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Contrasting epidemiology and genetic variation of *Plasmodium vivax* infecting Duffy-negative individuals across Africa

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ABSTRACT

Objectives: Plasmodium vivax malaria was thought to be rare in Africans who lack the Duffy blood group antigen expression. However, recent studies indicate that *P. vivax* can infect Duffy-negative individuals and has spread into areas of high Duffy negativity across Africa. Our study compared epidemiological and genetic features of *P. vivax* between African regions.
Methods: A standardized approach was used to identify and quantify *P. vivax* from Botswana, Ethiopia, and Sudan, where Duffy-positive and Duffy-negative individuals coexist. The study involved sequencing the Duffy binding protein (DBP) gene and inferring genetic relationships among *P. vivax* populations across Africa.
Results: Among 1215 febrile patients, the proportions of Duffy negativity ranged from 20–36% in East Africa to 84% in southern Africa. Average *P. vivax* prevalence among Duffy-negative populations ranged from 9.2% in Sudan to 86% in Botswana. Parasite density in Duffy-negative infections was significantly lower than in Duffy-positive infections. *P. vivax* in Duffy-negative populations were not monophyletic, with *P. vivax* in Duffy-negative and Duffy-positive populations sharing similar DBP haplotypes and occurring in multiple, well-supported clades.
Conclusions: Duffy-negative Africans are not resistant to *P. vivax*, and the public health significance of this should not be neglected. Our study highlights the need for a standardized approach and more resources/training directed towards the diagnosis of vivax malaria in Africa.

Introduction

Plasmodium vivax malaria was previously thought to be rare or absent in African populations who lack the Duffy blood group antigen expression (Miller et al., 1976; Howes et al., 2011). 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 (Tournamille et al. 1995; King et al., 2011). However, recent studies have reported several cases of *P. vivax* infection in Duffy-negative people in different parts of Africa (Zimmerman, 2017; Gunalan et al., 2018), including countries where Duffy negativity is predominant (Brazeau et al., 2018; Mendes et al., 2011; Motshoge et al., 2016; Niangaly et al., 2017; Russo et al., 2017) (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 (Niang et al.,

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Table 1
Summary of *P. vivax* infections with available Duffy blood group information in African countries, according to 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	1931	41/205 (20%)[2]	nPCR	205/1931 (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	2063	914/1878 (48.7%)	nPCR	285/2063 (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	3624[22]	N.A.	nPCR	179/3624 (5%)	169/179 (94.4%)	N.A.	Motshoge T, et al. BMC Inf Dis 2016;16:520
Cameroon	NA	Yes	485	*	nPCR	201/485 (41.4%)	8/201 (4%)[12]	8/8 (100%)*	Ngassa Mbenda HG and Das A. PLoS ONE 2014;9(8):e103262
Cameroon	2008–09	No	269	*	nPCR	87/267 (32.3%)	13/87 (14.9%)[13,14]	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	2009–13	Yes	263	NA	nPCR	164/263 (62.3%)	4/164 (2.4%)[24]	NA	Niang M, et al., Malar J, 2015;14:281
Senegal	2010–11	No	48 (×4)^[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	NA	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]	NA	nPCR	499/499 (100%)	4/499 (0.8%)[23]	NA	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; NA: not available; [§]conventional PCR of genes PvCOI and PvDBP.
[1]two *Pf-Pv* co-infections; [2]Duffy-Ag available only among *Plasmodium spp* pos; [3]33 *Pf-Pv* co-infections; [4]two *Pf-Pv* co-infections; [5]one *Pf-Pv* co-infection; [6]31 children, Duffy neg affected by malaria enrolled in a precedent study (anemia study); [7]only *Pv* pos analyzed (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*, five *Pf-Pm*, nine *Pv-Pm*, one *Pv-Po*, one *Pf-Pv-Pm*, one *Pf-Pv-Pm-Po*); [10]37 co-infections (25 *Pf-Pv*, nine *Pv-Pm*, one *Pv-Po*, one *Pf-Pv-Pm*, one *Pf-Pv-Pm-Po*); [11]nine *Pf-Pv* and one *Pf-Pv-Pm* co-infection; [12]two *Pf-Pv* co-infections; [13]three *Pf-Pv* and one *Pf-Pv-Pm* co-infection; [14]Duffy-Ag assessed among 228 participants (including all those infected); [15]two *Pf-Pv* co-infections; [16]four *Pf-Pv* co-infections; [17]four *Pf-Pv* co-infections; [18]48 school children followed over 2 years (four samples for each child, 192 total samples analyzed) [19]only *Plasmodium spp* pos sample analyzed; [20]four *Pf-Pv* co-infections; [21]all *Plasmodium* pos children aged 2 months–10 years; [22]all children aged 2–12 years; [23]all four were co-infections — three *Pf-Pv* and one *Pv-Pm*; [24]two *Pf-Pv* co-infections.

2018; Oboh et al., 2020; Poirier et al., 2016). These reports indicate that the endemic range of *P. vivax* has extended beyond East Africa and has spread into areas of very high Duffy negativity (Gunalan et al., 2018; Twohig et al., 2019). 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 with *P. falciparum*, *P. vivax* has a broader temperature tolerance and an earlier onset of gametocyte development, and can form dormant hypnozoites, causing relapse (Livingstone, 1984), enabling *P. vivax* to spread through the diverse African climate and outcompete *P. falciparum* (Battle et al., 2019). Primaquine and 8-aminoquinoline are antimalarials effective in clearing hypnozoites and preventing relapses, but they can promote hemolysis in subjects with G6PD deficiency (Baird, 2019). These factors make *P. vivax* malaria difficult to control and eliminate, highlighting the concern that these 'new' *P. vivax* strains

that infect Duffy-negative hosts could spread through much of Africa and result in substantial, negative public-health and economic impacts.

There is a major knowledge gap regarding *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 (Cowman et al. 2017; Kumar and Tolia, 2019). 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 (Gunalan et al., 2013; Kumar and Tolia, 2019). In *P. vivax*, only a single *P. vivax* ligand–receptor interaction has so far been studied in any detail — that involving *P. vivax* duffy binding protein (*PvDBP*1). A 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 (Gunalana et al., 2016). Salvador (Sal) I *P. vivax* infects squirrel monkeys without *PvDBP*1 binding to squirrel monkey erythrocytes (Gunalan et al., 2019). Furthermore, EBP/DBP2 region II, a paralog of *PvDBP*1, has been shown to bind to Duffy-positive and Duffy-negative human erythrocytes at low frequency (Gunalana et al., 2016; Ntumngia et al., 2016), despite being deleted in Sal-I *P. vivax* (Hester et al., 2013). Recently, reticulocyte-binding protein RBP2b of *P. vivax* has been shown to bind to a transferrin receptor in the reticulocytes (Gruszczyk et al., 2018). 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

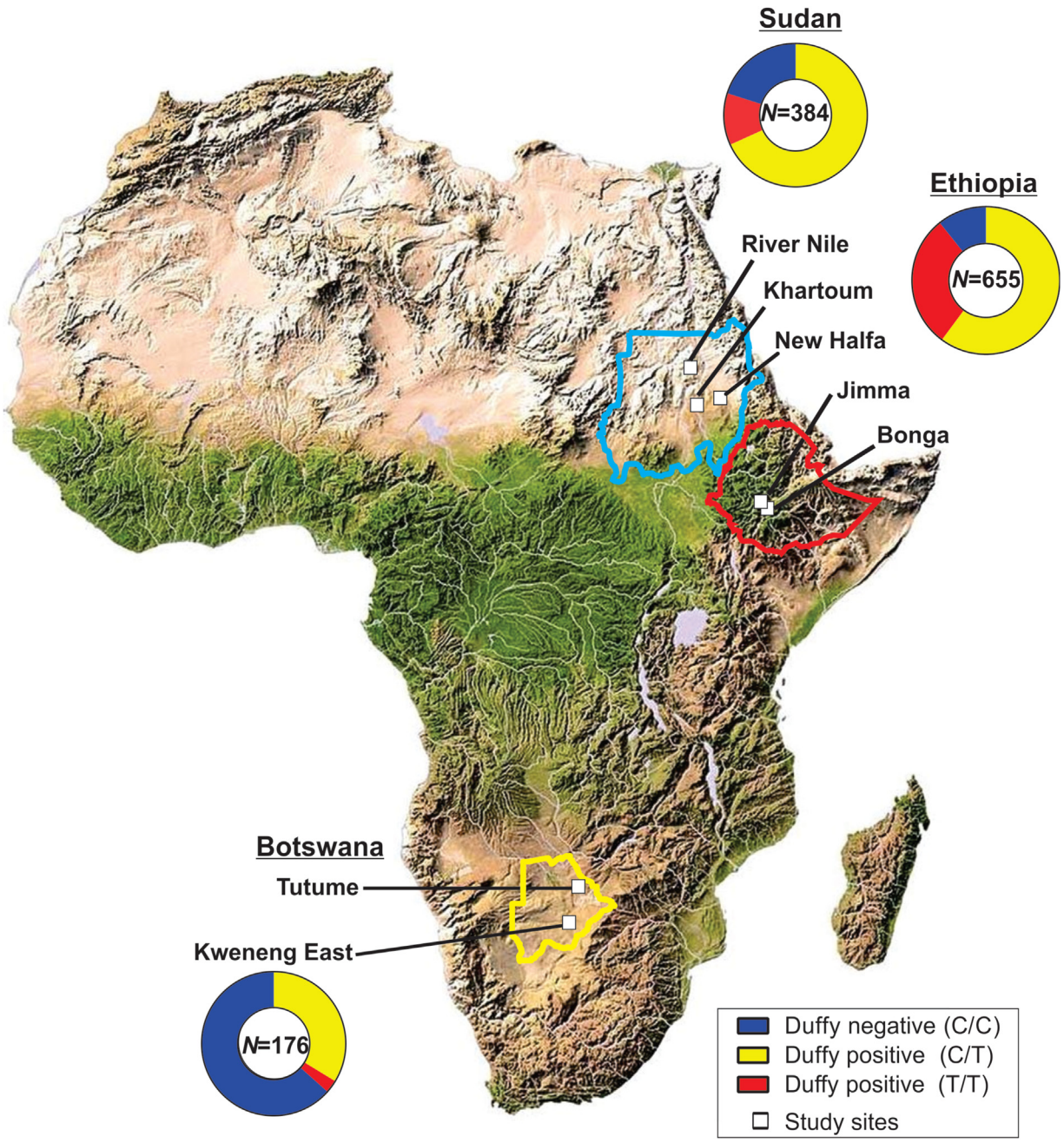

Figure 1. Map showing the distribution of study sites and the Duffy status of febrile patients included in the present study.

entomological and serological studies, community surveys, and clinical records (Gunalan et al., 2018; Twohig et al., 2019), the documentation of *P. vivax* infections 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 are no comprehensive genetic or epidemiological data for *P. vivax* in Africa available to National Malaria Programs or the World Health Organization for assessing impacts and guiding control strategies. Therefore, our study 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 gaps in our understanding of how widespread this phenomenon is, thus impacting malaria control strategies and highlighting the important effect of *P. vivax* as a cause of anemia.

Materials and methods

Study sites and sample collection

In total, 1215 febrile patients were collected from seven study sites in three countries: (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 from each participant were blotted on Whatman 3MM filter paper. Parasite DNA was extracted from dried blood spots using the Saponin/Chelex method (Bereczky et al., 2005). Eluted DNA was used for PCR diagnosis, quantification, and genotyping of malaria parasites.

Molecular screening of P. vivax

Parasite gene copy number was estimated using the SYBR Green detection method (Lo et al., 2015) 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: parasite density$_{sample}$ = $2^{E\times(40\text{-}Ctsample)}$. The differences in the log-transformed parasite densities between samples among the study sites were assessed for significance by one-tailed *t*-tests.

Duffy blood group genotyping

For all febrile patients, the qPCR-based TaqMan assay was first employed 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 1100-bp fragment of the *DARC* gene was further amplified using published primers (Menard et al., 2010). PCR products were sequenced to confirm the *Fy* genotypes.

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

The *PvDBP* sequences of four Duffy-positive and four Duffy-negative *P. vivax* samples were obtained from Botswana, 107 Duffy-positive and nine Duffy-negative *P. vivax* samples from Ethiopia, and 53 Duffy-positive and 16 Duffy-negative *P. vivax* samples from Sudan (Genbank accession number: MZ062224-MZ062409). 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). The Duffy statuses 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). The nucleotide and haplotype diversity of *PvDBP* sequences in Duffy-negative and Duffy-positive samples was further examined using DnaSP v6.12.03.

Results and discussion

Contrasting proportions of Duffy-negative patients and P. vivax *prevalence*

Duffy genotyping shows different proportions of Duffy-negative individuals among febrile patients in Botswana, Ethiopia, and Sudan (Figure 1). In Botswana, the proportion of Duffy-negatives was 83.5% (147/176) among febrile patients (Figure 1). In Kweneng East, our qPCR analyses indicated that 3% (nine out of 301) of the febrile patients were *P. vivax* positive. Among these, eight were Duffy-negative (C/C) and one was Duffy-positive (T/C) (Table 2; Supplementary File 3). In

Table 2
Comparison of *P. vivax* infection rates 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%)

Tutume, 6.8% (12/176) of the febrile patients were detected with *P. vivax*, and 10 of these were Duffy-negative.

Vivax malaria was first reported in asymptomatic children in a survey during the 2012–2013 transmission season (Motshoge et al., 2016). The average rate of asymptomatic *P. vivax* cases was 4.7%, but with large variation among districts. Compared with 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 (Motshoge et al., 2016).

In Ethiopia, the proportion of Duffy-negatives was 35.9% (235/655) among febrile patients (Figure 1), which was similar to our earlier finding in Asendabo indicating that 35.1% (137/390) of the general population was Duffy-negative (Lo et al., 2015). Among the 358 febrile patient samples collected in Jimma, 36% (129/358) were Duffy-negatives (Figure 1) and 37.4% (134/358) were detected with *P. vivax* (Table 2). Around 11.9% (16/134) of the confirmed *P. vivax* infections were in Duffy-negatives. In Bonga, 30.3% (125/413) of the febrile patients were detected with *P. vivax* and 3.2% (4/125) of these were from Duffy-negatives (Table 2). For these 20 Duffy-negative *P. vivax* infections, microscopy and PCR (nested and quantitative) indicated that 16 were single infections and four were mixed with *P. falciparum*.

Vivax malaria is a significant problem in Ethiopia (Lo et al., 2015; Woldearegai et al., 2013). Our previous study showed the asymptomatic prevalence of *P. vivax* to be 5.9% (23/390) in Asendabo, with Duffy-negatives accounting for 8.7% (2/23) of the *P. vivax* infections (Lo et al., 2015). A lower proportion of Duffy-negativity in febrile patients and in the general population in Ethiopia, as compared with Botswana, is consistent with the ethnic diversity and complex admixture history in East Africa (Hollfelder et al., 2017; Pickrell et al., 2014).

In Sudan, the proportion of Duffy-negatives 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 four of the 101 *P. vivax* samples were mixed with *P. falciparum*. The highest rate of *P. vivax* infection was observed in River Nile, where 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 were *P. vivax* positive and Duffy-negatives accounted for 14.3% (1/7) of these infections (Table 2). Across the country, there has been an increase in *P. vivax* detection and reports in recent years (Albsheer et al., 2019). Our findings indicated that the infection rate in Duffy-negative individuals varied among study sites.

Historical movement and genetic admixture explain the 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-based studies of African populations have refined earlier models of the continent's history and its impact on the genetic diversity of its inhabitants (Choudhury et al., 2020). Our data, showing a Duffy-negative rate of 83.5% among febrile patients in Botswana (Figure 1), are consistent with the Bantu expansion and admixture theories (Choudhury et al., 2020; Grollemunda et al., 2015). The Bantu expansion and population admixture were two major historical events that shaped 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 (Grollemunda et al., 2015). A component of Bantu ancestry (probably Duffy negative) has been found in the southern African Khoisan, who originated from mostly Duffy-positive ancestors (Hamblin et al., 2002; Petersen et al., 2013). The Duffy-negative allele from the Bantu of West/Central Africa may have reached the south of the continent within the last 750 years and mixed with the indigenous Khoisan, resulting in a variable Khoisan ancestry (Busby et al., 2016; Schuster et al., 2010).

While the direction of the Bantu expansion is still under debate, there is evidence that the Bantu migrated towards East Africa, where other ethnic groups such as the Cushitic and Nilotic dominated, potentially around 2000 years ago (Pickrell et al., 2014). Our data, showing a Duffy-negative rate of 20–36% in southwestern Ethiopia and eastern Sudan (Figure 1), are consistent with the complex admixture history. The Ethiopian and Sudanese population, with an admixture of several Eurasian ancestries and some Nilotic and Semitic-Cushitic components, migrated south after the Bantu expansion 2000–5000 thousand years ago (Hollfelder et al., 2017; Pickrell et al., 2014). Many population groups in Sudan are dominated by Nilotic and Eurasian admixtures, with a minimal West African component. One such exception is the Afro-Asiatic speaking Hausa population in the Middle-Eastern Sudan, who migrated from West Africa within the past 300 years (Hollfelder et al., 2017). These migrations could have spread *P. vivax* from West/Central to other parts of Africa.

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

In Botswana, Ethiopia, and Sudan, Duffy-positive and Duffy-negative individuals coexist. *P. vivax* parasite density in Duffy-negative infected individuals was found to be significantly lower than in the Duffy-positive infected individuals, regardless of geographical differences (Figure 2). Duffy-positive individuals with heterozygous C/T and homozygous T/T were not significantly different in terms of parasitemia. Both genotypes showed significantly higher parasitemia than Duffy-negative C/C. The Duffy-negative *P. vivax* samples in Ethiopia and Sudan showed a greater range of parasitemia than those in Botswana. This may have been due to differences in sample size (Figure 2; Supplementary File 3). In very few cases the asexual parasites were detected by microscopy in Duffy-negative individuals. For example, among the

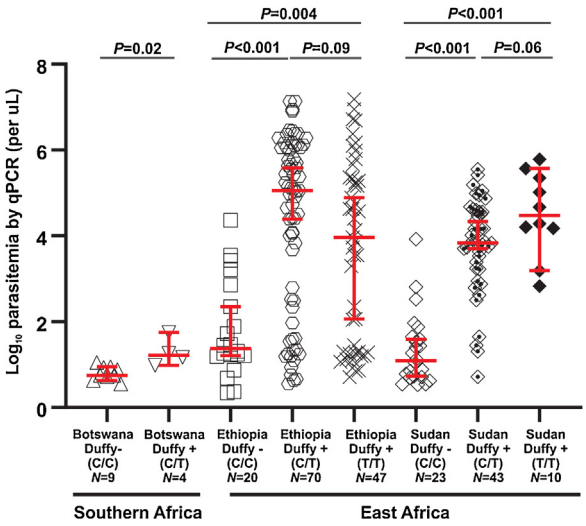

Figure 2. Comparison of *P. vivax* parasitemia levels, based on quantitative PCR assays, between Duffy-negative and Duffy-positive symptomatic infections among different geographical regions in Africa. Variations in parasitemia among samples are presented as boxplots, showing median and interquartile range values.

20 *P. vivax* infections identified in Duffy-negative patients from Ethiopia, only four were microscopic-positive and they all showed a relatively higher level of parasitemia compared with the submicroscopic infections. The Duffy-negative individuals who were infected with *P. vivax* were mostly submicroscopic 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 (Naing et al., 2014). Moreover, *P. vivax* can cause anemia during chronic, undetected infections (Niangaly et al., 2017). Other severe clinical manifestations include multi-organ dysfunction associated with anemia and thrombocytopenia, and spontaneous abortions, premature births, and low birth weights in pregnant women (Naing et al., 2014). 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.

Low parasitemia observed in Duffy-negative infections might suggest a low invasion capability of *P. vivax* in Duffy-negative individuals. A recent study showed 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 (Gunalana et al., 2016). Moreover, Sal-I *P. vivax* infects squirrel monkeys without *PvDBP*1 binding to squirrel monkey erythrocytes (Gunalan et al., 2019). These findings suggest that there are other Duffy-independent pathways that enable erythrocyte invasion. For example, EBP/DBP2 region II has been shown to bind to Duffy-positive and Duffy-negative human erythrocytes at low frequencies (Gunalana et al., 2016; Ntumngia et al., 2016). CD71 (transferrin receptor 1, TfR1) has been shown to bind readily to the reticulocyte-binding proteins (PvRBP2b) according to *in vitro* experiments (Chan et al., 2020; Gruszczyk et al., 2018). Given that 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, which may explain the considerably low parasitemia observed in Duffy-negative *P. vivax* infections (Figure 2). Furthermore, a recent transcriptomic study indicated that genes belonging to the tryptophan-rich antigen and merozoite surface protein families were highly expressed in the *Saimiri*-infected *P. vivax*, in which erythrocytes did not bind to DBP1 from the Belem isolate of *P. vivax* (Gunalan et al., 2019). There is growing evidence that members of the tryptophan-rich antigen gene family are involved in erythrocyte invasion (Zeeshan et al., 2015). Various other invasion ligands may also mediate the recognition and invasion of reticulocytes, providing a potential mechanism for variations in reticulocyte preference (Baquero et al., 2017; Moreno-Pérez et al., 2017). Successful schizont development has been shown to be associated with an increase in younger reticulocytes in Indian *P. vivax* isolates (Lim et al., 2016). 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 (Mayor and Alano, 2015), resulting in lower detectable parasitemia. Future studies should clarify the expression and roles 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 3). These clades did not show clear geographical boundaries, 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 the *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 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 levels of genetic variation, although the Duffy statuses of these samples were unclear (Table 3).

These findings offered a hypothesis on the origin of Duffy-negative *P. vivax*, but *PvDBP* could be biased by selection or have limited resolution. Extensive phylogenetic analyses using whole-genome sequences of Duffy-negative *P. vivax* from West/Central, southern, and East Africa, together with existing data on *P. vivax*-like isolates in African apes, are needed to adjudicate these origin hypotheses.

Previous studies have indicated that *P. vivax* in Southeast Asia and South America evolved in a clade of parasites that infected African monkeys (Loy et al., 2018). *P. vivax* in African apes might present a substantial parasite reservoir from which Duffy-positive and Duffy-negative human infections have arisen. There are two

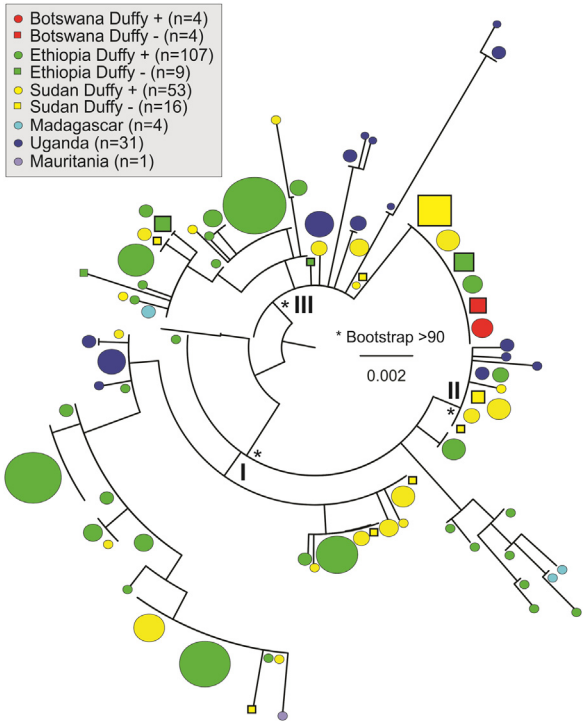

Figure 3. Phylogeny based on *PvDBP* sequences, showing multiple sources/origins of Duffy-negative *P. vivax* in Africa. The reference *P. vivax* strain PVP01, isolated from an Indonesian patient, was used as an outgroup. The size of the symbol indicates the sample size for each *PvDBP* haplotype. No clear differentiation was observed between the Duffy-negative and Duffy-positive *P. vivax*. Instead, they were nested within one another, suggesting similar DBP haplotypes.

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

Region	Country	Duffy status	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)

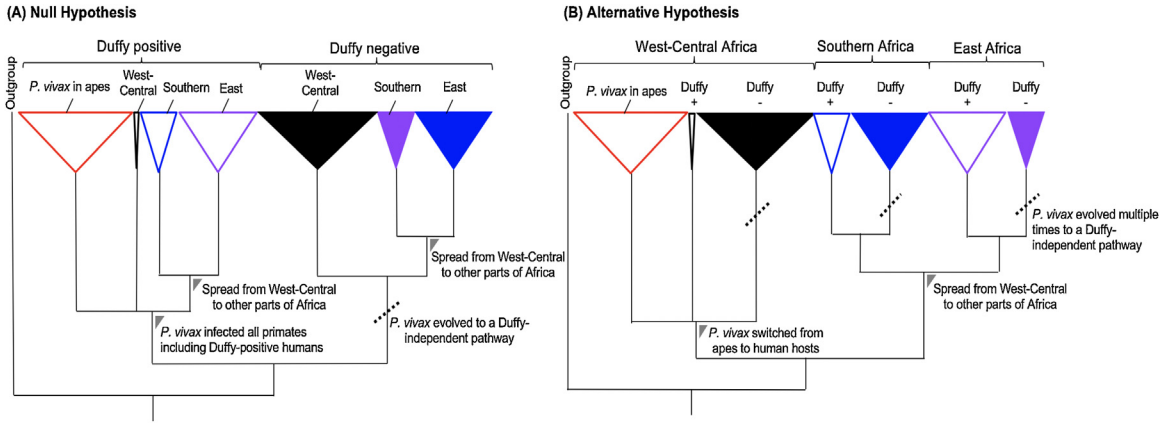

Figure 4. Hypothetical models illustrating the genetic origin of *P. vivax* in Duffy-negative Africans in a phylogenetic context. (A) The null hypothesis proposed that the ancestral *P. vivax* infected all African primates, including apes and Duffy-positive humans, forming a monophyletic clade. One of these ancestral lineages evolved to a Duffy-independent pathway (dotted line) and subsequently spread to different parts of Africa via human migration. (B) An alternative hypothesis proposed 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. 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 (dotted line), forming separate monophyletic clades.

hypotheses concerning the origin of *P. vivax* in Duffy-negative Africans (Figure 4). The first hypothesis proposes that the ancestral *P. vivax* infected all African primates including apes and Duffy-positive humans (Liu et al., 2014) (Figure 4A). One of these ancestral lineages evolved along a Duffy-independent pathway and subsequently spread to different parts of Africa via human migration (Choudhury et al., 2020; Grollemunda et al., 2015). The geographical overlap between apes and humans, for example in Cameroon and the Democratic Republic of Congo, suggests a West/Central African origin of *P. vivax* in Duffy-negatives (Liu et al., 2014). The second hypothesis proposes 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 (Prugnolle et al., 2013). It is possible that the Duffy-negative *P. vivax* observed today across Africa represent separate lineages that were derived multiple times, independently from Duffy-positive individuals (Figure 4B). Previous phylogenies based on nuclear genes and partial mitochondrial genomes have revealed incongruent genetic relationships (Liu et al., 2014; Prugnolle et al., 2013), possibly due to incomplete lineage sorting or a lack of phylogenetic signal (Maddison and Knowles, 2006). Moreover, no African *P. vivax* isolates from Duffy-positive or Duffy-negative individuals were included. Future studies should employ a genome-based phylogenetic approach and molecular dating analyses to clarify the origin of *P. vivax* in Africa.

Conclusions

With an increasing number of *P. vivax* cases reported in Duffy-negative individuals, as well as across the continent, vivax malaria is no longer a rare occurrence, but a growing and possibly widespread phenomenon in Africa. To the best of our knowledge, this study was the first to use 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 Duffy-negative infections may suggest a less efficient but continuously evolving invasion mechanism, leading to a more serious public health impact in Africa in coming years. The genetic relatedness based on *PvDBP* sequences suggest that similar strains are shared between Duffy-negative and Duffy-positive populations, although the transmission capability of *P. vivax* in Duffy-negative individuals remains 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. At the public health front-end, there should be more resources and training allocated to the diagnosis and treatment of vivax malaria, given its unique ability to cause 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.

Authors' contributions

EL, GR, MMAH, DY, and GMP conceived and designed the study. EL, GR, BR, GBDD, MMAH, DY, and GMP collected the samples. EL, KP, DK, KG, and GMP collected and analyzed the data. EL, GR, KG, LHM, and GMP wrote the paper. All authors read and approved the final manuscript.

Ethical approval

Scientific and ethical clearance was granted 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 the 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.

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Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2021.05.009.

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