Diversity, host switching and evolution of *Plasmodium vivax* infecting African great apes

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***Plasmodium vivax* is considered to be absent from Central and West Africa because of the protective effect of Duffy negativity. However, there are reports of persons returning from these areas infected with this parasite and observations suggesting the existence of transmission. Among the possible explanations for this apparent paradox, the existence of a zoonotic reservoir has been proposed. May great apes be this reservoir? We analyze the mitochondrial and nuclear genetic diversity of *P. vivax* parasites isolated from great apes in Africa and compare it to parasites isolated from travelers returning from these regions of Africa, as well as to human isolates distributed all over the world. We show that the *P. vivax* sequences from parasites of great apes form a clade genetically distinct from the parasites circulating in humans. We show that this clade's parasites can be infectious to humans by describing the case of a traveler returning from the Central African Republic infected with one of them. The relationship between this *P. vivax* clade in great apes and the human isolates is discussed.**

emergence | transfer | malaria | sylvatic | origin

The malaria agent *Plasmodium vivax* is the second most prevalent *Plasmodium* species in humans, responsible for tens of millions of cases every year all over the world (1). Among the five recognized *Plasmodium* species infecting humans, *P. vivax* has the broadest geographical distribution (1). However, *P. vivax* remains an enigmatic species, whose biology and evolution are little known, compared to *Plasmodium falciparum*.

Recently, *P. vivax*-related parasites, along with a set of other *Plasmodium* species (2–5), have been identified in great apes (gorillas and chimpanzees) in Africa, but their phylogenetic relationships to the human *P. vivax* was not clearly established. However, this information could help resolve an apparent paradox.

P. vivax is considered to be virtually absent in humans in West and Central Africa, where more than 95% of the human population is supposed to be resistant to it because of the absence of the Duffy antigen (Duffy negativity) on the surface of their red blood cells (6). However, there are several reports of Duffy positive travelers returning from these regions of the world infected with *P. vivax* (7–10) and other studies have reported findings that suggest a possible transmission of the parasite in this region (11, 12). Several hypotheses have been proposed to account for this apparent paradox. Some authors suggested that despite high frequencies of Duffy negativity, the frequency of Duffy positive blood groups (~1%–5%) may be high enough to maintain a certain level of transmission of *P. vivax* in these areas (13, 14). Alternatively, it has been proposed that *P. vivax* could use other pathways to enter human red blood cells and that Duffy negativity might no longer be a barrier to infection and transmission (12, 15, 16). Furthermore, *Plasmodium ovale* may have been misdiagnosed as *P. vivax*, given that the microscopic distinction of these two species is difficult (17). Finally, some authors have proposed that *P. vivax* transmission could be the consequence of a zoonotic transfer from a wild reservoir (18). In this context, might great apes be this reservoir?

In this article, we analyze, based on the complete mitonchondrial genome as well as two nuclear genes, the phylogenetic position and the relationships to human isolates of *P. vivax*-like pathogens circulating in great apes in Central Africa. We show that the ape *P. vivax* isolates form a clade genetically distinct from the human *P. vivax* one and that the ape clade can be a possible source of human infections. Our results also bring into question the evolution and origin of *P. vivax* in humans.

Results

Phylogenetic and Genetic Analyses of *P. vivax*-Like Parasites from Great Apes. To distinguish the evolutionary and genetic relationships of the parasites isolated in great apes (Fig. S1) from those infecting humans all over the world, we performed phylogenetic as well as median-joining haplotype network (MJN) analyses of human and great ape *P. vivax* based on mitochondrial and nuclear sequences [type A *18S* rRNA gene (*18S*) and the *Circumsporozoite* gene, *Csp*].

Mitochondrial sequences showed that the parasites isolated from great apes were genetically divergent from those infecting humans and some of them formed a distinct clade (Figs. 1*A* and 2*A* and Fig. S2). Indeed, with the exception of the *P. vivax* parasite extracted from a chimpanzee coming from the Democratic Republic of the Congo (3), which falls inside the human haplotype diversity (DRCG sample, see Figs. 1*A* and 2*A* and Figs. S1 and S2), the other *P. vivax* extracted from great apes in Gabon and Uganda clustered together, outside the worldwide diversity found in human *P. vivax*. The alignment and comparison of the complete mitochondrial sequences of this genetic clade with more than 200 sequences of human *P. vivax* from Africa, Asia,

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. KC750255–KC750258 (*Cyt b*), KC750259–KC750262 (*COX I*), JX444719–JX444726 (mitochondrial genome), KC750241–KC750246 (*18S*), and KC750247–KC750254 (*Csp*)].

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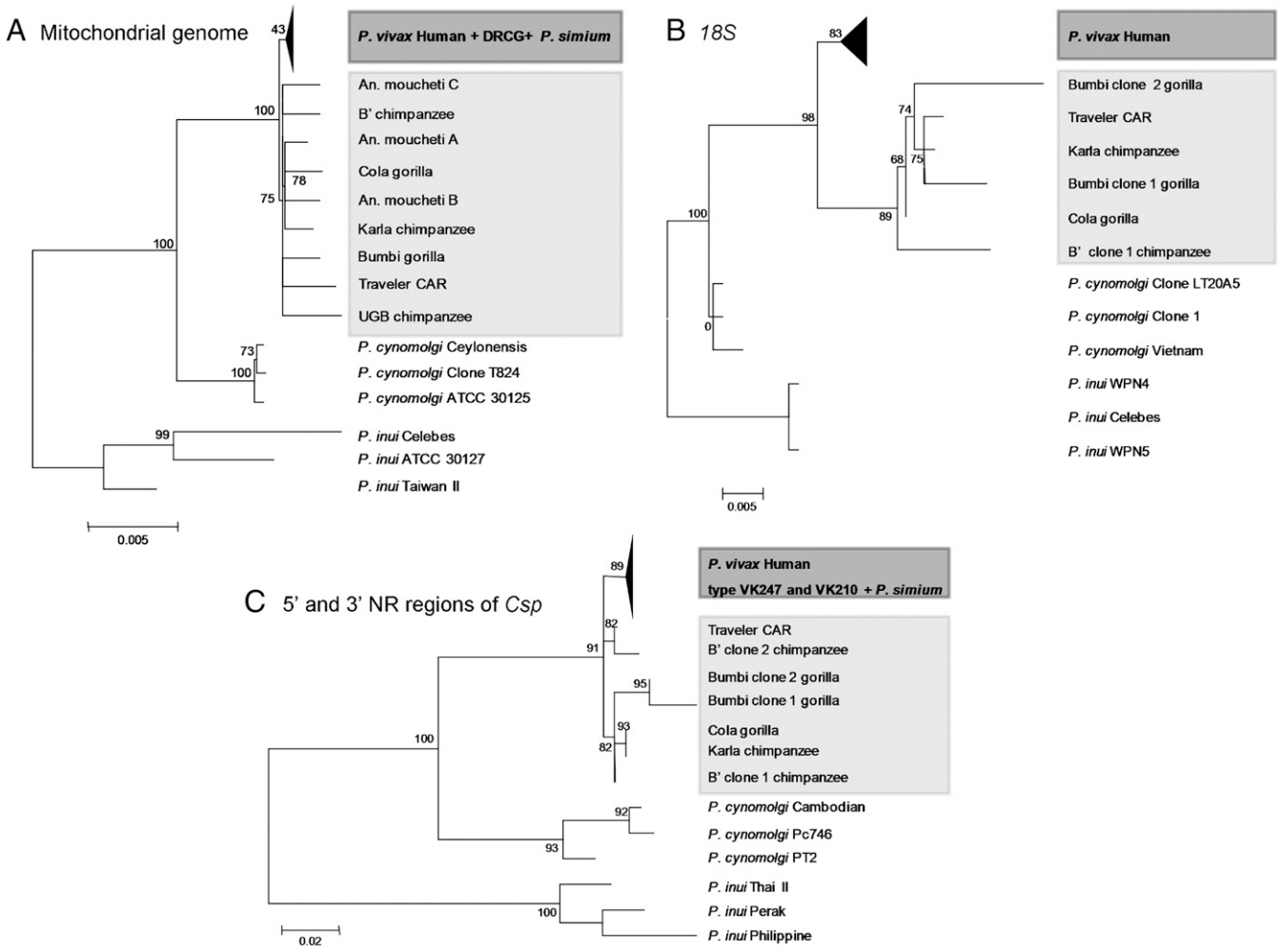

Fig. 1. Phylogenetic relationships among *P. vivax* isolates. (*A*) Based on the 5880-bp section of the mitochondrial genome; (*B*) the 810-bp section of the *18S* gene type A; and (*C*) the 230-bp section of the 5′ and 3′ regions of the *Csp* gene. Bootstrap values are indicated at the nodes. (Scale bars, number of substitutions per site.) For names, sample collection locations, host, and GenBank accession nos. of all sequences including those produced in this study, see Table S1.

and South America showed that it is more genetically diverse than the human clade (Table 1). In addition, this revealed that three SNPs are strictly specific to this clade (see Fig. S3 for positions of the SNPs on the mitochondrial genome) and can thus be used to discriminate it from the human *P. vivax* clade.

For *18S* sequences, single and mixed infections were detected. Two distinct haplotypes were obtained from gorilla Bumbi (clones 1 and 2). As for the mitochondrial genome, *18S* sequences yielded a very similar picture. Sequences found in great apes were genetically divergent from the sequences found in humans worldwide, constituting a yet unknown genetic clade (Figs. 1*B* and 2*B* and Fig. S4). Again *18S* sequences showed more genetic diversity than the human ones (Table 1). Moreover, one SNP was specific to the great ape isolates (Fig. S3).

In *P. vivax*, *Csp* consists of two flanking, highly conserved and nonrepetitive (NR) regions (5′ and 3′ NR), embracing a central repeat (CR) region consisting of numerous repetitions of short oligopeptides (amino acid motifs) (Fig. S5). The CR region varies in the number of amino acid motifs, and there is also sequence variation among the oligopeptides (19). Between the 3′ NR and the CR regions, there is an insertion region (IR) of relatively simple structure (Fig. S5). The *Csp* sequences obtained in our study included all *Csp* regions. Over the whole sequence, two distinct haplotypes were found in chimpanzee B′ (clones 1 and 2) and gorilla Bumbi (clones 1 and 2), whereas the other individuals harbored only one haplotype (chimpanzee Karla and gorilla Cola).

Because the *Csp* sequences show high polymorphism in the number of repeats in the CR region, only the nonrepetitive regions (5′ and 3′ NR) were used for phylogenetic analyses (20). Results showed that the ape sequences were genetically divergent from the ones circulating in humans and fell outside their haplotype diversity (Figs. 1*C* and 2*C* and Fig. S6). Two ape-specific nonsynonymous SNPs were found in the 5′ NR region (Fig. S3). Finally, as for the other genes, the diversity observed in the ape *Csp* NR regions was higher than the diversity found in the human isolates (Table 1).

Regarding the nature of the repeated amino acid motifs in the CR region, human isolates are of two types, VK 210 and VK 247, distinguished by several diagnostic SNPs in the 5′, 3′, and IR regions (19). In great apes, all isolates displayed SNPs and motifs related to the VK 247 *Csp* type in human *P. vivax* (Table S2).

Phylogenetic and Genetic Analyses of *P. vivax*-Like Parasites from Mosquitoes. One *P. vivax* isolate was found in a pool of forest-dwelling anopheline mosquitoes (species *Anopheles moucheti*) trapped in an area inhabited by great apes in the Park of La Lékédi (Gabon, Fig. S1). From this sample, three distinct mitochondrial haplotypes (*An. moucheti* clones A–C) were found. Although the haplotypes differed by several point mutations,

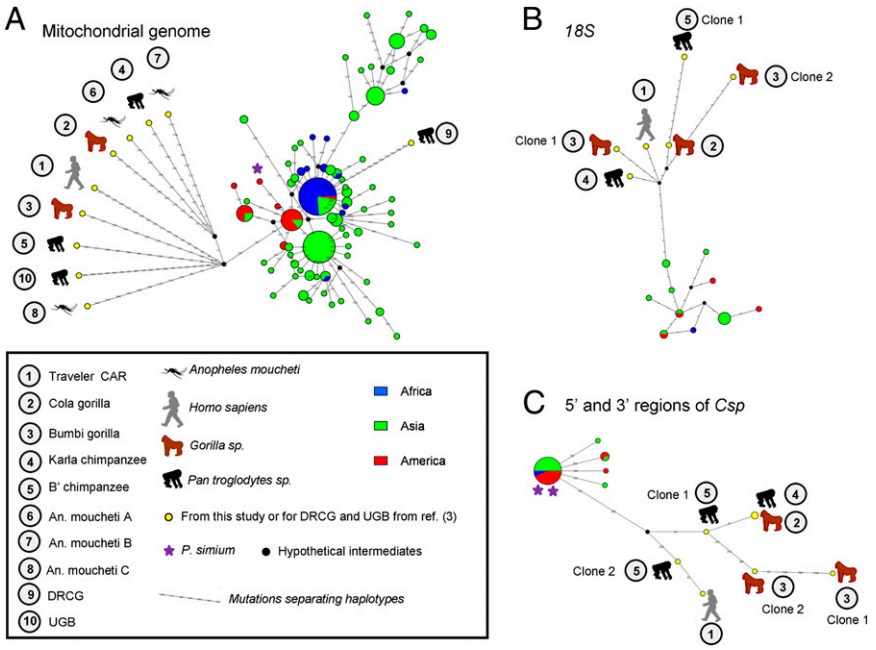

Fig. 2. Haplotype networks constructed using NETWORK 4.5.0. (*A*) Mitochondrial genome. Network was built using 226 *P. vivax* sequences (218 previously published (3, 11, 29), and eight samples from this work). (*B*) The *18S* gene. Network was built using the sequences from Table S1. (*C*) The 3′ and 5′ NR regions of the *Csp* gene. Network was built using the sequences from Table S1. Node sizes (circles) are proportional to haplotype frequencies. Node colors indicate geographic origin. For all information concerning the sequences used and produced in this study, refer to Table S1.

they all shared the three SNPs specific to the ape isolates (Fig. S3). The phylogenetic and MJN analyses confirmed that they belonged to the same clade as the ape parasites (Figs. 1*A* and 2*A*). *18S* and *Csp* genes could not be sequenced from this sample due to an insufficient amount of DNA.

Phylogenetic and Genetic Analyses of *P. vivax*-Like Parasites from Travelers. Five *P. vivax* samples isolated from travelers coming back from West and Central African countries were examined (Fig. S1). We first amplified and sequenced two portions of their mitochondrial genome (*Cytochrome b*, *Cyt b* and *Cytochrome oxidase 1*, *COX1*) to characterize the nature of the diagnostic SNPs distinguishing the human and ape *P. vivax* clades (accession nos. *Cyt b*, KC750255–KC750258; *COX I*, KC750259–KC750262). We obtained interpretable sequences for all samples except for the traveler returning from Guinea. Three samples out of four displayed the SNPs specific to the human *P. vivax* clade. For the other one (Fig. S1, sample 1, traveler from the Central African Republic, traveler CAR), all SNPs were identical to those harbored by ape *P. vivax*-like parasites (Fig. S3). Phylogenetic as well as MJN analyses based on the mitochondrial genome but also on *18S* and *Csp* sequences confirmed its position within the ape *P. vivax* clade (Figs. 1 and 2 and Figs. S2, S4, and S6). This parasite was isolated from a traveler coming back from the Central African Republic who had stayed most of his time (>15 d) in a forest inhabited by great apes (sample 1, Fig. S1). This traveler had the typical symptoms of a *P. vivax* malarial infection as described below.

Description of Case. The traveler was a 32-y-old Caucasian male returning from a 1-mo stay in the southwest of the Central African Republic (3°06′55.75″–3°01′48.79″N to 16°07′59.57″–16°10′42.05″E), where he spent 18 d working in a forest. The patient had no antecedent of malarial infections and, except for a 2-mo stay in the same region in 2005, he did not travel in any other malaria-endemic area. During his stay in Africa, he did not take any antimalarial chemoprophylaxis.

He was admitted to the hospital 12 d after his return, with asthenia and anorexia but without fever. His symptoms started 4 d before admission, in the evening, with fever, important shivers, sweats, myalgias, arthralgias. A second similar episode occurred the third night following the first one. Throughout this time, the patient complained of asthenia and anorexia accompanied by vomiting.

At admission, clinical examination was normal with a particular lack of hepatomegaly and splenomegaly. Blood tests showed thrombocytopenia (platelet count, 105 Giga/L), no anemia

Table 1. Comparison of the sequence diversity observed in the worldwide samples of human *P. vivax* with the *P. vivax*-like parasites

	Human *P. vivax*		Ape isolates		All isolates	
Sequence	*n*	π ± SD	*n*	π ± SD	*n*	π ± SD
Mitochondrial genome	216	0.00069 ± 0.00003	5	0.00451 ± 0.00066	9	0.00436 ± 0.0004
18S	17	0.00375 ± 0.00047	5	0.01492 ± 0.0037	6	0.0135 ± 0.0034
CSP 5′–3′ NR regions	33	0.00165 ± 0.00051	6	0.01737 ± 0.00490	7	0.01863 ± 0.00475

P. vivax-like parasites come from (*i*) apes only except DRCG (ape isolates) or (*ii*) apes, mosquitoes, and traveler CAR (all isolates). Sequence diversity is given for the mitochondrial genome, *18S*, and the 5′–3′ NR regions of *Csp*, respectively. *n*, number of sequences analyzed; π, average number of substitutions between pairs of sequences.

(hemoglobin, 168 g/L), and a normal white blood cell count and formula. Some slight liver abnormalities were observed (serum alanine aminotranferase 109 International Unit (IU)/L normal range (N) < 45; aspartate aminotransferase 51 IU/L, n < 35; gamma-glutamyltransferase 132 IU/L, n < 60; alkaline phosphatase 308 IU/L, n < 280). The lactic dehydrogenase was abnormal (503 IU/L, reference range <450) and an inflammatory syndrome was also noted with C-reactive protein at 73 mg/L (n < 5). Blood sugar and creatinine were normal. Chest radiography was normal.

A malarial infection was suspected and several diagnostic tests were carried out but only the real-time PCR (21) confirmed a malarial infection with *P. vivax* (PCRs were positive for the *Plasmodium* genus and for *P. vivax* species and negative for *P. falciparum*, *P. ovale*, and *Plasmodium malariae* species). Thin blood smear examination as well as the quantitative buffy coat (QBC) test were always negative.

Other laboratory tests had eliminated several possible diagnoses. For bacterial infections: urine and blood cultures were negative, a search for *Legionella pneumophila* urinary antigen was negative, and serodiagnosis for *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Chlamydiae psittaci*, and *Chlamydia trachomatis* infections were also negative. For viral infections: serogiagnostic for HIV-1/2, hepatitis A, B, C, and E, cytomegalovirus, and dengue infections were negative and for Epstein Barr virus, it was in favor of an old infection now healed.

The patient was treated with only a 900-mg oral dose of chloroquine at day 1 in two divided doses (600 and 300 mg), and then 300 mg at day 2 and day 3 and 1 g of paracetamol at day 2. Treatment was well tolerated and the patient rapidly recovered without any relapse to date.

Because of epidemiology, clinical symptoms, biological results, and the total cure after specific antimalarial treatment, the diagnosis of an acute *P. vivax* infection was highly probable even if symptomatic malaria cases with very low parasitaemia are not so frequent.

Discussion

Distinct *P. vivax* Phylogenetic Clade in Great Apes. Our results demonstrate the existence of a distinct *P. vivax* phylogenetic clade circulating in great apes in Africa that does not fall within the currently known diversity of *P. vivax* circulating in humans. Its geographical distribution as well as its host range in Central Africa are large as it was found in animals from both Gabon and Uganda (Fig. S1), in two subspecies of chimpanzees (*Pan troglodytes troglodytes* and *Pan troglodytes schweinfurthii*), and in one subspecies of gorilla (*Gorilla gorilla gorilla*). It was also found in a Caucasian European traveler returning from a forested zone from the southwestern part of the Central African Republic. *An. moucheti*, which is known as a forest mosquito (22), is very likely one of the vector species mediating transmission of this *P. vivax* clade. Because all nonhuman hosts that were found infected by this clade (chimpanzees, gorillas, and the mosquito species *An. moucheti*) are forest-dwelling animals, we will hereafter refer to it as the "sylvatic" *P. vivax* clade.

Sylvatic *P. vivax* Clade as a Possible Source of Infection in Humans. Our study demonstrates that the sylvatic *P. vivax* clade can be at the origin of human infections in Central Africa. We cannot be certain that the *P. vivax* isolate belonging to this clade and found in a European traveler is a zoonosis (i.e., a nonhuman to human transmission) and that the only reservoirs are great apes. Nevertheless, several elements suggest that it could indeed be a case of ape-to-human *Plasmodium* transmission.

During his stay (1 mo) in the Central African Republic, the patient spent most of his time (~18 d) working in a forest located west of the Sangha River, close to the border of Cameroon. This forest in the Central African Republic is home to populations of great apes reported to be infected by *P. vivax*-like pathogens (4). *An. moucheti*, which is one of the candidate vectors of this sylvatic *P. vivax* clade, is very likely present in this area. This species, which is highly antropophilic and represents a major vector of human *Plasmodium* species in forest-dwelling human populations (23), could serve as a perfect bridge vector between apes and humans. Finally, previous experimental works have underlined the possibility of transmission of *P. vivax*-like pathogens from apes to humans and vice versa (24). Indeed, at the beginning of the 20th century, a series of cross-infection experiments involving ape and human *Plasmodium* were performed (24). Among these ape *Plasmodium* was *P. schwetzi*. At that time, when only morphological diagnostics were available, this parasite was considered to be either an equivalent of *P. vivax* or *P. ovale* for great apes (25), and was even considered a subspecies of *P. vivax*. This parasite was then experimentally transmitted from apes to humans several times (24). In at least one of these cases, it is very likely that *P. schwetzi* was effectively a *P. vivax* parasite because transmissions were successful only if the recipients were Caucasians but not if they were Africans, perhaps due to the Duffy negativity of the latter (18, 24, 26).

The existence of one or several animal reservoirs for *P. vivax* is, therefore, one possible explanation for the reported cases of *P. vivax* transmission observed in West and Central Africa. Nevertheless, great ape-to-human transmission cannot account for all observed cases, because some human *P. vivax* infections were observed in areas where great apes are absent (e.g., sample 13, Fig. S1). Other explanations should thus be considered, like the existence of other reservoirs, a transmission maintained by Duffy positive individuals, or the ability of the parasite to infect red blood cells using other pathways.

We note that the mitochondrial *Cyt b* gene of *Plasmodium simium*, a platyrrhine parasite, is identical to the *Cyt b* gene of *P. vivax* (27) belonging to the human clade identified in the present paper. There is evidence of multiple (at least two) transmissions between humans and platyrrhine monkeys in South America, although the direction of transmission has not been determined (27).

Additional global studies in humans, primates, and vectors in West and Central Africa, as well as in South America, will be required to test these different hypotheses.

Humans as Source of Infections for Apes? Our study reveals the possibility of natural transfers of *P. vivax* the other way around, that is, from humans to apes. Indeed, among the ape *P. vivax* of which complete mitochondrial genomes were characterized, one clearly clustered with the human clade, exhibiting its three specific SNPs (DRCG, sample 9, Fig. S1). Its sequence differs from the human haplotypes, but it is likely that some of the substitutions that distinguish this chimpanzee sequence from the human ones could be due to PCR misincorporations (28). This parasite was isolated from an orphan *P. t. troglodytes* in the Democratic Republic of the Congo (3). There are previous reports of transfers of human *Plasmodium* to great apes, especially when the animals lived in close contact with humans, which was likely the case here, for at least part of the life of this orphan (3). Alternatively, this genetic clade might naturally circulate among wild populations of great apes. A more thorough genetic analysis of *P. vivax* infections in wild animals using noninvasive methods (5) should allow to distinguish between the two alternatives.

On Origins of Sylvatic and Human *P. vivax* Clades. What is the origin of the sylvatic clade? What are the links between the sylvatic and the human *P. vivax* clades? Considering that the *P. vivax* lineage is of Asian origin (29), Fig. 3 outlines four different possible scenarios. In scenario A (Fig. 3*A*), the human clade expanded out of Asia and the sylvatic clade derived from a transfer of parasites from human to great apes in Africa. Under this scenario, the sylvatic clade should display less diversity than the human clade and be included in it. This is not what is observed for any of the genes studied here (Table 1 and Figs. 1 and 2). Scenario A is therefore inconsistent with data regarding the origin of the sylvatic clade. However, such model of transmission from humans to primates would be consistent with what is observed for *P. simium* in South America because it is genetically very close to human

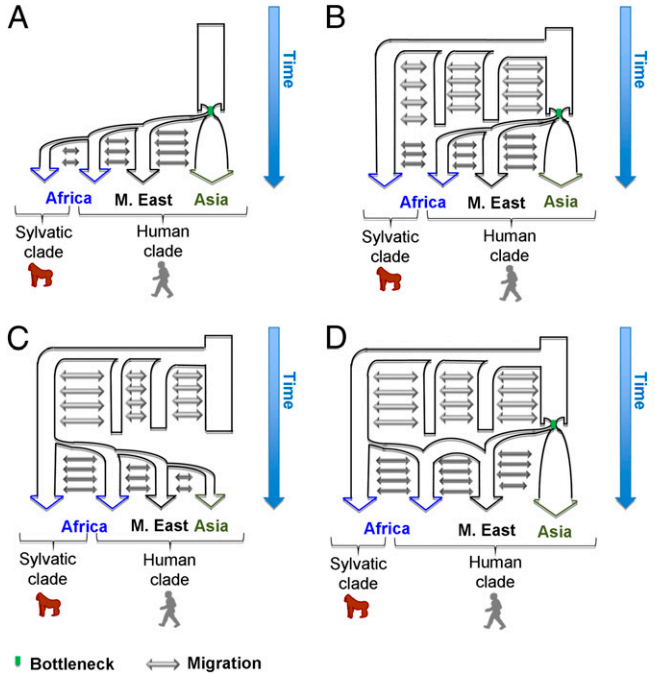

Fig. 3. Alternative scenarios (*A–D*) for the evolution of the sylvatic and the human *P. vivax* clades. (*A*) The sylvatic *P. vivax* clade recently derived from the human clade, which has an Asian origin. For scenarios *B–D*, the sylvatic clade derived from an Asian clade a long time ago. (*B*) The human clade recently derived from the Asian clade. (*C*) The human clade recently derived from the sylvatic clade. (*D*) The human clade is both of Asian and African origin. Detailed description of these scenarios is provided in *Discussion, On the Origins of Sylvatic and Human P. vivax Clades*). Lineage size is proportional to population size. M. East, Middle East.

P. vivax and is clearly included within the diversity of the human clade (27) (Figs. 1 and 2 and Figs. S2, S4, and S6).

In all other scenarios (Fig. 3 *B–D*), the sylvatic clade was considered to have been introduced in Africa more anciently, which is consistent with the high genetic diversity observed in the clade (Table 1). What differs between the different scenarios is the relationship between the human and the sylvatic clades. In scenario B (Fig. 3*B*), we hypothesized that the human *P. vivax* clade expanded out of Asia from an archaic Asian population that passed through a bottleneck. From there, human *P. vivax* progressively colonized the rest of the world following a succession of founding events. This model should translate into: (*i*) a decrease of genetic diversity from Asia to Africa in the human *P. vivax* clade; (*ii*) a global lower genetic diversity in the human *P. vivax* clade compared with the sylvatic one based on the fact that, in this scenario, the human clade originated from a small *P. vivax* Asian population; and (*iii*) the coexistence of two divergent clades in Africa. Regarding point *i*, some worldwide population genetic data are consistent with this expectation (11). For point *ii*, all sequences (mitochondrial, *18S* and *Csp*) displayed consistent genetic patterns (Table 1). However, for point *iii*, mitochondrial and *18S* sequences gave consistent genetic trees (Fig. 1 *A* and *B*), whereas for *Csp*, tree topology would be more consistent with scenario C (see below).

In scenario C, the human *P. vivax* population evolved from the sylvatic clade in Africa and then progressively colonized the rest of the world. From such a scenario, we may expect: (*i*) the genetic diversity in the human clade to be lower than the diversity observed in the sylvatic clade; (*ii*) a decrease of genetic diversity from Africa to Asia; (*iii*) the human *P. vivax* diversity should be included in the diversity of the sylvatic *P. vivax* clade. For this scenario, if some available genetic data seem inconsistent with point *ii* (11), the topology of the phylogeny observed for *Csp* could become consistent with this scenario if we consider that a radiation happened in the African sylvatic clade and that one lineage gave rise to the human *P. vivax* clade. This could also be the case for the mitochondrial sequences if we consider that the haplotype DRCG obtained from a chimpanzee is part of the sylvatic clade and naturally circulates in great apes in Africa.

Finally, we propose a last possible scenario (Fig. 3*D*), where the human clade would have a double origin, Asian and African. In this scenario, some human haplotypes should be closer to the African sylvatic clade, others to the Asian human one. None of the current data are consistent with this scenario. However, we think that current available data are still insufficient to robustly test for these different scenarios (Fig. 3 *B–D*). Indeed, although mitochondrial and *18S* sequences point toward scenario B as the most likely scenario, the low number of samples analyzed in our study may give a biased image of the true diversity of the sylvatic clade, which could actually be higher and completely embrace the human *P. vivax* diversity. Similarly, for *Csp*, which codes for an antigenic protein recognized by the host immune system (19), processes of selection could obscure the true relationships between the different lineages. Finally, there is still a lack of information regarding the genetic diversity and its distribution in human *P. vivax* populations all over the world, especially in Africa, to confront data in the different scenarios. More extensive analyses of the genetic diversity of the *P. vivax* infecting great apes as well as humans worldwide and more specifically in Africa are therefore necessary to resolve the origin and evolution of these different clades.

Conclusions

Our study demonstrates the circulation of two distinct genetic clades of *P. vivax* in Africa: the sylvatic *P. vivax* clade, which was discovered in great apes in Africa and the "human clade" which infects humans worldwide. We also show that these two clades can be potential sources of infection for both apes and humans in West and Central Africa and that *An. moucheti*, a sylvatic vector, could likely participate in the transfer of strains from these clades among the diverse host species. The existence of a possible zoonotic reservoir of *P. vivax* for humans raises concerns about the possibility of eradicating the parasite from human populations.

Materials and Methods

African *P. vivax*-Like Samples from Nonhuman Primates. Four *P. vivax*-like parasites were identified by molecular diagnostic (see below) from four great apes (two chimpanzees and two gorillas) during a continuous survey of wildlife *Plasmodium* infections carried out in Gabon by the Centre International de Recherches Médicales de Franceville (CIRMF) (Fig. S1). Details regarding the samples can be found in *SI Materials and Methods*.

African *P. vivax*-Like Samples from Infected Mosquitoes. One *P. vivax* isolate was identified by molecular diagnostic (see below) from sylvatic anopheline mosquitoes trapped with CDC light traps in the forest of the Park of La Lékédi in Gabon (~10 km from the village of Bakoumba, Haut Ogooué Province, 1°47′48.08″S, 12°59′11.85″E) (Fig. S1). More details regarding this sample can be found in *SI Materials and Methods*.

***P. vivax* Samples from European Travelers Returning from West and Central Africa.** We studied five *P. vivax* diagnosed by the Parasitolgy-Mycology department of the Toulouse University Hospital (France) in European travelers (Caucasians or Africans) returning from West or Central African countries. Among these five travelers, two (one African, one Caucasian) came back from Cameroon, one from Togo/Burkina Faso (Caucasian), one from Guinea (African), and one from the Central African Republic (Caucasian) (Fig. S1). Each of these travelers was diagnosed with a *P. vivax* infection following hospital admission after returning from the African country they visited. As carried out routinely in the department, identification was made by QBC test and examination of a thin blood smear. Real-time PCRs specific for the four *Plasmodium* species found in Africa (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) were systematically performed (21).

DNA Extraction, PCR Conditions, and Sequencing of Samples from Great Apes and Mosquitoes. For each sample (blood or mosquitoes), total DNA (*Plasmodium* and host) was isolated and purified using the DNeasy blood and

tissue kit (Qiagen) according to the manufacturer's instructions. *P. vivax* diagnostics in great apes and mosquitoes were performed by amplifying and sequencing the *Plasmodium Cytochrome b* (*Cyt b*) gene as described in ref. 30. We then sequenced the entire mitochondrial genome of the different *P. vivax* isolates (from great apes and mosquitoes). The amplification was performed following the protocol detailed in ref. 3. PCR products were cloned in the PGem-T vector (Promega) and sequenced by Eurofins. Two additional genes were sequenced from the isolates of great apes (*18S* and *Csp*). Both genes were amplified using nested PCRs as detailed in *SI Material and Methods*. PCR products were cloned using the TOPO TA cloning kit (Invitrogen). DNA sequencing was performed by Eurofins.

DNA Extraction, PCR Conditions, and Sequencing of Samples from European Travelers. Traveler samples were molecularly studied after the great ape samples. Following preliminary results obtained on the comparison of the mitochondrial genome of the parasites from great apes with those of humans showing the existence of three single nucleotide polymorphisms (SNPs) specific to the ape isolates (*Results*), two regions of the mitochondrial genome including these diagnostic SNPs were first amplified and sequenced. One sequence covered part of the *Cyt b* gene and included one SNP, the other covered part of the *Cytochrome c oxydase I* (*COX I*) gene and included two diagnostic SNPs. The first *Cyt b* sequence was amplified using a nested PCR, with conditions identical to those reported in ref. 30, except that the second round was performed with the following primers (PvCYTB1/F 5′-TGGAATTACGGTTCCTTTTAGAA-3′,

PvCYTB2/R 5′-GTAATCATAATGTGTTCGTCTATAA-3′). For *COX I*, we applied the same protocol as in ref. 30.

For the only traveler sample that displayed the SNPs specific to the ape *P. vivax*-like parasites (the one from the traveler returning from the Central African Republic, sample 1 Fig. S1), the complete mitochondrial genome as well as portions of the *18S* and *Csp* genes were amplified and sequenced as previously detailed.

Phylogenetic Tree. For phylogenetic analyses, we used different sources of *P. vivax* mitochondrial, *18S*, and *Csp* sequences for comparison with the sequences obtained in our study (Table S1). For ape *P. vivax*, two other complete mitochondrial sequences were retrieved from ref. 3. (GenBank accession nos.: *P. vivax* chimpanzee DRCG_GQ355480.1; chimpanzee UGB_GQ355481.1) (Fig. S1). For all phylogenies, *Plasmodium cynomolgi* and *Plasmodium inui* sequences were used as outgroups. *P. simium* sequences were added when available. Sequences were aligned using MUSCLE (31) and phylogenetic trees were constructed using maximum likelihood (ML) methods. The most likely DNA tree and corresponding bootstrap support values were obtained by PhyML (freely available at the ATGC bioinformatics platform www.atgc-montpellier.fr) using nearest neighbor interchange (NNI) branch swapping and 100 bootstrap replicates (32).

Haplotype Median-Joining Network Construction. Because traditional phylogenetic methods may fail to accurately reconstruct the relationships between related sequences (33), sequences from parasites isolated from chimpanzees, gorillas, sylvatic mosquitoes, and European travelers, as well as those obtained from humans, were used to construct a haplotype MJN using the software NETWORK 4.5.0 (Fluxus). For the mitochondrial network, more human sequences were used than for the phylogeny. Indeed, all sequences produced in refs. 29 and 11 were added. For the *18S* and *Csp* networks, the same sequences as those included in the phylogenies were used. In these networks, human isolates were assigned to their continental location (Africa, Asia, and America).

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