conventional (bulk) PCR

Fecal samples were first screened for *Plasmodium cytB* sequences (956 bp) as described[10], but using modified PCR conditions as well as a different second round reverse primer to generate a 166 bp longer amplicon. Nested PCR was performed using DW2 (5'-TAATGCCTAGACGTATTCCTGATTATCCAG -3) and DW4 (5'-TGTTTGCTTGGGAGCTGTAATCATAATGTG -3) in the first round, and Pfcytb1 (5'-CTCTATTAATTTAGTTAAAGCACA -3) and PLAS2a (5'-GTGGTAATTGACATCCWATCC -3) in the second round of PCR. For the first round, 2.5μl of fecal DNA was used in a 25μl reaction volume, containing 0.5μl dNTPs (10mM of each dNTP), 20pmol of each primer (DW2 and DW4), 2.5μl PCR buffer, and 0.25μl Expand Long Template enzyme mix (Roche). Cycling conditions included an initial denaturation step of 2 minutes at 94°C, followed by 15 cycles of denaturation (94°C, 10 sec), annealing (45°C, 30 sec), and elongation (68°C, 2 min), followed by 35 cycles of denaturation (94°C, 10 sec), annealing (48°C, 30 sec), and elongation (68°C, 2 min; with 15 seconds increments for each successive cycle), followed by a final elongation step of 10 min at 68°C. For the second PCR round, 1μl of the first round product was used in 25μl reaction volume, containing 0.5μl dNTPs (10mM of each dNTP), 20pmol of each primer (Pfcytb1 and PLAS2a), 2.5μl PCR buffer, and 0.25μl Expand Long Template enzyme mix. Cycling conditions included an initial denaturation step of 2 min at 94°C, followed by 60 cycles of denaturation (94°C, 10 sec), annealing (52°C, 30 sec) and elongation (68°C 1 min), followed by a final elongation step of 10 min at 68°C. Amplified products were gel purified and sequenced directly to confirm *Plasmodium* infection.

Samples positive for *Plasmodium* cytB sequences were then screened for apicoplast *clpC* (390 bp) and nuclear *ldh* (772 bp) sequences. Amplification of the *clpC* fragment was performed as described[9], but using modified PCR conditions and a different second round reverse primer to generate a 117 bp longer amplicon. Nested PCR was performed using primers TFM1421+ (5'- AAAACTGAATTAGCAAAAATATTA -3) and TFM1423RC (5'-CGAGCTCCATATAAAGGAT -3) in the first round, and CLPCF1 (5'-TCTAAACAATTATTTGGTTCTG -3) and CLPCR2 (5'-GTTAATCTATTTARTAATTCHGGTTTAA -3') in the second round of PCR. Amplification of the *ldh* fragment was also performed as described[28,32,33], but using different PCR conditions. Nested PCR was performed using primers JNB272 (5'-ATGGCACCAAAAGCAAAAAT -3) and JNB273 (5'-GCCTTCATTCTSYTAGTTTCAGC -3) for the first round, and LDH1 (5'-GGNTCDGGHATGATHGGAGG -3) and Fv2n (5'- AACRASAGGWGTACCACC -3) for the second round. PCR conditions were the same as described for the *cytB* fragment. Amplified products were gel purified and sequenced directly to confirm *Plasmodium* infection.

Finally, *cytB* positive samples were subjected to nested PCR, aiming to amplify larger fragments (3.4kb and 3.3kb in length, respectively), which together spanned the entire *Plasmodium* mitochondrial genome (Supplementary Fig. 1a). The 3.4kb fragment was amplified using Pf936p (5'-GAGAAAAATGYAATCCWGTWACACAATA-3') and DW4 in the first round, and Pf1031p (5'-GATGCAAAACATTRWCCTAATAAGTA-3') and PLAS2a in the second round of PCR. The 3.3kb fragment was amplified using McytP (5'-TATCCAAATCTATTAAGTCTTG-3') and Pf1916n (5' –GCGTTCGTTCTTATAGTGTAGGC-3 ') in the first round, and Pf4450p (5'-

CTGTTCCTATTATATGGTTTATGTGTGC-3') and Pf1880n (5'-CCTTTAATGTAGTTTCCTCACAGCTT-3') in the second round of PCR. For the first round of amplification, 2.5μl of fecal DNA was used in a 25μl reaction volume, containing 0.5μl dNTPs (10mM of each dNTP), 20pmol of each first round primers, 2.5μl PCR buffer, and 0.25μl Expand Long Template enzyme mix (Roche). Cycling condition included an initial denaturation step of 2 min at 94°C, followed by 15 cycles of denaturation (94°C, 10 sec), annealing (45°C, 30 sec) and elongation (68°C, 4 min), followed by 35 cycles of denaturation (94°C, 10 sec), annealing (48°C, 30 sec) and elongation (68°C, 4 min; with 15 seconds increments for each successive cycle), followed by a final elongation step of 10 min at 68°C. For the second round of amplification, 2μl of the first round PCR product was used in a 50μl volume, containing 1μl dNTPs (10mM of each dNTP), 20pmol of each second round primers, 5μl PCR buffer, and 0.5μl Expand Long Template enzyme mix. Cycling condition included an initial denaturation step of 2 min at 94°C, followed by 60 cycles of denaturation (94°C 10 sec), annealing (52°C, 30 sec) and elongation (68°C, 4 min), followed by a final elongation step of 10 min at 68°C. Amplified products were gel purified, but only a small fragment was sequenced to confirm *Plasmodium* infection.

Single genome amplification (SGA) of *Plasmodium* sequences from fecal DNA

To derive sequences suitable for phylogenetic analyses, a subset of bulk PCR positive chimpanzee (n=80) and gorilla (n=55) fecal samples was subjected to SGA analyses[21,22]. According to a Poisson distribution, the DNA dilution that yields PCR products in no more than 30% of wells contains one amplifiable template per positive PCR more than 80% of the time. Fecal DNA was thus endpoint diluted in 96-well plates, and the dilution that yielded less than 30% positive wells was used to generate between 1 and 40 different SGA sequences per sample (Supplementary Table 5). The same primers and PCR conditions used for bulk amplification of *cytB*, mtDNA-3.4kb, mtDNA-3.3kb, *clpC* and *ldh* fragments were also used for SGA analyses. Amplification products were gel purified, and sequenced directly using Sequencher version 4.6 (Gene Codes Corporation). Sequences that contained double peaks as an indicator of more than one amplified template were discarded.

Sensitivity and specificity of *Plasmodium* nucleic acid detection in fecal samples

To estimate the sensitivity of the diagnostic *cytB* PCR test, we determined the proportion of PCR positive specimens from *Plasmodium* infected apes that were sampled more than once on the same day. Other replicate samples were excluded since the duration of natural ape *Plasmodium* infections is unknown. The sensitivity of *Plasmodium* nucleic acid detection was then calculated as the fraction of positive tests per total number of samples tested (Supplementary Table 4). Including data from 32 such apes, we estimated the sensitivity to be 57% (with confidence limits determined assuming binomial sampling). It should be noted that this approach led to a systematic (albeit small) overestimation of the assay sensitivity, since it did not account for infected apes that yielded only negative replicate samples. Moreover, *Plasmodium* detection in fecal samples is very likely less sensitive than in blood, as is the case in urine and saliva[17]. Thus, the prevalence rates in Table 1 and Supplementary Table 1 should be interpreted as minimum estimates of *Plasmodium* infection rates in wild apes. The specificity of fecal *Plasmodium* detection was 1.00, since all amplification products were sequence confirmed.

Ape *Plasmodium* prevalence estimations

For sites where the number of sampled chimpanzees was known (Supplementary Table 1), *Plasmodium* prevalence rates were estimated based on the proportion of infected individuals. For each ape, the probability that it would be detected as being infected, if it was truly infected, was calculated taking into consideration the sensitivity of the diagnostic PCR test and the number of samples analyzed, with 95% confidence limits determined assuming binomial sampling. For the remaining field sites where the number of sampled individuals was not known, prevalence rates were estimated based on the number of fecal samples, but correcting for specimen degradation and oversampling. As shown in Supplementary Table 2, microsatellite analysis of 1,027 fecal samples indicated an average degradation factor of 13%. Microsatellite analyses also provided a quantitative estimate of oversampling. Because of regional differences in sample collection, oversampling values were calculated separately for the different ape species and subspecies. As shown in Supplementary Table 3, central chimpanzees, western gorillas, eastern chimpanzees, eastern gorillas and bonobos were each assumed to have been sampled on average 1.77, 1.84, 3.74, 2.01 and 1.84 times, respectively. Using these corrections, the proportion of *Plasmodium* infected chimpanzees was estimated for each field site, again taking into account the sensitivity of the diagnostic test. From these determinations, prevalence rates and their confidence limits were calculated.

Phylogenetic analyses

Ape derived *Plasmodium* sequences were aligned with human and simian reference sequences using CLUSTAL W[34]. Sites that could not be aligned unambiguously were excluded. Trees were constructed from mitochondrial *cytB* sequences (956 bp, Supplementary Figs. 2 and 3; 240 bp, Supplementary Fig. 8), apicoplast *clpC* sequences (390 bp, Supplementary Fig. 4), nuclear *ldh* sequences (772 bp, Supplementary Fig. 5), and mitochondrial half genomes (3,361 bp, Supplementary Fig. 6; 3,277 bp, Supplementary Fig. 7). In addition, trees were constructed from mitochondrial coding (Fig. 3a) and non-coding regions (Fig. 3b). Deduced CoxI, CoxIII, and CytB protein sequences were concatenated into a single 980 amino acid sequence. The non-protein coding portion of the mtDNA-3.3kb fragment comprised 2,447 nucleotides following the removal of ambiguous sites. Phylogenetic trees were inferred using PhyML[30]. The class of evolutionary model was chosen using ModelTest[35], and parameters were iteratively estimated in PhyML, using the GTR+I+G model for nucleotide and the LG+I+G[36] model for amino acid sequence trees. Bootstrap values were calculated with 100 replicates[37]. Posterior probability values were calculated using MrBayes[29], using an average standard deviation of partition frequencies < 0.01 as a convergence diagnostic. A neighbor-joining phylogenetic tree (Supplemental Fig. 8) was calculated with CLUSTAL W, using the Kimura 2-parameter model of evolution with bootstrap support based on 1000 bootstrap replicates[34,37].

Nucleotide sequence accession numbers

All new SGA derived ape *Plasmodium* sequences have been submitted to GenBank, with accession numbers listed in Supplementary Table 6.

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Figure 1. Location of ape study sites

Field sites are shown in relation to the ranges of three subspecies of the common chimpanzee (*P. t. ellioti*, magenta*; P. t. troglodytes*, red*; and P. t. schweinfurthii*, blue), western (*Gorilla gorilla*, red stripes) and eastern (*Gorilla beringei*, black stripes) gorillas, and the bonobo (*Pan paniscus*, orange) in sub-Saharan Africa. Forested areas are indicated in green, while arid areas are shown in yellow (map courtesy of Lilian Pintea, The Jane Goodall Institute). Circles, squares and hexagons identify field sites where chimpanzees, gorillas, or both species were sampled, respectively (ovals indicate bonobo collection sites). Sites where ape malaria was detected are highlighted in yellow, with red lettering indicating that both chimpanzees and gorillas were infected.

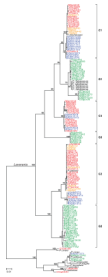

Figure 2. Phylogeny of *Plasmodium* parasites from wild-living chimpanzees and western gorillas

A representative subset of 146 SGA-derived *Plasmodium* mitochondrial cytochrome B sequences (956 bp) is shown in relation to human and simian *Plasmodium* reference sequences (for accession numbers see Supplementary Tables 6 and 7). The full set of 697 SGA-derived ape *Plasmodium* sequences is analyzed in Supplementary Fig. 3. Sequences are color-coded, with capital letters indicating the field site (Fig. 1) and lower case letters denoting species and subspecies origin (ptt: *P. t. troglodytes*, red; pte: *P. t. ellioti*, orange; pts: *P. t. schweinfurthii*, blue; gor: *G. g. gorilla*, green). C1-C3 and G1-G3 represent chimpanzee and gorilla specific *Plasmodium* species, respectively, all of which are included within the subgenus *Laverania*18. The tree was inferred using maximum likelihood methods[30]. Bootstrap values (above 70%) are indicated for major nodes only (the scale bar represents 0.01 substitutions per site).

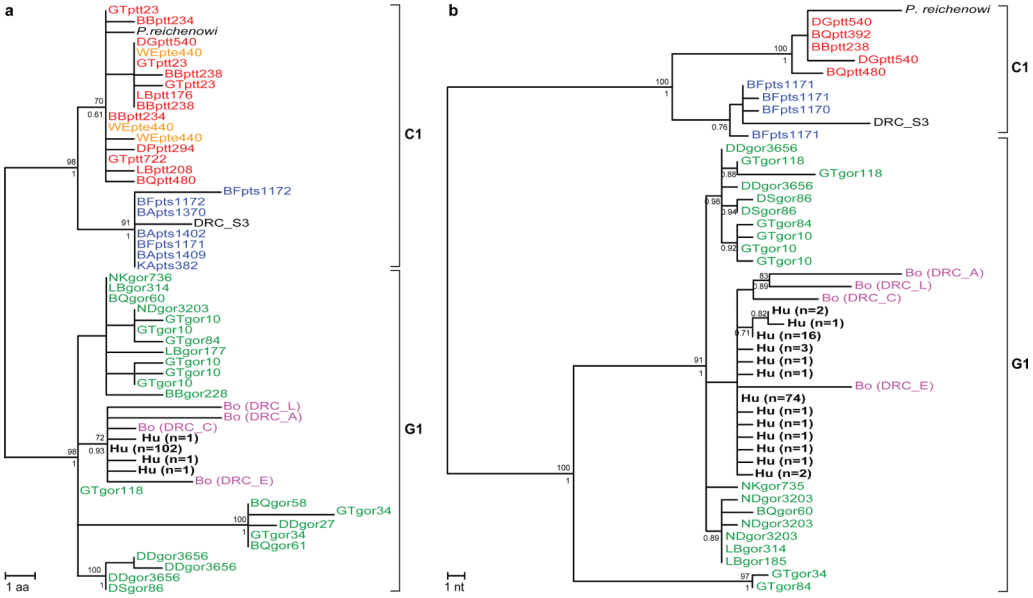

Figure 3. Evolutionary relationships of ape and human *Plasmodium* parasites in mitochondrial coding and non-coding regions

a,b, Phylogenetic trees of SGA-derived mitochondrial (**a**) CytB, CoxI and CoxIII protein (980 amino acids) and (**b**) non-overlapping nucleotide sequences (2,501 bp) of the closest chimpanzee (C1) and gorilla (G1) relatives of human *P. falciparum* (see Supplementary Fig. 1a for the position of the corresponding 3.4 kb and 3.3 kb SGA amplicons). *Plasmodium* sequences are labeled and color-coded as in Fig. 2, except for sequences derived from captive bonobos[11] (Bo), which are shown in magenta. Human *P. falciparum* (Hu) and chimpanzee reference sequences are depicted in black, with the number of human sequences representing the same haplotype shown in parentheses (for accession numbers see Supplementary Table 7). The trees were inferred using maximum likelihood methods[30]; numbers at nodes indicate bootstrap (above) and posterior probability (below) values, respectively (only values above 70 and 0.7 are shown). The scale bars represent 1 amino acid (aa) and one nucleotide (nt) substitution per site, respectively.

Table 1

Prevalence of *Plasmodium spp.* infection in wild-living African apes

Species/Subspecies	Field sites[a]	Fecal samples tested	Fecal samples positive[b]	*Plasmodium spp.* Prevalence (CI)[c]
Nigeria-Cameroon chimpanzee *(Pan troglodytes ellioti)*	9	119	19	32% (23%-46%)
Central chimpanzee *(Pan troglodytes troglodytes)*	13	612	147	48% (44%-53%)
Eastern chimpanzee *(Pan troglodytes schweinfurthii)*	24	1,096	187	34% (30%-40%)
Western lowland gorilla *(Gorilla gorilla gorilla)*	20	659	120	37% (32%-41%)
Eastern lowland gorilla *(Gorilla beringei graueri)*	3	146	0	0% (0%-4%)
Bonobo *(Pan paniscus)*	2	107	0	0% (0%-6%)

[a] Field sites are listed in Supplementary Table 1 and their location is shown in Fig. 1.

[b] Fecal samples were tested for *Plasmodium* mitochondrial (*cytB*) DNA by diagnostic PCR; all amplification products were sequenced to confirm *Plasmodium* infection.

[c] Prevalence of *Plasmodium* infection (%) with brackets indicating 95% confidence intervals (CI). Values were estimated based on the proportion of PCR positive fecal samples, correcting for specimen degradation (Supplementary Table 2), repeat sampling (Supplementary Table 3), and the sensitivity of the diagnostic test (Supplementary Table 4). Note that these prevalence rates represent minimum estimates, since the extent to which infected apes shed *Plasmodium* DNA into their feces is not known.

Table 2

Genetic diversity within different *Laverania* species

Species	*cytB*[a]				3.4 kb[a]				3.3 kb[a]			
	n	mean	median	max	n	mean	median	max	n	mean	median	max
C1	58	0.0055	0.0031	0.0136	25	0.0069	0.0024	0.0148	9	0.0035	0.0052	0.0060
G1(gor/all)[b]	51	0.0085	0.0021	0.0251	31	0.0058	0.0015	0.0193	19	0.0029	0.0016	0.0108
G1(gor/main)[b]	40	0.0018	0.0011	0.0073	26	0.0012	0.0012	0.0027	17	0.0015	0.0012	0.0040
G1(hum)[b]	105	0.0005	0	0.0021	105	0.0003	0.0003	0.0015	105	0.0002	0	0.0012
C2	109	0.0077	0.0021	0.0262	39	0.0078	0.0024	0.0202	16	0.0044	0.0020	0.0112
G2	92	0.0031	0.0031	0.0115	25	0.0032	0.0036	0.0071	36	0.0015	0.0016	0.0036
C3	48	0.0029	0.0031	0.0094	13	0.0035	0.0030	0.0071	8	0.0026	0.0028	0.0044
G3	23	0.0017	0.0021	0.0042	14	0.0010	0.0009	0.0033	4	0	0	0

[a]Pairwise distances were determined for SGA derived *cytB*, mtDNA-3.4 kb, mtDNA-3.3 kb sequences for each of the six *Laverania* species as shown in Supplementary Figs. 3, 6 and 7, respectively. The number of sequences (n) analyzed for each clade is indicated. Sequences were aligned and gapstripped to remove ambiguous sites. Alignments were then used to determine the mean, median and maximum (max) sequence distances. Identical sequences from different samples were included (identical sequences from the same sample were excluded). The sequence overlap between the mtDNA-3.3 kb and mtDNA3.4 kb fragments was removed from the mtDNA-3.3 kb comparisons.

[b]The *P. falciparum* clade (G1) was subdivided into three separate groups: G1(gor/all) included all gorilla *P. falciparum* sequences, G1(gor/main) included only the closest gorilla relatives of human *P. falciparum*, and G1(hum) included only human *P. falciparum* sequences. The comparisons show that human *P. falciparum* strains exhibit much lower diversity than the various clades of ape-derived strains (except for G3, where only very few sequences were available for the 3.3kb fragment); in particular, the G1(gor/main) clade exhibits substantially higher diversity than the G1(hum) clade.