

# A monkey's tale: The origin of *Plasmodium vivax* as a human malaria parasite

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The high prevalence of Duffy negativity (lack of the Duffy blood group antigen) among human populations in sub-Saharan Africa has been used to argue that *Plasmodium vivax* originated on that continent. Here, we investigate the phylogenetic relationships among 10 species of *Plasmodium* that infect primates by using three genes, two nuclear ( $\beta$ -tubulin and cell division cycle 2) and a gene from the plastid genome (the elongation factor Tu). We find compelling evidence that *P. vivax* is derived from a species that inhabited macaques in Southeast Asia. Specifically, those phylogenies that include *P. vivax* as an ancient lineage from which all of the macaque parasites could originate are significantly less likely to explain the data. We estimate the time to the most recent common ancestor at four neutral gene loci from Asian and South American isolates (a minimum sample of seven isolates per locus). Our analysis estimates that the extant populations of *P. vivax* originated between 45,680 and 81,607 years ago. The phylogeny and the estimated time frame for the origination of current *P. vivax* populations are consistent with an "out of Asia" origin for *P. vivax* as hominoid parasite. The current debate regarding how the Duffy negative trait became fixed in Africa needs to be revisited, taking into account not only human genetic data but also the genetic diversity observed in the extant *P. vivax* populations and the phylogeny of the genus *Plasmodium*.

Duffy | genetic diversity | host-switch

Almost 60 years ago, Haldane (1) proposed that human malaria might act as a selective force on human populations. Until recently, tests of this hypothesis were hampered by a limited amount of data and the lack of objective methods of phylogenetic reconstruction.

Initial phylogenetic investigations using molecular approaches have focused primarily on the origin of *Plasmodium falciparum*, the agent of malignant tertian malaria, and its relationship to other human and animal malaria parasite species (2–8). Two major conclusions were drawn from these studies. First, each of the four *Plasmodium* species parasitic to humans arose independently as human pathogens and, second, *Plasmodium reichenowi*, a parasite of the chimpanzee, is the species that shares the most recent common ancestor with *P. falciparum* (3, 6, 8). These findings have led to vigorous debate about the origin and age of the extant populations of *P. falciparum* (9–14). Until now, there has been limited information about the origin of *P. vivax*, the major and most prevalent human malaria parasite outside of sub-Saharan Africa.

Recent discussions on the origin of *P. vivax* have been driven for the most part by the analysis of indirect evidence without strong phylogenetic data. One of the earliest hypotheses placed the origin of *P. vivax* in Southeast Asia, together with other *Plasmodium* species parasitic in nonhuman primates (15). The argument was supported by the abundance of simian malaria parasite species in this region and the observation that several of the macaque parasites shared morphological and biological characteristics with *P. vivax* (15–17). However, this "out of Asia" hypothesis has not been generally accepted in recent years.

Particularly, arguments based merely on species abundance to identify "centers of origin" are questionable because they do not consider the rapid radiation of species in limited areas (6) or habitat changes that may affect the distribution and abundance of species. In contraposition, the high prevalence of Duffy negativity (lack of the Duffy blood group antigen or FY\*O) among human populations in sub-Saharan Africa has been used to support an African origin for *P. vivax* (16–18).

The Duffy blood group (FY) is a transmembrane glycoprotein that is also a chemokine receptor (19, 20). It has three blood types; two, FY\*A and FY\*B, correspond to functional protein, whereas the third, FY\*O, fails to express a product on the red blood cell surface because of a promoter mutation. The Duffy antigen/chemokine receptor (also referred as DARC) is also an erythrocyte receptor targeted by *P. vivax* as its gateway to invading the red blood cell. Thus, Duffy negative (FY\*O) homozygotes do not express the FY\*A or FY\*B proteins and are completely protected against *P. vivax* infection (17, 19, 20). The specificity of the Duffy–*vivax* interaction suggests that *P. vivax* could have been in contact with the African human population, allowing that selection imposed by the parasite to drive FY\*O to fixation (17); thus, it is possible that *P. vivax* originated out of Africa, carried by any of the hominoid lineages that had their origin there, including modern humans. However, given that *P. vivax* does not exhibit high levels of virulence in terms of mortality rates, it is not likely to be such a strong selective factor (18). This hypothesis leaves open the possibility that the FY\*O in Africa could have been fixed by another process (selection due to another pathogen or chance) and then became a barrier against a subsequent introduction of *P. vivax* (18).

An earlier phylogenetic study using the mitochondrial cytochrome *b* gene provided the first molecular suggestions about the origin of *P. vivax*. The estimated gene phylogeny indicated the following: (i) Asian primate malarias, including *P. vivax*, were apparently part of a recent species radiation (6); and (ii) there was a more ancient African origin for the lineage leading to the extant primate malarial species found in Southeast Asia (6). Specifically, parasites from Africa such as *Plasmodium gonderi* were placed at the base of the phylogeny as sister taxa of a monophyletic group that includes all existing Southeast Asian nonhuman primate parasites together with the human parasite, *P. vivax* (6). This phylogenetic information coincides with the origin and radiation of the various primate groups that are malaria hosts in Southeast Asia (21). This phylogenetic study also made less parsimonious that *P. vivax* could originate from a platyrrhine monkey parasite in South America such as *Plas-*

Abbreviations: DHFR, dihydrofolate reductase; FY, Duffy blood group; ML, maximum likelihood; Myr, million years; TMRCA, time to the most recent common ancestor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY639953–AY640007).

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**Table 1. *Plasmodium* species with their host, geographic range, and two life history traits: periodicity and their capacity for relapse**

Species	Natural hosts	Geographic range	Periodicity	Relapse
<i>P. vivax</i>	<i>Homo sapiens</i>	Tropical, subtropical, and temperate regions	Tertian	Yes
<i>P. hylobati</i>	<i>Hylobati moloch</i>	Indonesia, Malaysia (Borneo)	Tertian	No
<i>P. cynomolgi</i>	<i>Macaca sinica</i> , <i>M. nemestrina</i> , <i>M. fascicularis</i> , <i>M. mulatta</i> , <i>M. radiata</i> , <i>Presbytis entellus</i> , <i>P. critatus</i>	Southeast Asia	Tertian	Yes
<i>P. fieldi</i>	<i>M. nemestrina</i> , <i>M. fascicularis</i>	Malaysia	Tertian	Yes
<i>P. simiovale</i>	<i>M. sinica</i>	Sri Lanka	Tertian	Yes
<i>P. inui</i>	<i>M. fascicularis</i> , <i>M. nemestrina</i> , <i>M. cyclopis</i> , <i>M. mulatta</i> , <i>M. sinica</i> , <i>M. radiata</i>	South and East Asia	Quartan	No
<i>P. knowlesi</i>	<i>M. fascicularis</i> , <i>M. nigra</i> , <i>M. nemestrina</i>	Southeast Asia	Quotidian	No
<i>P. coatneyi</i>	<i>M. fascicularis</i>	Malaysia, Philippines	Tertian	No
<i>P. fragile</i>	<i>M. radiata</i> , <i>M. mulatta</i> , <i>Presbytis spp</i>	Southern India, Sri Lanka	Tertian	No
<i>P. gonderi</i>	<i>Cercocebus atys</i> , <i>Cercopithecus spp.</i>	Central Africa	Tertian	No

*modium simium*. Indeed, *P. simium* is identical to several isolates of *P. vivax* (22) and most likely originated because of a human-to-primate host switch (6).

It is expected that the lineage from which primate malaria radiated in Southeast Asia should appear ancestral (basal) in the phylogeny (17). However, the data from the cytochrome *b* gene was not able to elucidate the finer evolutionary relationships existing among the primate malaria parasites in Southeast Asia. Thus, the estimated phylogeny was compatible with the introduction of malaria parasites into the region from Africa by any primate lineage, among them early hominoids such as *Homo erectus* or more recently by *Homo sapiens*. Because of this lack of resolution in the phylogenetic tree, we further studied the phylogenetic relationships among 10 *Plasmodium* species in primates (including *P. vivax*) by using three new genes, two nuclear and a gene from the plastid genome.

Our data are not compatible with an early African *Homo–P. vivax* association. Furthermore, this investigation supports the notion that *P. vivax* originated from a primate malaria parasite in Southeast Asia, most likely a species infecting macaques.

## Materials and Methods

Table 1 shows the species included in this study with some of their biologic characteristics and geographic distributions; additional information is available in refs. 23 and 24. Phylogenetic analysis was based on two nuclear genes,  $\beta$ -tubulin and cell division cycle 2 (*CDC-2*); and one plastid gene, the elongation factor Tu (*TufA*). The genes were amplified by PCR using the following pairs of primers: AL1508, GAA AA(A/G) GA(A/G) GA(T/C) (G/C)AA GG(A/C) AT(T/C) CC(A/G) TCA AC with AL1509, CC(A/G) AA(A/G) TCI GC(A/G) ATT TTT AAT TCI CC for *CDC-2*; AL1499, GGI CA(A/G) TG(T/C) GGI AA(T/C) CA(A/G) AT(T/A) GGT GCI AA(A/G) TT(T/C) TGG GA, with AL1500, (C/T)TC IGT (A/G)AA (C/T)TC CAT (T/C)TC (G/A)TC CAT for  $\beta$ -tubulin; and AL1447, GGI CAT GTA GAT CAT GGI AAA ACT AC, with AL1448, AT(A/T) AT(A/T) CCT GCT CCT AT(A/T) for *TufA*. "I" codes for inosine. The amplification conditions for  $\beta$ -tubulin were as follows: first, 1 min at 94°C, followed by 30 cycles with 0.5 min of denaturation at 94°C, annealing at 45°C for 0.5 min, and elongation at 72°C for 1.5 min. After 30 cycles, a final elongation step at 72°C for 3.0 min was carried out. In the cases of *CDC-2* and *TufA*, the amplification conditions were, first, 4 min at 94°C, followed by 30 cycles with 0.5 min of denaturation at 94°C, annealing at 45°C for 0.5 min, and elongation at 72°C for 1.0 min. After 30 cycles, a final elongation step at 72°C for 3.0 min was carried out. The amplified products

were purified, cloned, and sequenced. Both strands were sequenced from at least two clones from two independent PCRs. The sequences were obtained by using the automated sequencer 3100 Genetic Analyzer (Applied Biosystems).

Sequence alignment was performed manually; gaps were not considered in the analysis. We performed phylogenetic analyses by using maximum likelihood (ML) methods based on an initial tree calculated by the neighbor-joining algorithm. The phylogenies were estimated with PAUP (Version 4.0 beta10) (25) and PAML (Version 3.13; ref. 26). The best substitution model was selected by likelihood ratio tests as implemented in MODEL TEST (Version 3.06; ref. 27) for each gene by using the initial neighbor-joining trees. In addition, the most inclusive model was implemented in PAML combining the information of the three genes to obtain a final ML phylogeny (28). Supporting values for the nodes of the ML phylogenies were obtained through a Monte Carlo Markov chain model as implemented in MR. BAYES (29).

We tested the expected phylogenies under the following two principal alternative scenarios for the origin of *P. vivax*: (i) a hominoid origin (as proposed in ref. 17) and (ii) a macaque origin (Fig. 1). Under the hominoid scenario, *P. vivax* should be a basal lineage in the phylogeny relative to the other species parasitic of primates in Southeast Asia (Fig. 1A). The alternative tree shown in Fig. 1B represents the macaque scenario (as estimated in this study). Existing data suggest that gibbon and orangutan parasites are derived from macaque parasites (this study and N. Wolfe, A.A.E., and A.A.L., unpublished data). Alternative phylogenies were compared by using the Shimodaira–Hasegawa test (30), which allows for multiple comparisons.

Once the best phylogeny was selected, the time to the most recent common ancestor (TMRCA) for *P. vivax* alleles was estimated for four gene loci from which no evidence for positive natural selection was found (31):  $\beta$ -tubulin (eight isolates from Colombia, Honduras, and Venezuela; two isolates from India, Thailand, Vietnam, and Sumatra); *CDC-2* (seven sequences: six isolates from the same areas as  $\beta$ -tubulin but India and the sequence AF136377 from the GenBank database), dihydrofolate reductase (*DHFR*; 11 isolates as reported by ref. 32 including French Guyana, Surinam, Burma, Cambodia, Indonesia, Madagascar, and Comoros Islands); and *Pvs25* (10 isolates from Colombia, Honduras, Venezuela, Nicaragua, Brazil, Indonesia, North Korea, Mauritania, and Papua New Guinea). The sampling covers the distribution of *P. vivax* including alleles from New World and Old World isolates (22). We followed a similar approach to that used by Hughes and Verra (11) and estimated  $R$ , the substitution rate per site per year, from time ( $t$ ) by using the equation  $t = D/2R$ , where  $D$  is the average genetic distance



**Table 2. Basic estimates for CDC-2,  $\beta$ -tubulin, DHFR, and Pvs25 alleles used for estimating time to the MRCA of *P. vivax***

Gene	<i>n</i>	<i>S</i>	$\eta$	No. of haplotypes	$\pi$ (SD)	F* test
DHFR	11	11	12	8	0.00421 (0.00074)	-1.350 (ns)
$\beta$ -tubulin	8	29	29	7	0.00446 (0.00120)	-1.183 (ns)
Pvs25	10	12	12	9	0.00431 (0.00077)	-1.903 (ns)
CDC-2	7	17	17	8	0.00845 (0.0011)	0.433 (ns)

*n*, number of sequences employed; *S*, number of segregating sites;  $\eta$ , total number of mutations;  $\pi$ , estimate of nucleotide diversity with Jukes and Cantor correction and its SD F\*, Fu and Li test (30); ns, not significant for  $\alpha = 0.10$ .

phylogeny that placed simian parasites as derived from *P. vivax* fitted the data less well; thus, *P. vivax* appeared as a species derived from a *Macaca* lineage.

We also investigated the most recent common ancestor of the extant populations of *P. vivax* by using four genes, from which information from several isolates was available (Table 2). There was no evidence of departure from neutrality on any of these genes (Table 2), and the assumption of a molecular clock was tested among the primate species as described previously. Isolates from Asia and the Americas were included in this sample. DHFR sequences are described in ref. 31. CDC-2,  $\beta$ -tubulin, and Pvs25 are reported in this study. We estimated the average divergence time among the *P. vivax* alleles sampled in this investigation by including one allele per locality, considering the broad distribution of *P. vivax*. As explained before, we estimated *R*, the mutation rate, from  $t = D/2R$ . We used the mutation rates estimated from the divergence among the simian parasites as previously described. The mutation rates obtained can be found in Table 3 and are comparable with those estimated for other eukaryotes (36, 37). We also estimated the mutation rates for all loci considering *P. vivax* as part of this radiation process, and they were comparable with the values obtained among macaque parasite species. However, in preferring to avoid the risk of a circular argument, we excluded *P. vivax* from the mutation rate estimations whenever possible. An average TMRCA for the divergence of the *P. vivax* alleles was estimated by weighting the average. The TMRCA obtained is between 45,680 and 81,607 years ago (Table 3). However, the times estimated ranged from 17,114 to 74,123 under the 1.4-Myr scenario and between 30,507 and 132,445 under the 2.5-Myr scenario. This time frame includes the accepted estimates for the introduction of *H. sapiens* in Southeast Asia (38); however, other hominoids were present such as *H. erectus* (21).

## Discussion

The primary result of our analysis is that *P. vivax* shares a recent common ancestor with the three major macaque parasite lineages (*P. cynomolgi*, *P. inui*, and *P. knowlesi*). This observation corroborates the fact that biologic traits have limited value for assessing phylogenetic relationships among *Plasmodium* species (6). However, a phylogeny is still indispensable for understanding their evolution. In the specific case of periodicity, for example, quotidian and quartan parasites (*P. knowlesi* and *P. inui*, respectively) are derived from tertian parasites such as *P. fragile*. In contrast, the origin of the capacity of relapse in *P. vivax* and related parasites could be a single event under the scenario of *P. vivax* and *P. cynomolgi* being sister taxa.

The phylogeny estimated in this study provides insights on the origin of *P. vivax* as a *Homo* parasite. Specifically, the out-of-Africa scenario for the origin of *P. vivax* is a less parsimonious hypothesis to explain the data presented in this investigation.

Two observations make the scenario of *Homo*-facilitated introduction of primate malaria into Southeast Asia unlikely. First, *P. vivax* should appear as a sister taxa of all Southeast Asian

primate parasites (Fig. 1A), which should form a monophyletic group (17). The phylogeny reported in this investigation is not consistent with this prediction because *P. vivax* appears as a species derived from a *Macaca* lineage of simian parasites. Furthermore, the data support the *P. vivax* lineage originating after the divergence of some of the extant lineages of macaque parasites, notably after the divergence of *P. fragile*/*P. knowlesi*. Additionally, a scenario of an early African origin for *P. vivax* and a subsequent *Homo* introduction in Southeast Asia also implies there would be less diversity within the derived species (in this case, the species parasitic to macaques and other nonhuman primates) than in the ancestral one, *P. vivax*. Contrary to expectations, the diversity within some macaque species such as *P. cynomolgi* and *P. inui* is higher than within *P. vivax*, which shows low genetic diversity (39, 40). Two ad hoc assumptions need to be made to make the genetic data compatible with a host switch from *Homo* to *Macaca*, as follows: (i) There were several extinction and recolonization events that we cannot document with the extant species; thus, the *P. vivax* lineage that survives today is only a derived one, whereas all of the others became extinct; and (ii) there was a recent bottleneck in this remaining *P. vivax* lineage so that its population size became smaller than *P. cynomolgi* and *P. inui* in macaques.

Based on our results, the most parsimonious hypothesis is that the lineage leading to the origin of *P. vivax* as a human pathogen was introduced into *Homo* in Asia by a species of *Plasmodium* parasitic to macaques (Fig. 1B). A host switch from *Macaca* sp. to *Homo* is readily possible and has been demonstrated by natural infections in modern humans with *P. knowlesi* under circumstances of natural transmission in mainland Malaysia and Borneo (41, 42). In addition, it has been postulated that *P. simiovale* may be found in humans, although the data available is from a single gene (43, 44). Host switches appear to be common phenomena in malaria parasites as demonstrated in avian and other primate malaria parasites (23, 44, 45). These findings are also congruent with phylogenetic studies of cestodes (*Taenia*) (46), hookworms (*Oesophagostomum*), and pinworms (*Enterobius*) (47) indicating secondary acquisitions of parasites by humans when they colonized Southeast Asia.

It is important to emphasize that the genera *Homo* and *Macaca* represent the two most successful primate expansions, and their geographical distributions overlapped, especially, during the late Pliocene and middle Pleistocene (0.7–2.5 Myr) (21), making possible the exchange of parasites in any direction.

It is worth noting that our proposal of a macaque origin for *P. vivax* is not based on the number of *Plasmodium* species parasitic to primates in Southeast Asia (16, 17) but on the fact that *Plasmodium* sp. parasitic to macaques are basal in the phylogeny that includes *P. vivax*. Under this scenario, the relatively low genetic diversity in *P. vivax* (40, 48) is the natural consequence of the colonization of hominoids by a macaque parasite lineage that later became *P. vivax*. A cautionary note is necessary at this point: a broader sample of *P. vivax* isolates is needed, particularly

**Table 3. TMRCA among *P. vivax* isolates estimated by  $t = D/2R$**

Genes	Length	<i>n</i>	Estimated		Estimated		Estimated	
			mutation rates for 1.4 Myr	TMRCA (1.4 Myr)	mutation rates for 2.1 Myr	TMRCA (2.1 Myr)	mutation rates for 2.5 Myr	TMRCA (2.5 Myr)
<i>DHFR</i>	709	11	$1.23 \times 10^{-7}$	17,114	$8.21 \times 10^{-8}$	25,639	$6.90 \times 10^{-8}$	30,507
<i>β-tubulin</i>	1185	8	$4.78 \times 10^{-8}$	46,653	$3.18 \times 10^{-8}$	70,126	$2.67 \times 10^{-8}$	83,521
<i>Pvs25</i>	660	10	$3.82 \times 10^{-8}$	56,414	$2.55 \times 10^{-8}$	84,510	$2.14 \times 10^{-8}$	100,701
<i>CDC-2</i>	775	7	$5.70 \times 10^{-8}$	74,123	$3.80 \times 10^{-8}$	111,184	$3.19 \times 10^{-8}$	132,445
Weighted average				45,680		68,512		81,607

Length, sequence length in base pairs; *n*, number of sequences. TMRCA estimates are based on the radiation of the major *Macaca* lineages as explained in the text. The estimated mutation rates are expressed in substitutions per site per year. Averages are weighted by the number of sequences employed for each locus.

isolates from Africa, where 10% of the malaria cases reported are *P. vivax* infections (49). This broader sample, together with more extensive molecular data, will allow elucidating the history of the extant populations of *P. vivax*. This investigation simply aims to underlay the inconsistency of the molecular data with an out-of-Africa origin of *P. vivax*.

A potential limitation in our analysis is the lack of a sample of *P. schwetzi*, a chimpanzee parasite that some authors consider closer to *P. vivax* but others described as a *P. ovale*-like parasite (23). No material of this parasite is available; thus, no data could be derived that challenges the evidence provided in this study. In addition, it is worth noting that the estimated time frames are consistent with both *H. sapiens* and *H. erectus* (21, 50). The role played by the dynamic of the hominoids in Southeast Asia could be very important in the evolution of *P. vivax*; however, we have no elements that allow us to speculate about the topic.

An Asian origin from a nonhuman primate raises questions about the hypothesis for fixation of the Duffy negativity in sub-Saharan Africa as the result of an ancient presence of *P. vivax* on that continent. We could speculate that the fixation of Duffy negativity was driven by selection from other *P. vivax*-like parasites because Asian malaria parasites, as a monophyletic group, derived from *Plasmodium* parasitic in primates in Africa as evidenced by *P. gonderi* and other malaria parasite species (6). However, the genetic signature of directional selection around FY\*O is still controversial, and there is no evidence of a long effect of positive selection in the gene encoding the Duffy blood group when several primates are studied (51). Current investigations show some evidence for a selective sweep leading to the fixation of FY\*O in Africa; however, the pattern is still unclear (52–54). Indeed, FY\*O fixation could have happened after the

onset of agriculture, when human population sizes increased and selection due to malaria could operate (54), a scenario that is still compatible with an Asian origin of *P. vivax*. Finally, but no less important, the fixation of Duffy negativity could be the outcome of other historic or selective processes. Given the available data about the Duffy blood group and this phylogenetic analysis, it appears that by using the high prevalence of FY\*O as evidence that *P. vivax* originated in Africa simply shows the inability of separating “current utility from reasons for origin” (55); that is, the fact that a trait is an adaptation today does not imply that it originated by natural selection. Our conclusion of an Asian origin is consistent with results from analyses of complete mitochondrial genomes (J. Mu, D. Joy, and X. Su, personal communication).

In summary, this investigation points to *P. vivax* being derived from ancestral macaque parasites when hominoids colonized Southeast Asia. Our results do not support that *P. vivax* was a *Homo* parasite before the expansion of the hominoids populations out of Africa. Thus the assumption that the high prevalence of Duffy negative is a consequence of a long *H. sapiens*–*P. vivax* association in Africa needs to be revisited.

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