Malaria Journal

RESEARCH

Open Access

Genetic diversity and population structure of *Plasmodium falciparum* across areas of varied malaria transmission intensities in Uganda

Alex Mwesigwa[1,2*], Stephen Tukwasibwe[3], Bryan Cummings[4], Hakiimu Kawalya[5], Shahiid Kiyaga[3,5,10], Stephen Okoboi[6], Barbara Castelnuovo[6], Everd Maniple Bikaitwoha[7], Joan N. Kalyango[1], Samuel L. Nsobya[3], Charles Karamagi[1], Pauline Byakika-Kibwika[8,9] and Joaniter I. Nankabirwa[1,3]

Abstract

Background Malaria remains a significant global health threat, with sub-Saharan Africa (SSA) bearing the highest burden of the disease. *Plasmodium falciparum* is the predominant species in the region, leading to substantial morbidity and mortality. Despite intensified control efforts over the last two decades, *P. falciparum* genetic diversity and multiplicity of infections (MOI) continue to pose significant challenges to malaria elimination in the region. This study assessed *P. falciparum* genetic diversity and population structure in areas with low, medium, and high malaria transmission intensities in Uganda.

Methods A total of 288 *P. falciparum*-positive samples from children (6 months to 10 years) and adults (≥ 18 years) living in Jinja (low transmission), Kanungu (medium transmission), and Tororo (high transmission) were genotyped using seven neutral microsatellite markers. Genetic diversity was assessed based on the number of alleles (N_a), allelic richness (Ar), and expected heterozygosity (H_e). Population structure was assessed using the fixation index, analysis of molecular variance (AMOVA), and clustering analysis.

Results High *P. falciparum* genetic diversity was observed across all study sites, with Kanungu exhibiting the highest mean H_e (0.81 ± 0.14), while Jinja and Tororo had lower mean H_e (0.78 ± 0.16). *P. falciparum* MOI varied significantly, with Tororo showing the highest mean MOI (2.5 ± 0.5) and 70% of samples exhibiting polyclonal infections, compared to Jinja's mean MOI of 1.9 ± 0.3 and 58% polyclonal infections. Significant multilocus linkage disequilibrium (LD) was noted ($p < 0.01$), ranging from 0.07 in Tororo to 0.14 in Jinja. Parasite population structure showed minimal genetic differentiation (F_{ST} ranged from 0.011 to 0.021) and a low AMOVA value (0.03), indicating high gene flow.

Conclusion This study demonstrates high *P. falciparum* genetic diversity and MOI but low population structure, suggesting significant parasite gene flow between study sites. This highlights the need for integrated malaria control strategies across areas with varying malaria transmission intensities in Uganda.

Keywords *Plasmodium falciparum*, Genetic diversity, Multiplicity of infection, Population structure and microsatellite markers

*Correspondence:
Alex Mwesigwa
mwesigwaalex@gmail.com
Full list of author information is available at the end of the article

Background

Despite the intensification of malaria control measures, malaria remains a significant health challenge, especially in sub-Saharan Africa (SSA). According to the World Malaria Report 2024, there were 263 million recorded malaria cases globally, with 246 million occurring in SSA in 2023 alone. Uganda accounted for 4.8% of global malaria cases and ranked as the third-highest contributor to malaria cases globally [1]. Over 90% of these cases are caused by *Plasmodium falciparum*, the species responsible for the highest levels of morbidity and mortality [2]. Furthermore, *P. falciparum* infections exhibit considerable genetic diversity, which contributes to anti-malarial drug resistance and complicates control and elimination efforts [3].

Plasmodium falciparum genetic diversity, which refers to variation within its populations, primarily arises from genetic recombination during sexual reproduction in the mosquito vector. This recombination leads to extensive allelic polymorphisms. Additionally, recombination events and epigenetic modifications contribute to antigenic variation during the asexual stages of the parasite within the human host [4–7]. Increased genetic recombination results in low linkage disequilibrium (LD), which refers to the non-random association of alleles at different loci within a population [8]. In *P. falciparum*, low LD is commonly observed due to frequent genetic recombination in the mosquito vector, allowing alleles at different loci to assort independently. This low LD facilitates the spread of beneficial traits, such as drug resistance, across various loci [3, 8, 9]. Therefore, monitoring LD is crucial for understanding the spread of drug resistance and the overall genetic diversity of the parasite—both of which are essential for developing effective malaria control strategies.

In malaria-endemic areas, individuals can experience repeated mosquito bites with different parasite strains, or a single mosquito bite may carry multiple strains, resulting in a condition known as multiplicity of infection (MOI) [10, 11]. *Plasmodium falciparum* MOI refers to the presence of multiple parasite strains within an individual [12]. This can occur through two mechanisms: superinfection, where a person is bitten by mosquitoes carrying different parasite strains, and co-transmission, where a single mosquito carries multiple strains. Both mechanisms lead to infection with more than one strain, though the transmission processes differ [13–15]. While genetic diversity can involve several distinct strains, it does not necessarily indicate that multiple strains are present within an individual at the same time.

The genetic diversity of *P. falciparum* strains complicates malaria control by increasing parasite virulence, disease severity, and contributing to drug resistance [16–18] Some strains may evolve greater resistance to anti-malarial drugs, making treatment more difficult [19–22]. Mutations in key genes, such as *pfcrt* (chloroquine resistance transporter), *pfmdr1* (multidrug resistance 1), and *pfk13* (kelch protein), drive anti-malarial drug resistance, which remains a major obstacle to malaria control and elimination in Uganda [23].

Malaria control strategies, including insecticide-treated bed nets (ITNs), indoor residual spraying (IRS), and chemotherapy, are essential components of malaria prevention [24, 25]. However, their effectiveness can be compromised by the development of insecticide resistance in mosquito populations [26]. Additionally, parasite genetic diversity and MOI, which are shaped by evolutionary processes such as genetic drift, mutation, natural selection, and migration, influence the effectiveness of these control measures and the spread of *P. falciparum* across different transmission settings [27, 28].

P. falciparum genetic diversity varies across individuals, transmission settings, and seasons, highlighting the need for continuous monitoring [29]. In areas with high malaria transmission, *P. falciparum* exhibits high genetic diversity and low genetic differentiation, while in low transmission areas, the parasites are less diverse [30–32]. Monitoring parasite genetic diversity and population structure is crucial for assessing malaria transmission dynamics, understanding connectivity between parasite populations, and evaluating the effectiveness of control interventions [28, 31, 33, 34]. This monitoring also helps in understanding malaria epidemiology and resistance patterns, which are vital for global malaria elimination strategies [12, 35, 36].

Although some studies have assessed the genetic diversity of *P. falciparum* in Uganda, most have focused on symptomatic children living in areas with moderate to high malaria transmission intensities [16, 37, 38]. No studies have examined the genetic diversity of *P. falciparum* across different malaria transmission settings (low, medium, and high) in Uganda, nor have any included asymptomatic individuals, who are also important reservoirs of malaria transmission. This gap highlights the need for a better understanding of how *P. falciparum* genetic diversity and population structure vary across these settings. This study aimed to assess the genetic diversity and population structure of *P. falciparum* in individuals from low, medium, and high malaria transmission areas. By comparing these settings, the study sought to understand how transmission intensity influences parasite gene flow and genetic diversity. These insights are crucial for implementing effective malaria control strategies and provide valuable

data to guide malaria elimination efforts in Uganda and other regions with similar malaria transmission patterns.

Methods

Study settings

The study utilized dried blood spot (DBS) samples collected between August 2011 and July 2017 from participants enrolled in cohort studies conducted in three sub-counties: Walukuba in Jinja District, Kihihi in Kanungu District, and Nagongera in Tororo District, under the Program for Resistance, Immunology, and Modelling of Malaria (PRISM) study. Walukuba is a relatively low-transmission, peri-urban area near Lake Victoria in the south-central part of the country. Kihihi is a rural area with moderate transmission intensity, bordering Bwindi Impenetrable National Park in the south-western part of the country. Nagongera is a rural area with high transmission intensity in the south-eastern part of the country, near the border with Kenya. Malaria transmission intensity in these regions was characterized

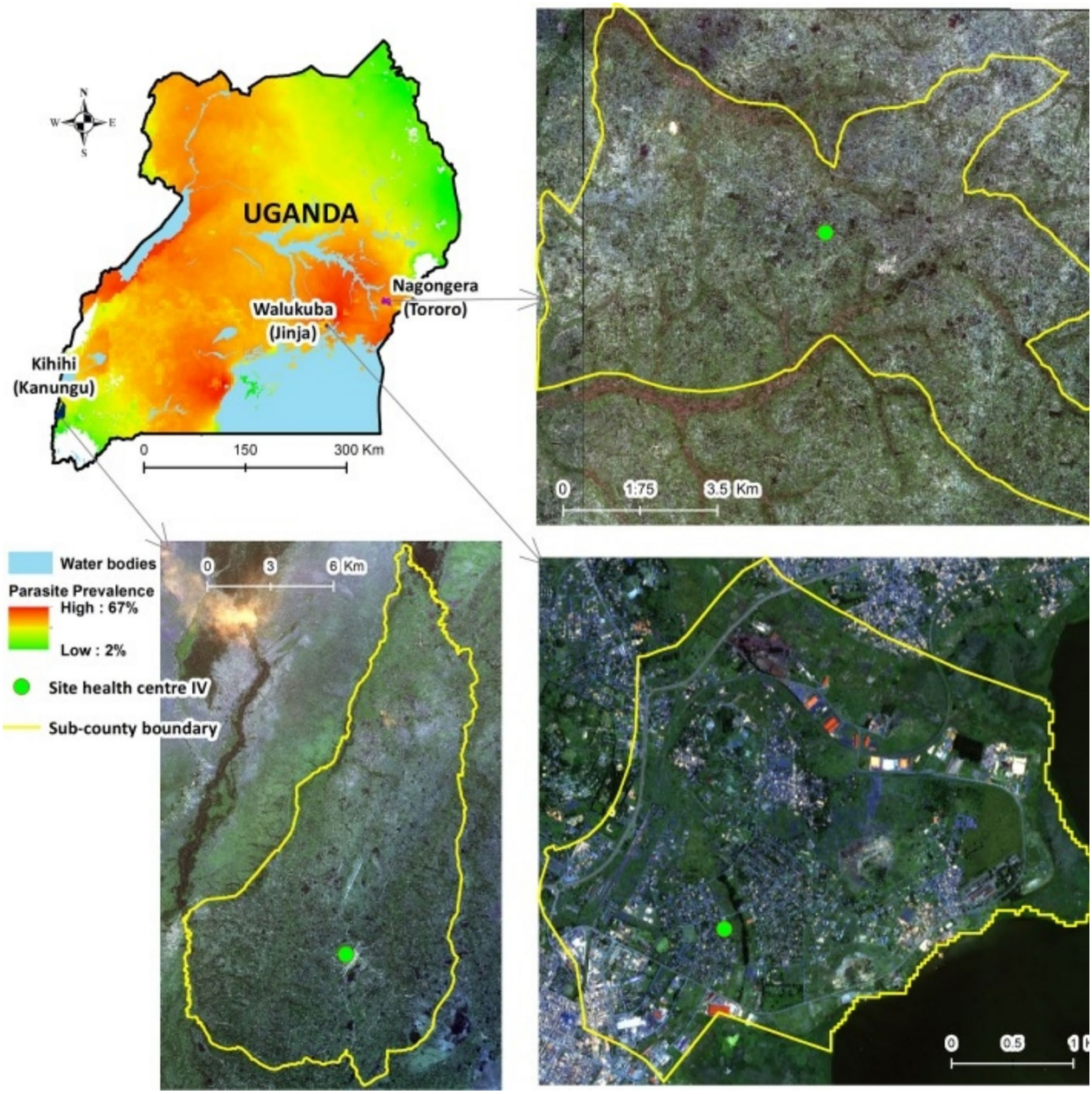

Fig. 1 Map of Uganda showing the malaria endemicity of the study sites at the time of sample collection. Adapted from Kamya et al., 2015 [40]

by the estimated entomological inoculation rates (EIR): 3.8 for Jinja (Walukuba), 26.6 for Kanungu (Kihihi), and 125 for Tororo (Nagongera) (Fig. 1) [39].

Study population

Details of the study population have been previously described [40]. Briefly, all households in each of the three sub-counties were enumerated and mapped, and 100 households were randomly selected to participate in the cohort. The selection of households was done irrespective of the presence of fever at the time of selection, to ensure that both symptomatic and asymptomatic individuals were included in the study. Within each selected household, both male and female children aged 0.5–10 years, as well as one primary adult caregiver (with no specific gender requirement), were enrolled in the study. The inclusion criteria were based on the household enumeration and mapping process, with no additional requirements beyond age for children and the selection of one primary caregiver per household. Study participants were encouraged to visit a dedicated study clinic open 7 days a week for all their medical care. Routine visits were conducted every 3 months and included a standardized evaluation and collection of blood by finger prick for thick blood smear, haemoglobin measurement, and DBS filter paper samples for molecular analysis. Study participants who had a fever (tympanic temperature > 38.0 °C) or a history of fever in the previous 24 h at the time of any clinic visit had a thick blood smear read immediately. If the thick blood smear was positive by light microscopy, the patient was diagnosed with malaria and managed according to national guidelines. The cohort was dynamic, with newly eligible children from participating households being enrolled, and participants who reached 11 years of age being excluded from further follow-up. Study subjects who missed their scheduled routine visits were visited at home and requested to come to the study clinic as soon as possible.

Sample size determination

To determine the required sample size, a formula for comparing two proportions was used:

$$n = \left[(Z\alpha/2 + Z\beta)^2 * \left(p_1(1-p_1)\right) + p_2(1-p_2)\right] / \left(p_1 - p_2\right)^2] = 95.7,$$

where Zα/2 is the critical value of the normal distribution at the 95% confidence level, which is 1.96; Zβ is the critical value of the normal distribution for a power of 80%, which is 0.84; $p_1 = 62\%$ is the moderate estimate of the proportion of multiple infections; and $p_2 = 80\%$ is the higher estimate of the proportion of multiple infections as per the studies from the East African region [7, 41]. Since there are three transmission settings, a total of $96 \times 3 = 288$ samples was required.

Laboratory methods

Determination of parasite density

Microscopy slides for parasite density and species detection were prepared using a 2% Giemsa solution and stained for 30 min, with both thick and thin blood films being prepared. Experienced microscopists examined the slides under a light microscope at 100 × oil immersion. *P. falciparum* parasite density was determined by counting asexual parasites against 200 leukocytes. The parasite density per µL of blood was calculated by multiplying the total parasite count by 40, assuming an average of 8000 leukocytes per µL of blood [42].

For quality control, each smear was independently read by two microscopists. Though discrepancies—defined as differences in species diagnosis, parasite density > 50%, or presence of parasites—were rare, any identified discrepancy prompted a review by a third trained and experienced microscopist. Final parasitaemia was determined by averaging the readings of the two microscopists or, in cases of disagreement, by averaging the third microscopist's reading with that of the closest of the initial two. In cases where the third microscopist's reading was significantly different, it was used as the final for determination of both parasite density, and species. To minimize discrepancies, all microscopists underwent thorough training on standardized techniques and parasite identification prior to the study, and regular assessments were conducted to ensure consistent performance. Slides were not blinded to the microscopists, but each microscopist independently read the slides without knowledge of the other's results.

Selection of the samples for molecular analysis

During the study period, a total of 1466 samples were collected from study participants: 495 from Walukuba (Jinja District), 396 from Kihihi (Kanungu District), and 575 from Nagongera (Tororo District). A total of 288 DBS filter paper samples, collected from *P. falciparum* infected individuals on the day of participant enrollment for parasite detection, were randomly selected for molecular analysis. These samples included 92 from Walukuba, 81 from Kihihi, and 115 from Nagongera. The samples were retrieved from the IDRC biorepository at the Central Public Health Laboratory in Butabika, Kampala, Uganda, where they had been stored at − 20 °C to maintain DNA integrity over time. To ensure that the samples were

representative of the broader study population, a stratified random sampling approach was employed. Selection criteria included *P. falciparum* mono-infection positivity and the availability of sufficient demographic and clinical data for each participant.

DNA extraction

P. falciparum genomic DNA was extracted from DBS using Chelex 100 Resin (Sigma-Aldrich, USA), following the method described by Musapa et al. [43]. The Chelex extraction method was chosen for its simplicity, cost-effectiveness, and efficiency in processing DBS samples. It effectively isolates *P. falciparum* DNA, reduces PCR inhibitors, and requires fewer reagents, offering a practical and reliable alternative to silica-based or column-based methods. Briefly, 6 mm discs were punched out from the DBS into 1.5 mL microcentrifuge tubes containing 1 mL of 1X phosphate-buffered saline (PBS) and incubated overnight at 4 °C. The punching machine was cleaned with DNase, and a clean blank piece of Whatman 3MM filter paper was pre-cut between samples to prevent cross-contamination. The discs were washed twice with 1 mL PBS and then boiled at 99 °C in 200 µL of 20% Chelex (Sigma-Aldrich, USA) in DNase/RNase-free water. After a final centrifugation step (14,000 × g for 1 min), the extracted DNA was transferred into a labelled 0.6 mL microcentrifuge tube with a 100 µL elution volume and then stored at − 20 °C until further use. *P. falciparum* detection and confirmation was performed through genotyping of *P. falciparum* 18S rRNA using nested PCR [44]. As an internal control, every eighth sample consisted of a blank filter paper sample that was cut, extracted, and processed alongside the field samples to identify any contamination that could lead to false positives.

Microsatellite genotyping

Microsatellite markers, including Poly-α, TA1, TA109, and PfPK2, exhibit a high level of variability and have been widely used to assess *P. falciparum* genetic diversity and population structure in malaria-endemic regions [9, 45]. *P. falciparum* genetic diversity and population structure were evaluated using a panel of seven neutral polymorphic microsatellites: TA1 (Chr6), Poly-α (Chr4), PfPK2 (Chr12), TA109 (Chr6), 2490 (Chr10), C2M34–313 (Chr2), and C3M69–383 (Chr3). These microsatellites were genotyped using primers labelled with HEX or 6-FAM (Additional file 1: Table S1). The microsatellites Poly-α, TA1, TA109, PfPK2, and 2490 were nested, while C2M34–313 and C3M69–383 were unnested.

For the nested PCR reactions, the primary reaction for each marker was carried out in a total volume of 15 µL, containing 10.5 µL of molecular-grade PCR water, 1.5 µL of 10 × reaction buffer, 0.3 µL of dNTPs (1.25 mM), 0.3 µL of Forward Primer (10 µM), 0.3 µL of Reverse Primer (10 µM), 0.25 µL of AmpliTaq Gold (5 U/µL), and 2 µL of DNA template. The Round 1 PCR conditions were as follows: 94 °C for 2 min, followed by 25 cycles of (94 °C for 30 s, 42 °C for 30 s, 40 °C for 30 s, 65 °C for 40 s), and ending with 65 °C for 2 min. The secondary reaction contained the same reagents as the primary reaction, with the addition of 0.3 µL of the labeled primer for each marker. A 2 µL aliquot of the primary reaction product was used in a final volume of 15 µL for the nested PCR reactions. The Round 2 PCR conditions were: 94 °C for 2 min, followed by 25 cycles of (94 °C for 20 s, 45 °C for 20 s, 65 °C for 30 s), and ending with 65 °C for 2 min.

PCR conditions for the C2M34–313 and C3M69–383 reactions were as follows: 94 °C for 2 min, followed by 5 cycles of (94 °C for 30 s, 50 °C for 30 s, 60 °C for 30 s), then 40 cycles of (94 °C for 30 s, 45 °C for 30 s, 60 °C for 30 s), and ending with 60 °C for 2 min. A 2 µL sample of the PCR product was then run on a 2% agarose gel to confirm amplification before being analysed on the sequencer. The amplified PCR products were transferred to safe-lock DNA amplicon storage tubes, securely wrapped in aluminum foil, and sent to Inqaba Biotec in South Africa for microsatellite fragment analysis using an ABI capillary electrophoresis platform.

Microsatellite analysis

Microsatellite fluorescent-labelled PCR products were analysed to determine their fragment lengths using the ABI 3730xl (ThermoFisher/Hitachi) genetic analyzer. Peak scoring was performed with GeneMarker HID V2.9.5 software. For samples with multiple peaks, the highest peak was defined as the dominant allele, while peaks with a height greater than 200 relative fluorescence units (RFU) and more than 20% of the dominant peak height were considered minor alleles. To ensure the quality and reliability of the data, the raw electropherogram data in.fsa files, generated by the ABI 3730xl, were first analyzed by the Inqaba Biotec team. A thorough review and revalidation of the data were then conducted using the.fsa files to ensure its accuracy before proceeding with the final analysis.

Data analysis

Participants' demographic and clinical data, including age, gender, parasite density, and haemoglobin levels, were extracted from the primary PRISM cohort database and exported to STATA version 17 (Stata Corp., College Station, TX, USA) for analysis. Microsatellite data were retrieved from the ABI 3730xl Genetic Analyzer. Subsequent genetic analysis was conducted only on samples

where at least five microsatellite markers were successfully amplified, using only the predominant alleles to minimize bias associated with analysing samples with multiple infections.

Plasmodium falciparum genetic diversity

P. falciparum genetic diversity, defined as variation in genetic composition [46] resulting from genetic recombination or mutation [47], was assessed in each parasite population from each study site by calculating the mean number of alleles (N_a), allele richness (Ar), allele frequency, the number of effective alleles (N_e) and expected heterozygosity (H_e) across each locus. These metrics were calculated from the predominant allele dataset using GENALEX 6.5 software [48]. The H_e, defined as the probability that two randomly selected clones from a population will carry distinct alleles at each marker locus, was calculated using ARLEQUIN software version 3.11 [49] with the formula:

$$H_e = [n/(n-1)]\left[1 - \Sigma pi^2\right],$$

where 'n' represents the number of isolates analysed and 'pi' is the frequency of the ith allele in a given population. H_e values range between 0 (no genetic diversity) and 1 (high genetic diversity) [9]. The average N_a, N_e, and H_e values for each study site were computed as the mean of the values from each locus. Significant differences in N_a, Ar, N_e, and H_e across study sites were detected using the Kruskal–Wallis test at $p < 0.05$.

Plasmodium falciparum MOI across the study sites

P.falciparum MOI defined as the number of distinct parasite genotypes coexisting within a given infection [12] was determined based on the maximum number of alleles per locus. Isolates with only one allele were considered monoclonal infections, whereas those with more than one allele were considered polyclonal [31]. The results were summarized by counting the number of participants exhibiting a certain number of clones and compared across study sites.

Plasmodium falciparum parasite population structure analysis

P. falciparum population structure across the three study sites was assessed based on Wright's F-statistics (F_{ST}), linkage disequilibrium, and cluster analysis. F_{ST} values were calculated using Arlequin 3.11 [49]. The F_{ST} values were classified as follows: 0 to 0.05 for low genetic variability, 0.05–0.15 for moderate genetic variability, 0.15–0.25 for high genetic differentiation, and > 0.25 for substantial genetic differentiation [50]. Inter- and intra-population variance was determined using analysis of molecular variance (AMOVA). Multilocus LD, measured as the standardized index of association (I_A^S), was calculated using LIAN version 3.5 [51] for the whole dataset. This index was calculated as:

$$I_A^S = (1/n - 1((VD/(VE) - 1),$$

where VE is the expected variance of the nth number of loci for which two individuals differ, and VD is the observed variance. The significance of I_A^S values was tested using the Monte Carlo method. The distance between isolates from the study sites was estimated through Principal Component Analysis (PCoA) using GENALEX 6.5 software [48] to assess parasite population substructure. Additionally, cluster analysis was performed using STRUCTURE v2.1 software [52].

Results

Characteristics of the study population

Of the 288 *P. falciparum* samples retrieved and genotyped using seven neutral microsatellites, 211 (73.3%) samples successfully amplified on at least five microsatellites and were included in the genetic analysis. Of these 211 samples, 109 (51.7%) were from male participants, and the majority (54%) were from participants less than five years of age. The parasite densities in the selected samples ranged from 48 to 1,600,000 parasites/μL. Participants from Kanungu had significantly higher mean temperatures (38.3 °C) compared to those from Tororo (37.9 °C) and Jinja (37.9 °C), while participants from Jinja had significantly lower parasite densities compared to those from other sites (Table 1).

Plasmodium falciparum genetic diversity

The mean number of alleles (N_a) observed was 11.29 ± 3.82 in Jinja, 13.43 ± 4.58 in Kanungu, and 12.57 ± 5.47 in Tororo. The markers C2M34–313 (Chr2), Poly-α, and TA1 exhibited the highest number of alleles, ranging from 13 to 21 across the three study sites. The mean expected heterozygosity (H_e) for *P. falciparum* was 0.78 ± 0.16 in Jinja, 0.81 ± 0.14 in Kanungu, and 0.78 ± 0.16 in Tororo (Table 2).

The mean number of effective alleles (N_e) was 6.13 ± 3.27 in Jinja, 6.85 ± 3.73 in Kanungu, and 6.59 ± 4.20 in Tororo. The mean allele richness (Ar) was 10.84 ± 3.76 in Jinja, 12.42 ± 4.24 in Kanungu, and 12.13 ± 5.59 in Tororo (Additional file 2: Table S2). A Kruskal–Wallis test revealed no significant differences in the number of alleles (N_a) or expected heterozygosity (H_e) values across the three study sites ($p > 0.05$). Similarly, no significant differences were observed for the effective

Table 1 Demographic characteristics of the cohort participants whose samples are included in the analysis

Characteristic	Overall N = 211	Jinja N = 69	Kanungu N = 71	Tororo N = 71	P value
Age in years					
< 5 years		35	43	37	
5–11 years		25	28	26	
≥ 18 years		9	0	8	
Gender (%)					
Male		53.6	50.7	50.7	
Mean axillary temperature, °C (SD)		37.9 ± 1.17	38.3 ± 1.3	37.9 ± 1.1	0.006
Geometric mean parasite density/μL (GSD)		41,367.9 ± 7372	109,734.5 ± 4833.4	277,424.6 ± 9245.1	< 0.001
Mean Hb g/dL (SD)		11.34 ± 1.89	10.36 ± 2.33	11.37 ± 1.84	< 0.001

Table 2 *P. falciparum* genetic diversity based on number of alleles, and expected heterozygosity

Locus	Jinja		Kanungu		Tororo	
	N_a	H_e	N_a	H_e	N_a	H_e
2490	6	0.47	6	0.52	5	0.47
Poly-α	13	0.89	15	0.91	16	0.91
C2M34–313	18	0.92	20	0.93	21	0.93
TA1	13	0.87	17	0.88	16	0.86
TA109	10	0.8	10	0.82	8	0.8
C3M69–383	9	0.69	13	0.82	12	0.7
PfPK2	10	0.84	13	0.78	10	0.82
Mean	11.29	0.78	13.43	0.81	12.57	0.78
SD	3.82	0.16	4.58	0.14	5.47	0.16

number of alleles (N_e) or allele richness (Ar) across the sites ($p > 0.05$).

Plasmodium falciparum MOI

The highest rate of polyclonal infection was observed in Tororo, with 50 out of 71 samples (70%). In Jinja, 40 out of 69 samples (58%) exhibited polyclonal infections, and in Kanungu, 40 out of 71 samples (56%) showed polyclonal infections. The highest polyclonal infection rates were recorded with marker C3M69–383, showing rates of 83% in Jinja, 80.3% in Kanungu, and 88.2% in Tororo. Conversely, the lowest rates of polyclonal infection were recorded with marker 2490, with rates of 33.3% in Jinja, 24.9% in Kanungu, and 46.5% in Tororo. The mean MOI varied slightly across the study sites: Jinja had a mean of 1.9 ± 0.3, Kanungu had a mean of 2.0 ± 0.05, and Tororo had a mean of 2.5 ± 0.05 (Table 3). Across the sites, the microsatellites C3M69–383 and TA1 identified more polyclonal infections (number of clones ≥ 2), while 2490 identified the most monoclonal infections. The Poly-α and PfPK2 markers recorded the maximum number of alleles [5] (Fig. 2).

Plasmodium falciparum parasite population structure between sites

Plasmodium falciparum genetic differentiation

The pairwise F_{ST} values between the three study sites revealed low genetic differentiation (< 0.05). The lowest genetic differentiation was observed between Jinja and Tororo (0.011), while the highest genetic differentiation was observed between Kanungu and Tororo (0.021) (Table 4).

Linkage disequilibrium (LD)

Multilocus Index of Association (I_A^S) analysis was performed to assess the non-random association of all complete haplotypes at the individual study sites. The LD ranged from 0.07 in Tororo to 0.14 in Jinja. Significant

Table 3 *P. falciparum* MOI in Jinja, Kanungu and Tororo

Locus	Jinja (n = 69)		Kanungu (n = 71)		Tororo (n = 71)	
	Percentage polyclonal	Mean MOI	Percentage polyclonal	Mean MOI	Percentage polyclonal	Mean MOI
2490	33.3	1.4	24.9	1.3	46.5	1.6
Poly-α	44.5	1.6	51.7	2.0	62.9	2.5
C2M34–313	73.2	2.1	31.9	1.4	54.5	2.1
TA1	69.1	2.2	81.7	2.4	80.7	2.6
TA109	47.6	1.9	43.8	1.8	74.6	2.7
C3M69–383	83.0	2.2	80.3	2.4	88.2	3.2
PfPK2	55.1	2.0	77.5	2.5	82.6	2.8
Mean	58	1.9	56	2.0	70	2.5
SD/*SE	*6.7	0.3	*9.0	0.5	*5.9	0.5

* Standard error (SE)

multilocus LD was observed across all study sites ($p < 0.01$) (Table 5).

Principal Component Analysis (PCoA) revealed no distinct clusters of parasites based on their geographic origins (Additional file 4: Fig. S4). Subsequent cluster analysis with STRUCTURE identified significant genetic admixture within the population. Parasite samples from all three regions displayed varying proportions of blue, orange, and black clusters, reflecting the complex genetic diversity among the isolates. The mixed genetic heritage of the parasites is evident from the overlapping and diverse color proportions within the columns, underscoring the intricate genetic landscape across the three study sites (Fig. 3).

Discussion

The current study utilized seven neutral microsatellite markers to characterize *P. falciparum* genetic diversity and population structure across three regions in Uganda with varying malaria transmission intensities. The influence of transmission intensity on *P. falciparum* genetic diversity is well-documented. High transmission areas typically exhibit greater genetic diversity and weaker population structure, while low transmission areas often show reduced genetic diversity, stronger LD, and more defined population structure [9, 30–32, 53, 54]. However, the findings of this study revealed high *P. falciparum* genetic diversity and MOI, with limited population structure across the study sites.

The mean number of alleles (N_a) observed in this study ranged from 11.29 to 13.34, higher than values reported in low malaria transmission areas such as Djibouti [55], but comparable to findings in high transmission regions of SSA, where N_a ranges from 5.3 to 13.5 [55, 56]. The high mean H_e (ranging from 0.78 to 0.81) suggests a large effective parasite population size, fostering genetic recombination and diversity [4, 57]. These values are consistent with reports from other high malaria transmission regions in SSA, such as Madagascar, where mean H_e ranged from 0.82 to 0.89 across different malaria transmission settings [58]. A study in Eswatini, a low transmission area, also observed a high mean H_e value (0.75) [59]. The persistence of higher genetic diversity in low malaria transmission areas may be explained by the influx of *P. falciparum*-infected individuals from high malaria-endemic regions. This influx tend to sustain genetic diversity even in areas with lower transmission rates. The observed high genetic diversity in this study poses challenges for malaria control programs, as it complicates immunity development, facilitates immune evasion, and promotes the emergence of drug-resistant strains [3]. These dynamics are particularly critical in high transmission areas, such as Uganda, where repeated exposure to diverse *P. falciparum* strains may hinder the development of immunity and reduce the effectiveness of vaccines and treatment regimens.

This study found significant variations in *P. falciparum* MOI across the study sites ($p < 0.001$), with Tororo (mean MOI = 2.5) exhibiting the highest values, followed by Kanungu (mean MOI = 2.1), and Jinja (mean MOI = 1.9). These results reflect the higher prevalence of polyclonal infections in the study areas, indicating high malaria transmission, despite Jinja being classified as a low transmission area. This pattern mirrors findings in other SSA countries. For instance, in Rwanda, higher MOI values were reported in high malaria transmission areas like Ruhaha (mean MOI = 2.13, polyclonal infection = 62%) compared to low transmission areas like Mubuga (mean MOI = 1.29, polyclonal infection = 26.9%) [35]. Increased MOI in high malaria transmission areas is typically due to frequent exposure to multiple *P. falciparum* strains [60, 61]. Conversely, low transmission areas often

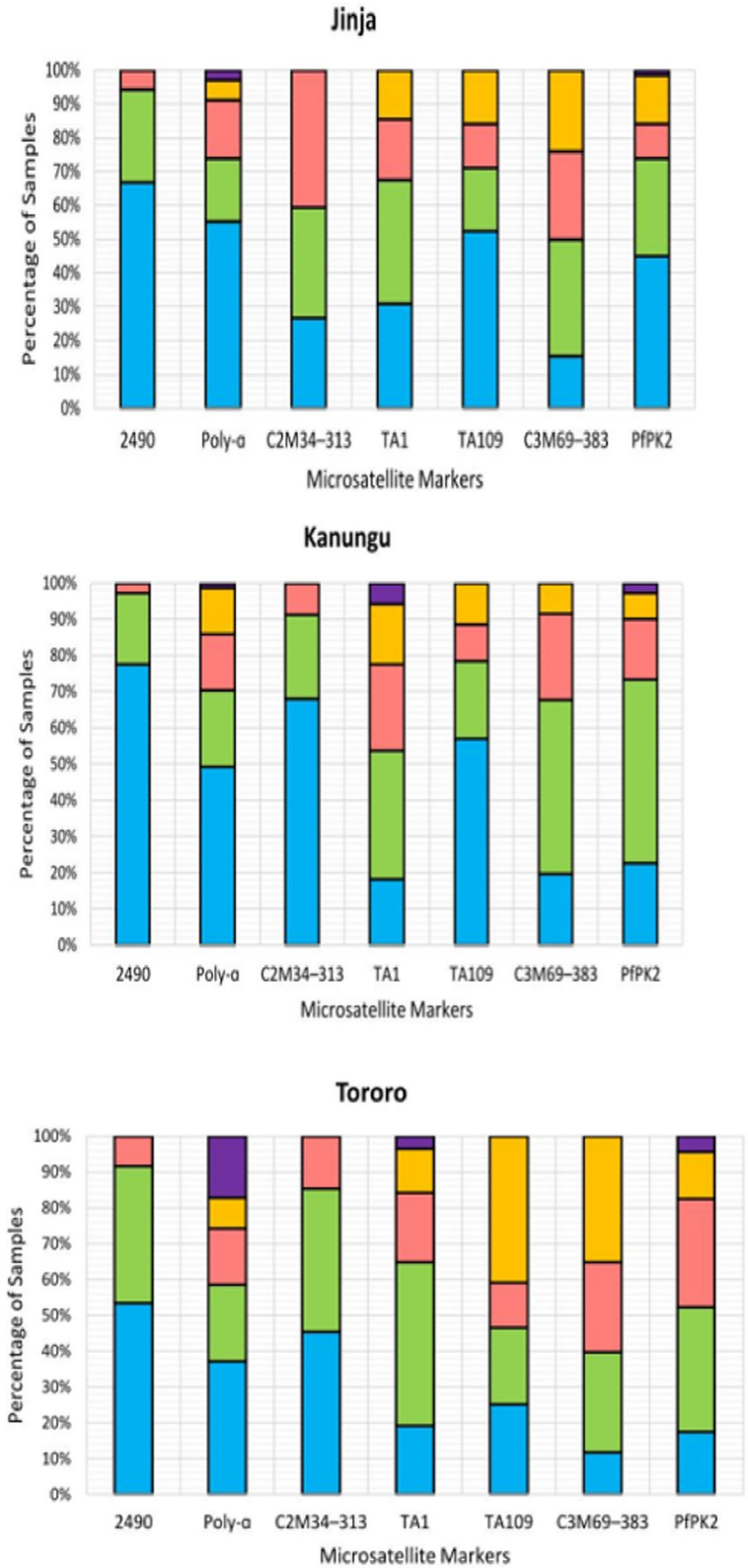

Fig. 2 Percentage of clones within each microsatellite marker with 1 clone (blue), 2 clones (green), 3 clones (red), 4 clones (yellow), and 5 clones (purple) in Jinja, Kanungu and Tororo. The vertical axis represents the percentage of samples while the horizontal axis represents the genetic markers

Table 4 Pairwise genetic differentiation (F_{ST}) among study sites

	Jinja	Kanungu	Tororo
Jinja	0	0.016	0.011
Kanungu	0.016	0	0.021
Tororo	0.011	0.021	0

The AMOVA result (0.03) indicated that most of the genetic variation (97%) in *P. falciparum* parasites is contained within populations, with only 3% of the variation explained by differences between subpopulations across study sites (Additional file 3: Table S3)

Table 5 Linkage disequilibrium analysis for *P. falciparum* populations obtained in each study site

Test factor	Jinja	Kanungu	Tororo
V_D	21.95	19.28	12.96
V_E	4.77	4.43	4.81
I^A_S	0.14	0.13	0.07
Var (V_D)	0.27	0.26	0.20
P. value	< 0.01	< 0.01	< 0.01

V_D = variance; V_E = expected variance if linkage equilibrium exists; I^A_S = Standardized Index of Association

exhibit monoclonal infections, likely due to fewer mosquito bites and reduced genetic recombination [62, 63]. The observation of higher MOI in Tororo suggests that malaria transmission rates are indeed high in this region, consistent with neighboring high transmission areas near Lake Victoria in Kenya, where polyclonal infections are more common [30]. Increased MOI also increases the likelihood of immune evasion and the emergence of drug-resistant strains [64].

Allele frequencies and genetic differentiation analysis revealed minimal genetic differentiation (F_{ST} ranging from 0.011 to 0.021), consistent with previous studies from other SSA malaria-endemic regions. For instance, *P. falciparum* populations in Kenya have shown low genetic differentiation (F_{ST} = 0.072) between high and low malaria transmission areas [65]. Principal Component Analysis (PCoA) in this study further supported the low F_{ST} values, indicating minimal geographic structuring of *P. falciparum* populations. These findings suggest high levels of gene flow among parasite populations across the study sites, a pattern that mirrors previous research in SSA regions where high gene flow across subpopulations has been attributed to factors like human migration and movement [30, 66, 67]. In Uganda, such gene flow may be facilitated by high rates of internal migration, as observed in other SSA countries, contributing to genetic similarities across *P. falciparum* populations in different malaria transmission settings.

LD is inversely correlated with malaria transmission intensity, with high transmission areas typically showing low LD values due to frequent genetic recombination [8, 9]. In this study, LD values ranged from 0.07 to 0.14, which are considered low, indicating that alleles at different loci are largely independent within the populations. This finding is consistent with studies from other high malaria transmission areas in SSA, such as Mali and Nigeria, where low LD values have been reported, suggesting high rates of recombination and genetic mixing [66, 67]. Similarly, a study in Kenya found a low genetic differentiation index (F_{ST} = 0.072, p = 0.01) between low and high malaria transmission areas [65]. The low LD and observed admixture pattern in this study suggest limited population structure, likely due to high levels of gene flow and recombination across *P. falciparum* populations [68]. Human movement across regions may also contribute to this genetic mixing, as *P. falciparum* strains spread between urban, rural, and border areas [30, 68, 69]. This interconnectedness of parasite populations underscores the need for malaria control strategies that take into account the genetic connectivity between parasite strains, advocating for regional rather than strictly local interventions.

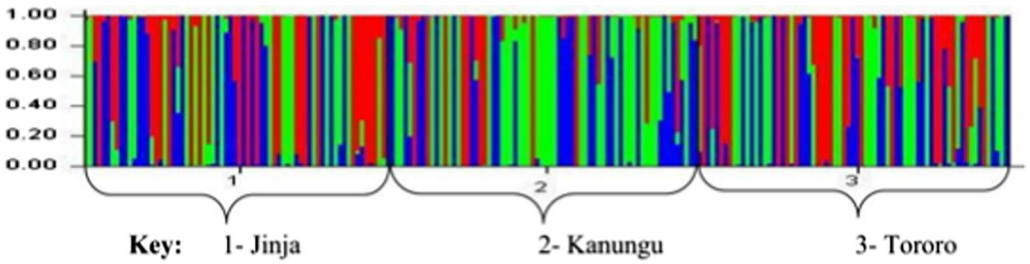

Fig. 3 The Bayesian bar plot illustrates the population structure of *P. falciparum* parasite isolates collected from three different regions with varying levels of malaria transmission: Jinja (low malaria transmission), Kanungu (medium malaria transmission), and Tororo (high malaria transmission). Each vertical bar in the plot represents an individual parasite isolate. Each bar is divided into colored segments, with colours (green, red, and blue) representing different genetic clusters of *P. falciparum*. The size of each coloured segment within a bar corresponds to the proportion of that genetic cluster in the sample.

Parasite density varied significantly between study sites, with higher parasite densities in high transmission areas, consistent with malaria transmission dynamics. In high transmission regions, more frequent cycles of transmission lead to higher parasite burdens in infected individuals. Studies from Uganda, including those near Lake Victoria, have similarly reported higher parasite densities in high transmission areas, correlating with increased disease severity and inflammatory responses [70, 71]. In contrast, low transmission areas such as parts of central Uganda typically show lower parasite densities due to fewer opportunities for infection. This further reinforces the relationship between parasite density and transmission intensity.

This study provides valuable insights into the genetic diversity and population structure of *P. falciparum* in Uganda across different malaria transmission settings. While the findings share similarities with studies from other African countries, such as Kenya, Rwanda, and Tanzania, the regional differences in transmission intensity observed in Uganda offer a unique perspective on the diversity and transmission dynamics of *P. falciparum*. However, this study has some limitations. It was conducted in only three regions, which limits the generalizability of the findings to other parts of Uganda with different ecological and epidemiological conditions. Additionally, the relatively small number of adults included in the study may have impacted the analysis for this group.

Implication for future research and policy

The study findings highlight the dynamic nature of *P. falciparum* genetic diversity and population structure across varying malaria transmission settings. High genetic diversity and MOI in high transmission areas pose challenges for immunity development, vaccine efficacy, treatment effectiveness, and the emergence of drug-resistant strains. It is crucial for malaria control strategies to consider genetic connectivity between parasite populations to optimize interventions and move toward malaria elimination. Future studies should employ longitudinal designs to examine temporal and seasonal changes in *P. falciparum* genetic diversity and population structure to better understand malaria transmission dynamics. Additionally, further investigations are needed to clarify the underlying causes of low LD, which could be influenced by factors such as population dynamics, genetic drift, or natural selection.

Conclusion

This study reveals high *P. falciparum* genetic diversity and MOI, with low population structure, across various malaria transmission settings in Uganda. These findings present significant challenges for malaria control in regions with differing transmission intensities. The high genetic diversity and prevalence of polyclonal infections complicate immunity development and hinder efforts to manage drug resistance. These challenges emphasize the need for region-specific malaria control strategies that account for the genetic connectivity of *P. falciparum* populations. Additionally, the study underscores the importance of continuous surveillance and the adaptation of control measures based on genetic data to ensure the sustainable elimination of malaria in Uganda.

Abbreviations

AMOVA	Analysis of molecular variance
DBS	Dried blood spot
EIR	Entomological inoculation rate
F_{ST}	Fixation Index
Hb	Haemoglobin
H_e	Expected heterozygosity
I_A^S	Index of Association
IRS	Indoor residual spraying
ITNs	Insecticide-treated bed nets
LD	Linkage disequilibrium
MOI	Multiplicity of infection
MSP	Merozoite surface protein
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PRISM	Programme for Resistance, Immunology and Modelling of Malaria
RFU	Relative Fluorescence Units
SSA	Sub-Saharan Africa

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-025-05325-6.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

Acknowledgements

Not applicable.

Author contributions

AM, SLN, PBK, CK, EMB and JIN conceived the idea, planned, and designed the study. AM, HK and SK performed the analysis. AM wrote the first draft of the manuscript JIN, BC, SO and ST critically revised the manuscript AM wrote the final draft of the manuscript. All authors reviewed the manuscript.

Funding

This study was supported by EDCTP3, the Infectious Diseases Institute (IDI) OPTIMAL study, and the Government of Uganda through the Makerere University Research and Innovation Fund (Mak-RIF PhD grants).

Availability of data and materials

Datasets generated or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study utilized secondary data without participant interaction. Study approval and a waiver of consent were provided by the Makerere University School of Medicine Institutional Review Board (# Mak-SOMREC-2021–152) and the Uganda National Council for Science and Technology (# HS2744ES).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Clinical Epidemiology Unit, School of Medicine, Makerere University College of Health Sciences, P. O. Box 7072, Kampala, Uganda. [2]Department of Microbiology and Immunology, School of Medicine, Kabale University, P. O. Box 314, Kabale, Uganda. [3]Infectious Diseases Research Collaboration (IDRC), P.O. Box 7475, Kampala, Uganda. [4]Malaria Research Program, Center for Vaccine Development and Global Health, University of Maryland School of Medicine, 655 W. Baltimore St, Baltimore, MA 21201, USA. [5]Department of Immunology and Molecular Biology, Makerere University College of Health Sciences, P. O. Box 7072, Kampala, Uganda. [6]Infectious Diseases Institute, College of Health Sciences, Makerere University, P. O. Box 7072, Kampala, Uganda. [7]Department of Community Health, School of Medicine, Kabale University, P. O. Box 314, Kabale, Uganda. [8]Department of Medicine, School of Medicine, Makerere University College of Health Sciences, P. O. Box 7072, Kampala, Uganda. [9]Mbarara University of Science and Technology, Mbarara, Uganda. [10]African Center of Excellence in Bioinformatics and Data Intensive Sciences, Kampala, Uganda.

Received: 30 September 2024 Accepted: 7 March 2025
Published online: 24 March 2025

References

1. WHO. World malaria report 2024. Geneva, World Health Organization; 2024.
2. WHO. World Malaria Report 2019. Geneva, World Health Organization; 2019.
3. Su X-Z, Lane KD, Xia L, Sá JM, Wellems TE. *Plasmodium* genomics and genetics: new insights into malaria pathogenesis, drug resistance, epidemiology, and evolution. Clin Microbiol Rev. 2019;32: e00019-19.
4. Jiang H, Li N, Gopalan V, Zilversmit MM, Varma S, Nagarajan V, et al. High recombination rates and hotspots in a *Plasmodium falciparum* genetic cross. Genome Biol. 2011;12:R33.
5. Takala S, Escalante A, Branch O, Kariuki S, Biswas S, Chaiyaroj S, et al. Genetic diversity in the Block 2 region of the merozoite surface protein 1 (MSP-1) of *Plasmodium falciparum:* additional complexity and selection and convergence in fragment size polymorphism. Infect Genet Evol. 2006;6:417–24.
6. Lapp Z, Obala AA, Abel L, Rasmussen DA, Sumner KM, Freedman E, et al. *Plasmodium falciparum* genetic diversity in coincident human and mosquito hosts. Mol Biol. 2022;13:e02277-e2322.
7. Abamecha A, El-Abid H, Yilma D, Addisu W, Ibenthal A, Bayih AG, et al. Genetic diversity and genotype multiplicity of *Plasmodium falciparum* infection in patients with uncomplicated malaria in Chewaka district, Ethiopia. Malar J. 2020;19:203.
8. Wang M, Jia T, Jiang N, Wang L, Hu X, Luo Z. Inferring linkage disequilibrium from non-random samples. BMC Genomics. 2010;11:328.
9. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol Biol Evol. 2000;17:1467–82.
10. Pinkevych M, Petravic J, Bereczky S, Rooth I, Färnert A, Davenport MP. Understanding the relationship between *Plasmodium falciparum* growth rate and multiplicity of infection. J Infect Dis. 2015;211:1121–7.
11. Zhong D, Koepfli C, Cui L, Yan G. Molecular approaches to determine the multiplicity of *Plasmodium* infections. Malar J. 2018;17:172.
12. Sondo P, Derra K, Rouamba T, Nakanabo Diallo S, Taconet P, Kazienga A, et al. Determinants of *Plasmodium falciparum* multiplicity of infection and genetic diversity in Burkina Faso. Parasit Vectors. 2020;13:427.
13. Alizon S. Parasite co-transmission and the evolutionary epidemiology of virulence. Evol. 2013;67:921–33.
14. Nkhoma SC, Nair S, Cheeseman IH, Rohr-Allegrini C, Singlam S, Nosten F, et al. Close kinship within multiple-genotype malaria parasite infections. Proc Biol Sci. 2012;279:2589–98.
15. Wong W, Griggs AD, Daniels RF, Schaffner SF, Ndiaye D, Bei AK, et al. Genetic relatedness analysis reveals the cotransmission of genetically related *Plasmodium falciparum* parasites in Thiès, Senegal. Genome Med. 2017;9:5.
16. Kiwuwa MS, Ribacke U, Moll K, Byarugaba J, Lundblom K, Färnert A, et al. Genetic diversity of *Plasmodium falciparum* infections in mild and severe malaria of children from Kampala, Uganda. Parasitol Res. 2013;112:1691–700.
17. Jensen AR, Adams Y, Hviid L. Cerebral *Plasmodium falciparum* malaria: the role of PfEMP1 in its pathogenesis and immunity, and PfEMP1-based vaccines to prevent it. Immunol Rev. 2020;293:230–52.
18. Narh CA, Ghansah A, Duffy MF, Ruybal-Pesántez S, Onwona CO, Oduro AR, et al. Evolution of antimalarial drug resistance markers in the reservoir of *Plasmodium falciparum* infections in the Upper East Region of Ghana. J Infect Dis. 2020;222:1692–701.
19. Mita T, Tanabe K. Evolution of *Plasmodium falciparum* drug resistance: implications for the development and containment of artemisinin resistance. Jpn J infect Dis. 2012;65:465–75.
20. Pumpaibool T, Arnathau C, Durand P, Kanchanakhan N, Siripoon N, Suegorn A, et al. Genetic diversity and population structure of *Plasmodium falciparum* in Thailand, a low transmission country. Malar J. 2009;8:155.
21. Al-Rumhi A, Al-Hashami Z, Al-Hamidhi S, Gadalla A, Naeem R, Ranford-Cartwright L, et al. Influx of diverse, drug resistant and transmissible *Plasmodium falciparum* into a malaria-free setting in Qatar. BMC Infect Dis. 2020;20:413.
22. Pasternak ND, Dzikowski R. PfEMP1: an antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite *Plasmodium falciparum*. Int J Biochem Cell Biol. 2009;41:1463–6.
23. Asua V, Conrad MD, Aydemir O, Duvalsaint M, Legac J, Duarte E, et al. Changing prevalence of potential mediators of aminoquinoline, antifolate, and artemisinin resistance across Uganda. J Infect Dis. 2021;223:985–94.
24. Okumu FO, Moore SJ. Combining indoor residual spraying and insecticide-treated nets for malaria control in Africa: a review of possible outcomes and an outline of suggestions for the future. Malar J. 2011;10:208.
25. Warrell DA, Watkins WM, Winstanley PA. Treatment and prevention of malaria. Essential malariology, 4edn: CRC Press; 2017. p. 268–312.
26. Oxborough RM, Chilito KCF, Tokponnon F, Messenger LA. Malaria vector control in sub-Saharan Africa: complex trade-offs to combat the growing threat of insecticide resistance. Lancet Planet Health. 2024;8:e804–12.
27. Mita T, Jombart T. Patterns and dynamics of genetic diversity in *Plasmodium falciparum*: what past human migrations tell us about malaria. Parasitol Int. 2015;64:238–43.
28. Gwarinda HB, Tessema SK, Raman J, Greenhouse B, Birkholtz L-M. Population structure and genetic connectivity of *Plasmodium falciparum* in pre-elimination settings of Southern Africa. Front Epidemiol. 2023;3:1227071.
29. Adjah J, Fiadzoe B, Ayanful-Torgby R, Amoah LE. Seasonal variations in *Plasmodium falciparum* genetic diversity and multiplicity of infection in asymptomatic children living in southern Ghana. BMC Infect Dis. 2018;18:432.
30. Mulenge FM, Hunja CW, Magiri E, Culleton R, Kaneko A, Aman RA. Genetic diversity and population structure of *Plasmodium falciparum* in Lake Victoria Islands, a region of intense transmission. Am J Trop Med Hyg. 2016;95:1077–85.
31. MohdAbdRazak MR, Sastu UR, Norahmad NA, Abdul-Karim A, Muhammad A, Muniandy PK, et al. Genetic diversity of Plasmodium falciparum populations in malaria declining areas of Sabah, East Malaysia. PLoS ONE. 2016;11: e0152415.
32. Iwagami M, Rivera PT, Villacorte EA, Escueta AD, Hatabu T, Kawazu S-I, et al. Genetic diversity and population structure of *Plasmodium falciparum* in the Philippines. Malar J. 2009;8:96.
33. Atuh NI, Anong DN, Jerome F-C, Oriero E, Mohammed NI, D'Alessandro U, et al. High genetic complexity but low relatedness in *Plasmodium*

falciparum infections from Western Savannah Highlands and coastal equatorial Lowlands of Cameroon. Pathog Glob Health. 2022;116:428–37.
34. Bei AK, Niang M, Deme AB, Daniels RF, Sarr FD, Sokhna C, et al. Dramatic changes in malaria population genetic complexity in Dielmo and Ndiop, Senegal, revealed using genomic surveillance. J Infect Dis. 2018;217:622–7.
35. Kateera F, Nsobya SL, Tukwasibwe S, Mens PF, Hakizimana E, Grobusch MP, et al. Malaria case clinical profiles and *Plasmodium falciparum* parasite genetic diversity: a cross sectional survey at two sites of different malaria transmission intensities in Rwanda. Malar J. 2016;15:237.
36. Shetty AC, Jacob CG, Huang F, Li Y, Agrawal S, Saunders DL, et al. Genomic structure and diversity of *Plasmodium falciparum* in Southeast Asia reveal recent parasite migration patterns. Nat Commun. 2019;10:2665.
37. Briggs J, Kuchta A, Murphy M, Tessema S, Arinaitwe E, Rek J, et al. Within-household clustering of genetically related *Plasmodium falciparum* infections in a moderate transmission area of Uganda. Malar J. 2021;20:68.
38. Agaba BB, Anderson K, Gresty K, Prosser C, Smith D, Nankabirwa JI, et al. Genetic diversity and genetic relatedness in *Plasmodium falciparum* parasite population in individuals with uncomplicated malaria based on microsatellite typing in Eastern and Western regions of Uganda, 2019–2020. Malar J. 2021;20:242.
39. Kilama M, Smith DL, Hutchinson R, Kigozi R, Yeka A, Lavoy G, et al. Estimating the annual entomological inoculation rate for *Plasmodium falciparum* transmitted by *Anopheles gambiae s.l.* using three sampling methods in three sites in Uganda. Malar J. 2014;13:111.
40. Kamya MR, Arinaitwe E, Wanzira H, Katureebe A, Barusya C, Kigozi SP, et al. Malaria transmission, infection, and disease at three sites with varied transmission intensity in Uganda: implications for malaria control. Am J Trop Med Hyg. 2015;92:903–12.
41. Mwesigwa A, Ocan M, Musinguzi B, Nante RW, Nankabirwa JI, Kiwuwa SM, et al. *Plasmodium falciparum* genetic diversity and multiplicity of infection based on msp-1, msp-2, glurp and microsatellite genetic markers in sub-Saharan Africa: a systematic review and meta-analysis. Malar J. 2024;23:97.
42. WHO. Basic malaria microscopy. Geneva, World Health Organization; 2010.
43. Musapa M, Kumwenda T, Mkulama M, Chishimba S, Norris DE, Thuma PE, et al. A simple Chelex protocol for DNA extraction from Anopheles spp. J Vis Exp. 2013;71:3281.
44. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg. 1999;60:687–92.
45. Wei G, Zhang L, Yan H, Zhao Y, Hu J, Pan W. Evaluation of the population structure and genetic diversity of *Plasmodium falciparum* in southern China. Malar J. 2015;14:283.
46. Tanabe K, Mita T, Jombart T, Eriksson A, Horibe S, Palacpac N, et al. *Plasmodium falciparum* accompanied the human expansion out of Africa. Curr Biol. 2010;20:1283–9.
47. Meyer CG, May J, Arez AP, Gil JP, Do RV. Genetic diversity of *Plasmodium falciparum*: asexual stages. Trop Med Int Health. 2002;7:395–408.
48. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics. 2012;28:2537–9.
49. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online. 2007;1:47–50.
50. Balloux F, Lugon-Moulin N. The estimation of population differentiation with microsatellite markers. Mol Ecol. 2002;11:155–65.
51. Haubold B, Hudson RR. LIAN 30: detecting linkage disequilibrium in multilocus data. Linkage analysis. Bioinformatics. 2000;16:847–8.
52. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155:945–59.
53. Larrañaga N, Mejía RE, Hormaza JI, Montoya A, Soto A, Fontecha GA. Genetic structure of *Plasmodium falciparum* populations across the Honduras-Nicaragua border. Malar J. 2013;12:354.
54. Bonizzoni M, Afrane Y, Baliraine FN, Amenya DA, Githeko AK, Yan G. Genetic structure of *Plasmodium falciparum* populations between lowland and highland sites and antimalarial drug resistance in Western Kenya. Infect Genet Evol. 2009;9:806–12.
55. Bogreau H, Renaud F, Bouchiba H, Durand P, Assi S-B, Henry M-C, et al. Genetic diversity and structure of African *Plasmodium falciparum* populations in urban and rural areas. Am J Trop Med Hyg. 2006;74:953–9.
56. Zhong D, Afrane Y, Githeko A, Yang Z, Cui L, Menge DM, et al. *Plasmodium falciparum* genetic diversity in western Kenya highlands. Am J Trop Med Hyg. 2007;77:1043–50.
57. Yuan L, Zhao H, Wu L, Li X, Parker D, Xu S, et al. *Plasmodium falciparum* populations from northeastern Myanmar display high levels of genetic diversity at multiple antigenic loci. Acta Trop. 2013;125:53–9.
58. Ralinoro F, Rakotomanga TA, Rakotosaona R, Doll Rakoto DA, Menard D, Jeannoda V, et al. Genetic diversity of *Plasmodium falciparum* populations in three malaria transmission settings in Madagascar. Malar J. 2021;20:239.
59. Roh ME, Tessema SK, Murphy M, Nhlabathi N, Mkhonta N, Vilakati S, et al. High genetic diversity of *Plasmodium falciparum* in the low-transmission setting of the Kingdom of Eswatini. J Infect Dis. 2019;220:1346–54.
60. Kidima W, Nkwengulila G. *Plasmodium falciparum msp2* genotypes and multiplicity of infections among children under five years with uncomplicated malaria in Kibaha, Tanzania. J Parasitol Res. 2015;2015: 721201.
61. Sondén K, Doumbo S, Hammar U, Vafa Homann M, Ongoiba A, Traoré B, et al. Asymptomatic multiclonal *Plasmodium falciparum* Infections carried through the dry season predict protection against subsequent clinical malaria. J Infect Dis. 2015;212:608–16.
62. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Saif-Ali R, Al-Mekhlafi AM, Surin J. Genetic diversity of *Plasmodium falciparum* isolates from Pahang, Malaysia based on MSP-1 and MSP-2 genes. Parasit Vectors. 2011;4:233.
63. Ghanchi NK, Mårtensson A, Ursing J, Jafri S, Bereczky S, Hussain R, et al. Genetic diversity among *Plasmodium falciparum* field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. Malar J. 2010;9:1.
64. Patgiri S, Sarma K, Sarmah N, Bhattacharyya N, Sarma D, Nirmolia T, et al. Characterization of drug resistance and genetic diversity of *Plasmodium falciparum* parasites from Tripura, Northeast India. Sci Rep. 2019;9:13704.
65. Ingasia LA, Cheruiyot J, Okoth SA, Andagalu B, Kamau E. Genetic variability and population structure of *Plasmodium falciparum* parasite populations from different malaria ecological regions of Kenya. Infect Genet Evol. 2016;39:372–80.
66. Nabet C, Doumbo S, Jeddi F, Konaté S, Manciulli T, Fofana B, et al. Genetic diversity of *Plasmodium falciparum* in human malaria cases in Mali. Malar J. 2016;15:353.
67. Ajogbasile FV, Kayode AT, Oluniyi PE, Akano KO, Uwanibe JN, Adegboyega BB, et al. Genetic diversity and population structure of *Plasmodium falciparum* in Nigeria: insights from microsatellite loci analysis. Malar J. 2021;20:236.
68. Touray AO, Mobegi VA, Wamunyokoli F, Herren JK. Diversity and multiplicity of *P. falciparum* infections among asymptomatic school children in Mbita, Western Kenya. Sci Rep. 2020;10:5924.
69. Gwarinda HB, Tessema SK, Raman J, Greenhouse B, Birkholtz L-M. Parasite genetic diversity reflects continued residual malaria transmission in Vhembe District, a hotspot in the Limpopo Province of South Africa. Malar J. 2021;20:96.
70. Ademolue TW, Aniweh Y, Kusi KA, Awandare GA. Patterns of inflammatory responses and parasite tolerance vary with malaria transmission intensity. Malar J. 2017;16:145.
71. Stresman GH, Stevenson JC, Ngwu N, Marube E, Owaga C, Drakeley C, et al. High levels of asymptomatic and subpatent *Plasmodium falciparum* parasite carriage at health facilities in an area of heterogeneous malaria transmission intensity in the Kenyan highlands. Am J Trop Med Hyg. 2014;91:1101–8.

Publisher's Note