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Contrasting Epidemiology and Genetic Variation of *Plasmodium vivax* Infecting Duffy Negatives across Africa

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Abstract

Recent studies indicated that *Plasmodium vivax* can infect Duffy-negative individuals, but the varied diagnostic and methodological approaches have limited our ability to characterize *P. vivax* across Africa. Here, we utilized a standardized approach to compare epidemiological and genetic attributes of *P. vivax* from Botswana, Ethiopia, and Sudan, where Duffy-positive and Duffy-negative individuals coexist. Among 1,215 febrile patients, the proportions of Duffy negativity range from 20-36% in East Africa to 84% in Southern Africa. Considerable differences were observed in *P. vivax* prevalence among Duffy-negative populations ranging from averaged 9.2% in Sudan to 86% in Botswana. *P. vivax* parasite density in Duffy-negative infections is significantly lower than in Duffy-positive infections. Phylogenetic analyses of 229 *PvDBP* sequences indicated that Duffy-negative *P. vivax* were not monophyletic but occurred in multiple well-supported clades, suggesting independent origins. Duffy-negative Africans are clearly not resistant to *P. vivax* and the public health significance should no longer be neglected.

Keywords: Plasmodium vivax; Duffy Negatives; Africa; Molecular epidemiology; Genetic relatedness

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Introduction

Plasmodium vivax malaria was previously thought to be rare or absent in African populations who lack the Duffy blood group antigen expression [1,2]. A point mutation (c.1-67T>C; rs2814778) in the GATA-1 transcription factor binding site of the Duffy antigen/receptor for chemokines (*DARC*) gene promoter alters erythroid expression, eliminating Duffy antigen expression on the surface of the red blood cells [3]. However, recent studies reported several cases of *P. vivax* infection in Duffy-negative people in different parts of Africa [5,6], including countries where Duffy-negatives are predominant [7-11] (**Table 1**). In addition, 29 African countries including six previously undocumented endemic countries (Benin, Comoros, Mozambique, Senegal, Zambia and Zimbabwe) have reported *P. vivax* clinical cases, infected vectors or asymptomatic parasitemia [12-14]. These reports indicate that the endemic range of *P. vivax* has extended beyond East Africa and penetrated into areas of very high Duffy-negativity [6,15]. While *P. falciparum* is considered to be the deadliest malaria parasite with the most severe clinical outcomes, *P. vivax* is more widespread and often associated with high levels of morbidity. Compared to *P. falciparum*, *P. vivax* has a broader temperature tolerance, an earlier onset of gametocyte development, and can form dormant hypnozoites causing relapse [16], enabling *P. vivax* to spread through the diverse African climate and outcompete *P. falciparum* [17]. Primaquine and 8-aminoquinoline are antimalarials effective in clearing hypnozoites and preventing relapses, but they may promote hemolysis in subjects with G6PD deficiency [18]. These factors make *P. vivax* malaria difficult to control and eliminate, highlighting the concern of this 'new' *P. vivax* strains that infect Duffy-negative hosts to spread through much of Africa and result in substantial, negative public health and economic impacts.

There is a major knowledge gap in the *P. vivax* invasion mechanisms in Duffy-negative erythrocytes. In *P. falciparum*, erythrocyte invasion involves multiple interactions between parasite ligands and host receptors, some of which have overlapping and partially redundant roles [19,20]. Several established invasion ligands from Erythrocyte Binding Antigens such as EBA-175, EBA-181/JESEBL and EBA-140/BAEBL and Reticulocyte binding homolog proteins such as RH1, RH2a, RH2b, RH4 and RH5 are used by *P. falciparum* for invasion [20,21]. In *P. vivax*, only a single *P. vivax* ligand-receptor interaction has so far been studied in any detail, *P. vivax* Duffy Binding Protein (*PvDBP*1). Previous study has shown that mutations in *PvDBP*1 region II unique to *P. vivax* in Duffy-negative people in Ethiopia did not lead to binding of Duffy-negative erythrocytes [22]. Salvador (Sal) I *P. vivax* infects Squirrel monkeys without *PvDBP*1 binding to Squirrel monkey erythrocytes [23]. Further, EBP/DBP2 region II, a paralog of *PvDBP*1, was shown to bind to Duffy-positive and Duffy-null human erythrocytes at low frequency [22,24], despite being deleted in Sal-I *P. vivax* [25]. Recently, reticulocyte binding protein RBP2b of *P. vivax* was shown to bind to transferrin receptor in the reticulocytes [26]. These findings suggested that there are other Duffy-independent pathways that enable erythrocyte invasion and explain the widespread phenomenon of *P. vivax* infections in Africa.

Despite the fact that several case reports from almost all countries across the African continent are emerging from various entomological and serological studies, community surveys, and clinical records [6,15], the documentation of *P. vivax* infections

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across Africa is diverse, context-specific, and primarily driven by the specific objectives of isolated clinical or epidemiological activities. The varied diagnostic and methodological approaches used across studies have limited our ability to identify distinct epidemiological characteristics of *P. vivax* between regions (**Table 1**). This situation is concerning because there is no comprehensive genetic and epidemiological data of *P. vivax* in Africa available to National Malaria Programs or World Health Organization to assess impacts and confer control strategies. Therefore, in this study, we utilized a standardized assay to examine the epidemiological attributes of *P. vivax* in three African countries where Duffy-positive and Duffy-negative individuals coexist. Specifically, we (1) compared the prevalence of Duffy negativity and *P. vivax* infections among countries; (2) compared *P. vivax* parasitemia between Duffy-negative and Duffy-positive infections collected from the same area; and (3) inferred the genetic relationships among the African *P. vivax* isolates. The epidemiological and genetic features of *P. vivax* from different parts of Africa will fill critical gap in understanding how widespread this phenomenon is impacting malaria control and the important effect of *P. vivax* as a cause of anemia.

Table 1. Summary of *P. vivax* infections with available Duffy blood group information in African countries from the literature. nPCR: Nested PCR of *P. vivax* 18S rRNA gene; qPCR: Quantitative real-time PCR of *P. vivax* 18S rRNA gene.

Country	Sample collection year	Symptoms	Sample size	Duffy negative, *n* (%)	Malaria diagnostic method	*Plasmodium spp* pos (%)	*P. vivax* pos (% of *P. spp*+)	*Pv*+ in Duffy neg (% of total *Pv*+)	Reference
East-Southern Africa									
Angola	2006-07	No	898 ^	*	nPCR	245/898 (28.9%)	7/245 (2.8%)[1]	7/7 (100%)*	Mendes C, et al. PLoS NTDs, 2011;5(6): e1192.
Ethiopia	2009	Yes	1,931	41/205 (20%)[2]	nPCR	205/1,931 (10.6%)	111/205 (54.1%)	3/111 (2.7%)	Woldearegai TG, et al. Trans R Soc Trop Med Hyg 2013;107:328-31.
Ethiopia	2013-14	Yes	416	94/416 (29.7%)	qPCR	331/416 (79.5%)	197/331 (59.5%)[3]	2/197 (1%)[4]	Lo E, et al. Malaria J, 2015;14:84.
Ethiopia	2013-14	No	390	139/390 (35.6%)	qPCR	73/390 (18.7%)	24/73 (32.9%)[5]	4/24 (16.6%)	Lo E, et al. Malaria J, 2015;14:84.
Kenya	1999-2000	Yes	31 ^[6]	31/31[8] (100%)	microscopy	31/31[6] (100%)	11/31 (35.4%)	9/11 (81.8%)	Ryan JR, et al. Am J Trop Med Hyg, 2006;75:575-81.
Madagascar	2006-07	Yes	183[7]	*	nPCR	183/183 (100%)[7]	183/183 (100%)[7]	17/183 (9.3%)*	Ménard D, et al. PNAS, 2010;107(13):5697-71.
Madagascar	2006-07	No	661 ^	476/661 (72%)	nPCR §	251/661 (38%)	86/251 (34.3%)[8]	42/86 (48.8%)	Ménard D, et al. PNAS, 2010;107(13):5697-71.
Madagascar	2014	No	2,063	914/1,878 (48.7%)	nPCR	285/2,063 (13.8%)[9]	137/285 (48.1%)[10]	44/914 (4.8%)	Howes RE, et al. Am. J. Trop. Med. Hyg., 2018;99(4):995-1002.
West-Central Africa									
Benin	2009-10	No	84 ^^	*	nPCR	25/84 (29.8%)	13/25 (52%)[11]	13/13 (100%)*	Poirier P, et al., Malar J, 2016;15:570.
Botswana	2012	No	3,624 [22]	N.A.	nPCR	179/3,624 (5%)	169/179 (94.4%)	N.A.	Matshoge T, et al. BMC Inf Dis 2016;16:520.
Cameroon	N.A.	Yes	485	*	nPCR	201/485 (41.4%)	8/201 (4%)[12]	8/8 (100%)*	Ngassa Mbenda HG & Das A. PLoS ONE 2014;9(8):e103262.
Cameroon	2008-09	No	269	*	nPCR	87/267 (32.3%)	13/87 (14.9%)[13]	6/13 (46.1%)*	Fru-Cho J, et al. Malaria J, 2014;13:170.
Cameroon	2012-13	Yes	484	224/228 (98.3%)[15]	nPCR	70/484 (14.4%)	27/70 (38.6%) [15]	27/27 (100%)	Russo G et al. Malaria J, 2017;16(1):74.
Democratic Republic of Congo	2013-14	No	292 ^	*	nPCR	194/292 (66.4%)	14/194 (7.2%)	14/14 (100%)*	Brazeau NF, et al. Am. J. Trop. Med. Hyg., 2018;99(5):1128–33.

Equatorial Guinea	2005	No	97	*	nPCR	84/97 (86.6%)	8/84 (9.5%)[16]	8/8 (100%)*	Mendes C, et al. PLoS NTDs, 2011;5(6):e1192.
Mali	2009-11	No	300 ^	*	qPCR	135/300 (15%)	25/135 (18.5%)	25/25 (100%)*	Niangaly A, et al. Am J Trop Med Hyg 2017;97(3):744-52.
Mauritania	2007-09	Yes	277	52/258 (20.1%)	qPCR	110/277 (39.7%)	110/110 (100%)	1/110 (0.9%)	Wurtz N et al. Malaria J, 2011;10:336.
Nigeria	2016-17	Yes	436	*	nPCR	256/436 (58.7%)	5/256 (1.9%)[17]	5/5 (100%)*	Oboh MA, et al., Malar J, 2018;17:439 and 2020;19:229.
Senegal	2009-2013	Yes	263	N.A.	nPCR	164/263 (62.3%)	4/164 (2.4%) [24]	N.A.	Niang et al., Malar J, 2015; 14: 281.
Senegal	2010-2011	No	48(x4)^[18]	48/48 (100%)	nPCR	74/192 (38.5%)	15/74 (20.3%)	5/5 (100%)	Niang M, et al. Trop Med & Hyg 2018;46:45.
Sudan	2009	Yes	126	*	nPCR	N.A.	48/126 (38.1%)	4/48 (8.3%)*	Abdelraheem MH, et al. Trans R Soc Trop Med Hyg 2016;110:258-60
Sudan	2016	Yes	992[19]	*	microscopy	992/992 (100%)[19]	190/992 (19.1%)[20]	34/190 (17.9%)*	Albsheer MMA, et al. Genes, 2019;10:437.
Uganda	2016	Yes	499 [21]	N.A.	nPCR	499/499 (100%)	4/499 (0.8%) [23]	N.A.	Asua, V. et al. Am J Trop Med Hyg. 2017;97:753-57.

* Duffy-Ag assessed only among *Pv* pos patients; ^ only children; ^^ only blood-donors; N.A. not available.
§ Conventional PCR of genes PvCOI and PvDBP.

[1] 2 *Pf-Pv* co-infections; [2] Duffy-Ag available only among *Plasmodium spp* pos; [3] 33 *Pf-Pv* co-infections; [4] 2 *Pf-Pv* co-infections; [5] 1 *Pf-Pv* co-infection; [6] 31 children Duffy neg affected by malaria enrolled in a precedent study (anemia study); [7] only *Pv* pos analysed (153 *Pv* mono-infections and 30 *Pf-Pv* co-infections); [8] 34 *Plasmodium* mixed-infections (species not specified); [9] 42 co-infections (25 Pf-Pv, 5 Pf-Pm, 9 Pv-Pm, 1 Pv-Po, 1 Pf-Pv-Pm, 1 Pf-Pv-Pm-Po); [10] 37 co-infections (25 Pf-Pv, 9 Pv-Pm, 1 Pv-Po, 1 Pf-Pv-Pm, 1 Pf-Pv-Pm-Po); [11] 9 *Pf-Pv* and 1 *Pf-Pv-Pm* co-infections; [12] 2 *Pf-Pv* co-infections; [13] 3 *Pf-Pv* and 1 *Pf-Pv-Pm* co-infections; [14] Duffy-Ag assessed among 228 participants (including all those infected); [15] 2 *Pf-Pv* co-infections; [16] 4 *Pf-Pv* co-infections; [17] 4 *Pf-Pv* co-infections; [18] 48 school children followed during 2 years (4 samples for each children, 192 total samples analyzed) [19] only *Plasmodium spp* pos sample analysed; [20] 4 *Pf-Pv* co-infections; [21] all Plasmodium pos children aged 2 months-10 years; [22] all children aged 2-12 years; [23] all 4 were co-infections: 3 *Pf-Pv* and 1 *Pv-Pm*; [24] 2 *Pf-Pv* co-infections

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Materials and Methods

Ethics statement

Scientific and ethical clearance was given by the institutional scientific and ethical review boards of Jimma University (Ethiopia), the Ethics Committee of the Institute of Endemic Diseases, University of Khartoum (Reference number: 9/2016), the Health Research and Development Division of the Botswana Ministry of Health and Wellness (Reference number: HPDME: 13/18/1), and University of North Carolina at Charlotte (USA). Written informed consent/assent was obtained from all consenting heads of households, parents/guardians (for minors under 18 years old), and each individual who participated in this study.

Study sites and sample collection

A total of 1,215 febrile patients were collected from seven study sites in three countries including (1) Jimma and Bonga in Ethiopia; (2) Khartoum, River Nile, and New Halfa in Sudan; and (3) Tutume and Kweneng East in Botswana (**Figure 1**). Finger-prick blood samples were obtained from patients who visited the health facilities. Thick and thin blood smears were prepared for microscopic screening. Three to four blood spots were blotted on Whatman 3MM filter paper from each participant. Parasite DNA was extracted from dried blood spots by the Saponin/Chelex method [27]. Eluted DNA was used for PCR diagnosis, quantification and genotyping of malaria parasites.

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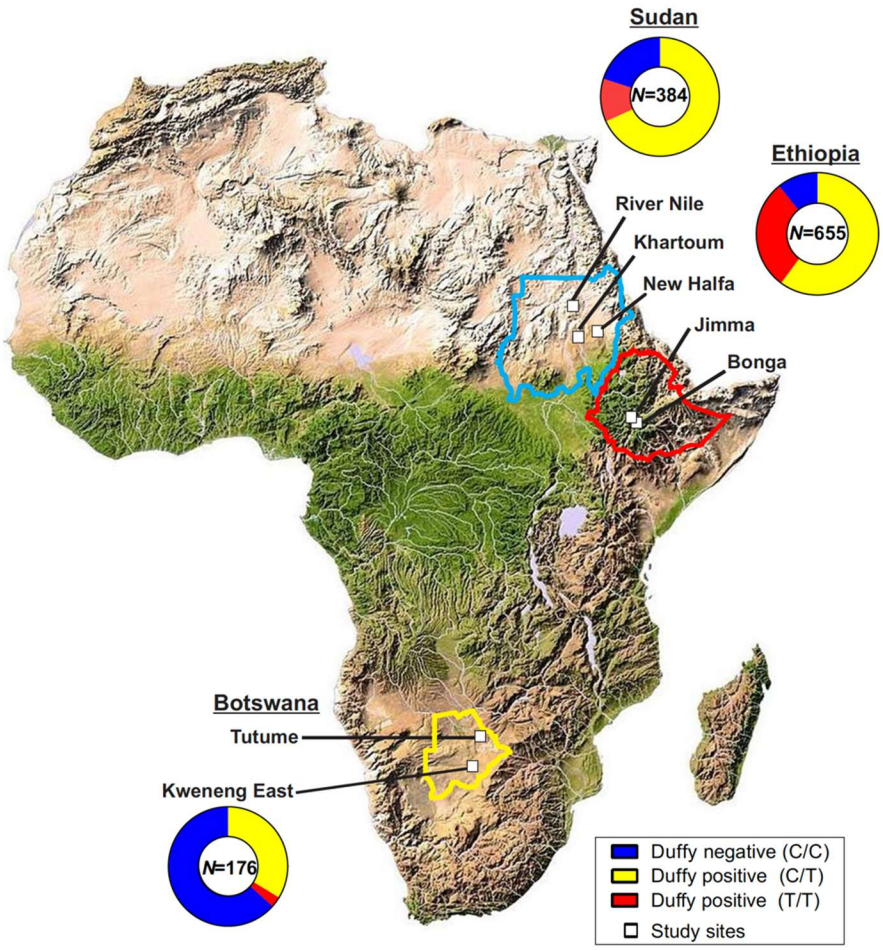

Figure 1. Map showing the distribution of study sites and the Duffy status of febrile patients included in the present study.

Molecular screening of *P. vivax*

Parasite gene copy number was estimated using the SYBR Green detection method [28] using *P. vivax*-specific primers that targeted the 18S rRNA genes (detail in **Supplementary File 1**). Each assay included positive controls of *P. vivax* Pakchong (MRA-342G) and Nicaragua (MRA-340G) isolates, in addition to negative controls. A standard curve was produced from a ten-fold dilution series of the *P. vivax* control plasmid to determine the amplification efficiency (E). Melting curve analyses were performed to confirm the specificity of gene amplifications. The mean threshold cycle (*Ct*) and standard error were calculated from three independent assays of each sample. The amount of parasite density in a sample was calculated using the follow equation: $\text{Parasite density}_{\text{sample}} = 2^{E \times (40 - Ct\text{sample})}$. The differences in the log-transformed parasite density between samples among the study sites were assessed for significance by one-tailed t-tests.

Duffy blood group genotyping

For all febrile patients, we first employed qPCR-based TaqMan assay to examine the point mutation (c.1-67T>C; rs2814778) of the *DARC* gene (**Supplementary File 1**). A no-template control was used in each assay. The *Fy* genotypes were determined by the allelic discrimination plot based on the fluorescent signal emitted from the allele-specific probes. For *P. vivax* positive samples, a 1,100-bp fragment of the *DARC* gene was further amplified using published primers [29]. PCR products were sequenced to confirm the *Fy* genotypes.

Phylogenetic analyses of *P. vivax* from Duffy negative and Duffy positive samples

We amplified and obtained *PvDBP* sequences of 4 Duffy-positive and 4 Duffy-negative *P. vivax* samples from Botswana, 107 Duffy-positive and 9 Duffy-negative *P. vivax* samples from Ethiopia, and 53 Duffy-positive and 16 Duffy-negative *P. vivax* samples from Sudan. These sequences were aligned with 36 previously published *P. vivax* isolates from other parts of Africa including Uganda (*n*=31), Madagascar (*n*=4), and Mauritania (*n*=1; **Supplementary File 2**). Duffy status of the published sequences are unknown. The *DBP* sequence of Sal-1 (NC_009911.1) and *EBP* sequence of *P. cynomolgi* (Y11396.1) were used as outgroups. Phylogenetic trees were reconstructed using the maximum likelihood method implemented in RAxML v8.0 with 500 bootstrap replicates to assess clade support (details in **Supplementary File 1**). We further examined the nucleotide and haplotype diversity of *PvDBP* sequences in Duffy-negative and Duffy-positive samples using DnaSP v6.12.03.

Results and Discussion

Contrasting proportion of Duffy-negatives and *P. vivax* prevalence

Duffy genotyping shows different proportions of Duffy-negative among febrile patients in Botswana, Ethiopia, and Sudan (**Figure 1**). In Botswana, the proportion of Duffy-negative was 83.5% (147/176) among febrile patients (**Figure 1**). Vivax malaria was first reported in asymptomatic children in a survey during the 2012-2013 transmission season [10]. The average rate of asymptomatic *P. vivax* cases was 4.7%, but with large variation among districts. In Kweneng East, our qPCR analyses indicated that 3% (9 out of 301) of the febrile patients were *P. vivax* positive. Among them, eight were Duffy-negative (C/C) and one was Duffy-positive (T/C) (**Table 2; Supplementary File 3**). In Tutume, 6.8% (12/176) of the febrile patients were detected with *P. vivax* and 10 of them were Duffy-negative. Compared to other parts of Botswana, Tutume and Kweneng East accounted for most of the *P. vivax* cases, with previously reported rates of 16.9% (54/320) and 13.6% (93/686), respectively [8].

Vivax malaria is a significant problem in Ethiopia [28,30]. The proportion of Duffy-negative was 35.9% (235/655) among febrile patients (**Figure 1**), similar to our earlier finding in Asendabo indicating that 35.1% (137/390) of the general population was Duffy-negative [28]. Among the 358 febrile patient samples collected in Jimma, 36%

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(129/358) were Duffy-negatives (**Figure 1**) and 37.4% (134/358) were detected with *P. vivax* (**Table 2**). About 11.9% (16/134) of the confirmed *P. vivax* infections were from Duffy-negatives. Likewise, in Bonga, 30.3% (125/413) of the febrile patients were detected with *P. vivax* and 3.2% (4/125) were from Duffy-negatives (**Table 2**). For these 20 Duffy-negatives *P. vivax* infections, microscopy, nested and quantitative PCRs indicated that 16 were single infections and four were mixed with *P. falciparum*. previous study has shown that the asymptomatic prevalence of *P. vivax* is 5.9% (23/390) in Asendabo and Duffy-negatives accounted for 8.7% (2/23) of the *P. vivax* infections [28]. A lower proportion of Duffy-negativity in febrile patients and the general population in Ethiopia, as compared to Botswana, is consistent with the ethnic diversity and complex admixture history in East Africa [31,32].

In Sudan, most cases of malaria are caused by *P. falciparum* but in recent years there has been an increase in *P. vivax* detection and reports [33]. The proportion of Duffy-negative was 20% (77/384) among febrile patients (**Figure 1**). Over a 6-month collection period between 2018 and 2019, 101 out of 831 febrile patients were confirmed as *P. vivax* positive by qPCR assays (**Table 2**). Further testing revealed that 4 of the 101 *P. vivax* samples were mixed with *P. falciparum*. The highest rate of *P. vivax* infection was observed in River Nile, of which 24.4% (52/213) of the febrile patients were confirmed with *P. vivax* and Duffy-negatives accounted for 3.8% (2/52) of these infections (**Table 2**). In Khartoum, 8% (42/525) of the febrile patients were *P. vivax*-positive and Duffy-negatives accounted for 9.5% (4/42) of these infections. In New Halfa, despite a smaller sample size, 7.5% (7/93) of the febrile patients was *P. vivax*-positive and Duffy-negatives accounted for 14.3% (1/7) of these infections (**Table 2**).

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Table 2 Comparison of *P. vivax* infection rate in Duffy-negative populations across different study sites in Botswana, Ethiopia, and Sudan based on febrile patient samples collected in this study.

Region	Country	Study site	Collection period	Type of collection	Total samples	Infection rate of *P. vivax*	Duffy-negative among *P. vivax* infections
Southern Africa							
	Botswana	Tutume	2017 - 2018	Symptomatic	176	12 (6.8%)	10 (83.3%)
		Kweneng East	2017 - 2018	Symptomatic	301	9 (3%)	8 (88.9%)
East Africa							
	Ethiopia	Jimma	April - October 2017	Symptomatic	358	134 (37.4%)	16 (11.9%)
		Bonga	October – November 2019	Symptomatic	297	76 (25.6%)	8 (10.5%)
	Sudan	River Nile	August 2018 – February 2019	Symptomatic	213	52 (24.4%)	2 (3.8%)
		Khartoum	August 2018 – February 2019	Symptomatic	525	42 (8%)	4 (9.5%)
		New Halfa	August 2018 – February 2019	Symptomatic	93	7 (7.5%)	1 (14.3%)

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Historical movement and genetic admixture explain distribution of Duffy-negative people in Africa

Historical human movement and human genetics are highly relevant to the distribution of Duffy-negative people and *P. vivax* in Africa. Recent genome-wide studies of African populations have refined earlier models of the continent's history and its impact on genetic diversity of its inhabitants [34]. The Bantu expansion and population admixture are two main historical events that shape the present distribution and genetic make-up of ethnic groups across Africa. The Bantu and Khoisan are two major ethnic groups in West-Central and Southern Africa, with the Bantu heartland in the region between southern Nigeria and Cameroon where malaria transmission was and still is endemic [35]. A component of Bantu ancestry (likely Duffy-negative) was found in the Southern African Khoisan, which were originally and mostly Duffy-positive ancestors [36,37]. The Duffy-negative allele from Bantu of West-Central Africa may have reached south of the continent within the last 750 years [38] (**Figure 2A**) and mixed with the indigenous Khoisan, resulting in a variable Khoisan ancestry [39]. Our data showing a Duffy negative rate of 83.5% among febrile patients in Botswana (**Figure 1**) is consistent with the Bantu expansion and admixture theories [34,35]. While the direction of the Bantu expansion is still in debate, there is evidence showing that the Bantu migrated towards East Africa where other ethnic groups such as the Cushitic and Nilotic dominated, potentially around 2,000 years ago [31] (**Figure 2A**). The Ethiopian and Sudanese population, with an admixture of several Eurasian ancestries and some Nilotic and Semitic-Cushitic components, also migrated south after the Bantu expansion 2-5 thousand years [31,32] (**Figure 2B**). Our data showing a Duffy negative rate of 20-36% in Southwestern Ethiopia and East Sudan (**Figure 1**) is consistent with the complex admixture history. Many population groups in Sudan are dominated by Nilotic and Eurasian admixtures with minimal West African component. One such exception is the Afro-Asiatic speaking Hausa population in the Middle Eastern Sudan, which have migrated from West Africa within the past 300 years [32]. These migrations could have spread *P. vivax* from West-Central to other parts of Africa.

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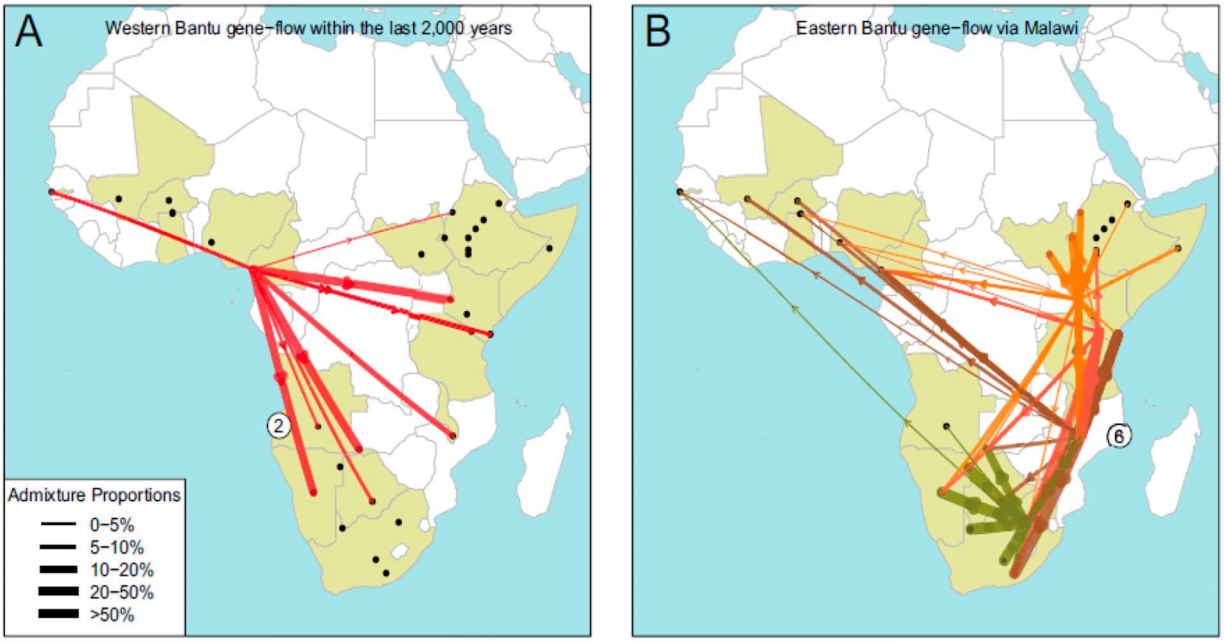

Figure 2. Bantu expansion and admixture according to two hypotheses [38].

Low parasitemia in symptomatic Duffy-negative *P. vivax* infections and implications on invasion mechanism

In Botswana, Ethiopia, and Sudan, Duffy-positive and Duffy-negative individuals coexist. *P. vivax* parasite density in Duffy-negative infected individuals is significantly lower than the Duffy-positive infected individuals, regardless of geographical differences (**Figure 3**). The Duffy-negative *P. vivax* samples in Ethiopia and Sudan showed a greater range of parasitemia variation than those in Botswana. This may be due to differences in sample size (**Figure 3; Supplementary File 3**). In very few cases the asexual parasites were detected by microscopy in Duffy-negative individuals. For example, among the 20 *P. vivax* infections identified in Duffy-negative patients from Ethiopia, only four were microscopic-positive and they all showed a relatively higher parasitemia compared to the submicroscopic infections. The Duffy-negative individuals who were infected with *P. vivax* were mostly submicrocopic and exhibited fever at the time of sample collection. Without highly sensitive diagnostic tools and vigorous on-site training and screening of *P. vivax* in different parts of Africa, the public health burden, economic impact, and severity associated with vivax malaria could have been vastly underestimated. The clinical spectrum of *P. vivax* malaria ranges from asymptomatic parasitemia and uncomplicated febrile illness to severe and fatal malaria [40]. Moreover, *P. vivax* can cause anemia during chronic undetected infections [10]. Other severe clinical manifestations include multiorgan dysfunction associated with anemia and thrombocytopenia, spontaneous abortions, premature and low birth weight in pregnant women [40]. These clinical features have mostly been described for Duffy-positive populations. It is unclear if the spectrum of clinical symptoms is different in Duffy-negative patients in Africa.

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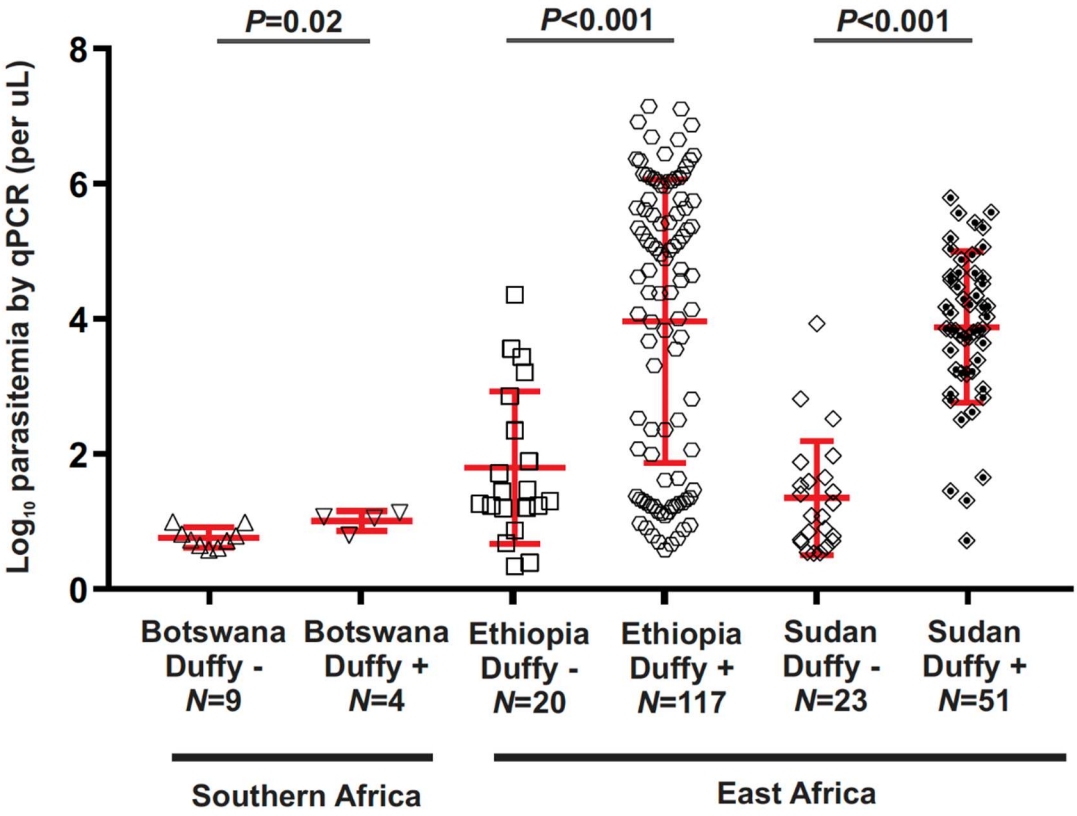

Figure 3. Comparison of *P. vivax* parasitemia based on quantitative PCR assays between Duffy negative and Duffy positive symptomatic infections among different geographical regions in Africa. Variations in parasitemia among samples were presented as boxplots showing the median and interquartile range values.

Low parasitemia observed in Duffy-negative individuals might suggest a low invasion capability of *P. vivax* in Duffy-negative individuals. Recent study has shown that mutations in *PvDBP*1 region II unique to *P. vivax* in Duffy-negative people in Ethiopia did not lead to binding of Duffy-negative erythrocytes [22]. Also, Sal-I *P. vivax* infects Squirrel monkeys without *PvDBP*1 binding to Squirrel monkey erythrocytes [23]. These findings suggested that there are other Duffy-independent pathways that enable erythrocyte invasion. For example, EBP/DBP2 region II has shown to bind to Duffy-positive and Duffy-negative human erythrocytes at low frequency [22,24]. CD71 (Transferrin Receptor 1, TfR1) has been shown to bind readily to the reticulocyte binding proteins (PvRBP2b) based on *in vitro* experiments [26,41]. Given reticulocytes constitute only a small fraction of all red blood cells, invasion via this RBP2b-TfR1 pathway may result in only a small number of infected erythrocytes and this may explain the considerably low parasitemia observed in Duffy-negative *P. vivax* infections (**Figure 3**). Further, recent transcriptomic study has also indicated that genes belonging to tryptophan-rich antigen and merozoite surface protein families were highly expressed in the *Saimiri*-infected *P. vivax*, of which erythrocytes did not bind to DBP1 from the Belem isolate of *P. vivax* [23]. There is growing evidence that members of the tryptophan-rich

antigen gene family are involved in erythrocyte invasion [42]. Various other invasion ligands may also mediate the recognition and invasion to reticulocytes, providing a potential mechanism for variations in reticulocyte preference [43,44]. Successful schizont development has been shown to be associated with increased younger reticulocytes in the Indian *P. vivax* isolates [45]. The low prevalence of schizonts in peripheral blood has led to the hypothesis that *P. vivax* could be sequestering in reticulocyte-rich zones such as the bone marrow [46], resulting in lower detectable parasitemia. Future studies should clarify the expression and role of various *P. vivax* ligand proteins and their respective receptors in Duffy-negative erythrocyte invasion.

Genetic relationships and origin hypotheses of *P. vivax* in Duffy-negative Africans

Maximum likelihood analyses of the African *P. vivax* isolates based on *PvDBP* indicated that *P. vivax* from Duffy-negative individuals were not monophyletic but found in multiple well-supported clades (clades I-III in **Figure 4**). These clades did not show clear geographical boundary but a mixture of *P. vivax* from different African countries. For instance, Duffy-negative *P. vivax* from Botswana, Ethiopia, and Sudan were closely related to Duffy-positive *P. vivax* from the same area, as well as to *P. vivax* from neighboring Uganda (clade II; bootstrap 91%). The Duffy-negative *P. vivax* were clustered together with the Duffy-positive ones without genetic distinction. The present data may imply that Duffy-negative and Duffy-positive individuals shared similar *P. vivax* strains possibly by the same ancestral origin or through recent transmission. The evolution of *PvDBP* region II could be also driven by functional selection rather than by geographical isolation. Interestingly, Duffy-negative *P. vivax* samples from Ethiopia and Sudan showed a higher nucleotide and haplotype diversity than the Duffy-positive ones, despite a smaller sample size (**Table 3**). Among all geographical isolates, *P. vivax* from Uganda and Madagascar had the highest level of genetic variation, though Duffy status of these samples are unclear (**Table 3**). These findings offered a hypothesis on the origin of Duffy-negative *P. vivax*, but *PvDBP* could be biased by selection or has limited resolution. Extensive phylogenetic analyses using whole genome sequences of Duffy-negative *P. vivax* from West-Central, Southern, and East Africa, together with the existing data of the *P. vivax*-like isolates in African apes are needed to adjudicate these origin hypotheses.

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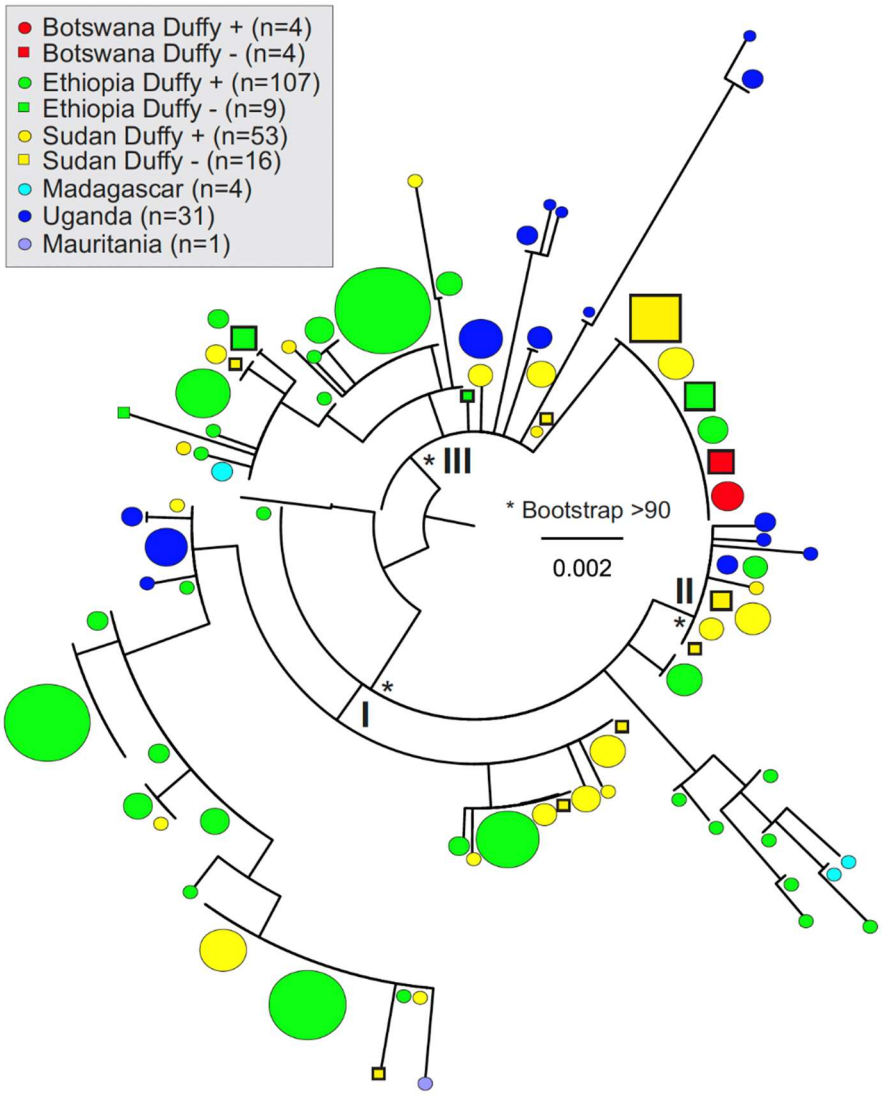

Figure 4. Phylogeny based on *PvDBP* sequences showing multiple source/origin of Duffy negative *P. vivax* in Africa. The reference *P. vivax* strain PVP01 isolated from an Indonesian patient was used as an outgroup. No clear differentiation was observed between the Duffy negative and Duffy positive *P. vivax* but nested within one another, suggestive of similar DBP haplotypes.

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Table 3 Nucleotide and haplotype diversity of *PvDBP* gene sequences between Duffy-negative and Duffy-positive samples from different African countries.

Region	Country	Duffy	Total samples	Number of polymorphic sites	Nucleotide diversity (SD)	Number of haplotypes	Haplotype diversity (SD)
Central Africa							
	Botswana	Duffy-positive	4	0	0	1	0
		Duffy-negative	4	0	0	1	0
East Africa							
	Ethiopia	Duffy-positive	107	9	1.51×10^{-3} (1.5×10^{-4})	11	0.762 (0.031)
		Duffy-negative	9	9	4.18×10^{-3} (1.0×10^{-4})	4	0.694 (0.147)
	Sudan	Duffy-positive	53	4	3.03×10^{-3} (2.5×10^{-4})	6	0.720 (0.039)
		Duffy-negative	16	17	5.59×10^{-3} (8.3×10^{-4})	8	0.758 (0.110)
	Uganda	-	31	28	6.51×10^{-3} (7.7×10^{-4})	17	0.933 (0.027)
	Madagascar	-	4	6	7.08×10^{-3} (2.0×10^{-4})	3	0.833 (0.222)

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Previous studies indicated that *P. vivax* in Southeast Asia and South America evolved in a clade of parasites that infect African monkeys [47]. *Plasmodium vivax* in African apes might present a substantial parasite reservoir from which Duffy-positive and Duffy-negative human infections arose from. There are two hypotheses concerning the origin of *P. vivax* in Duffy-negative Africans (**Figure 5**). The first hypothesis posits that the ancestral *P. vivax* infected all African primates including apes and Duffy-positive humans [48] (**Figure 5A**). One of these ancestral lineages evolved to a Duffy-independent pathway and subsequently spread to different parts of Africa via human migration [34,35] (**Figure 2**). The geographical overlap between apes and humans, e. g, in Cameroon and the Democratic Republic of Congo suggest a West-Central African origin of *P. vivax* in Duffy-negatives [48]. The second hypothesis posits that the ancestral *P. vivax* infected only non-human primates in Africa until some of the lineages crossed the species barrier and gave rise to the parasite population currently infecting Duffy-positive humans [49]. It is possible that Duffy-negative *P. vivax* observed today across Africa represent separate lineages that were derived multiple times independently from Duffy-positive individuals (**Figure 5B**). Previous phylogenies based on nuclear genes and partial mitochondrial genomes revealed incongruent genetic relationships [48,49], possibly due to incomplete lineage sorting or lack of phylogenetic signal [50]. Moreover, no African *P. vivax* isolates from Duffy-positive and Duffy-negative individuals were included. Future studies should employ genome-based phylogenetic approach and molecular dating analyses to clarify the origin of *P. vivax* in Africa.

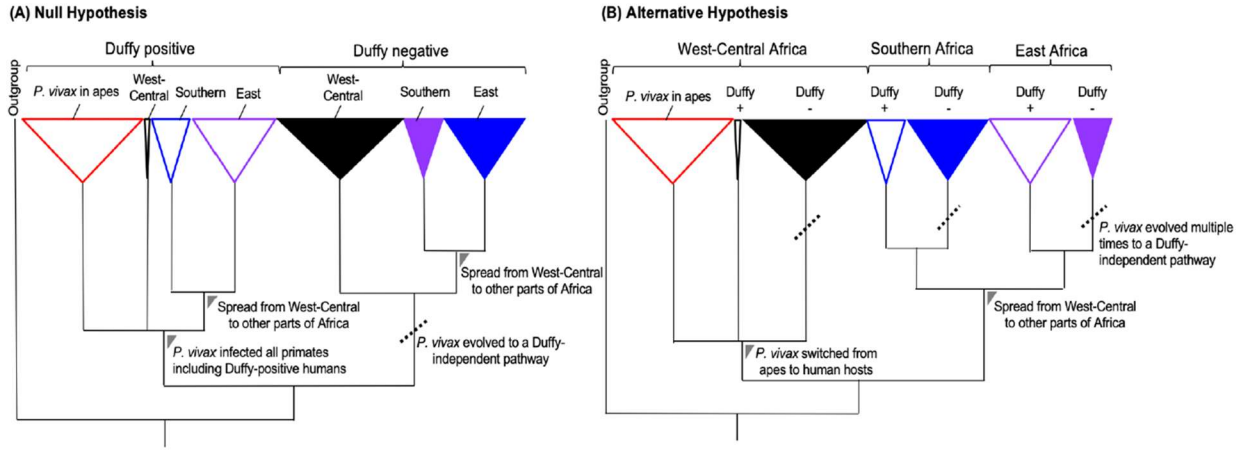

Figure 5. Hypothetical models illustrating the genetic origin of *P. vivax* in Duffy-negative Africans in a phylogenetic context.

Conclusions

With the increasing number of *P. vivax* cases reported in Duffy-negative individuals as well as across the continent, vivax malaria is no longer a rare but a growing and possibly widespread phenomenon in Africa. To the best of our knowledge, this paper is

the first using a standardized approach to characterize and compare the epidemiological and genetic features of Duffy-negative *P. vivax* from different parts of Africa. The generally low parasitemia observed in the Duffy-negative infections may suggest a less efficient but continuously evolving invasion mechanism that allows a greater negative public health impact in Africa in coming years. The genetic relatedness based on *PvDBP* sequences suggested similar strains shared between Duffy-negative and Duffy-positive populations, though the transmission capability of *P. vivax* in Duffy-negative individuals is still unclear. Further investigations are needed to unveil the invasion and transmission mechanisms of these infections. These data would help predict the scale of disease spread and improve existing malaria control measures, beyond *P. falciparum* in Africa. On the public health front-end, there should be more resources and training allocated to diagnosis and treatment of vivax malaria, given its unique ability in causing relapse and other longer-term health problems such as anemia in asymptomatic infections. Duffy-negative Africans are not resistant to *P. vivax* infection and the public health significance of vivax malaria in Africa should no longer be neglected.

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Biography

Dr. Lo is an Assistant Professor at University of North Carolina at Charlotte. Her lab focuses on evolutionary genomics of malaria parasites, host-parasite interactions, and molecular epidemiology of malaria in African countries.

Supplementary Files

Supplementary File 1. Detailed protocols of *P. vivax* screening, Duffy genotyping, and *PvDBP* sequencing.

Supplementary File 2. Genbank accession number and geographical location of *PvDBP* sequences included in the present study.

Supplementary Fie 3. Duffy genotype and parasite density based on quantitative PCR assay of Duffy negative *P. vivax* samples.

References

1. Miller LH, Mason SJ, Clyde DF, McGinniss MH. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy. *N Engl J Med* 1976;295:302-304.

2. Howes RRE, Patil APAAP, Piel FBF, Nyangiri OAOOA, Kabaria CWCCW, Gething PW, et al. The global distribution of the Duffy blood group. Nat Commun. 2011;2:266.

3. Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. *Nat Genet* 1995;10:224-228.

5. Zimmerman PA. *Plasmodium vivax* Infection in Duffy-Negative People in Africa. *Am J Trop Med Hyg* 2017;97:636-638.

6. Gunalan K, Niangaly A, Thera MA, Doumbo OK, Miller LH. *Plasmodium vivax* Infections of Duffy-negative erythrocytes: Historically undetected or a recent adaptation? *Trends Parasitol* 2018;34:420-429.

7. Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, et al. Duffy negative antigen is no longer a barrier to *Plasmodium vivax*--molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis* 2011;5:e1192.

8. Motshoge T, Ababio GK, Aleksenko L, Read J, Peloewetse E, Loeto M, et al. Molecular evidence of high rates of asymptomatic *P. vivax* infection and very low *P. falciparum* malaria in Botswana. *BMC Infect Dis* 2016;16:520.

9. Russo G, Faggiono G, Paganoti GM, Djeunang Dongho GB, Pomponi A, De Santis R, et al. Molecular evidence of *Plasmodium vivax* infection in Duffy negative symptomatic individuals from Dschang, West Cameroon. *Malar J* 2017;16:74.

10. Niangaly A, Gunalan K, Ouattara A, Coulibaly D, MS´a J, Adams M, Travassos MA, et al. *Plasmodium vivax* infections over 3 years in Duffy blood group negative Malians in Bandiagara, Mali. *Am J Trop Med Hyg* 2017;97:744-752.

11. Brazeau NF, Whitesell AN, Doctor SM, Keeler C, Mwandagalirwa MK, Tshefu AK, et al. *Plasmodium vivax* Infections in Duffy-negative individuals in the Democratic Republic of the Congo. *Am J Trop Med Hyg* 2018;99:1128-1133.

12. Poirier P, Doderer-Lang C, Atchade PS, Lemoine JP, de l'Isle MC, Abou-Bacar A, et al. The hide and seek of Plasmodium vivax in West Africa: report from a large-scale study in Beninese asymptomatic subjects. *Malar J* 2016;15:570.

13. Niang M, Sane R, Sow A, Sadio BD, Chy S, Legrand E, Faye O, et al. Asymptomatic *Plasmodium vivax* infections among Duffy-negative population in Kedougou, Senegal. *Trop Med Health* 2018;46:45.

14. Oboh MA, Singh US, Ndiaye D, Badiane AS, Ali NA, Bharti PK, Das A. Presence of additional *Plasmodium vivax* malaria in Duffy negative individuals from Southwestern Nigeria. *Malar J* 2020;19:229.

15. Twohig KA, Pfeffer DA, Baird JK, Price RN, Zimmerman PA, Hay SI, et al. Growing evidence of *Plasmodium vivax* across malaria endemic Africa. *PLoS Negl Trop Dis* 2019;13: e0007140.

16. Livingstone FB. The Duffy blood groups, vivax malaria, and malaria selection in human populations: a review. Hum Biol. 1984;56:413–25.

17. Battle KE, Lucas TCD, Nguyen M, Howes RE, Nandi AK, Twohig KA, et al. Mapping the global endemicity and clinical burden of Plasmodium vivax, 2000–17: a spatial and temporal modelling study. *Lancet* 2019;394:P332-343.

18. Baird JK. 8-Aminoquinoline therapy for latent malaria. *Clin Microbiol Rev* 2019;32:e00011-19.

19. Cowman A, Tonkin CJ, Tham WH, Duraisingh MT. The molecular basis of erythrocyte invasion by malaria parasites. *Cell Host Microbe* 2017;22:232-245.

20. Kumar H, Tolia NH. Getting in: The structural biology of malaria invasion. *PLoS Pathog* 2019; 15: e1007943.

21. Gunalan K, Gao X, Yap SS, Huang X, Preiser PR. The role of the reticulocyte-binding-like protein homologues of *Plasmodium* in erythrocyte sensing and invasion. *Cell Microbiol* 2013;15:35-44.

22. Gunalana K, Lo E, Hostetler JB, Yewhalaw D, Mu J, Neafsey DE, Yan G, Miller LH. Role of *Plasmodium vivax* Duffy-binding protein 1 in invasion of Duffy-null Africans. *Proc Natl Acad Sci USA* 2016;113:6271.

23. Gunalan K, Sá JM, Barros RRM, Anzick SL, Caleon RL, Mershon JP, et al. Transcriptome profiling of *Plasmodium vivax* in Samira monkeys identifies potential ligands for invasion. *Proc Natl Acad Sci USA* 2019;116: 7053–61.

24. Ntumngia FB *et al.*, A novel erythrocyte binding protein of *Plasmodium vivax* suggests an alternate invasion pathway into Duffy-positive reticulocytes. *MBio* 2016;7:e01261-01216.

25. Hester J, Chan ER, Menard D, Mercereau-Puijaion O, Barnwell J, Zimmerman PA, Serre D. De novo assembly of a field isolate genome reveals novel *Plasmodium vivax* erythrocyte invasion genes. *PLoS Negl Trop Dis* 2013;7:e2569.

26. Gruszczyk J, Kanjee U, Chan LJ, Menant S, Malleret B, *et al.* Transferrin receptor 1 is a reticulocyte-specific receptor for *Plasmodium vivax*. *Science* 2018;359:48-55.

27. Bereczky S, Martensson A, Gil JP, Farnert A. Short report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of Plasmodium falciparum. Am J Trop Med Hyg. 2005;72:249–51.

28. Lo EYY, Delenasaw Y, Zhong D, Zemene E, Degefa T, Tushune K, et al. Molecular epidemiology of *Plasmodium vivax* and *Plasmodium falciparum* malaria among Duffy-positive and Duffy-negative populations in Ethiopia. *Malar J* 2015;14:84-94.

29. Menard D, Barnadas C, Bouchier C, Henry-Halldin C, Gray LR, Ratsimbasoa A, et al. *Plasmodium vivax* clinical malaria is commonly observed in Duffy-negative Malagasy people. *Proc Natl Acad Sci USA* 2010;107:5967-5971.

30. Woldearegai TG, Kremsner PG, Kun JF, Mordmuller B. *Plasmodium vivax* malaria in Duffy-negative individuals from Ethiopia. *Trans R Soc Trop Med Hyg* 2013;107:328-31.

31. Pickrell JK, Patterson N, Loh PR, Lipson M, Berger B, Stoneking M, Pakendorf B, Reich D. Ancient west Eurasian ancestry in southern and eastern Africa. *Proc Natl Acad Sci USA* 2014;111:2632-7.

32. Hollfelder N, Schlebusch CM, Günther T, Babiker H, Hassan HY, Jakobsson M. Northeast African genomic variation shaped by the continuity of indigenous groups and Eurasian migrations. *PLoS Genet* 2017;13:e1006976.

33. Albsheer M, Pestana K, Ahmed S, Elfaki M, Gamil E, Ahmed SM, et al. Distribution of Duffy Phenotypes among *Plasmodium vivax* Infections in Sudan. *Genes* 2019;10:437.

34. Choudhury A, Aron S, Botigué LR, Sengupta D, Botha G, *et al.* High-depth African genomes inform human migration and health. *Nature* 2020;586:741–748.

35. Grollemunda R, Branforda S, Bostoenb K, Meadea A, Vendittia C, Page M. Bantu expansion shows that habitat alters the route and pace of human dispersals. *Proc Natl Acad Sci USA* 2015;112:13296-301.

36. Hamblin MT, Thompson EE, Di Rienzo A. Complex signatures of natural selection at the Duffy blood group locus. *Am J Hum Genet* 2002;70:369-383.

37. Petersen DC, Libiger O, Tindall EA, Hardie R-A, Hannick LI, Glashoff RH, et al. Complex patterns of genomic admixture within Southern Africa. *PLoS Genet* 2013;9:e1003309.

38. Busby GBJ, Band G, Le QS, Jallow M, Bougama E, Mangano VD, et al. Malaria genomic epidemiology network. Admixture into and within sub-Saharan Africa. *eLife* 2016;5:e15266.

39. Schuster SC, Miller W, Ratan A, Tomsho LP, et al. Complete Khoisan and Bantu genomes from southern Africa. *Nature* 2010;463:943–947.

40. Naing C, Whittaker MA, Nyunt Wai V, Mak JW. Is Plasmodium vivax Malaria a Severe Malaria? A Systematic Review and Meta-Analysis. *PLoS Negl Trop Dis* 2014;8:e3071.

41. Chan LJ, Dietrich MH, Nguitragool W, Tham WH. Plasmodium vivax Reticulocyte Binding Proteins for invasion into reticulocytes. *Cell Microbiol* 2020;22:e13110.

42. Zeeshan M, Tyagi RK, Tuagi K, Alam MS, Sharma YD. Host-parasite interaction: selective Pv-fam-a family proteins of *Plasmodium vivax* bind to a restricted number of human erythrocyte receptors. *J Infect Dis* 2015;211:1111-20.

43. Moreno-Pérez DA, Baquero LA, Chitiva-Ardila DM, Patarroyo MA. Characterising PvRBSA: an exclusive protein from *Plasmodium* species infecting reticulocytes. *Parasites Vectors* 2017;10:243.

44. Baquero LA, Moreno-Pérez DA, Garzón-Ospina D, Forero-Rodríguez J, Ortiz-Suárez HD, Patarroyo MA. PvGAMA reticulocyte binding activity: predicting conserved functional regions by natural selection analysis. *Parasites Vectors* 2017;10: 251.

45. Lim C, Pereira L, Saliba KS, Mascarenhas A, Maki JN, Chery L, Gomes E, et al. Reticulocyte preference and stage development of *Plasmodium vivax* isolates. *J Infect Dis* 2016;214:1081–1084.

46. Mayor A, Alano P. Bone marrow reticulocytes: a Plasmodium vivax affair? *Blood* 2015;125:1203-1205.

47. Loy DE, Plenderleith LJ, Sundararaman SA, Liu W, Gruszczyk J, Chen YJ, et al. Evolutionary history of human *Plasmodium vivax* revealed by genome-wide analyses of related ape parasites. *Proc Natl Acad Sci USA* 2018;115:E8450-8459.

48. Liu W, Li Y, Shaw KS, Learn GH, Plenderleith LJ, Malenke JA, Sundararaman SA, et al. African origin of the malaria parasite *Plasmodium vivax*. *Nat Commun* 2014;5:3346.

49. Prugnolle F, Rougeron V, Becquarta P, Berryd A, Makangab B, Raholaa N, et al. Diversity, host switching and evolution of *Plasmodium vivax* infecting African great apes. *Proc Natl Acad Sci USA* 2013;110:8123-8128.

50. Maddison WP, Knowles LL. Inferring phylogeny despite incomplete lineage sorting. *Syst Biol* 2006;55:21-30.