

Wild Chimpanzees Infected with 5 *Plasmodium* Species

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Data are missing on the diversity of *Plasmodium* spp. infecting apes that live in their natural habitat, with limited possibility of human-mosquito-ape exchange. We surveyed *Plasmodium* spp. diversity in wild chimpanzees living in an undisturbed tropical rainforest habitat and found 5 species: *P. malariae*, *P. vivax*, *P. ovale*, *P. reichenowi*, and *P. gaboni*.

Despite ongoing and, in some regions, escalating morbidity and mortality rates associated with malaria-causing parasites, the evolutionary epidemiology of *Plasmodium* spp. is not well characterized. Classical studies of the blood pathogens of primates have found protozoa resembling human malaria parasites in chimpanzees and gorillas (1); however, these studies were limited to microscopy, negating conclusions regarding evolutionary relationships between human and ape parasites. Recent studies that used molecular approaches showed that captive and wild chimpanzees (*Pan troglodytes*) and lowland gorillas (*Gorilla gorilla*), as well as captive bonobos (*Pan paniscus*), harbor parasites broadly related to *P. falciparum* (2–5); wild and captive gorillas and captive bonobos and chimpanzees are sometimes infected with *P. falciparum* itself (4–6). Further, captive chimpanzees and bonobos have been shown to have malaria parasites related to human *P.*

ovale and *P. malariae* (6–8); *P. vivax* has been identified in various monkeys and 1 semiwild chimpanzee (5,9). Recently, *P. knowlesi*, a simian malaria species, became the fifth human-infecting species (10), highlighting the possibility of transmission of new *Plasmodium* spp. from wild primates to humans.

The Study

To investigate the prevalence of different *Plasmodium* spp. in wild great apes living in their natural habitat (tropical rainforests), we analyzed tissue samples from 16 wild West African chimpanzees that died primarily of anthrax or respiratory disease in Taï National Park, Côte d'Ivoire. A generic real-time PCR that detects all known *Plasmodium* spp. was used to test all samples for the parasite. Sequence analysis of the *CytB* gene and small subunit rRNA genes was conducted for real-time PCR-positive samples to determine the strain present; 1,140 bp of the *CytB* gene and 765 bp of the *18S* gene of the *Plasmodium* genome were amplified by classic PCR. Resulting products were sequenced either directly or after cloning for rRNA gene and when initial sequence information showed the possible presence of 2 different species (Table).

Phylogenetic analyses of sequences obtained confirmed the presence of 5 species: *P. reichenowi* and *P. gaboni*, which had been found previously (2,3); but also *P. vivax*, *P. ovale*, and *P. malariae*-like strains (Figures 1, 2). The most prevalent species was *P. reichenowi* (6/16), which had representatives in subclusters *P. gaboni* and *P. reichenowi*. The other species were rare, seen only 1 (*P. ovale* and *P. vivax*) or 2 (*P. malariae*) times. Two chimpanzees showed co-infections with multiple *Plasmodium* spp. (Figures 1, 2), 1 infected with *P. reichenowi* and a *P. malariae*-like strain and the other with *P. reichenowi* and *P. gaboni*.

Is the observed high prevalence of *Plasmodium* spp. typical for wild chimpanzees or related to reduced immune function associated with the severe infection that was the primary cause of death in each case? To investigate this question, we tested DNA extracted from fecal samples of apparently healthy chimpanzees collected over the past 8 years ($n = 30$) (11) of the same study population by using the generic real-time PCR followed by amplification of the *CytB* gene. Of these samples, 21 (70%) were positive for *Plasmodium* spp. by real-time PCR. Because of low copy numbers in feces, phylogenetic analyses were limited to 2 samples in which *P. reichenowi* of the *P. gaboni* subcluster was confirmed.

To determine if the observed high prevalence of plasmodia was a site- or chimpanzee subspecies-specific phenomenon, we tested 30 randomly selected fecal samples of individually known apparently healthy wild Eastern chimpanzees from the Budongo Forest in Uganda. Overall prevalence of *Plasmodium* spp. was lower than in West African

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Table. Tissue and fecal samples from wild chimpanzees examined for *Plasmodium* species, Tai National Park, Cote d'Ivoire, and Budongo Forest, Uganda*

Type of sample and name or species of chimpanzee	Genetic sequence copies/mg tissue	<i>Plasmodium</i> species detected	GenBank accession nos.
Necropsy			
Loukoum	530	<i>P. gaboni</i>	GU815507 (<i>CytB</i>), GU815523 (<i>18S</i>)
Noah	50	<i>P. gaboni</i>	GU815508 (<i>CytB</i>), GU815524 (<i>18S</i>)
Orest	2.2 x 10	<i>P. gaboni</i>	GU815509 (<i>CytB</i>), GU815525 (<i>18S</i>)
Candy	65	<i>P. reichenowi</i>	GU815510 (<i>CytB</i>), GU815526 (<i>18S</i>)
Atra	100	<i>P. reichenowi</i>	GU815511 (<i>CytB</i>)
Louise	160	<i>P. reichenowi</i>	GU815512 (<i>CytB</i>), GU815527 (<i>18S</i>)
EastChip 06	105	<i>P. reichenowi</i> , <i>P. gaboni</i>	GU815512–13 (<i>CytB</i>)
Olduvai	130	<i>P. reichenowi</i> , <i>P. malariae</i>	GU815514–15 (<i>CytB</i>), GU815528–29 (<i>18S</i>)
Leo	850	<i>P. malariae</i>	GU815516 (<i>CytB</i>), GU815530 (<i>18S</i>)
Kady	105	<i>P. ovale</i>	GU815517 (<i>CytB</i>), GU815531 (<i>18S</i>)
Sagu	760	<i>P. vivax</i>	GU815518 (<i>CytB</i>), GU815532 (<i>18S</i>)
Dorry	Neg		
Virunga	Neg		
Ophelia	Neg		
Akruba	Neg		
Akwaba	Neg		
Fecal samples, n = 30			
Positive qPCR results			
<i>P. t. verus</i>	21 (2)	<i>P. gaboni</i>	GU815519 (<i>CytB</i>)
<i>P. t. schweinfuthii</i>	12 (3)	<i>P. reichenowi</i> , <i>P. gaboni</i>	GU815520–22 (<i>CytB</i>)

*All chimpanzees were *Pan troglodytes verus* from Tai except *P. t. schweinfuthii* chimpanzees, which were from Budongo Forest. Parentheses indicate the number of samples for which sequences were obtained and used for phylogenetic tree analyses. Neg, negative; qPCR, quantitative PCR.

chimpanzees but still relatively high (40%); *P. reichenowi* and *P. gaboni* were identified in 3 samples.

Our results demonstrate that the prevalence of different *Plasmodium* spp. in wild chimpanzees is similar to that of untreated human populations in sub-Saharan Africa (www.who.int/malaria). Throughout sub-Saharan Africa, *P. falciparum* is more predominant in humans than are other *Plasmodium* spp. Considering the lack of clinical signs of malaria in chimpanzees from which fecal samples were collected and those that had died of respiratory dis-

ease or anthrax, *Plasmodium* spp. infections appear to be asymptomatic or at least nonlethal in wild chimpanzees. However, signs of illness are rarely observed in wild primates because infected animals often mask weakness to maintain social position and avoid attack by predators (12). Recently developed technologies for the noninvasive determination of temperature in wild chimpanzees may enable more effective examination of the relationship between the primary clinical feature of malaria (i.e., cyclical fevers) and *Plasmodium* spp. infection (13).

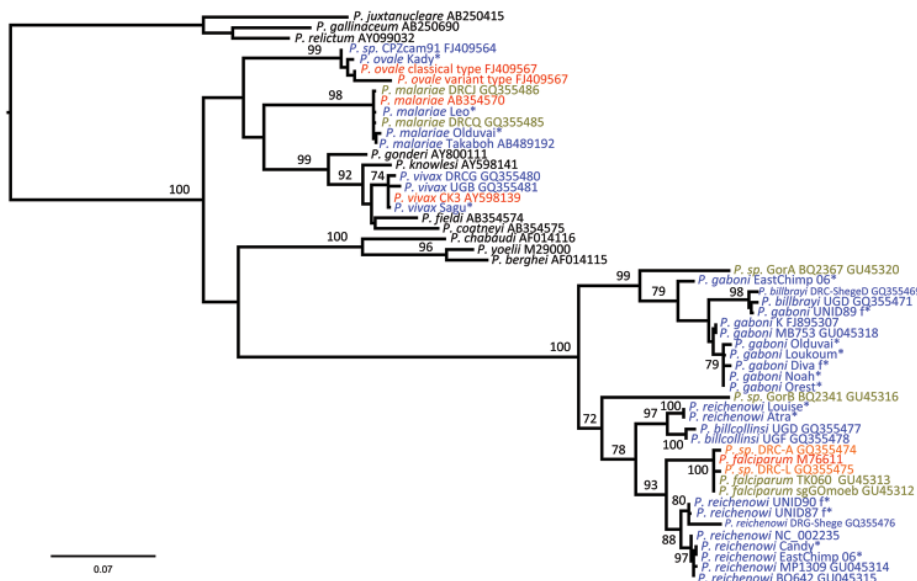


Figure 1. Maximum-likelihood trees of *Plasmodium* spp. obtained from the analysis of a 1,087-bp *CytB* alignment. Blue indicates sequences determined from chimpanzee hosts, green, bonobos, gray, gorillas, and red, humans; black indicates sequences obtained from nonprimate hosts. *Plasmodium* spp. sequences derived from chimpanzees in this study are marked with an asterisk. Bootstrap values are shown when ≥ 70 . The tree was rooted using avian plasmodium sequences. Accession numbers of all sequences used are shown in the Table. Scale bar indicates nucleotide substitutions per site.

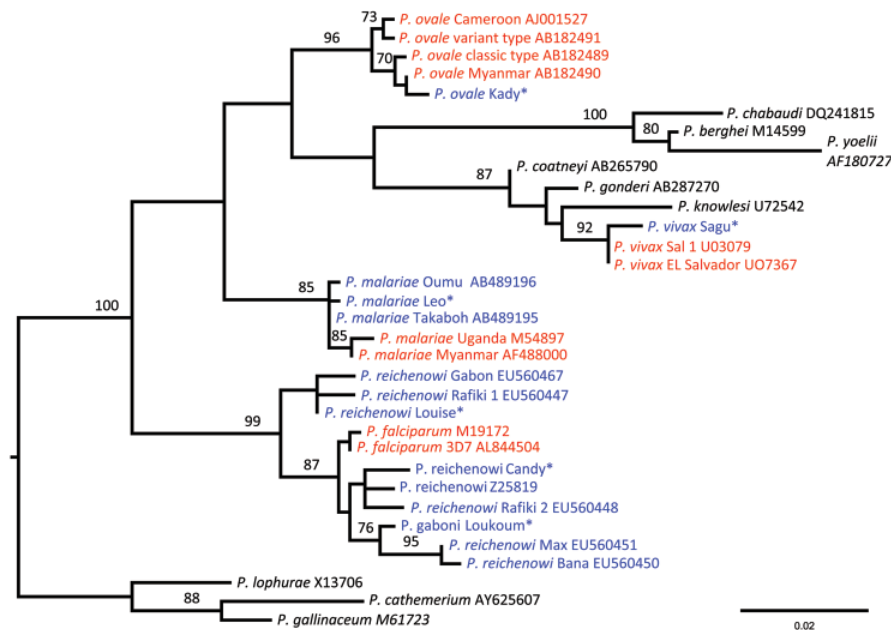


Figure 2. Maximum-likelihood tree of *Plasmodium* spp. obtained from the analysis of a 621 bp-long 18S alignment. Blue indicates sequences determined from chimpanzee hosts; green, bonobos; gray, gorillas; and red, humans. Black indicates sequences obtained from nonprimate hosts. *Plasmodium* spp. sequences derived from chimpanzees in this study are marked with an asterisk. Bootstrap values are shown when ≥ 70 . The tree was rooted using avian plasmodium sequences. Accession numbers of all sequences used are shown in the Table. Scale bar indicates nucleotide substitutions per site.

P. ovale was previously described from captive chimpanzees and *P. malariae* from captive chimpanzees and captive bonobos have been described (5–8). Our study results demonstrate that *P. malariae* and *P. ovale* occur in wild chimpanzees that inhabit pristine contiguous forest with extremely limited exposure to humans, suggesting the natural existence of these parasites in wild great apes.

Because of a Duffy-negative condition in 95%–99% of the human population in western and central continental Africa, transmission of *P. vivax* does not seem to occur. However, *P. vivax* infections are common in travelers returning from these areas (11). Even though we cannot totally exclude the possibility of introduction of *P. vivax* in the chimpanzee population through humans, our discovery of *P. vivax* in wild chimpanzees living exclusively within their natural habitat suggests that wild African apes may be a natural reservoir.

Our study shows the existence of *P. reichenowi* and related strains in wild chimpanzees as described for chimpanzees and gorilla by others (2–4,6). Infections with strains of the *P. reichenowi* group (sometimes referred to as the species *P. gaboni*, *P. billbrayi*, and *P. billcollinsi*) appear to occur widely in wild and captive great apes in Africa with some variation between chimpanzee subspecies from biogeographically distinct sites. The wild chimpanzees examined demonstrated no infections with classic human *P. falciparum*. This lack of infection is likely caused by low human presence in their habitat and, consequently, few or no infected vectors, low sample size, or a missing receptor in chimpanzees (14). More investigations are needed because recently *P. falciparum* infections have been described for 2 captive chimpanzees (6). The situation is

clearer for captive and wild lowland gorillas (*Gorilla gorilla*) for which infections and receptors have recently been described (4). Infections have also been documented for captive bonobos (5).

Conclusions

Previous examination of the role of our closest phylogenetic relatives, the great apes, in the evolution and persistence of human plasmodia has been limited by a lack of data from wild ape populations where opportunities for human-mosquito-ape malaria exchange are minimal. Interpretation of patterns of malaria infection in captive ape populations, such as sanctuaries and zoos, must consider the ample opportunities for human-to-ape transmission of such parasites, negating the opportunity to investigate the evolutionary origins and public health-related risks of these parasites. Conversely, our examination of these parasites in wild chimpanzees with no contact to the periphery of the rainforest habitat (online Technical Appendix Figure, www.cdc.gov/EID/content/16/12/1956-Techapp.pdf) demonstrates that these apes are most likely naturally infected with *P. ovale*, *P. vivax*, and *P. malariae*, 3 types of plasmodia rarely observed in humans of the region. Whether wild great apes are the origin or reservoirs of these *Plasmodium* types requires further investigation. These results may have implications for global efforts to eradicate malaria in humans, including vaccine development based on animal variants of human parasites.

Addendum

While this article was in press, Liu et al. published a study showing strong evidence that *P. falciparum* originat-

ed in gorillas (15). Their study also recovered other plasmodia, complementing our findings. As recommended in our conclusions section, the Liu et al. study was based on a large number of samples from wild great apes.

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Mr Kaiser is a PhD candidate at the Robert Koch-Institute. He specializes in the development of PCR-based detection methods for various pathogens.

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Technical Appendix

Methods

Ethics Statement

No living nonhuman primates were used for this study. Tissue samples were obtained from animals that died of various causes (see sample collection) in the Tai National Park, Côte d'Ivoire. Noninvasive samples (feces) were collected without disturbing animal behavior or interfering with them. Tissue samples were exported from Côte d'Ivoire under permission of the CITES authorities and fecal samples were exported according to the regulations of each country.

Sample Collection

Spleen samples of wild chimpanzees that died of anthrax or respiratory disease in Tai National Park, Côte d'Ivoire were collected using single-use equipment and appropriate safety measures (1–4). In addition, over a period of 8 years, fecal samples were collected from known living individuals of the same chimpanzee population and within a 12-month period in 2007 from individually known wild chimpanzees of various communities living in the Budongo Forest area, Uganda. Samples were collected using single-use gloves avoiding any contamination with human pathogens. All samples from Tai National Park were preserved in liquid nitrogen. Samples from Budongo Forest were preserved in RNAlater (QIAGEN, Hilden, Germany) and later frozen.

DNA Extraction, Amplification, and Sequencing

DNA was extracted from the spleen of 16 chimpanzees using the DNeasy Blood and Tissue Kit (QIAGEN). DNA was extracted from 30 fecal samples from each of the chimpanzee sites by using the EURx GeneMATRIX Stool DNA Purification Kit (Roboklon, Berlin, Germany). An in-house real time qPCR, *P.sp.* 18S F (5'-gTT TCT gAC CTA TCA gCT TTT gAT gT-3'), *P.sp.* 18S R1 (5'-CTg CCT TCC TTA gAT gTg gTA CCT A-3') and

P.sp. TM (5'-YAK-CAg gCT CCC TCT CCg gAA TCg AAC-BBQ-3'), was used to analyze samples for the presence of *Plasmodium* spp. Each sample was analyzed in duplicate. The detection limit was 10 copies of target DNA per reaction. qPCR positive samples were used for classic PCRs aiming at amplifying 1140bp of *cytB* gen and 765bp fragment of the small subunit rRNA gen for phylogenetic analysis.

For each PCR reaction, 5 μ L 10 x buffer, 3 μ L MgCl₂ 50mM, 4 μ L dNTPs 10 mM, 1 μ L each primer 10 μ M, 0.5 μ L (2.5 U) Platinum Taq (Invitrogen, Carlsbad, CA, USA) and 5 μ L DNA were used. Reaction volume was adjusted with water (Molecular Biology Grade Eppendorf) to 50 μ L. The reaction was run in a thermocycler (Mastercycler epgradient, Eppendorf) at 95°C for 2 min, 10 cycles at 95 °C for 15 sec, at 68 °C for 15 s and at 72 °C for 70 s with reducing the annealing temperature 1 °/cycle and 35 cycles at 95 °C for 15 sec, at 58 °C for 15 s, and at 72 °C for 70 s. The final extension was 72 °C for 5 min.

The following primers were used: P.sp. *CytB* F (5'-TgC CTA gAC gTA TTC CTg ATT ATC CAg-3') and P.sp. *CytB* R (5'-CTT gTg gTA ATT gAC ATC CWA TCC-3') to amplify *cytB* gene and P.sp. 18S F (5'-gTT TCT gAC CTA TCA gCT TTT gAT gT-3') and P.sp. 18S R (5'-TCT gAT CgT CTT CAC TCC CTT AAC-3') to amplify 18S gene. PCR products were separated by 1.5% agarose gel electrophoresis, purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and sequenced with the ABI PRISM Big Dye Terminator cycle sequencing kit, according to the manufacturer's protocol. Sequences were determined using the ABI 310 automated DNA sequencer and analyzed with the ABI PRISM DNA Sequencing Analysis Software (Version 3.7, Applied Biosystems).

Phylogenetic Analysis

Plasmodium sequences determined in this investigation were compared with published sequences using the BLAST network program from the National Center for Biotechnology Information (NCBI). *CytB* and 18S sequences were aligned to a selection of published sequences using multiple sequence alignment (MUSCLE) (5) as implemented in SeaView (6) and T-Coffee, which was run on a dedicated webserver (www.tcoffee.org). Conserved blocks were selected from the best alignment (as determined by visual inspection) by using Gblocks (7), which was run on the Gblocks webserver (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The resulting alignments were haplotyped by using Fabox. The overall process came to generating a 1087bp-long *CytB* alignment comprising 54 taxa and a 621bp-long 18S alignment composed of 33 taxa, which served as input for further analyses. Likelihoods of models of evolution (JC, HKY and GTR;

+F; +I, +G, +I+G) were estimated and then compared according to the Akaike information criterion by using jModelTest (8). The models of evolution to which the datasets were a better fit were GTR+I+G for CytB and GTR+G for 18S.

Maximum likelihood (ML) phylogenetic trees were finally estimated under these models using PhyML (9) as implemented on a dedicated webserver (<http://www.atgc-montpellier.fr/phyml/>). Equilibrium frequencies, topology, and branch lengths were optimized and the tree search was realized by using a combination of hill-climbing algorithms (NNI & SPR). Branch robustness was assessed by nonparametric bootstrapping (500 pseudo-replicates).

Sequence Info Used from the Public Database

For CytB: M76611, FJ409566, FJ409567, AB354570, AY598139, GU045312 - GU045316, GU045318, GU045320, GQ355469, GQ355471, GQ355474 – GQ355478, GQ355480, GQ355481, GQ355485, GQ355486, NC_002235, FJ895307, FJ409564, AB489192, AY598141, AB354574, AB354575, AY800111, M29000, AF014115, AF014116, AB250415, AB250690, AY099032.

For 18S: M19172, AL844504, U07367, U03079, AB182489 - AB182491, AJ001527, M54897, AF488000, Z25819, EU560447, EU560448, EU560450, EU560451, EU560467, U72542, AB265790, AB287270, AB489195, AB489196, M14599, AF180727, DQ241815, X13706, M61723, AY625607.

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Technical Appendix Figure. Satellite image of habitat of wild chimpanzees investigated in Tai National Park, Côte d'Ivoire. The shaded areas show the maximum extension of the territories of the communities studied. Pristine forest is dark green.