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Genetic diversity and population structure of *Plasmodium falciparum* in Kenyan–Ugandan border areas

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Summary Kenya has, in the last decade, made tremendous progress in the fight against malaria. Nevertheless, continued surveillance of the genetic diversity and population structure of *Plasmodium falciparum* is required to refine malaria control and to adapt and improve elimination strategies. Twelve neutral microsatellite loci were genotyped in 201 *P. falciparum* isolates obtained from the Kenyan–Ugandan border (Busia) and from two inland malaria-endemic sites situated in western (Nyando) and coastal (Msambweni) Kenya. Analyses were done to assess the genetic diversity (allelic richness and expected heterozygosity, [H_e]), multilocus linkage disequilibrium (I_S^A) and population structure. A similarly high degree of genetic diversity was observed among the three parasite populations surveyed (mean $H_e = 0.76$; $P > 0.05$). Except in Msambweni, random association of microsatellite loci was observed, indicating high parasite out-breeding. Low to moderate genetic structure ($F_{ST} = 0.022–0.076$; $P < 0.0001$) was observed with only 5% variance in allele frequencies observed among the populations. This study shows that the genetic diversity of *P. falciparum* populations at the Kenyan–Ugandan border is comparable to the parasite populations from inland Kenya. In addition, high genetic diversity, panmixia and weak population structure in this study highlight the fitness of Kenyan *P. falciparum* populations to successfully withstand malaria control interventions.

keywords *Plasmodium falciparum*, microsatellites, genetic diversity, population structure, malaria, Kenya

Introduction

Malaria is a major public health problem accounting for about 219 million morbidities and 435 000 mortalities reported in 2017 globally [1]. Ninety per cent of the disease burden occurs in sub-Saharan Africa, in particular in children below 5 years. In Kenya, malaria is the second leading cause of morbidity and mortality [2]. The continuous fight against malaria has led to a remarkable reduction in disease incidence and fatalities in recent years [3–5]. These achievements are largely attributed to the improved malaria control policy, including widespread use of artemisinin-based combination therapies (ACTs) and the scale-up of mosquito vector control [3–5].

Malaria treatment guidelines have undergone numerous changes in the last three decades. In the late 1990s, sulfadoxine–pyrimethamine (SP) replaced chloroquine (CQ) as first-line treatment for uncomplicated *P. falciparum* malaria in Kenya, due to treatment failure and the advent of CQ resistance [6]. SP efficacy was short-lived, also due to the rapid development of drug resistance. Since 2006, artemether–lumefantrine (AL) has been the antimalarial drug of choice in Kenya [7, 8] and in other countries of sub-Saharan Africa. The changes in drug policy have elicited notable changes in *P. falciparum* population genetics. For instance, the frequency of non-synonymous mutations in *P. falciparum*'s kelch 13 gene has increased, even though these mutations are not associated with delayed parasite clearance [9], whereas the frequency of distinct single-nucleotide polymorphisms conferring resistance to CQ and SP has declined after the withdrawal of these drugs in 1999

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and 2006 respectively [10, 11]. Continued SP use in intermittent preventive treatment in pregnancy (IPTp) has recently been linked to the resurgence of SP resistance markers in Kenya [10, 11]. The shift from CQ and SP to AL in uncomplicated malaria treatment has increased the genetic diversity of the Kenyan *P. falciparum* population because of the reduced drug selection pressure [12].

Plasmodium falciparum genetic diversity was expected to decline with malaria transmission and disease prevalence reduction. However, this has not been the case despite the intensified deployment of vector control measures such as use of insecticide-treated nets (ITNs) and insecticide residual spraying (IRS). Analyses conducted 5 and 10 years after ITN introduction showed no change in the genetic diversity and population structure of *P. falciparum* populations from inland Kenya [13, 14]. Increasing asymptomatic malaria prevalence with higher gametocytaemias [15], mosquito vector resistance to pyrethroids [16], changing mosquito biting behaviour [17, 18] and high gene flow [19–21] are some of the prominent factors informing the resilience of *P. falciparum* genetic variability and population structure.

Plasmodium falciparum genetic factors are also dependent on transmission intensity. *P. falciparum* populations from low malaria transmission settings such as South America and Southeast Asia are characterised by low genetic diversity with strong linkage disequilibrium (LD) and defined structures of parasite populations [22]. In sub-Saharan high transmission regions, *P. falciparum* strains exhibit high genetic diversity, non-defined population structures and weak LD. Nevertheless, parasite inbreeding and subtle population fragmentation have been observed in a few high transmission areas despite the prevailing high genetic diversity, in particular in Senegal, Niger, Republic of Djibouti, Zimbabwe, Republic of Congo and Kenya [20, 22–24].

The level of genetic diversity and panmixia contributes to the fitness of *P. falciparum* to counter malaria control interventions such as candidate malaria vaccines [25] as well as the emergence and dispersal of antimalarial drug-resistant parasites. Insight into the genetic diversity and population structure of *P. falciparum* populations is vital to refine and effectively implement malaria control and elimination strategies. This study describes the genetic diversity and population structure of *P. falciparum* populations from a distinct region of the Kenyan–Ugandan border (Busia) in comparison to *P. falciparum* populations from two inland areas in Kenya, namely Msambweni in coastal Kenya and Nyando in western Kenya.

Methods

Ethical considerations

The study was approved by the Scientific & Ethics Review Unit (SERU) of Kenya Medical Research Institute (KEMRI) Nairobi (KEMRI/SERU/0152/3250 and SSC2276). Written informed consent was obtained from the parents/guardians of all children. All experiments were performed in accordance with good laboratory practice guidelines.

Study sites and study samples

The study was conducted in malaria-endemic areas situated in western (Busia and Nyando) and coastal (Msambweni) Kenya regions (Figure 1). Busia is located in Busia County and borders Uganda; Nyando is located in Kisumu County and Msambweni in Kwale county. According to the national population census of 2009, the population of Busia is 743 946 of Kisumu 968 909 and of Kwale 649 931 people [26]. Agriculture and fishing are the main economic activities of these counties owing to their proximity to Lake Victoria (Busia and Kisumu Counties) and the Indian Ocean (Kwale County).

Malaria transmission is perennial in the three study areas with peak incidences shortly after the rainy seasons (March to June and October to November). The prevalence of malaria in western Kenya ranges from 20% to 40% [27], whereas in the coastal region, it ranges between 5% and 20% [28]. Most of the malaria cases are due to *P. falciparum* infection. Co-infections of *P. falciparum* and other *Plasmodium* species (*P. malariae* and/or *P. ovale*) occur as well [29]. *P. vivax* is not present. Three *Anopheles* mosquito vectors transmit malaria in Kenya sympatrically; these include *A. gambiae sensu stricto*, *A. arabiensis* and *A. Funestus* [30]. In the coastal region, however, *A. merus* is an important secondary vector for malaria transmission [30].

Two hundred and one *P. falciparum* isolates obtained from febrile children visiting health facilities in 2013 (Msambweni), 2015 (Nyando) and 2016 (Busia) were analysed in this study (Figure 1). Patients from all study sites were recruited during the rainy season. Parasitaemias ranged from >2000 to 200 000 parasites/μl as assessed by microscopy. Genomic DNA was extracted from dried blood spots on filter papers, except for Busia (EDTA whole blood), using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. A PCR method targeting the *P. falciparum* 18S ribosomal RNA (rRNA) gene was

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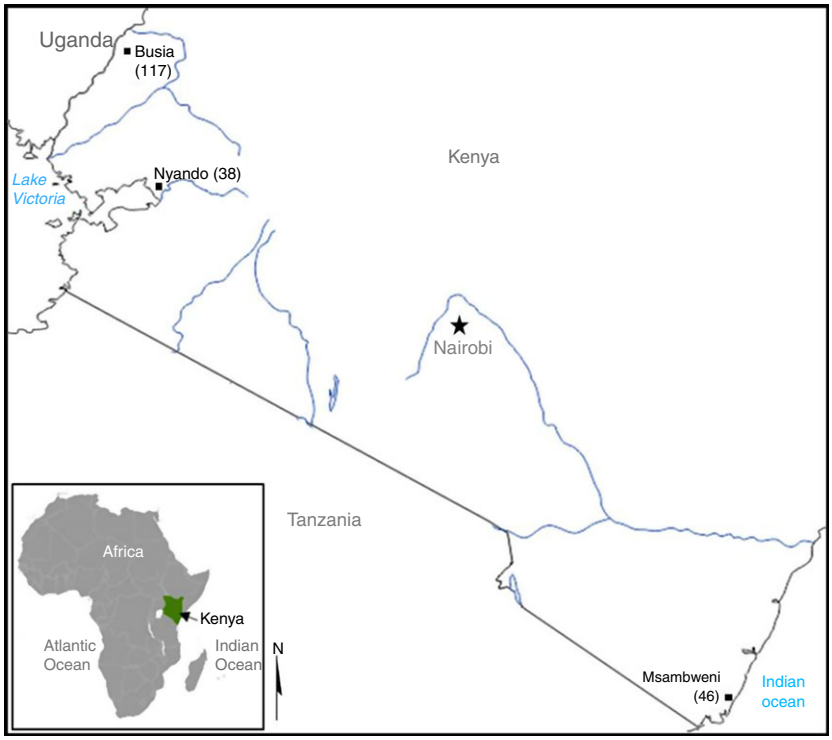

Figure 1 Map of Kenya showing study sites. The sample size of each study site is shown in parentheses. Black lines: country borders, blue lines: rivers, star: the capital city of Kenya. [Colour figure can be viewed at wileyonlinelibrary.com]

performed to confirm *P. falciparum* positivity as described previously [31].

Plasmodium falciparum microsatellite loci genotyping

Twelve neutral microsatellite (MS) loci were used to genotype *P. falciparum* isolates using a hemi-nested PCR method described previously [32, 33]. The MS loci included Poly-α (Chromosome, Chr, 4), TA81(Chr 5), TA42 (Chr 5), TA87 (Chr 6), TA1 (Chr 6), TA109 (Chr 6), TA40 (Chr 10), 2490 (Chr 10), ARA2 (Chr 11), PfG377 (Chr 12), PfPK2 (Chr 12) and TA60 (Chr 13). From each sample, FAM, HEX and PET-labelled PCR products were pooled together with GeneScan™ 500 Liz size standard (Applied Biosystems, Foster City, CA, USA) for capillary electrophoresis by the 3130xl Genetic Analyzer (Applied Biosystems) platform. The Peak Scanner™ software version 1.0 (Applied Biosystems) was used to visualise the electropherograms and automatically determine MS allele lengths relative to the GeneScan™ 500 Liz size standard. MS alleles per locus were scored if the electropherogram peak height was ≥ 200 relative fluorescent units (RFU). Where more than one peak was observed per MS locus, only the minor peak(s) with ≥ 30% height of the predominant allele peak was/were scored as additional allele(s). *P. falciparum* strain 3D7 genomic DNA and PCR grade water were used as a positive and negative controls, respectively, for each run.

Data analysis

As the asexual stages of *P. falciparum* are haploid, the number of multiple *P. falciparum* parasite strains in a given individual was determined by the number of alleles he/she carries among the investigated MS loci [32]. In brief, a single or monoclonal infection is defined by the observation of a single allele across all MS loci, whereas multiple or polyclonal infections are defined by the observation of > 1 allele in at least one MS locus. The MS locus with the highest number of alleles was used to determine the number of multiple infections of a sample. Kruskal–Wallis tests were applied to compare the mean number of infections between study sites. Chi-square tests were used to compare the proportion of multiple infections between study sites.

ARLEQUIN software version 3.5.2.2 (http://cmpg.unibe.ch/software/arlequin35/) was used to analyse the genetic diversity and population structure of Kenyan *P. falciparum* isolates using the predominant alleles. Genetic diversity was estimated by the allele frequencies, number of alleles per MS locus (A), allelic richness (A_R) and the expected heterozygosity (H_e). Since A is

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dependent on the sample size, A_R was calculated using the hierfstat package of the R software, based on the smallest sample size, to normalise data. H_e is the probability that any two MS locus alleles observed in a population are from different *P. falciparum* isolates. H_e was determined by using the formula $H_e = \frac{n}{n-1}\left(1 - \sum pi^2\right)$ where *pi* was the frequency of the different alleles of a given MS locus and *n* was the number of samples. The non-random association of MS loci was estimated by the multilocus linkage disequilibrium (LD). Multilocus LD was determined by the index of association (I_S^A) using LIAN software, http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query [34]. The paired Wright's fixation index (F_{ST}) and analyses of molecular variance (AMOVA) were used to assess the population structure of Kenyan *P. falciparum* isolates. The sum of genetic variability between parasite populations was classified and interpreted as follows: low differentiation, (F_{ST} value: 0–0.05), moderate differentiation (F_{ST} value: >0.05–0.15), great differentiation (F_{ST} value: >0.15–0.25) and very great differentiation (F_{ST} value: >0.25) [35]. Nei's genetic distance was used to assess the genetic relatedness among populations. Significant differences were observed at *P*-values < 0.05.

Results

Plasmodium falciparum genetic diversity

A total of 201 *P. falciparum* isolates were genotyped using 12 neutral microsatellite (MS) loci. High allelic polymorphism was observed with an overall mean of 10.14 ± 1.07 alleles (Table 1). The total number of different alleles circulating at the various study sites did not differ significantly. Furthermore, the difference between the total number of different alleles and the mean allelic richness did not reach statistical significance. This showed that the sample size difference among study sites did not influence the determination of allelic polymorphism. On average, the MS locus TA40 had the highest number of alleles (17.33), while loci PfG377 and 2490 were the least polymorphic ones.

The genetic diversity of Kenyan *P. falciparum* isolates was estimated by calculating the expected heterozygosity (H_e) based on the allele frequencies of the predominant alleles as described in the Methods section. The overall H_e ranged from 0.45 (TA42) to 0.90 (TA40) and the mean H_e was 0.76 (Table 1). The mean H_e of individual study sites was not statistically different. Poly-α had the highest H_e (0.92 in Busia) whereas 2490 and PfG377 in Busia, as well as TA42 in Nyando, had the lowest H_e (0.4). Figure 2a,b show the distribution and frequency of alleles of the 12 MS loci. A few alleles were observed to be predominant in at least one study site compared to the others. Since the blood stages of *P. falciparum* are haploid, we used the MS locus with the highest number of alleles per sample to determine the number of infections per sample and calculated the mean number of infections as well as the proportion of samples with polyclonal infections as described in the Methods section. The overall mean of the number of infections was 1.8 (95% CI: 1.6–1.8) and the overall proportion of polyclonal

Table 1 Genetic diversity of *Plasmodium falciparum* isolates from western (Busia and Nyando) and coastal (Msambweni) Kenya

	$A(A_R)$			H_e			Mean		
Loci	Busia	Nyando	Msambweni	Busia	Nyando	Msambweni	*A*	A_R	H_e
Poly-α	18 (18)	18 (13.35)	11 (10.39)	0.92	0.88	0.85	15.67	13.91	0.88
PfPK2	14 (10)	10 (10.22)	13 (12.22)	0.87	0.73	0.90	12.33	10.81	0.83
TA81	10 (10)	10 (8.04)	8 (7.62)	0.85	0.87	0.80	9.33	8.55	0.84
ARAII	11 (9)	9 (8.52)	8 (7.82)	0.81	0.82	0.86	9.33	8.45	0.83
TA87	13 (10)	10 (10.62)	10 (9.77)	0.88	0.87	0.88	11	10.13	0.87
TA40	23 (18)	18 (14.53)	11 (10.47)	0.89	0.91	0.89	17.33	14.33	0.90
TA42	7 (7)	7 (5.24)	7 (6.42)	0.47	0.41	0.47	7	6.22	0.45
2490	5 (6)	6 (3.54)	6 (5.77)	0.42	0.58	0.67	5.67	5.14	0.56
TA1	13 (13)	13 (10.28)	9 (8.79)	0.86	0.87	0.87	11.67	10.69	0.87
TA60	10 (6)	6 (8)	7 (6.65)	0.80	0.83	0.78	7.67	6.88	0.80
TA109	13 (7)	7 (8.32)	8 (7.6)	0.81	0.78	0.81	9.33	7.64	0.80
PfG377	6 (5)	5 (4.5)	5 (4.5)	0.43	0.57	0.54	5.33	4.77	0.51
Mean	12 (10)	10 (8.77)	9 (8.19)	0.75	0.76	0.78	10.14	8.96	0.76
SEM	1.5 (1.3)	1.27 (0.95)	0.67 (0.63)	0.056	0.045	0.041	1.07	0.90	0.046

A, number of alleles; A_R, allelic richness; H_e, unbiased expected heterozygosity; SEM, standard error of mean, *P*-value > 0.05.

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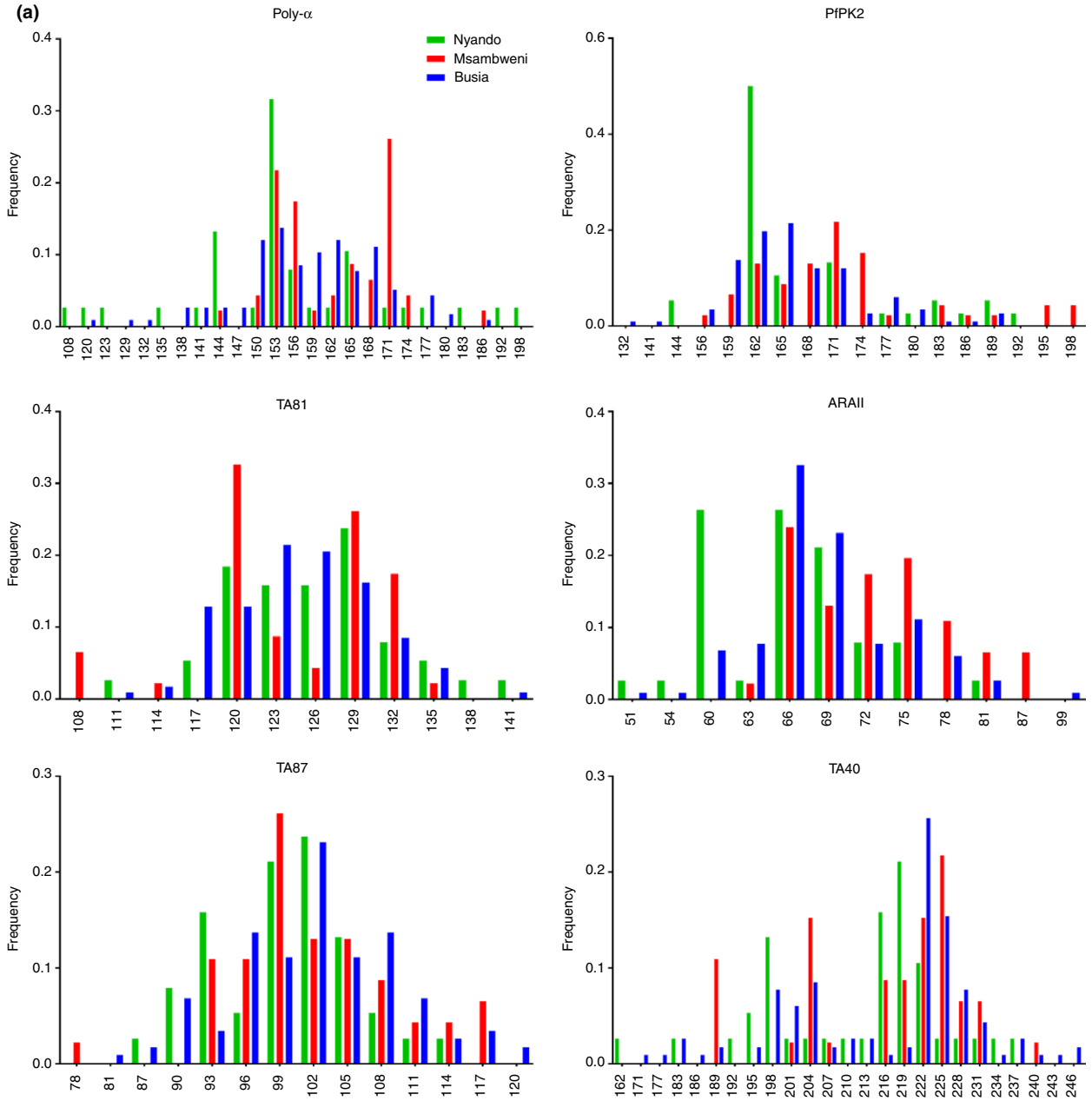

Figure 2 Allele frequency and size variability of *Plasmodium falciparum* neutral microsatellite loci. *X*-axis is the normalised allele size (base pairs) and *y*-axis is the standardised proportion per locus. [Colour figure can be viewed at wileyonlinelibrary.com]

infections was 51%. The mean of the number of infections and proportion of polyclonal infections was similar among the study sites ($P > 0.05$). The mean of the number of infections among the samples from Nyando, Busia and Msambweni was 1.6 (95% CI 1.3–1.9), 1.9 (95% CI 1.7–2.1) and 1.6 (95% CI 1.3–1.8) respectively. 45% of samples from Nyando, 56% from Busia and 43% from Msambweni had *P. falciparum* polyclonal infections.

Multilocus index of association analysis was performed to assess the non-random association of all MS loci in

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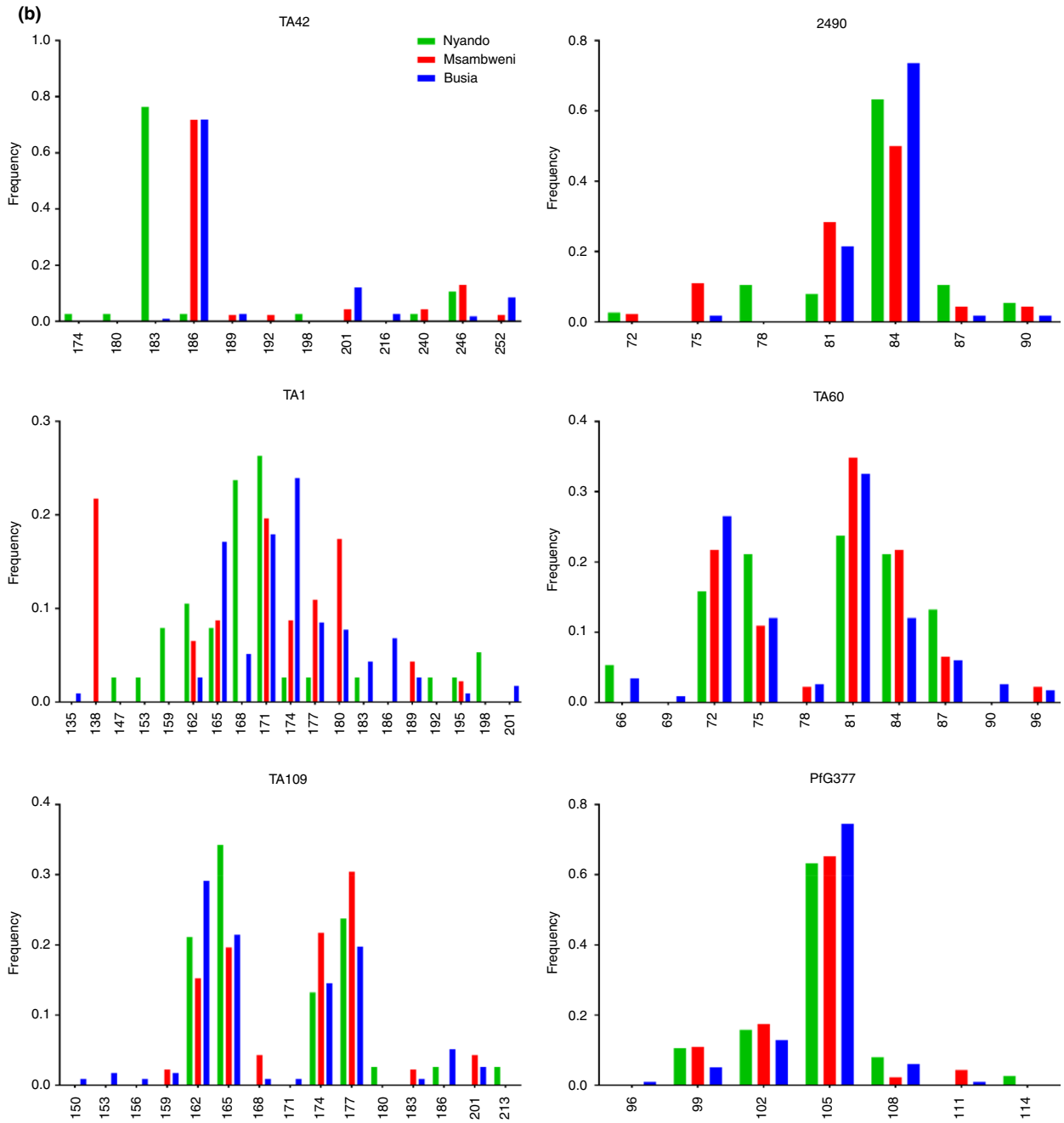

Figure 2 Continued

the individual study sites. Statistical significance of linkage disequilibria (LD) was tested using 10 000 Monte Carlo simulations. The LD ranged from 0 in Busia to 0.04 in Msambweni. Significant multilocus LD was only observed in Msambweni (Table 2). This resulted from the repeated occurrence of four MS haplotypes. Three MS haplotypes were shared by two isolates (4%) each, whereas the remaining haplotype was shared by three

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Table 2 Multilocus linkage disequilibrium in *Plasmodium falciparum* isolates from Kenya

Test factor	Busia	Nyando	Msambweni
V_D	1.90	2.05	2.64
V_E	1.84	1.91	1.86
I_S^A	0.00	0.01	0.04
P-value	0.19	0.196	0.0001

Statistical significance at *P*-value < 0.05. I_S^A, index of association calculated by Monte Carlo simulations at 10 000 per mutations under the null hypothesis $V_D = V_E$; V_D, observed variance; V_E, expected variance.

isolates (6%). There were no matching MS haplotypes in Busia and Nyando, indicating that these parasite populations are panmictic.

Genetic structure analyses performed by paired Wright's fixation index (F_{ST}) showed a significantly low genetic differentiation in Busia *vs*. Msambweni (F_{ST} = 0.022; $P < 0.0001$) and significant moderate genetic differentiation in Busia *vs*. Nyando (F_{ST} = 0.076; $P < 0.0001$) as well as in Nyando *vs*. Msambweni (F_{ST} = 0.079; $P < 0.0001$). This shows that there were differences in the frequency of alleles among the study sites. Analysis of molecular variance (AMOVA) also demonstrated significant genetic structuring of Kenya *P. falciparum* isolates (*P* = 0.01). Overall, 95% of the variance in allele frequencies observed was within populations, whereas 5% of the variance was observed across populations (Table 3). The highest allele frequency variance (8%) among paired study sites was observed in Busia *vs*. Nyando and Nyando *vs*. Msambweni, and the lowest allele frequency variance (2%) occurred in Busia *vs*. Msambweni (Table 4), indicating that the Kenyan *P. falciparum* populations studied are closely related. A short Nei's genetic distance and a high gene flow (number of migrants [Nm]) determined here support these findings. The Nei's genetic distance between Busia and Nyando was 0.294, 0.072 between Busia and Msambweni and 0.34 between Nyando and Msambweni. The overall Nm was 9.08 (Table 3). Interestingly, the Nm between Busia and Msambweni was 3.7-fold higher than between Busia and Nyando as well as between Nyando and Msambweni (Table 4).

Table 3 Analysis of molecular variance in Kenyan *Plasmodium falciparum* population (Busia, Nyando and Msambweni)

Source of variation	df	Sum of squares	Mean squares	Estimated variance	Percentage of variance (%)	Nm
Among Population	2	37.94	18.97	0.25	5	
Among individual	198	900.83	4.55	4.55	95	9.08
Within individual	201	0.00	0.00	0.00	0	
Total	401	938.77		4.80	100	

df, degrees of freedom; Nm, number of migrants; *P*-value = 0.01.

Table 4 Analysis of molecular variance in paired *Plasmodium falciparum* populations from Kenya

Populations	Source of variation	df	Sum of squares	Mean squares	Estimated variance	Percentage of variance (%)	Nm
Busia and Nyando	Among population	1	25.97	25.97	0.37	8	
	Among individual	153	691.15	4.52	4.52	92	6.04
	Within individual	155	0	0.00	0.00	0	
	Total	309	717.12		4.89	100	
Busia and Msambweni	Among population	1	11.26	11.26	0.10	2	
	Among individual	161	731.8	4.55	4.55	98	22.35
	Within individual	163	0	0	0	0	
	Total	325	743.06		4.65	100	
Nyando and Msambweni	Among population	1	21.03	21.03	0.39	8	
	Among individual	82	378.7	4.62	4.62	92	5.86
	Within individual	84	0	0.00	0	0	
	Total	167	399.73		5.01	100	

df, degrees of freedom; Nm, number of migrants; *P*-value = 0.01.

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Discussion

Malaria control strategies have changed tremendously over the last three decades with the sole aim of reducing the disease burden and safeguard gains, particularly in sub-Saharan Africa where 90% of the global disease burden occurs [30, 36]. The introduction and/or withdrawal of malaria interventions have shaped *P. falciparum* genetic factors. Insight into the genetic diversity and population structure of *P. falciparum* populations is vital for refinement of malaria control and elimination strategies. This study describes the genetic diversity and population structure of *P. falciparum* in the Kenyan–Ugandan border region (Busia, Busia County) in comparison to two inland malaria-endemic sites of Kenya, Nyando (western Kenya) and Msambweni (coastal Kenya).

Unexpectedly, we observed a lower proportion of polyclonal infections (51%) than previously reported in western Kenya. In 2018, Zhong *et al.* reported the prevalence of polyclonal infections to be 75% in western Kenya [37]. This was a slight reduction from the ≥ 80% prevalence reported by previous studies [13, 14, 19, 38]. Since the prevalence of polyclonal infections is proxies of malaria transmission, our findings suggest that malaria transmission intensity has decreased in line with the lower malaria prevalence in Kenya over the last three decades [4]. However, this trend has not been observed in the genetic diversity of the Kenyan *P. falciparum* population as exemplified in the present and in previous studies [13, 14, 19, 20, 38].

High *P. falciparum* genetic diversity has remained unchanged in Kenya even after the introduction of artemisinin-based combination therapy (ACT) and insecticide-treated nets (ITN) [12–14]. In fact, the parasite genetic diversity increased after ACTs were introduced [12]. Explanations for the persisting high genetic diversity are the increasing number of asymptomatic malaria cases with higher gametocytaemias, vector resistance against pyrethroids which sustain transmission and the removal of antimalarial drug selection pressure following the replacement of the less effective sulphadoxine–pyrimethamine in 2006 by the highly efficacious ACT [8, 12, 15, 16, 39, 40]. It is also worth noting that the *P. falciparum* genetic diversity reported here is similar to the genetic diversity of parasite populations from other countries in sub-Saharan Africa [22, 41], and it is higher than the genetic diversity of populations from low malaria-endemic settings in the Pacific Region, Southeast Asia and South America [22, 42–46].

Multilocus linkage disequilibrium (LD) analysis revealed that *P. falciparum* populations in the Kenyan–Ugandan border region and in Nyando are panmictic. This was demonstrated by the lack of a significant index of association (I_S^A). In contrast, a significant I_S^A was observed in the *P. falciparum* population from Msambweni with the repeated occurrence of four MS haplotypes. This indicates inbreeding in Msambweni and respective absence at the Kenyan–Ugandan border and in Nyando. The level of multilocus non-random association in this study is lower than previously reported in areas with intense transmission, including Kenya [13, 14, 20, 22, 38]. The recent increase (2011–2014) and subsequent decline (from 2015 on) of malaria prevalence are likely to have altered the frequency of minor alleles [4, 5], thus contributing to the lower LD observed here. This is demonstrated by significant multilocus LD in Asembo, western Kenya before deployment of ITNs in 1996, followed by non-significant and significant multilocus LDs in 2001 and 2007, respectively [13, 14]. The lower parasite prevalence in coastal (10–29%) and western (≥30%) Kenya offers an alternative explanation for the observed significant multilocus LD in Msambweni, which was not seen in Busia and Nyando [4]. Apparently, parasite inbreeding increases with a reduction transmission [22].

The distance from Busia to Nyando is 163 km, and from Busia to Msambweni, it is 987 km. However, our data show that the parasite populations from these areas are not isolated as previously described in Africa [22]. This becomes apparent by low to moderate genetic differentiation and Nei's genetic distance of parasite populations (Busia and Msambweni) separated by the longest geographical distance. These results are also supported by the low allele frequency variance (2%) in Busia *vs*. Msambweni compared to 8% in both Busia *vs*. Nyando and Nyando *vs*. Msambweni. In 2016, Ingasia *et al.* reported similar findings in Kenyan *P. falciparum* populations separated by a similar geographical distance (≈1000 km) as populations from Busia and Msambweni which have a low genetic differentiation (F_{ST} = 0.027; $P < 0.0001$) [20]. Busia is a socio-economically important gateway to Uganda, a landlocked country. It accounts for most of human travel between Kenya and Uganda. Therefore, parasite migration via humans is the likely reason for the limited genetic structure observed here [20, 47]. The high parasite migration (Nm > 3) in this study shows that there are limited barriers to hinder genetic flow, particularly between the Kenyan–Ugandan border region and Msambweni. Studies conducted in the islands of Lake Victoria and Vanuatu demonstrate human-mediated *P. falciparum* dispersal and gene flow [21, 48]. The existence of high *P. falciparum* effective population sizes in sub-Saharan Africa offers an additional explanation for the weak parasite population structure observed in this study [22].

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In conclusion, this study shows that the genetic diversity of *P. falciparum* populations in the Kenyan–Ugandan border area and inland Kenya is similar. The high panmixia and limited genetic population structure highlight the genetic fitness of Kenyan *P. falciparum* populations to counter the impact of current malaria control interventions. Therefore, continued surveillance of *P. falciparum* genetics and prompt refinement of malaria control and elimination strategies is needed to accelerate the realisation of a malaria-free Kenya.

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