MAKERERE  **UNIVERSITY**

Assessment of *Plasmodium falciparum* genetic diversity, multiplicity of infection, and algorithms for distinguishing recrudescence from reinfection in antimalarial drug efficacy studies in Uganda

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DECLARATION

I, Alex Mwesigwa, hereby declare that this thesis, titled "Assessment of *P. falciparum* genetic diversity, multiplicity of infection, and algorithms for distinguishing recrudescence from reinfection in antimalarial drug efficacy studies in Uganda", is my original work and has not been submitted for any degree or examination at any other university.

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DEDICATION

I dedicate this work to my beloved parents, Mr. Evaristo Karymarwaki and Mrs. Victoria Karymarwaki, in recognition of their invaluable guidance and unwavering support throughout my academic journey.

I also dedicate this work to my wife, Fatima Dianah, and our children: Moris Agaba, Daniel Mwebaze, Anabel Akampa, and Joshua Mwesigwa, for their love, patience, and encouragement.

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TABLE OF CONTENTS

DECLARATION i

DEDICATION i

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES ii

LIST OF FIGURES iii

LIST OF APPENDICES v

LIST OF ABBREVIATIONS vi

OPERATIONAL DEFINITIONS ix

LIST OF PUBLICATIONS FROM THIS PhD PROJECT x

ORGANISATION OF THE THESIS xii

ABSTRACT xiii

CHAPTER ONE: INTRODUCTION 1

1.1 Background 1

1.2 Statement of the problem 4

1.3 Justification of the study 5

1.4 Significance of the Study 7

1.5 Research questions 7

1.6 Study objectives 9

1.7 Study hypotheses 9

1.8 Conceptual frame work 10

CHAPTER TWO: LITERATURE REVIEW 13

2.1 Burden and etiology of malaria 13

2.2 *P. falciparum* genome and genetic diversity 14

2.3 *P. falciparum* genetic diversity and MOI in varied malaria transmission settings 15

2.4 *P. falciparum* genetic diversity and MOI in symptomatic versus asymptomatic infections 18

2.5 Temporal changes in *P. falciparum* genetic diversity and MOI 19

2.6 Distinguishing *P. falciparum* recrudescence from reinfection and the algorithms for classification 20

2.7 Molecular tools for genotyping of Plasmodium parasites 22

CHAPTER THREE: MATERIALS AND METHODS 24

3.1 Study designs 24

3.2 Sub-study I: Systematic review and meta-analysis to describe the status of *P. falciparum* genetic diversity and MOI based on *msp-1, msp-2, glurp* and microsatellite genetic markers in SSA (Objective 1) 25

3.3. Sub-study II: Evaluation of *P. falciparum* genetic diversity and MOI in relation to malaria transmission intensity, clinical disease status (symptomatic versus asymptomatic), and temporal trends (Objectives 2, 3, and 4) 29

3.3.1 Populations 32

3.3.2 Selection criteria 32

3.3.3 Sample size determination 33

3.3.4 Study variables 34

3.4 Sub-study III. Evaluation of genotyping algorithms to distinguish recrudescence from reinfection (objective 5) 34

3.4.1 Populations 36

3.4.1.1 Target population 36

3.4.1.2 Accessible population 36

3.4.1.3 Study population 36

3.4.2 Selection criteria 37

3.4.3 Sample size determination 37

3.4.4 Study variables 37

3.5 General laboratory methods for objectives 2–5 38

3.5.1 Determination of parasite density 38

3.5.2 Selection of the samples for molecular analysis 39

3.5.3 DNA extraction 40

3.5.4 Detection and confirmation of *P. falciparum* 41

3.5.5 Microsatellite genotyping 41

3.5.6 Microsatellite analysis 42

3.5.7 *P. falciparum msp-1, msp-2* and *glurp* genotyping 43

3.5.8 Determination of *P. falciparum* genetic diversity 44

3.5.9 Determination of *P. falciparum* MOI 44

3.5.10 *P. falciparum* multi-locus linkage disequilibrium and genetic differentiation analysis 45

3.5.11 *P. falciparum* population cluster analysis 46

3.5.12 Recrudescence and reinfection classification 46

3.6 Statistical analyses 46

3.7 Ethical approval 48

CHAPTER FOUR: RESULTS 49

4.1 Review status of *P. falciparum* genetic diversity and MOI based on *msp-1, msp-2, glurp* and microsatellite genetic markers in SSA 49

4.2 Evaluation of *P. falciparum* genetic diversity and MOI in areas of low, medium, and high transmission intensities in Uganda 57

4.3 Comparison of *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases 62

4.4 Evaluation of temporal changes in *P. falciparum* genetic diversity and MOI across three sites with varying (low, medium and high) malaria transmission intensities 71

4.5 Performance of different genotyping markers and three algorithms (3/3 match-counting, ≥2/3 match-counting, and the Bayesian approach) for distinguishing P. falciparum recrudescence from reinfection 76

CHAPTER FIVE: DISCUSSION 83

5.1 Discussion 83

5.2 Strengths and limitations of the study 90

CHAPTER SIX: CONCLUSIONS, RECOMMENDATIONS AND IMPLICATIONS . 93

6.1 Conclusions and recommendations 93

6.2 Study implications for future research and policy 94

REFERENCES 98

APPENDICES 127

LIST OF TABLES

Table 1. Demographic and clinical characteristics of study participants from Jinja, Kanungu and Tororo included in the analysis of *P. falciparum* genetic diversity and MOI……...55

Table 2. *P. falciparum* genetic diversity among participants from Jinja, Kanungu and Tororo……56

Table 3. *P. falciparum* MOI among participants from Jinja, Kanungu and Tororo……..57

Table 4. Demographic and clinical characteristics of study participants included in the comparison of *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria infections……60

Table 5. The genetic diversity of *P. falciparum* in asymptomatic, symptomatic uncomplicated, and symptomatic complicated individuals……64

Table 6. *P. falciparum* MOI in asymptomatic, symptomatic uncomplicated, and symptomatic complicated individuals…….66

Table 7. Demographic and clinical characteristics of study participants included in the analysis of temporal changes in *P. falciparum* genetic diversity and MOI across areas with low, medium, and high malaria transmission intensities……69

Table 8. *P. falciparum* genetic diversity between 2011-2012 and 2015-2016 across study sites…….70

Table 9. Genetic diversity of the *msp1*, *msp2, glurp* and microsatellite markers a cross Aduku, Arua and Masafu ……..74

LIST OF FIGURES

Figure 1. Conceptual framework showing factors associated with *P. falciparum* genetic diversity and MOI……10

Figure 2. Map showing malaria endemicity of the study sites at the time of sample collection……..28

Figure 3. PRISMA flow diagram for identification and screening of included articles……..47

Figure 4. Forest plot representing the pooled mean expected heterozygosity of *P. falciparum* infection across 17 studies that reported measures of dispersion (CI and SD) for mean expected heterozygosity in malaria-affected countries in SSA, sorted by region……49

Figure 5. Forest plot representing the pooled mean MOI of *P. falciparum* infection across 32 studies that reported measures of dispersion (CI and SD) for mean MOI in malaria-affected countries in SSA, sorted by region……...51

Figure 6. Forest plot representing the pooled prevalence of *P. falciparum* polyclonal infections reported by 48 studies from malaria-affected countries in SSA, sorted by region..53

Figure 7. The Bayesian bar plot illustrates the population structure of *P. falciparum* parasite isolates collected from Jinja, Kanungu and Tororo study sites……59

Figure 8. The allele frequencies of the genotyped markers assessed among individuals with asymptomatic, symptomatic uncomplicated and complicated malaria infections……...61

Figure 9. Changes in *P. falciparum* mean He values between 2011-2012 and 2015-2016 study periods across sites.

Figure 10. Changes in *P. falciparum* mean MOI and the percentage of polyclonal infections between 2011-2012 and 2015-2016 study periods Jinja, Kanungu and Tororo……...72

Figure 11. Distribution of the *P. falciparum* MOI across Aduku, Arua and Masafu study sites……..73

Figure 12. Comparison of the number of recrudescences classified by ≥2/3, 3/3, and Bayesian algorithm……..78

LIST OF APPENDICES

APPENDIX I. Publication from objective 1: To review articles on the status of *P. falciparum* genetic diversity and MOI by examining their geographical distribution, prevalence of polyclonal infections, and associated factors in malaria-endemic regions of SSA.

APPENDIX II. Publication from objective 2: To evaluate *P. falciparum* genetic diversity and MOI across areas with low, medium, and high malaria transmission intensities in Uganda.

APPENDIX III. Publication from objective 3: To compare *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases in Uganda.

APPENDIX IV. Publication from objective 4: To investigate temporal changes in *P. falciparum* genetic diversity and MOI across areas with low, medium, and high malaria transmission intensities in Uganda.

APPENDIX V. Publication from objective 5: To assess the performance of polymorphic molecular markers (microsatellites, *msp-1, msp-2*) and three genotyping algorithms (3/3 match-counting, ≥2/3 match-counting, and a Bayesian approach) in distinguishing between recrudescence and reinfection.

APPENDIX VI. Data abstraction tool

APPENDIX VII. IDRC Administrative clearance

APPENDIX VIII. SOMREC Approval

APPENDIX IX. UNCST Approval

APPENDIX X. Standard Operating Procedures (SOPs)

LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
AL	Artemether/-Lumefantrine
AMOVA	Analysis of Molecular Variance
CDC	Centre for Disease Control
DBS	Dried Blood Spot
DP	Dihydroartemisinin-piperaquine
DRC	Democratic Republic of Congo
EIR	Entomological Inoculation Rate
F_{ST}	Fixation Index
GRADE	Grading of Recommendations, Assessment and Development and Evaluations
GSD	Geometric Standard Deviation
Hb	Hemoglobin
H_e	Expected heterozygosity
HRP2	Histidine Rich Protein 2
I_A^S	Index of Association
IDRC	Infectious Diseases Research Collaboration
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Nets

JBI	Joanna Briggs Institute's
LD	Linkage Disequilibrium
LLINs	Long-Lasting Insecticidal Nets
MOI	Multiplicity of Infection
MSP	Merozoite Surface Protein
N_a	Number of alleles
N_e	Number of effective alleles
NR	Not Reported
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PfEMP1	*P. falciparum* Erythrocyte Membrane Protein 1
PRISM	Programme for Resistance, Immunology and Modelling of Malaria
PRISMA-P	Reporting Items for Systematic Review and Meta-Analysis for Protocols
RFU	Relative Fluorescence Units
RoB	Risk of Bias
SD	Standard Deviation
SSA	Sub Saharan Africa
STREGA	Strengthening the Reporting of Genetic Association Studies
STROBE	Strengthening the Reporting of Observational Studies in Epidemiology

STRs	Short Tandem Repeats
TES	Therapeutic Efficacy Studies
WHO	World Health Organization

OPERATIONAL DEFINITIONS

***P. falciparum* genetic diversity**: The extent of genetic variation within *P. falciparum* populations.

Multiplicity of infection (MOI): The number of distinct parasite clones simultaneously infecting an individual.

Polyclonal infections: The occurrence of multiple distinct parasite genotypes within an individual's single malaria infection episode.

Malaria transmission intensity: The rate at which malaria is spread within a population over a defined period, often measured by metrics like entomological inoculation rate or parasite prevalence.

Genetic recombination: The process by which genetic material is rearranged between different DNA molecules, leading to new combinations of genes that can increase genetic diversity within a population.

Recrudescence: The recurrence of parasites in the bloodstream that originate from those that survived initial treatment, rather than from a new infection.

Areas of varied malaria transmission intensity: Areas with different levels of malaria transmission, including low, medium, and high transmission intensities.

Malaria infection profile: The degree of *P. falciparum* infection, which can be classified into three levels: asymptomatic, symptomatic uncomplicated, and symptomatic complicated.

Microsatellites: Short tandem repeat sequences (STRs) of nucleotides that are abundant in the non-coding regions of the *P. falciparum* genome.

LIST OF PUBLICATIONS FROM THIS PhD PROJECT

1. **Mwesigwa A**, Ocan M, Musinguzi B, Nante RW, Nankabirwa JI, Kiwuwa SM, Kinengyere AA, Castelnuovo B, Karamagi C, Obuku EA, Nsobya SL, Mbulaiteye SM, Byakika-Kibwika P. *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. doi: 10.1186/s12936-024-04925-y.
2. **Mwesigwa A,** Tukwasibwe, S, Cummings, Kawalya H, Kiyaga S, Okoboi S, Castelnuovo B, Bikaitwoha EM, Kalyango JN, Nsobya SL, Karamagi C, Pauline Byakika-Kibwika P, Nankabirwa JI, Genetic diversity and population structure of *Plasmodium falciparum* across areas of varied malaria transmission intensities in Uganda. *Malar J* **24**, 97 (2025). https://doi.org/10.1186/s12936-025-05325-6
3. **Mwesigwa A**, Ocan M, Cummings B, Musinguzi B, Kiyaga S, Kiwuwa SM, Okoboi S, Castelnuovo B, Bikaitwoha EM, Kalyango JN, Karamagi C, Nankabirwa JI, Nsobya SL, Byakika-Kibwika P. *Plasmodium falciparum* genetic diversity and multiplicity of infection among asymptomatic and symptomatic malaria-infected individuals in Uganda. *Trop Med Health*. 2024; 52:86. doi: 10.1186/s41182-024-00656-7.
4. **Mwesigwa A**, Kiwuwa SM, Musinguzi B, Kawalya H, Katumba JD, Baguma A, Mutuku IM, Adebayo IA, Nsobya SL, Byakika-Kibwika P, Kalyango JN, Karamagi C, Nankabirwa JI. Temporal changes in *Plasmodium falciparum* genetic diversity and multiplicity of infection across three areas of varying malaria transmission intensities in Uganda. *Trop Med Health*. 2024; 52:103. doi: 10.1186/s41182-024-00672-7.
5. **Mwesigwa A**, Golumbeanu M, Jones S, Cantoreggi SL, Musinguzi B, Nankabirwa JI, Bikaitwoha EM, Kalyango JN, Karamagi C, Plucinski M, Nsobya SL, Nsanzabana C,

Byakika-Kibwika P. Assessment of different genotyping markers and algorithms for distinguishing *Plasmodium falciparum* recrudescence from reinfection in Uganda. *Sci Rep*. 2025; 15:4375. doi: 10.1038/s41598-025-88892-7.

ORGANISATION OF THE THESIS

This thesis is organised into six chapters

Chapter 1 introduces the research topic, providing background information, a clear statement of the problem, justification for the study, research questions, objectives, hypotheses, and the conceptual framework guiding the investigation. **Chapter 2** presents a comprehensive literature review, covering the global and regional malaria burden, the *Plasmodium falciparum* genome and its genetic diversity, the concept of multiplicity of infection (MOI), and the molecular tools commonly used for *P. falciparum* genotyping. **Chapter 3** describes the general and specific methods employed to address each research objective, including study designs, data sources, laboratory procedures, and analytical approaches. **Chapter 4** presents the results corresponding to each objective. **Chapter 5** discusses the key findings in the context of existing literature, highlighting their significance, and critically examines the study's strengths and limitations. **Chapter 6** concludes the thesis by summarizing the main findings and presenting recommendations for future research and policy, with an emphasis on their implications for malaria control and surveillance.

ABSTRACT

Background: Despite renewed control efforts, malaria remains a major public health problem, contributing significantly to morbidity and mortality, especially in endemic countries. Effective control is hindered by several factors, including the high genetic diversity of *Plasmodium falciparum*, the presence of multiple parasite clones within a single host (referred to as multiplicity of infection (MOI)), and the persistence of parasite strains despite treatment (recrudescence). This PhD study aimed to assess *P. falciparum* genetic diversity and MOI with in sub-Saharan Africa (SSA), with a specific focus on areas of varying malaria transmission intensity in Uganda. Additionally, we evaluated the performance of different microsatellite markers and genotyping algorithms in distinguishing recrudescence from reinfection, an essential component of antimalarial drug efficacy monitoring.

Methods: This study employed a combination of systematic review, meta-analysis, and secondary data analysis using both cross-sectional and longitudinal studies. To evaluate *P. falciparum* genetic diversity and MOI across different geographical regions in SSA (Objective 1), I conducted a systematic review and meta-analysis of published articles. The review focused on studies using common molecular markers, including genes of merozoite surface protein 1 and 2 (*msp-1, msp-2*), and microsatellites. The review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. To determine how *P. falciparum* genetic diversity and MOI vary by malaria transmission intensity, clinical disease status (asymptomatic versus symptomatic), and over time (Objectives 2, 3, and 4), I performed secondary analyses of data and dried blood spot (DBS) filter paper samples from comprehensive surveillance studies conducted between 2011 and 2017 in areas of varying transmission intensities in Uganda. To assess the performance of microsatellite markers and three genotyping algorithms (3/3 match-counting, ≥2/3 match-

counting, and the Bayesian approach) in distinguishing between recrudescence and reinfection (Objective 5), I analyzed data and DBS filter paper samples from a therapeutic efficacy study (TES) conducted between September 2018 and February 2019 at three health centers located in high malaria transmission regions of Uganda. Demographic and clinical data were retrieved from primary study databases, while genetic data were generated through genotyping of *P. falciparum* isolates using the highly polymorphic markers *msp-1*, *msp-2*, and microsatellites. Genomic DNA was extracted from DBS samples using Chelex 100 Resin (Sigma-Aldrich, USA). Genotyping of *msp-1, msp-2*, and *glurp* was performed using nested PCR, with amplicon sizing on the QIAxcel Advanced System (QIAGEN). For microsatellites, seven loci (TA1, Poly-α, PfPK2, TA109, 2490, C2M34–313, and C3M69–383) were amplified using fluorescently labeled primers, and allele sizing was conducted via high-resolution capillary electrophoresis on an ABI 3730 Genetic Analyzer. *P. falciparum* genetic diversity was assessed by calculating allele count, effective allele number, allelic richness, and expected heterozygosity (H_e). *P. falciparum* MOI was estimated by determining the number of distinct genotypes per infection. Population structure was assessed using multilocus linkage disequilibrium (LD), genetic differentiation, analysis of molecular variance (AMOVA), and clustering analysis. Statistical comparisons were made using the Kruskal–Wallis test, Chi-square test, or Fisher's exact test, as appropriate. Temporal trends were analyzed using the Jonckheere–Terpstra test, while genotyping algorithm performance was assessed using the Wilcoxon signed-rank test. For all analyses, 95% confidence intervals and/or standard deviations were reported where applicable, with statistical significance set at $p < 0.05$.

Results: The systematic review and meta-analysis showed significant regional variation in *P. falciparum* genetic diversity and MOI across SSA. The pooled mean H_e was 0.65 (95% CI:

0.51–0.78), and the pooled mean MOI was 2.09 (CI: 1.88–2.30), with 63% (CI: 56–70%) of cases showing polyclonal infections. When *P. falciparum* genetic diversity was compared between areas with different malaria transmission intensities in Uganda, no statistically significant variation was observed across sites ($p > 0.05$), though Kanungu (a low transmission site) showed the highest mean H_e (0.81 ± 0.14), while the mean H_e was slightly lower and similar in both Jinja and Tororo (both 0.78 ± 0.16). Regarding MOI, Tororo, a high-transmission site, exhibited a significantly higher mean MOI (2.5 ± 0.5) and polyclonal infection rate (70%) compared to Kanungu (MOI: 2.0 ± 0.05; polyclonal rate: 56%) and Jinja (MOI: 1.9 ± 0.3; polyclonal rate: 58%) ($p = 0.004$). Genetic structure analysis indicated minimal differentiation (F_{ST}: 0.011–0.021). When comparing *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic participants, both groups exhibited high genetic diversity (H_e 0.79–0.81). Polyclonal infections were common in both groups (58.5% in symptomatic and 63% in asymptomatic). Asymptomatic participants had a higher mean MOI (2.10) than symptomatic individuals (1.92), though the difference was not statistically significant ($p = 0.33$). Temporal analyses showed a slight decline in H_e from 0.79 to 0.76, with polyclonal infections remaining above 50% throughout the period. Finally, the 3/3 match-counting algorithm identified significantly fewer recrudescence cases compared to the ≥2/3 match-counting and Bayesian algorithms ($p < 0.001$).

Conclusions: This study shows significant regional variation in *P. falciparum* genetic diversity and MOI within SSA. within Uganda, genetic diversity of *P. falciparum* did not differ significantly across sites, although MOI varied significantly by transmission intensity. Persistently high genetic diversity, minimal population differentiation, and sustained polyclonal infections were observed across clinical disease status and time, reflecting ongoing transmission pressures rather than uniform regional variation. Discrepancies in

recrudescence classification between match-counting and Bayesian algorithms highlight the need for standardized PCR correction practices. These findings underscore the need for tailored malaria control strategies, including targeted interventions for asymptomatic carriers and continuous molecular surveillance to adapt to evolving transmission dynamics. Furthermore, the study emphasizes the importance of using robust analytical algorithms to accurately distinguish recrudescence from reinfection, an essential component of effective antimalarial drug efficacy monitoring.

CHAPTER ONE: INTRODUCTION

1.1 Background

Malaria remains a significant global health challenge. In 2023 alone, an estimated 263 million cases were reported worldwide, with 246 million occurring in Sub-Saharan Africa (SSA). The region also accounted for approximately 569,000 of the 597,000 global malaria-related deaths (1). *Plasmodium falciparum* is the predominant parasite species in SSA, responsible for over 90% of infections and associated with the most severe and treatment-resistant forms of the disease (1). Its transmission is sustained by highly efficient mosquito vectors, particularly female *Anopheles gambiae* and *Anopheles funestus*, which are well adapted to human environments and capable of maintaining intense transmission cycles (2, 3).

Uganda ranks third globally in malaria burden, contributing 4.8% of global cases and recording the highest incidence in East and Southern Africa (1). Despite intensified malaria control efforts, malaria is the leading cause of morbidity and mortality in Uganda, accounting for roughly 40% of outpatient visits, 25% of hospital admissions, and 14% of inpatient deaths (4). However, efforts to control malaria are increasingly challenged by the widespread resistance to insecticides especially pyrethroids used in long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) (5), and the emergence of resistance to artemisinin-based combination therapies (ACTs), which threatens recent gains in malaria prevention and control (6).

Adding to these challenges is the remarkable genetic diversity of *P. falciparum*, which allows the parasite to evade host immune responses and rapidly adapt to control interventions (7). The *P. falciparum* genome spans approximately 23 megabases (Mb) across 14 chromosomes

(8) and encodes several multigene families including *var*, *rifin*, and *stevor*, that contribute to antigenic variation and immune evasion (8, 9). A key example is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), is a highly polymorphic protein that enables infected red blood cells to modify their surface antigens, helping the parasite avoid immune detection (10).

This extensive antigenic diversity is primarily generated through sexual recombination in the mosquito vector, and further amplified by gene mutations and epigenetic mechanisms (11-14). These processes contribute to high allelic polymorphism and low linkage disequilibrium (LD), enabling the rapid spread of beneficial mutations, including those conferring drug resistance (9, 15, 16). Therefore, understanding the population structure and genetic diversity of *P. falciparum* is critical for guiding malaria surveillance, resistance monitoring, and vaccine development.

In high malaria transmission regions of SSA, including Uganda, *P. falciparum* infections are frequently polyclonal, meaning a single host can harbor multiple genetically distinct parasite strains (17-20). This phenomenon is referred to as multiplicity of infection (MOI), which indicates the number of unique parasite clones present within an individual (21, 22). *P. falciparum* MOI can arise through superinfection, when a person receives multiple infective mosquito bites, each introducing different strains or through co-transmission, where a single mosquito introduces several but unique parasite strains in one bite (23-25). High MOI is associated with increased parasite virulence, disease severity, and a higher likelihood of antimalarial drug resistance emergence (20, 26-28). Additionally, high MOI elevates the risk of recrudescence (29) which can go undetected and perpetuate transmission (30). MOI and parasite genetic diversity are therefore useful descriptive metrics of transmission intensity,

providing insight into parasite population dynamics across different regions and time periods without implying a direct measurement of intervention impact.

Parasite genetic diversity and MOI are both shaped by evolutionary forces including mutation, natural selection, genetic drift, and gene flow (31). These parameters vary across geographic regions, seasons, and host immunity profiles (32). Tracking these dynamics provides critical insights into whether malaria transmission is driven by local parasite lineages or the introduction of new strains (33-35), which is essential for designing localized and effective intervention strategies (36-38).

In resource-constrained settings such as Uganda, *P. falciparum* genotyping has traditionally relied on antigen-coding molecular markers like *msp-1*, *msp-2*, and *glurp* (39, 40). WHO has suggested replacing *glurp* with neutral microsatellite markers in TES to improve genotyping accuracy in high-transmission settings. While widely used, these markers are subject to immune selection, potentially limiting their effectiveness in capturing the full extent of parasite genetic diversity (41). In contrast, neutral microsatellite markers such as Poly-α, TA1, TA109, and PfPK2 are not influenced by host immunity and provide a more feasible and relatively cost-effective approach for assessing genetic variation in resource-limited settings, considering the required laboratory infrastructure, technical expertise, and processing time (15, 42, 43). Their widespread distribution across the genome provides deeper insight into parasite population structure (44). Although high-resolution tools like whole-genome sequencing offer comprehensive data, their use in SSA is limited by cost and infrastructure requirements (45).

Despite the recognized importance of parasite genetic diversity in malaria control, most previous studies in SSA and Uganda have employed cross-sectional designs or limited markers, often focusing on symptomatic children (46, 47). While single cross-sectional studies provide important snapshots of parasite population structure, they may not adequately

capture temporal changes in genetic diversity and MOI. By incorporating repeated time points, this study allows assessment of temporal dynamics, improving the relevance of findings for surveillance and national malaria control program monitoring. This current study expands on these by including both symptomatic and asymptomatic individuals across regions with different transmission intensities and over two distinct time periods, allowing more current and descriptive estimates of MOI and genetic diversity. Additionally, while therapeutic efficacy studies (TES) have historically relied on *msp-1*, *msp-2*, and *glurp* to distinguish recrudescence from reinfection, *glurp* has been suggested by WHO to be replaced with microsatellites for higher accuracy, and this study focused on MSP markers and neutral microsatellites**.** The study also evaluated the performance of microsatellite markers alongside *msp-1* and *msp-2* genes and three genotyping algorithms for differentiating recrudescence from reinfection. The findings provided important insights into parasite gene flow, contributing to more targeted and effective malaria surveillance, control, and intervention strategies.

1.2 Statement of the problem

Despite global efforts, including the WHO's goal of achieving malaria elimination by 2030, *P. falciparum* malaria remains a critical public health threat in Uganda. It accounts for approximately 40% of outpatient visits, 25% of hospital admissions, and 14% of hospital deaths (4). In malaria endemic regions, infections are characterized by high genetic diversity and MOI, with parasite strains varying across individuals, populations, transmission settings, and over time (48, 49). While high genetic diversity and MOI have been associated with increased virulence and disease severity in previous studies (26, 47), the findings of this study indicated no significant difference in MOI and genetic diversity between symptomatic and

asymptomatic individuals, suggesting that high diversity alone may not directly translate to increased clinical severity in the study populations.

The ongoing evolution of *P. falciparum* strains is particularly concerning in high-transmission settings like Uganda, where numerous strains co-circulate. Infection transmission dynamics are influenced by host immunity, demographic factors, and asymptomatic carriers (50, 51). Asymptomatic infections, which often go undetected, serve as reservoirs for ongoing transmission, highlighting the public health significance of MOI and genetic diversity beyond clinical disease outcomes. Environmental factors, such as seasonal rainfall and temperature variations, affect mosquito breeding and vector density, further driving transmission. Human behavior, such as inconsistent use of preventive tools, limited access to care, and delayed treatment, also plays a significant role in disease persistence (52, 53).

Despite the heavy burden of malaria in SSA, comprehensive data on the genetic diversity and MOI of *P. falciparum* remain limited, particularly in capturing temporal dynamics, asymptomatic carriers, and variation across different transmission settings in Uganda. Although previous studies have reported parasite diversity and MOI across wide geographic areas using microsatellite markers, many were cross-sectional, focused on symptomatic individuals, or used a limited set of markers, limiting generalizability and the ability to monitor changes over time**.** There is also a critical gap in understanding the performance of different genetic markers and algorithms in distinguishing recrudescence from reinfection, particularly in high malaria transmission areas, where accurate differentiation is vital for effective treatment and control.

Therefore, the focus of this thesis was to assess the status and temporal dynamics of *P. falciparum* genetic diversity and MOI within SSA, with a particular focus on areas with

varying malaria transmission intensities in Uganda. It also assessed the performance of different genetic markers and algorithms in distinguishing recrudescence from reinfection, which is essential for evaluating treatment outcomes and guiding targeted malaria control strategies.

1.3 Justification of the study

Uganda's Malaria Control and Elimination Strategic Plan (2021–2025) aims to reduce malaria morbidity by 50% and mortality by 75% by 2025. Achieving these targets requires enhanced surveillance, stratified interventions, and a deeper understanding of parasite dynamics. Despite these efforts, Uganda remains one of the countries bearing a disproportionately high malaria burden within SSA, with *P. falciparum* accounting for over 90% of the cases (54).

In areas of intense transmission, infections often consist of multiple and genetically diverse *P. falciparum* strains (17, 55, 56). This diversity is closely linked to transmission intensity, treatment outcomes, and the emergence and spread of drug resistance. However, data on parasite genetic diversity and MOI across different transmission settings within Uganda remain limited, particularly regarding temporal dynamics and asymptomatic infections. The parasite genetic diversity and MOI differences between asymptomatic and symptomatic infections also remain incompletely understood. Moreover, few studies have explored temporal changes in parasite diversity and MOI over time within the same settings, limiting the ability to assess the sustained impact of malaria control measures.

There is ongoing global debate regarding the limitations of antigen-based markers such as MSP1, MSP2, and glurp in distinguishing recrudescence from reinfection due to strong immune selection acting on these loci. While WHO continues to recommend the use of

MSP1, MSP2, and at least one highly polymorphic microsatellite marker (such as Poly-α) for molecular correction in therapeutic efficacy studies (TES), concerns remain regarding their discriminatory power in high-transmission settings. Although advanced methods such as amplicon deep sequencing and whole-genome sequencing offer higher resolution, their application in this study was constrained by cost, infrastructure requirements, bioinformatics capacity, and feasibility within routine programmatic and resource-limited settings. Therefore, this study utilized a combination of MSP markers and neutral microsatellites, together with multiple genotyping algorithms, to enhance discriminatory accuracy and address known limitations of individual markers. Furthermore, the study evaluated the performance of different markers and analytical approaches to strengthen molecular correction strategies in high-transmission contexts.

1.4 Significance of the study

This study contributes significantly to understanding *P. falciparum* genetic diversity and MOI as indicators of malaria transmission dynamics and control effectiveness within Uganda. By analyzing parasite populations across varying epidemiological settings and over time, it underscores the importance of robust, context-appropriate genotyping tools and analytical approaches.

By comparatively evaluating MSP markers, neutral microsatellites, and different genotyping algorithms, the study provides evidence to inform optimization of molecular correction methods within the framework currently recommended by WHO, while acknowledging the evolving landscape toward more advanced sequencing technologies. The application of improved molecular correction strategies, including Bayesian analytical approaches, enhanced drug efficacy assessments and supported evidence-based policy-making.

Furthermore, the findings have informed the identification of malaria hotspots, clarified parasite gene flow patterns, and supported targeted, context-specific interventions. While the study does not directly identify malaria hotspots, it offers valuable information on parasite diversity that can inform future hotspot mapping and surveillance strategies. Ultimately, the insights gained from this research strengthen malaria surveillance systems, improve interpretation of treatment outcomes, and support Uganda's malaria control and elimination efforts, while contributing to broader discussions on genotyping strategies in high-burden, resource-limited settings.

1.5 Research questions

1. What is the current status of *P. falciparum* genetic diversity and MOI in malaria-endemic regions of SSA?

2. How do *P. falciparum* genetic diversity and MOI differ in regions with varying malaria transmission intensities in Uganda?

3. What are the differences in *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases in Uganda?

4. What are the temporal changes in *P. falciparum* genetic diversity and MOI among individuals with malaria infection living in areas of varied malaria transmission intensity in Uganda?

5. How effective are polymorphic molecular markers (*msp-1*, *msp-2*, and microsatellites) in distinguishing between recrudescence and reinfection, and how do the 3/3 match-counting, ≥2/3 match-counting, and Bayesian genotyping algorithms compare in classifying recurrent *P. falciparum* infections?

1.6 Study objectives

1.6.1 General objective

To assess the status of *P. falciparum* genetic diversity and multiplicity of infection within sub–Saharan Africa, with a specific focus on areas with varying malaria transmission intensities in Uganda and the performance of different genetic markers and algorithms in distinguishing recrudescence from reinfection.

1.6.2 Specific objectives

1. To review articles on the status of *P. falciparum* genetic diversity and multiplicity of infection by examining their geographical distribution, prevalence of polyclonal infections, and associated factors in malaria-endemic regions of SSA.
2. To evaluate *P. falciparum* genetic diversity and multiplicity of infection across areas with low, medium, and high malaria transmission intensities in Uganda.
3. To compare *P. falciparum* genetic diversity and multiplicity of infection between asymptomatic and symptomatic malaria cases in Uganda.
4. To investigate temporal changes in *P. falciparum* genetic diversity and multiplicity of infection across areas with low, medium, and high malaria transmission intensities in Uganda.
5. To assess the performance of polymorphic molecular markers (microsatellites, *msp-1, msp-2*) and three genotyping algorithms (3/3 match-counting, ≥2/3 match-counting, and a Bayesian approach) in distinguishing between recrudescence and reinfection.

1.7 Study hypotheses

1. *P. falciparum* genetic diversity and multiplicity of infection vary significantly with geographical region, clinical presentation, and transmission intensity.

2. *P. falciparum* genetic diversity and multiplicity of infection differ according to malaria transmission settings in Uganda.

3. *P. falciparum* genetic diversity and multiplicity of infection differ between asymptomatic and symptomatic malaria cases in Uganda.

4. Temporal changes in transmission intensity are associated with shifts in *P. falciparum* genetic diversity and multiplicity of infection.

5. Microsatellites and the genes *msp1* and *glurp* differ in their performance in distinguishing recrudescence from reinfection, and Bayesian algorithm classifies *P. falciparum* recrudescence more accurately than the multiplicity of infection match-counting algorithm.

1.8 Conceptual frame work

This conceptual framework illustrates the interrelationship between *P. falciparum*, the human host, mosquito vectors, environmental conditions, and intervention-related factors in shaping parasite genetic diversity and MOI. Intervention exposures (e.g., ACT use, vector control, surveillance strength, and drug pressure), vector characteristics (density, species composition, insecticide resistance, and feeding behavior), environmental conditions (temperature, rainfall, urbanization, altitude, and ecological zones), and human/host factors (immune status, symptomatic versus asymptomatic infections, LLIN use, adherence to medication, and mobility) collectively influence malaria transmission intensity (low, medium, or high).

Transmission intensity in turn drives parasite genetic diversity and MOI. High MOI and parasite diversity, fueled by frequent recombination, sustained transmission, asymptomatic carriage, and environmental and behavioral factors, may contribute to treatment failure, emergence of drug-resistant strains, misclassification of recrudescence and reinfection, and persistent transmission. Inadequate surveillance systems can further exacerbate these challenges.

Understanding these interconnected dynamics is essential for strengthening malaria surveillance, improving molecular correction strategies, guiding targeted and stratified interventions, and supporting malaria control and elimination efforts, particularly in high-burden, resource-limited settings such as Uganda and the broader sub-Saharan Africa region (Figure 1).

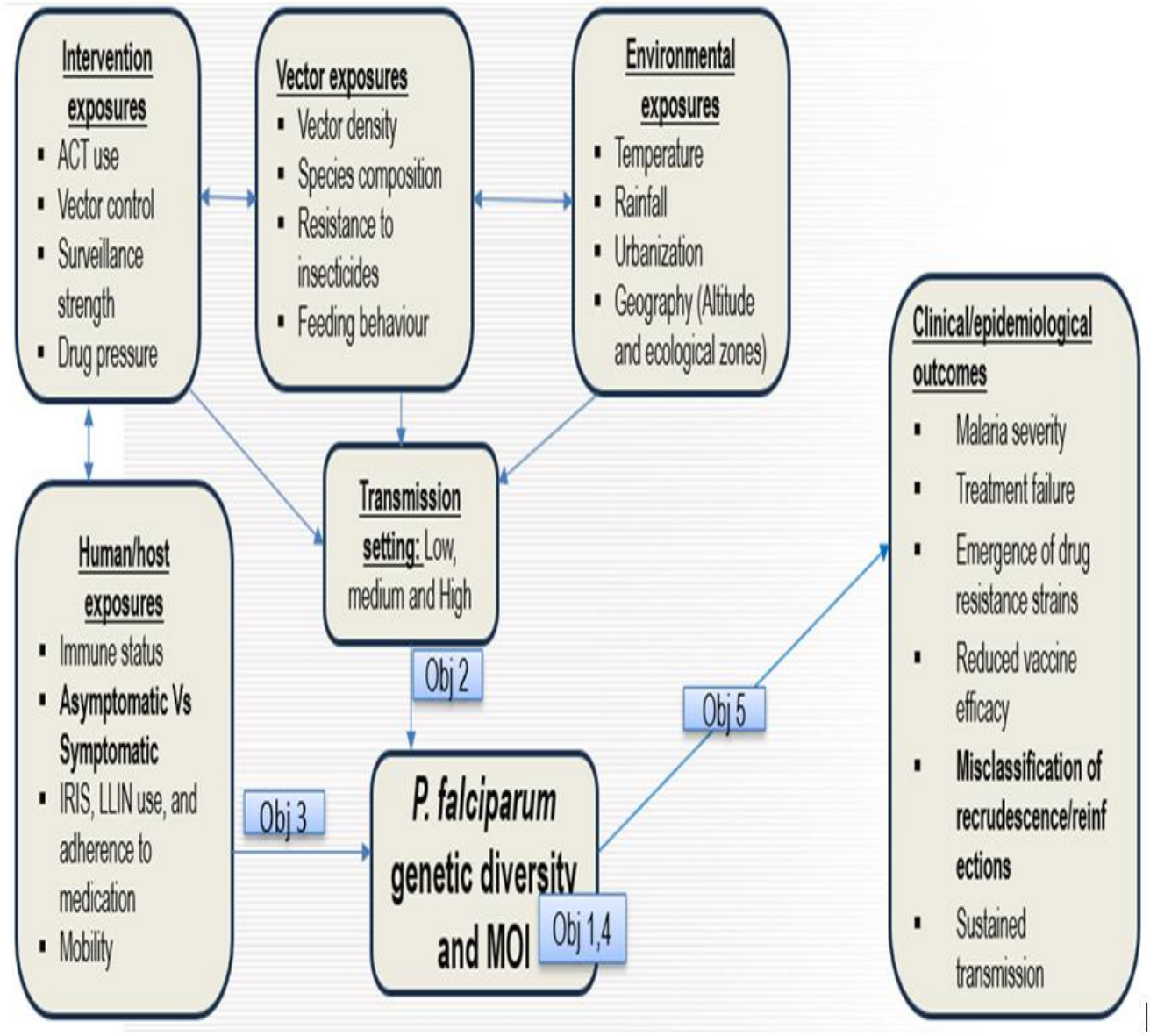


Figure 1. Conceptual framework illustrating the interrelationships between intervention, vector, environmental, and human/host exposures and their influence on malaria transmission setting, P. falciparum genetic diversity, and MOI, and subsequent clinical and epidemiological outcomes.

CHAPTER TWO: LITERATURE REVIEW

2.1 Burden and etiology of malaria

Malaria continues to pose a significant public health challenge in Uganda, primarily due to its high transmission intensity and widespread geographic distribution. Over 90% of malaria cases are caused by *P. falciparum* (54), the most virulent and life-threatening of the five *Plasmodium* species (*P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale*, and *P. knowlesi)* known to infect humans (57). Transmission is perennial but peaks during the two rainy seasons: March to May and September to November when mosquito breeding intensifies (58). The primary vectors are female *Anopheles* mosquitoes, with *An. gambiae* being the most dominant species, followed by *An. funestus* and *An. arabiensis* (59).

Malaria control strategies in Uganda align with those implemented across SSA and include the use of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), intermittent preventive treatment in pregnancy (IPTp), and prompt treatment with artemisinin based combination therapies (ACTs) (60). However, these efforts are increasingly compromised by the spread of insecticide-resistant mosquito vectors, particularly to pyrethroids (5) and the emergence of drug resistance in *P. falciparum* (6).

Plasmodium species have a complex life cycle involving two hosts: humans (intermediate hosts) and female *Anopheles* mosquitoes (definitive hosts). The cycle begins when an infected mosquito bites a human and releases sporozoites into the bloodstream via its saliva (61). The sporozoites travel to the liver, invade hepatocytes, and undergo asexual multiplication through schizogony, developing into hepatic schizonts. The schizonts rupture, releasing merozoites into the hepatic veins (62, 63). Merozoites invade red blood cells (erythrocytes), replicate asexually, and develop into erythrocytic schizonts. Schizonts eventually rupture, releasing more merozoites that infect additional erythrocytes (62). A

subset of merozoites differentiates into dormant sexual forms of male and female gametocytes.

The *P. falciparum* gametocyte development progresses through five stages. Early-stage gametocytes typically sequester in the bone marrow, spleen, and other organs to evade immune clearance (64, 65). The mature gametocyte infected erythrocytes return to peripheral circulation, where they can be taken up by a mosquito or destroyed by the host immunity (65, 66). During a blood meal, female *Anopheles* mosquitoes ingest gametocytes concentrated in skin capillaries (67). In the inside the mosquito, gametocytes exit erythrocytes; male gametes fertilize female gametes, forming diploid zygotes (50). The zygotes undergo meiosis to form motile ookinetes, which penetrate the mosquito's midgut epithelium and develop into oocysts. The oocysts mature into sporoblasts, producing sporozoites through budding, which migrate to the mosquito's salivary glands, becoming ready for transmission during the next blood meal. Mosquitoes become infective 7 to 10 days after ingesting gametocytes (67).

The *P. falciparum* exhibits considerable genetic diversity, shaped by sexual recombination within the mosquito vector. This diversity contributes to complex infection dynamics in endemic regions. The Multiplicity of infection (MOI), which is the number of genetically distinct parasite clones within a single host reflects transmission intensity, host immunity, and vector density (68, 69). In SSA, high MOI and genetic complexity are typically observed in high-transmission areas, while in low transmission areas tend to show lower MOI (70). This variability highlights the need for region specific approaches to parasite population monitoring and malaria control.

2.2 *P. falciparum* genome and genetic diversity

The genome of *P. falciparum*, spanning approximately 23 megabases (Mb) across 14 chromosomes, encodes numerous genes essential for host–parasite interactions (8). A key

example is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a highly polymorphic surface antigen that enables the parasite to alter the surface properties of infected red blood cells, thereby evading host immune responses (10). In addition, the genome contains several multigene families such as *var*, *rif*, and *stevor* which play crucial roles in red blood cell invasion and immune modulation (8, 9). These genes encode hypervariable antigens and virulence factors that are exported to the surface of infected erythrocytes and serve as targets for naturally acquired immunity (8, 71). This extensive genetic variation allows *P. falciparum* to adapt rapidly to host immunity and environmental pressures, contributing to its persistence and potential resistance to treatment.

The high genetic diversity observed in *P. falciparum* is primarily driven by sexual recombination in the mosquito vector and spontaneous mutations (72, 73), leading to extensive allelic polymorphism. Antigenic variation is further enhanced by recombination and epigenetic mechanisms during the parasite's asexual stages in the human host (11-14). Frequent recombination results in low linkage disequilibrium (LD), the non-random association of alleles at different loci which is characteristic of *P. falciparum* populations (16). This low LD reflects independent assortment of alleles, enabling the rapid spread of beneficial traits such as drug resistance (9, 15, 16). Monitoring LD and genetic diversity is therefore critical for understanding parasite population dynamics and informing malaria surveillance, resistance monitoring, and control strategies.

2.3 *P. falciparum* genetic diversity and MOI in varied malaria transmission settings

The diversity of *P. falciparum* strains complicates malaria control by increasing parasite virulence and disease severity (20, 26, 27). Additionally, parasite genetic diversity and MOI, shaped by genetic drift, mutation, natural selection, and migration influence the effectiveness

of control measures and the spread of *P. falciparum* (31, 74). Monitoring parasite genetic diversity is therefore essential for assessing malaria transmission dynamics, understanding population connectivity, and evaluating the impact of interventions (74-77). It also provides critical insights into malaria epidemiology, which are vital for informing global elimination strategies (19, 78, 79). Although these associations are well established, many previous studies were cross-sectional and facility-based, often limited to symptomatic infections, with limited integration of asymptomatic reservoirs and temporal analyses. Differences in laboratory markers, sampling strategies, and analytical approaches have also limited comparability across studies and settings.

P. falciparum genetic diversity varies across individuals, transmission settings, and seasons. High-transmission areas typically harbor *P. falciparum* populations with greater genetic diversity and low population differentiation, whereas low-transmission areas tend to exhibit lower diversity (77, 80, 81). High transmission intensity promotes frequent outcrossing and genetic recombination, leading to increased diversity (82). Consequently, individuals in such regions are often infected with multiple genetically distinct parasite clones (83, 84). In contrast, monoclonal infections are more common in low-transmission settings (91, 92). Efforts to suppress *P. falciparum* transmission, such as in Ethiopia (85) and Senegal (86), have resulted in reduced parasite genetic diversity. However, exceptions exist, for example, Eswatini, despite being a low-transmission setting, shows high genetic diversity due to the frequent importation of infections (87). Similarly, some studies have found no significant differences in genetic diversity between high and low malaria transmission settings (88). Peak malaria transmission typically occurs shortly after the rainy season, rather than during the rains themselves, which influences the temporal patterns of genetic diversity and MOI.

A positive correlation between parasite density and both *P. falciparum* genetic diversity and MOI has been observed in Rwanda and Ethiopia (89, 90). In contrast, low-density infections in Ethiopia tend to have reduced genetic diversity and MOI (91). However, other studies in Ethiopia have reported no clear relationship between parasite density and MOI (14). Human movement also plays a key role in shaping MOI. In Nigeria, areas with significant migration between regions of varying transmission intensity exhibit higher MOI (92). Such movement facilitates the introduction of new parasite strains (87, 93, 94) and contributes to low genetic differentiation among populations (93). Age-related patterns in genetic diversity and MOI are inconsistent across regions. Some studies have reported a positive correlation between age and genetic diversity in Equatorial Guinea (91), while others in Ethiopia and Côte d'Ivoire have found no such association (14, 90, 95). Generally, MOI tends to be higher in children in high-transmission settings and declines with age, as seen in Ghana (96). However, this trend is not universally observed.

Malaria control interventions such as insecticide-treated nets (ITNs), indoor residual spraying (IRS), and antimalarial drug use have been shown to reduce transmission and, consequently, genetic diversity in several settings, including Senegal and the Thai–Burma border (regions of Thailand and Myanmar) (97, 98). Nonetheless, some studies, such as those conducted in Eswatini, report high MOI even after a substantial decline in transmission (87).

Transmission intensity directly influences genetic diversity. In high-transmission areas, *P. falciparum* populations exhibit greater allelic richness and diversity due to larger effective population sizes and frequent recombination (15). Uganda provides a unique setting for such studies due to its diverse transmission profiles, ranging from holoendemic regions in the east to hypoendemic areas in the southwest (99-101). Empirical studies in Uganda have confirmed a positive correlation between transmission intensity and MOI, mirroring patterns observed

elsewhere in SSA (102). However, previous Ugandan studies have not consistently evaluated symptomatic and asymptomatic infections, temporal shifts in parasite populations, and comparative genotyping performance within a unified framework. This thesis addresses these gaps by integrating multi-setting comparisons, inclusion of asymptomatic infections, and temporal assessment using standardized molecular approaches.

2.4 *P. falciparum* genetic diversity and MOI in symptomatic versus asymptomatic infections

P. falciparum infection ranges from asymptomatic parasitemia to severe disease (103, 104). These outcomes are influenced by factors such as host immunity, transmission intensity, and parasite genetic diversity (105-107). The genetic profile of *P. falciparum* associated with clinical outcomes can inform treatment strategies and improve understanding of why some patients develop severe disease while others remain asymptomatic (108). Multiple *P. falciparum* genotypes often result in higher MOI, which may increase proinflammatory cytokine release contributing to severe malaria (109, 110). However, immunity to specific clones can protect against clinical malaria (111). Previous exposure to similar strains may provide immunity, reducing the risk of severe malaria (112, 113). In contrast, infection with novel strains increases the likelihood of symptomatic disease (114).

Studies have indicated that genetic diversity and MOI of *P. falciparum* are correlated with malaria severity and are greater in symptomatic infections (91, 115) than in asymptomatic infections (116). However, other studies have reported high genetic diversity and MOI in both asymptomatic and symptomatic malaria infections (18, 117-119), although some have found no significant differences (120). These inconsistencies may reflect variation in study design, age structure of study populations, transmission intensity, and genotyping methods used.

Furthermore, many studies have not systematically incorporated asymptomatic carriers in community-based surveys alongside clinical cases, limiting comprehensive comparison of parasite population structure by clinical status.

High *P. falciparum* genetic diversity in asymptomatic malaria poses challenges for parasite elimination, as asymptomatic infections are often untreated and serve as reservoirs of transmission (121). This persistence is due to acquired immunity to genetically similar infections (114). Asymptomatic carriers often harbor long-term polyclonal infections, contributing silently to transmission and parasite diversity (122). Symptomatic cases, on the other hand, may be dominated by a single or a few clones, often those capable of evading host immunity (123). Several studies have reported significantly higher MOI in asymptomatic individuals compared to symptomatic ones, especially among older children and adults in endemic areas (124, 125). These findings highlight the hidden role of asymptomatic infections in maintaining parasite diversity.

2.5 Temporal changes in *P. falciparum* genetic diversity and MOI

Temporal variations in *P. falciparum* genetic diversity and MOI often reflect changes in transmission resulting from control interventions such as insecticide-treated nets and indoor residual spraying. Reductions in transmission intensity are typically associated with declines in MOI and allelic richness (126) while resurgence can lead to renewed genetic complexity (127). *P. falciparum* genetic diversity is maintained by large parasite populations over time (82). Both genetic diversity and MOI are valuable indicators for tracking changes in malaria transmission intensity and assessing the effectiveness of interventions (98, 128, 129). Studies show that *P. falciparum* genetic diversity varies based on individual host factors, population characteristics, transmission settings, and parasite prevalence (17, 48, 130, 131). The MOI, in particular, has been found to decrease over time as malaria transmission intensity declines

(33). However, longitudinal evaluations within the same geographic settings using comparable molecular tools remain limited, particularly in Uganda, constraining the ability to directly link intervention scale-up to sustained changes in parasite population structure.

In regions of high malaria transmission, such as Kenya, high *P. falciparum* genetic diversity (H_e ~ 0.78) has been consistently observed (132). Conversely, countries like the Kingdom of Eswatini, with reduced malaria transmission, also report high genetic diversity, largely due to the importation of parasites from neighboring countries with higher transmission (133). Additionally, in Grande Comore Island, a significant decrease in *P. falciparum* MOI was observed following the introduction of artemisinin-based combination therapy (ACT) (134). However, studies from the Democratic Republic of the Congo (DRC) (135), and Kilifi County, Kenya (136), did not report a temporal decline in MOI, despite a marked decrease in malaria positivity rates. This inconsistency underscores the complexity of transmission dynamics, especially in regions like Uganda, where transmission intensity varies considerably (137). Although longitudinal studies in Uganda remain limited, regional data suggest that control efforts can shift parasite population structures over time, affecting diagnostics, drug efficacy, and vaccine performance (128).

2.6 Distinguishing *P. falciparum* recrudescence from reinfection and the algorithms for classification

Artemether-lumefantrine (AL) is the first-line treatment for uncomplicated malaria, with artemisinin rapidly reducing parasite density and lumefantrine preventing recrudescence (138). However, the growing drug resistance, particularly in *P. falciparum* poses a major threat to malaria control efforts, highlighting the need for continuous monitoring of treatment efficacy (139, 140). In response, the WHO recommends conducting therapeutic efficacy studies (TES) every two years to monitor the effectiveness of antimalarial treatments (141, 142). A central challenge in TES is accurately distinguishing recrudescence from reinfection

(142-144). This process, known as molecular correction, is essential for correctly interpreting treatment outcomes. WHO recommends genotyping using highly polymorphic markers such as *msp-1, msp-2,* and *glurp* to differentiate between recrudescence and reinfection (145). However, increasing evidence indicates limitations of antigen-based markers, particularly glurp, due to strong immune selection and reduced allelic diversity in high-transmission settings, potentially leading to misclassification of treatment outcomes. This has contributed to global debate and revised WHO guidance recommending replacement of *glurp* with at least one highly polymorphic microsatellite marker such as Poly-α (146), which offers improved discriminatory power (147). Although amplicon deep sequencing and whole-genome sequencing provide higher resolution and improved detection of minority clones, their routine application remains limited in many endemic settings due to cost, infrastructure, and bioinformatics requirements. Therefore, this study combined MSP markers with neutral microsatellites and applied multiple classification algorithms, including Bayesian probabilistic models (148), to enhance molecular correction accuracy while maintaining feasibility within routine surveillance frameworks in Uganda.

In addition to molecular markers choice, the classification algorithm plays a key role in molecular correction. The WHO recommends the 3/3 match-counting algorithm, which defines recrudescence as the recurrence of infections with identical alleles at all three genotyping loci on both day 0 and day of failure sampling days (146), While this stricter approach increases specificity, it may misclassify true recrudescences as reinfections, particularly in high-transmission areas where genetic diversity and multiple clones are common. To address these limitations, Bayesian statistical models have been introduced as more robust alternatives (148). These models incorporate allele frequencies and MOI to calculate the probability of a recurrence being a recrudescence versus a reinfection. This

probabilistic approach enhances accuracy, particularly in settings with complex transmission patterns.

2.7 Molecular tools for genotyping of *Plasmodium* parasites

Genotyping techniques are essential for differentiating between parasite clones, offering valuable insights into the population genetics of *Plasmodium* parasites, and assessing the effectiveness of drug treatments or vector interventions. Four main tools are commonly used: *msp-1, msp-2,* and *glurp* typing, microsatellite analysis, molecular DNA barcodes, and genome-wide sequencing (149).

Merozoite surface protein 1 and 2 genes

The *msp-1* and *msp-2* genes have been widely used to study genetic diversity in malaria parasites (14). These tools are recommended by the WHO, and are especially useful in low-income countries to determine the MOI in *P. falciparum* and evaluate antimalarial drug efficacy, helping distinguish recrudescent infections from new ones (150-152). Many studies in Africa, including Uganda, have used these markers to assess genetic diversity and MOI (47, 153). However, these markers have limitations, such as undergoing immune selection, making them unsuitable for assessing parasite genetic structure (89, 90, 154). Additionally, their limited number may underestimate true diversity, leading to the recommendation of microsatellites which are neutral markers for better population genetics assessments (155).

Microsatellites

Microsatellite markers, which consist of tandem repeats of 2–6 base pairs, are highly polymorphic and commonly found in non-coding regions of the *P. falciparum* genome (156)**.** These molecular markers are abundant in the Plasmodium genome, with common repeat sequences such as [TA]n, [T]n, and [TAA]n (44). The high variability makes them ideal for distinguishing different parasite strains. Additionally, microsatellites are neutral with no

phenotypic effects, and their diversity allows them to provide valuable insights into the population structure and genetic diversity of *P. falciparum* (145, 146). A range of 5–14 microsatellite markers, such as Polyα, TA42, TA81, and TA109, are used in studies of genetic diversity and MOI (22).

These markers are particularly effective for investigating population structure and transmission dynamics (157) and have been widely applied in *P. falciparum* genomic studies (158, 159)**.** In Uganda, previous studies relying on *msp-1* and *msp-2* markers may have underestimated the true genetic diversity and MOI of *P. falciparum*, since these markers are under immune selection. Microsatellite markers, being highly polymorphic and selection-neutral (22), and widely distributed throughout the genome (44), offer a more accurate understanding of parasite diversity and transmission dynamics.

In conclusion, variations in *P. falciparum* genetic diversity across studies highlight the need for a systematic review for region-wide comparisons among SSA countries with varying malaria transmission settings. Such comparisons could provide insights into transmission patterns and control effectiveness. Conflicting findings on the relationship between genetic diversity, transmission intensity, MOI, and parasite density emphasize the need for standardized methodologies and larger, more comprehensive studies. Limited long-term data hinder understanding of how genetic diversity and MOI evolve with control measures and fluctuating transmission. While asymptomatic individuals are key reservoirs, the role of genetic diversity in these infections remains underexplored. Further research is needed to understand how genetic diversity in asymptomatic cases contributes to transmission, especially in low-transmission areas. Despite increasing use of microsatellites, many SSA malaria-endemic countries still rely on *msp-1* and *msp-2*, which have lower polymorphism and are prone to immune selection.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study designs

General overview

This PhD thesis is structured around three interrelated sub-studies designed to address five key objectives concerning *P. falciparum* genetic diversity and MOI across SSA, with a particular focus on Uganda. **Sub-study I** employed a systematic review and meta-analysis to evaluate the status of *P. falciparum* genetic diversity and MOI across SSA, based on commonly used molecular markers: *msp-1*, *msp-2*, *glurp*, and microsatellites (Objective 1). The analysis focused on the geographical distribution of *P. falciparum* genetic diversity, the prevalence of polyclonal infections, and associated factors. **Sub-study II** assessed *P. falciparum* genetic diversity and MOI in relation to malaria transmission intensity, clinical disease status (symptomatic versus asymptomatic), and temporal trends (Objectives 2, 3, and 4). This sub-study utilized metadata and archived *P. falciparum*-positive dried blood spot (DBS) filter paper samples collected during comprehensive surveillance studies conducted between 2011 and 2017 in three Ugandan regions with different malaria transmission intensities. **Sub-study III** assessed the performance of microsatellite markers alongside *msp1* and *msp1* and three genotyping algorithms (3/3 match-counting, ≥2/3 match-counting, and a Bayesian approach) in distinguishing between recrudescence and reinfection (Objective 5). This sub-study utilized metadata and archived *P. falciparum*-positive DBS filter paper samples from a TES conducted between September 2018 and February 2019 at three health centers located in high malaria transmission regions of Uganda.

3.2 Sub-study I: Systematic review and meta-analysis to describe the status of *P. falciparum* genetic diversity and MOI based on *msp-1, msp-2, glurp* and microsatellite genetic markers in SSA (Objective 1)

3.2.1 Study design

The systematic review and meta-analysis was conducted using the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (160).

Protocol registration: The review protocol was registered in PROSPERO (#CRD42021267661).

3.2.2 Review question

The data was reviewed for *P. falciparum* genetic diversity and MOI in SSA based on *msp-1, msp-2, glurp*, and microsatellite genetic markers from articles published from January 2000 through May 2023. This period was chosen because access to malaria genomic technologies was reasonably high, yielding representative data in the regions sampled (73). In addition, the period coincides with rapid decline in malaria incidence in SSA (161). The specific objectives of the review were to: a) Characterize the geographical distribution of *P. falciparum* genetic diversity in SSA; b) Determine the prevalence of *P. falciparum* polyclonal infections in SSA; and c) Identify factors associated with *P. falciparum* genetic diversity and MOI in SSA.

3.2.3 Search strategy and information sources

A systematic search was conducted by an experienced librarian (AAK), utilized PubMed, EMBASE, EBSCOhost, Web of Science, and the first 50 pages of Google Scholar after searching several pages and found no more relevant articles. Additionally, citation lists of the identified articles were searched for additional relevant articles (31, 105, 162-165). The

search terms included keywords such as *'Plasmodium falciparum*,' '*P. falciparum* genetic diversity, '*P. falciparum* multiplicity of infection,' and 'sub-Saharan Africa'.

3.2.4 Eligibility

3.2.4.1 Inclusion criteria

- Articles published in English,
- Study design, i.e., observational (cross-sectional/survey, case control, and cohort) or randomized clinical trials (RCTs),
- Minimum required data elements: country, sample size, calendar year(s) when the study was conducted, and detailed laboratory methods used to genotype markers for genetic diversity or MOI,
- Detailed methods for determining *P. falciparum* genetic diversity and MOI, including mean H_e, allele frequencies, and mean MOI or percentage of multiple infections.

3.2.4.2 Exclusion criteria

- Absence of key terms '*P. falciparum* genetic diversity and or MOI' in the title and or abstract,
- Studies using experimental animals,
- Review articles, case reports, case series, or editorials,
- Use of inappropriate laboratory molecular methods (DNA extraction, PCR and then fragment analysis).

3.2.5 Article screening and data extraction

The articles were deduplicated using EndNote software *version* X9. Subsequently, the unique articles underwent screening by two independent reviewers (AM and RWN), who also

performed data abstraction using predetermined review criteria. Abstracted data was compared, and any disagreements were resolved through discussion.

Harmonized extracted data included:

- Study characteristics (author name, article title, publication year, country, malaria transmission setting, and study design); participant characteristics (sample size, age group, and malaria clinical category),
- Malaria diagnosis and genotyping (malaria diagnosis method, name of genotyped markers, and PCR fragment analysis method),
- Outcome results on *P. falciparum* genetic diversity and MOI based on mean H_e, allele frequencies of selected genotyped genetic markers, and the prevalence of polyclonal infections or mean MOI, respectively. Data on factors associated with *P. falciparum* genetic diversity and MOI were also extracted.

3.2.6 Data analysis

Meta-analysis was performed using the random effects model (DerSimonian and Laird approach) in STATA (version 17, Stata Corporation, College Station, TX). Forest plots included only studies that reported measures of dispersion, such as SD or CI for the respective effect sizes (Mean MOI and or Mean H_e) to enable computation of standard error for use in meta-analysis using STATA. Pooled estimates for *P. falciparum* genetic diversity and MOI were generated, sorted by region. Additionally, patterns of *P. falciparum* genetic diversity and MOI were assessed according to malaria clinical categories (asymptomatic and symptomatic malaria infection) (117) and specific genetic marker(s) used to evaluate *P. falciparum* genetic diversity and MOI (149).

3.2.7 Heterogeneity analysis

Heterogeneity across the studies was assessed using the chi-squared test and Cochran's Q statistic, with a 5% level of statistical significance (166), and the I-squared (I^2) statistic (167). An I^2 statistic of 25% indicates low heterogeneity, 50% indicates moderate heterogeneity, and >75% indicates high heterogeneity (168).

3.2.8 Risk of bias and quality of evidence assessment

Risk of bias (RoB) in the selected articles was independently evaluated by two reviewers (AM, RWN) using an RoB assessment tool adapted from Joanna Briggs Institute's (JBI) critical appraisal tools (169). The quality of evidence was determined by two independent reviewers using the Grading of Recommendations, Assessment, Development, and Evaluations (GRADE) guidelines (170). RoB assessment covered five domains: study design and limitations, inconsistency in selected articles, indirectness of the evidence, imprecision, and publication bias. Studies scoring 0 to 1, 2 to 3, 4 to 5, and at least 6 were judged to be very low, low, moderate, and high-quality studies, respectively.

3.2.9 Publication bias

Publication bias was assessed by visualizing the asymmetry of the funnel plot and examining the presence and distribution of dots in the plot (171). Egger's statistical test was performed to assess the asymmetry of the funnel plot. A statistically significant result ($p < 0.05$) in Egger's test indicates that the funnel plot asymmetry is due to small-study effects (172). All data analysis was conducted using STATA *version* 17 software package (Stata Corporation, College Station, TX).

3.2.10 Missing data

Variables that were missing from included articles were recorded as not reported (NR). Authors of articles with missing data were contacted for additional information, but only a small number (5/12: 41.67%) responded.

3.3. Sub-study II: Evaluation of *P. falciparum* genetic diversity and MOI in relation to malaria transmission intensity, clinical disease status (symptomatic versus asymptomatic), and temporal trends (Objectives 2, 3, and 4)

Su-study II utilized data and *P. falciparum*-positive DBS filter paper samples collected during the comprehensive surveillance studies conducted under PRISM study, conducted from August 2011 to July 2017. The metadata and DBS samples were obtained from the Resistance, Immunology, and **S**urveillance of Malaria (PRISM) study, which was carried out in three regions of Uganda with varying malaria transmission intensities: Jinja (low transmission), Kanungu (moderate transmission), and Tororo (high transmission) (Figure 2). The objectives of this sub-study were to assess the genetic diversity and MOI of *P. falciparum* across varying transmission settings in Uganda; compare genetic diversity and MOI between asymptomatic and symptomatic malaria cases; and investigate temporal changes in *P. falciparum* genetic diversity and MOI over time points.

Description of the PRISM Study

The PRISM study enrolled participants from three sub-counties in Uganda, selected to represent varying levels of malaria transmission intensity (137). These included Walukuba**,** a peri-urban area near Lake Victoria in south-central Uganda, classified as a low-transmission setting; Kihihi**,** a rural area located near Bwindi Impenetrable National Park in the southwest, representing a moderate-transmission area; and Nagongera**,** a rural area in the southeast near

the Kenyan border, categorized as a high-transmission area. Transmission intensities at these sites were assessed using entomological inoculation rates (EIR): 3.8 for Walukuba (Jinja), 26.6 for Kihihi (Kanungu), and 125 for Nagongera (Tororo), as measured at the start of the PRISM study (Figure 2) (137).

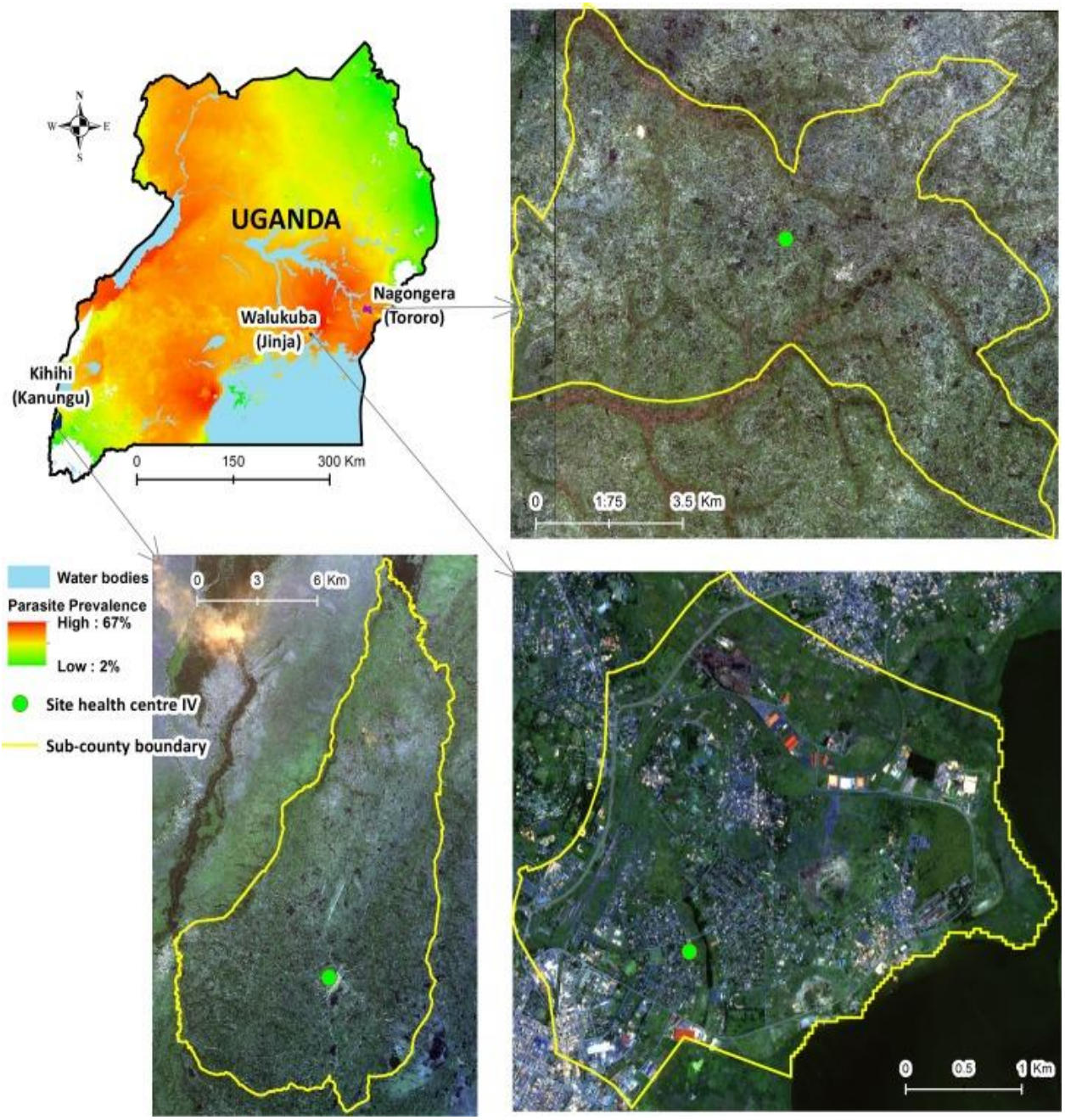


Figure 2. Map of Uganda showing the malaria endemicity of the study sites at the time of sample collection. Adapted from Kamya *et al.* 2015 (137).

The PRISM study enumerated and mapped all households in each of the three selected sub-counties. From these, 100 households were randomly selected to participate in the cohort, regardless of whether malaria infection was present at the time of selection. This approach ensured the inclusion of both symptomatic and asymptomatic individuals. 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 cohort. 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. 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.

The PRISM cohort estimated the incidence of malaria among children and adults living in three different epidemiological settings with varying levels of malaria transmission intensity. Also measured the prevalence of asymptomatic parasitemia and anemia among children and adults in the same settings. Additionally, determined the age and exposure-dependent acquisition of antibody responses to malaria antigens, and assessed the association between antimalarial antibodies and protection from malaria. Further identified and characterized interactions between agricultural development and malaria, and evaluated the associations between overnight travel within Uganda and the risk of malaria infection.

3.3.1 Populations

3.3.1.1 Target population

Children and adults living in low, medium and high malaria transmission areas of Uganda covered by the primary studies.

3.3.1.2 Accessible population

Children and adults who were enrolled in the primary PRISM study (2011–2017), conducted in low, medium and high malaria transmission areas of Uganda.

3.3.1.3 Study population

All children and adults enrolled in the PRISM study in low, medium and high malaria transmission areas of Uganda and who fulfilled the selection criteria.

3.3.2 Selection criteria

3.3.2.1 Inclusion

- Children aged 6 months to < 10 years and adults aged ≥18 years
- Individuals with a documented fever (tympanic temperature >38.0°C) or a history of fever within the previous 24 hours, as well as asymptomatic individuals
- A positive thick blood smear for *P. falciparum*
- Availability of DBS filter paper
- Participants who had provided consent for use of samples for future molecular studies
- Participants with sufficient demographic and clinical data.

3.3.2.2 Exclusion

- Mixed infections
- Missing data as per medical records.

3.3.3 Sample size determination

The minimum sample size required for each objective was determined as follows:

In **objective 2**, the aim was to evaluate *P. falciparum* genetic diversity and MOI across areas with low, medium, and high malaria transmission intensities in Uganda. To determine the required sample size, a formula for comparing two proportions was used, considering p_1=62% as the moderate estimate of the proportion of multiple infections and p_2=80% as the higher estimate, based on studies from the East African region (14, 18). The critical value Zα/2 corresponded to a 5% level of significance, and Zβ was the critical value for a power of 80%.

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

For three transmission settings, a total of 96×3=288 samples were required.

In **objective 3**, the aim was to compare *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases in Uganda. We applied a formula for determining the sample size for two proportions, considering p_1 as the proportion of individuals with multiple *P. falciparum* infections among asymptomatic malaria cases (63.3%) and p_2 as the proportion of individuals with multiple *P. falciparum* infections among symptomatic malaria cases (80.6%) (173).

$$n = [(Z\alpha/2+Z\beta)2 * (p_1(1-p_1) + p_2(1-p_2)) / (p_1-p_2)2] =74.$$

For this study objective, three levels of *P. falciparum* infection severity were considered: asymptomatic, symptomatic uncomplicated, and symptomatic complicated. A total of 74 samples were selected from each category, resulting 222 samples in total.

In **objective 4**, the aim was to investigate temporal changes in *P. falciparum* genetic diversity and MOI across areas with low, medium, and high malaria transmission intensities in Uganda. We used the formula for determining the sample size for two proportions considering p_1 and p_2 as the proportions of polyclonal infection at two time points: 50.5% and 69.2%, respectively (174).

$$n = [(Z\alpha/2+Z\beta)2 * (p_1(1-p_1) + p_2(1-p_2)) / (p_1-p_2)2] = 104.4.$$

For the three sites, a total sample size of 105*3= 315 was required.

3.3.4 Study variables

The study utilized both demographic/clinical and genetic variables:

- **Demographic/Clinical variables**: Age, gender, parasite density, hemoglobin levels, and body temperature.
- **Genetic diversity indices**: Number of alleles, allelic richness, number of effective alleles, and expected heterozygosity.
- **Multiplicity of infection (MOI) metrics**: Mean MOI and proportion of polyclonal infections.
- **Population structure metrics**: Multilocus linkage disequilibrium, genetic differentiation (F_{ST}) and analysis of molecular variance (AMOVA).

3.3.5 Data collection

Demographic and clinical data: These were extracted from the PRISM database, including age, gender, hemoglobin levels, body temperature, and parasite density. Data were managed using Microsoft Excel and were double-checked to ensure completeness and accuracy.

Genetic data: Genetic data was obtained through molecular analysis of *P. falciparum* isolates. DNA was extracted and analyzed using polymorphic microsatellites (TA1, Poly-α, PfPK2, TA109, 2490, C2M34–313, and C3M69–383). PCR-based genotyping was performed to assess *P. falciparum* genetic diversity, determine MOI, and to evaluate population structure.

3.4 Sub-study III. Evaluation of genotyping algorithms to distinguish recrudescence from reinfection (Objective 5)

This sub-study aimed to assess the performance of polymorphic molecular markers (microsatellites, *msp-1*, *msp-2*) and three genotyping algorithms (3/3 match-counting, ≥2/3 match-counting, and a Bayesian approach) in distinguishing between recrudescence and reinfection. The performance of various genotyping markers and algorithms for distinguishing *P. falciparum* recrudescence from reinfection was evaluated using stored DBS filter paper samples collected from children aged 6 months to 10 years with recurrent uncomplicated malaria. These children were enrolled in a TES assessing the efficacy of artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP), conducted between September 2018 and February 2019 in three high malaria endemic areas: Aduku, Arua, and Masafu in Uganda.

Background description of the Therapeutic Efficacy Study (TES)

The Therapeutic Efficacy Study (TES), assessing the efficacy of artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP), was conducted from September 2018 to February 2019 at three high-transmission health facilities in Uganda: Aduku Health Centre IV in Kwania District (Northern Uganda), Arua Regional Referral Hospital in Arua District (Northwestern Uganda), and Masafu District Hospital in Busia District (Eastern Uganda). These sites experience perennial malaria transmission with high intensity. According to the 2018–2019 malaria indicator survey, parasite prevalence among children aged 0–59 months was 13% in Kwania District, 22% in Arua District, and 21% in Busia District (175). The TES enrolled children aged 6 months to 10 years who had uncomplicated *P. falciparum* malaria confirmed by microscopy. During the study, DBS filter paper samples were collected for parasite genotyping. These samples were later used to distinguish between *P. falciparum* recrudescence and reinfection in cases of recurrent parasitemia.

3.4.1 Populations

3.4.1.1 Target population

Children living and high malaria transmission areas of Uganda covered by the TES.

3.4.1.2 Accessible population

Children who were enrolled in the primary TES conducted in high malaria transmission areas Uganda.

3.4.1.3 Study population

All children enrolled in the TES who experienced recurrent infections.

3.4.2 Selection criteria

3.4.2.1 Inclusion

- Children aged 6 months to 10 years who had recurrent uncomplicated *P. falciparum* malaria
- A positive thick blood smear for *P. falciparum*
- Availability of DBS filter paper
- Participants who had provided consent for use of samples for future molecular studies
- Participants with sufficient demographic and clinical data.

3.4.2.2 Exclusion

- Mixed infections
- Missing data as per medical records.

3.4.3 Sample size determination

All DBS filter paper samples collected from children aged 6 months to 10 years with recurrent uncomplicated *P. falciparum* malaria were analyzed. A total of 179 paired samples collected at the time of initial diagnosis and at recurrence were genotyped, including 123 from the artemether-lumefantrine (AL) arm and 56 from the dihydroartemisinin-piperaquine (DP) arm. Since the objective was to genotype all available isolates from participants with recurrent parasitemia, no additional sample size calculation was necessary beyond the original inclusion criteria of the TES. This sample size was considered sufficient to address the study objective, as all eligible recurrent cases were included and a comprehensive molecular analysis was conducted. However, formal power calculations were not performed

retrospectively, as the analysis was exploratory and based on an existing dataset from a well-characterized clinical trial.

3.4.4 Study variables

Parasite genetic diversity indices (e.g., number of alleles, allelic richness, number of effective alleles, expected H_e); MOI metrics (mean MOI, proportion of polyclonal infections); number of recrudescent infections.

Data were obtained by genotyping *P. falciparum* isolates from individuals with recurrent parasitemia during TES, using polymorphic markers such as *msp-1*, *msp-2*, *glurp*, and neutral microsatellites.

3.5 General laboratory methods for objectives 2–5

Laboratory assays, including malaria microscopy and parasite genotyping including PCR were conducted at the Molecular Biology Research Laboratory of the Infectious Diseases Research Collaboration (IDRC) in Kampala, Uganda.

3.5.1 Determination of parasite density

Microscopy slides for parasite density and species detection were prepared using a 2% Giemsa solution and stained for 30 minutes, with both thick and thin blood films being prepared. Experienced microscopists examined the slides under a compound light microscope at 100× oil immersion. Although these parasite density and species detection methods were originally performed in previous studies, we obtained the existing data for this analysis and independently verified parasite densities in 10% of the samples prior to molecular analysis to ensure data reliability. *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 8,000 leukocytes per μL of blood (176). For quality control, each smear was independently read by two microscopists. Though discrepancies defined as differences in species diagnosis, parasite density >25%, or presence of parasites were rare, any identified discrepancy prompted a review by a third microscopist. Final parasitemia 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 determination for 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. Microscopists were not blinded to the slides, but each microscopist independently read the slides without knowledge of the other's results.

3.5.2 Selection of the samples for molecular analysis

For **Objectives** 2–4, a stratified random sampling approach was employed, guided by the calculated sample size for each objective. This strategy ensured that the molecular analysis included participants from various study sites and captured a wide range of demographic and clinical characteristics, thereby representing the broader study population. Stratification was based on key variables, including age, gender, infection profile, geographical location, and study period, to achieve a balanced and representative sample.

For **Objective 5**, all available samples from participants with recurrent parasitemia were retrieved for molecular analysis.

All selected samples were obtained from the IDRC Biorepository at the Central Public Health Laboratory in Butabika, Kampala, Uganda, where they had been stored at –20°C to preserve DNA integrity. The retrieved DBS filter paper samples were linked to participants' demographic and clinical information using their cohort ID, along with the corresponding date and year of sample collection.

3.5.3 DNA extraction

P. falciparum genomic DNA was extracted from DBS filter paper samples using Chelex 100 Resin (Sigma-Aldrich, USA), following the method described by Musapa *et al.*(177). The Chelex extraction method was chosen for its simplicity, cost-effectiveness, and efficiency in processing 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 filter paper samples into prelabelled 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 x 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. 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.

3.5.4 Detection and confirmation of *P. falciparum*

Detection and confirmation of *P. falciparum* was performed through genotyping of the *P. falciparum* 18S rRNA gene using nested PCR (178). Briefly, two rounds of PCR amplification were conducted to enhance sensitivity and specificity. In the first round, primers targeting conserved regions of the *Plasmodium* genus amplified a broad segment of the 18S rRNA gene, common to all *Plasmodium* species. In the second round, nested primers specific to *P. falciparum* amplified a targeted region of the gene, confirming the presence of *P. falciparum*. The PCR products were visualized on a 1.5% agarose gel stained with SYBR Green (Invitrogen) and prepared in 1× TAE buffer. The products were compared to a 100 bp DNA ladder (Thermo Fisher Scientific), which allowed the identification of DNA fragments between 100–1,000 base pairs. The expected amplicon size for *P. falciparum* was 120–200 base pairs. A distinct band at the expected size confirmed the presence of *P. falciparum* and validated the specificity of the nested PCR assay.

3.5.5 Microsatellite genotyping

A panel of seven neutral polymorphic microsatellites of *P. falciparum*, including TA1 (Chr6), Poly-α (Chr4), PfPK2 (Chr12), TA109 (Chr6), 2490 (Chr10), C2M34–313 (Chr2), and C3M69–383 (Chr3), was genotyped using primers labelled with HEX or 6-FAM. The microsatellites Poly-α, TA1, TA109, PfPK2, and 2490 were nested, while C2M34–313 and C3M69–383 were not nested (Appendix X).

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 minutes, followed by 25 cycles of (94°C for 30 seconds, 42°C for 30 seconds, 40°C for 30 seconds, 65°C for 40 seconds), and ending with 65°C for 2 minutes. 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 minutes, followed by 25 cycles of (94°C for 20 seconds, 45°C for 20 seconds, 65°C for 30 seconds), and ending with 65°C for 2 minutes.

PCR conditions for the C2M34–313 and C3M69–383 reactions were as follows: 94°C for 2 minutes, followed by 5 cycles of (94°C for 30 seconds, 50°C for 30 seconds, 60°C for 30 seconds), then 40 cycles of (94°C for 30 seconds, 45°C for 30 seconds, 60°C for 30 seconds), and ending with 60°C for 2 minutes (Appendix X). A 2 µL sample of the PCR product was then run on a 2% agarose gel to confirm amplification before being analyzed 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.

3.5.6 Microsatellite analysis

Microsatellite fluorescent-labelled PCR products were analysed using an Applied Biosystems ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) to determine their lengths and at Inqaba Biotec in South Africa. The peaks were scored using GeneMarker HID V2.9.5 software. For samples that produced more than one peak, the highest peak was defined as the dominant allele. Additional peaks were classified as minor alleles if their peak heights exceeded 200 relative fluorescence units (RFU) and were > 20% of the height of the dominant peak. This threshold was used to identify minor alleles, which may represent clones

present at lower frequencies but still contribute to the genetic diversity of the infection. The identification of these additional minor alleles, including third and fourth alleles, was based on the relative peak heights at each microsatellite locus. Peaks that met these criteria were recorded as distinct 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. We then conducted a thorough review and revalidation of .fsa files to ensure data accuracy before proceeding with the final analysis. Subsequent genetic analysis was performed on samples with successful amplification of at least five microsatellite markers. To minimize bias from multiple infections, only the predominant alleles were included. Samples with incomplete, poor-quality amplification, or those failing to amplify one or more markers were excluded to ensure data accuracy.

3.5.7 *P. falciparum msp-1, msp-2* and *glurp* genotyping

Parasite genotyping was conducted using nested PCR as described by Congpuong K *et al.,* 2014 (179) adjusting the PCR reaction mixture to 25 µL (Appendix XI). DNA from *P. falciparum* reference strains (3D7, and HB3) was used in each run as positive control (180). A 3µL sample of the PCR product was then analysed on a 2% agarose gel to confirm the amplification.

PCR products were analysed by capillary electrophoresis using the QIAxcel DNA High-Resolution Kit (Qiagen) on the QIAxcel Advanced System (Qiagen, Hilden, Germany). PCR products for the *msp-1* and *msp-2* allele families were analysed following the AM420 protocol, utilising the QX DNA Size Marker 50–800 bp and the QX Alignment Marker 15 bp/1 kb (Qiagen, Hilden, Germany). Nested PCR products for *glurp* were analysed using the same protocol with the QX DNA Size Marker 100 bp–2.5 kb and the QX Alignment Marker 15 bp/3 kb (Qiagen, Hilden, Germany). Band sizes were determined using the QIAxcel

ScreenGel software (Version 1.5.0.16, Qiagen, Hilden, Germany). The height cutoff for minority clones was set at 10% of the dominant peak for *msp-1* and *msp-2*, and 20% for *glurp* (181).

3.5.8 Determination of *P. falciparum* genetic diversity

The genetic diversity of *P. falciparum*, usually resulting from genetic recombination (72), was assessed in each parasite population from each study site by calculating the: mean number of alleles (N_a), allele richness (Ar), allele frequency, 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 (182). Expected heterozygosity (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 (183) with the formula:

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

where 'n' represents the number of isolates analyzed and 'pi' is the frequency of the i^{th} allele in a given population. The H_e values range between 0 (no genetic diversity) and 1 (high genetic diversity) (15). The mean N_a, N_e, and H_e values for each study site were computed from each locus.

3.5.9 Determination of *P. falciparum* MOI

P. falciparum MOI was defined as the number of distinct parasite genotypes coexisting within a given infection (78). Isolates with only one allele were considered monoclonal infections, while those with more than one allele were classified as polyclonal infections (77). The MOI for each infection was determined by identifying the highest number of alleles observed across any of the microsatellite markers used in the analysis. This maximum allele count was

considered the MOI for that particular infection. To assess the parentage of polyclonal infections, we counted the number of isolates with more than one allele for each microsatellite marker that successfully amplified. The results were summarized by counting the number of participants exhibiting a certain number of clones and compared across study sites, infection profiles and overtime.

3.5.10 *P. falciparum* multi-locus linkage disequilibrium and genetic differentiation analysis

Differentiation analysis was done using Wright's F-statistics (F_{ST}) values using Arlequin 3.11 (183). 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 (184). Inter- and intra-population variance was determined using analysis of molecular variance (AMOVA). Multilocus linkage disequilibrium (LD), measured as the standardized index of association (I_A^S), was calculated using LIAN version 3.5 (185) for the whole dataset. This index was calculated using the formula:

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

where VE is the expected variance of the n^{th} 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 Coordinates Analysis (PCoA) using GENALEX 6.5 software (182) to assess parasite population substructure.

3.5.11 *P. falciparum* population cluster analysis

Cluster analysis was performed using STRUCTURE v2.1 software (186).

3.5.12 Recrudescence and reinfection classification

Three algorithms namely: the 3/3 match counting algorithm, the ≥2/3 match counting algorithm, and a Bayesian approach (146, 148), were applied to several datasets, each combining different genotyped microsatellites with *msp-1*, and *msp-2*. The 3/3 method classified an infection as recrudescence if one or more alleles occurred at all three genotyped markers on both day 0 and the day of the recurrent malaria infection. In contrast, a ≥2/3 match counting algorithm classified an infection as a recrudescence if one or more alleles were present in at least two of the three genotyped markers on both day 0 and the recurrence day. The Bayesian algorithm utilized allele information from all samples to estimate the probability of recrudescence for each case. A low probability indicated a greater likelihood of reinfection, whereas a probability closer to 1 suggested a high likelihood of recrudescence (148). This approach highlighted how different probability thresholds can influence the sensitivity (true positive rate) and specificity (true negative rate) of the algorithm. Specifically, a lower threshold may lead to the identification of more recrudescences, thereby enhancing sensitivity, while a higher threshold can improve specificity by reducing false positives. To convert these probabilities into a binary classification of recrudescence versus reinfection, thresholds of 0.7 and 0.8 were applied sequentially. The outcomes of all three algorithms were then compared to assess their effectiveness in identifying recrudescences.

3.6 Statistical analyses

Data quality checks were conducted prior to analysis to identify and resolve any inconsistencies. All statistical analyses were performed using STATA version 17 (StataCorp,

College Station, TX). Descriptive statistics, including means and proportions, were used to summarize baseline characteristics of predictor variables for each study objective.

To evaluate *P. falciparum* genetic diversity and MOI across areas of varying malaria transmission intensity in Uganda, genetic diversity indices, number of alleles (N_a), effective number of alleles (N_e), allelic richness (Ar), and expected heterozygosity (H_e), along with MOI indices (mean MOI and polyclonal infection rates), were compared across sites representing low, moderate, and high malaria transmission intensities using Kruskal–Wallis tests, Chi-square tests, or Fisher's exact tests, as appropriate. Population structure was assessed via linkage disequilibrium (LD) analysis, with the significance of the multilocus index of association (I_A^S) tested using the Monte Carlo method. Pairwise genetic differentiation (F_{ST}) values were computed to quantify variation between populations. Principal Coordinate Analysis (PCoA) was used to visualize genetic distances, and STRUCTURE v2.1 was employed to investigate clustering patterns among parasite populations.

To compare *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases, genetic diversity indices (N_a, N_e, allele frequencies, and H_e) and MOI indices (mean MOI and polyclonal infection rates) were compared among individuals with asymptomatic, symptomatic uncomplicated, and symptomatic complicated malaria. Appropriate tests: Kruskal–Wallis, Chi-square, or Fisher's exact were used depending on the data type and distribution. Population structure differences were again evaluated using LD and I_A^S, with Monte Carlo significance testing. Pairwise F_{ST} values were also calculated to assess genetic differentiation between infection profiles.

To evaluate changes in *P. falciparum* genetic diversity and MOI over time, comparisons of N_a, N_e, H_e, mean MOI, and the proportion of polyclonal infections were made across different time points using the Kruskal–Wallis test. Temporal trends in these indices were further analyzed using the Jonckheere–Terpstra test.

To assess performance of molecular markers and genotyping algorithms for distinguishing recrudescence from reinfection, Spearman's rank correlation was used to examine the relationship between mean H_e and mean MOI. The performance of the three genotyping algorithms (3/3 match-counting, ≥2/3 match-counting, and a Bayesian approach) in classifying recrudescence was compared using the Wilcoxon signed-rank test.

For all analyses, 95% confidence intervals (CIs) and/or standard deviations (SDs) were reported where applicable. Statistical significance was defined as $p < 0.05$.

3.7 Ethical approval

The PhD study received ethical approval from the Makerere University School of Medicine Research and Ethics Committee (Mak-SOMREC-2021-152) and the Uganda National Council for Science and Technology (Ref No: HS2744ES). Only samples from participants who had provided prior consent for future use of their biological specimens were included in this analysis. Samples from participants who had not consented were removed from the repository and were therefore not available for use. Permission to use the stored samples was also obtained from the respective principal investigators (PIs). As the study involved no direct interaction with participants, a waiver of consent was granted by SOMREC.

CHAPTER FOUR: RESULTS

4.1 Review status of *P. falciparum* genetic diversity and MOI based on *msp-1, msp-2, glurp* and microsatellite genetic markers in SSA

The systematic review and meta-analysis showed wide variations in *P. falciparum* genetic diversity and MOI across different countries as well as regions in SSA. The review included 52 studies, which collectively analyzed 11,640 genotyped parasite isolates from 9,062 symptomatic and 2,578 asymptomatic *P. falciparum* infections.

Among the articles reviewed, a large proportion (75%) were from studies that employed cross-sectional designs, while others utilized RCTs or cohort study designs. The predominant genetic markers used to genotype parasites were the antigen-coding loci, especially *msp-1* and/or *msp-2*, in 76.9% (40/52) of the articles. Microsatellites were used in about 19% of the articles, and a small number used a combination of markers, including microsatellites and SNPs. The review also highlighted the imbalance in research focus, with most studies being conducted in high-transmission areas and mainly among symptomatic individuals.

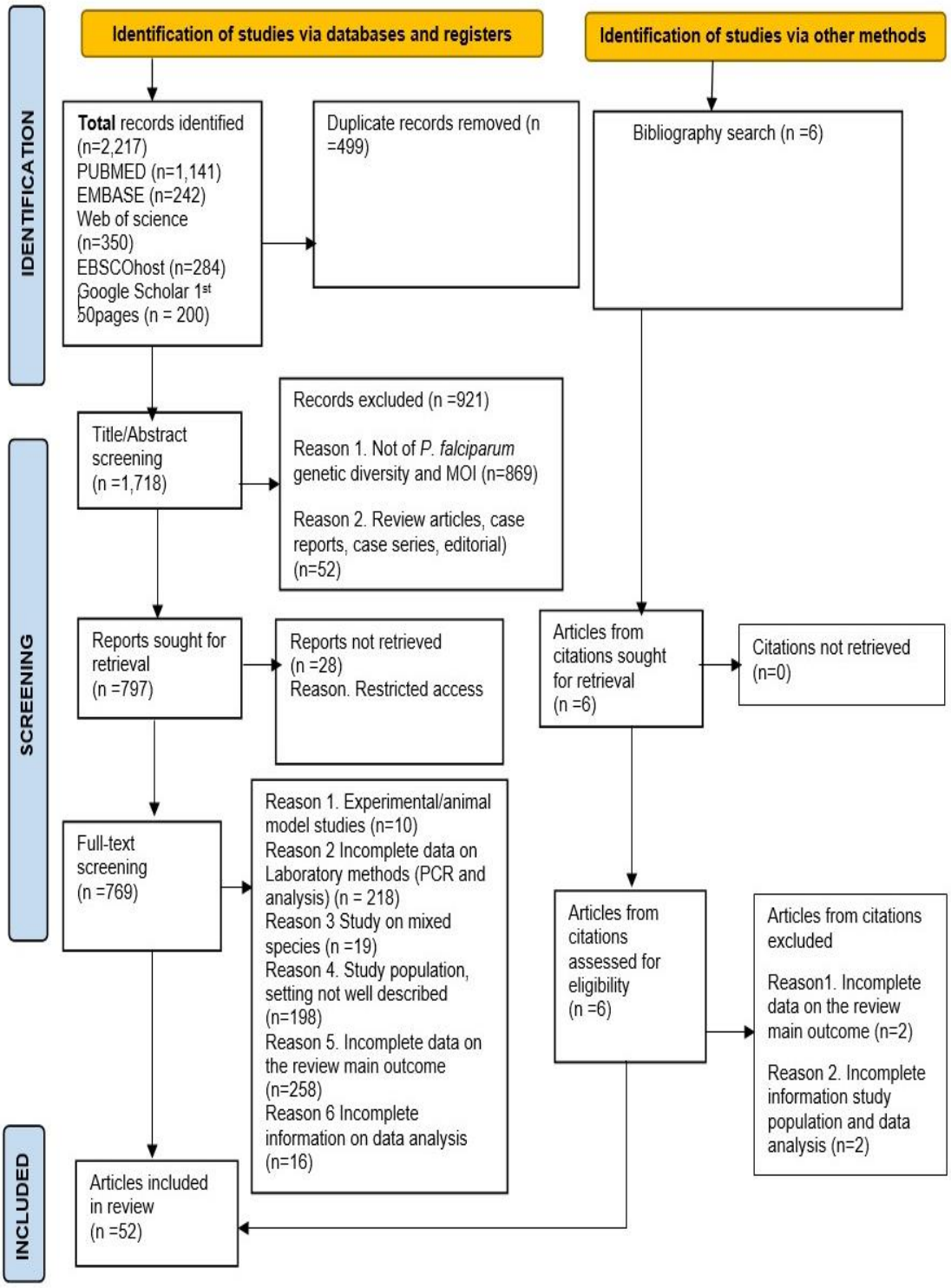


Figure 3. *PRISMA flow diagram for identification and screening of included articles*

P. falciparum genetic diversity in SSA

In areas with high malaria transmission, such as Equatorial Guinea, the frequencies of *msp-1* alleles K1, MAD20, and RO33 were notably high, 96.07%, 96.09%, and 70.78%, respectively (91). In contrast, in Ethiopia, a country with moderate transmission, these frequencies were much lower, 20.8%, 4.2%, and 4.2%, respectively (14). The pooled allele frequencies for *msp-1* alleles K1, MAD20, and RO33 across studies were 61%, 44%, and 40%, respectively, showing considerable regional variation across SSA. Specifically, in Eastern Africa, the pooled frequencies were 46%, 42%, and 31%; in Central Africa, they were 74%, 51%, and 48%; and in West Africa, the frequencies were 67%, 43%, and 44%, respectively.

Similarly, the frequency of the *msp-2* gene I/C 3D7 and FC27 alleles varied widely. For example, the frequency of the I/C 3D7 allele ranged from 15.9% to 98.3% in Ethiopia (14, 85). The FC27 allele frequency ranged from 10.3% in Ethiopia (187) to 98.9% in high-transmission areas of Benin (164). The pooled allele frequency for I/C 3D7 was 61%, while for FC27 it was 55%. Regionally, the pooled allele frequency for I/C 3D7 was 60% in East Africa, 67% in Central Africa, and 61% in West Africa. For FC27, the pooled allele frequencies were 55% in East Africa, 67% in Central Africa, and 50% in West Africa.

For the *glurp* gene, allele frequencies also demonstrated significant variation. These ranged from 39.53% in Nigeria (188) to 97.6% in Uganda (20). The overall pooled allele frequency for *glurp* across all studies was 66%, with regional differences: 90% in East Africa and 70% in West Africa.

The mean expected H_e varied significantly by region. In Ethiopia, the mean H_e was 0.09 (85), and the highest (0.93) was in the Democratic Republic of Congo (DRC) (189). The overall pooled mean H_e across all studies was 0.65 (95% CI: 0.51–0.78). Regionally, the pooled mean H_e values were 0.58 (95% CI: 0.29–0.86) in East Africa, 0.84 (95% CI: 0.81–0.86) in

Central Africa, 0.74 (95% CI: 0.73–0.75) in Southern Africa, and 0.69 (95% CI: 0.62–0.75) in West Africa (Figure 4).

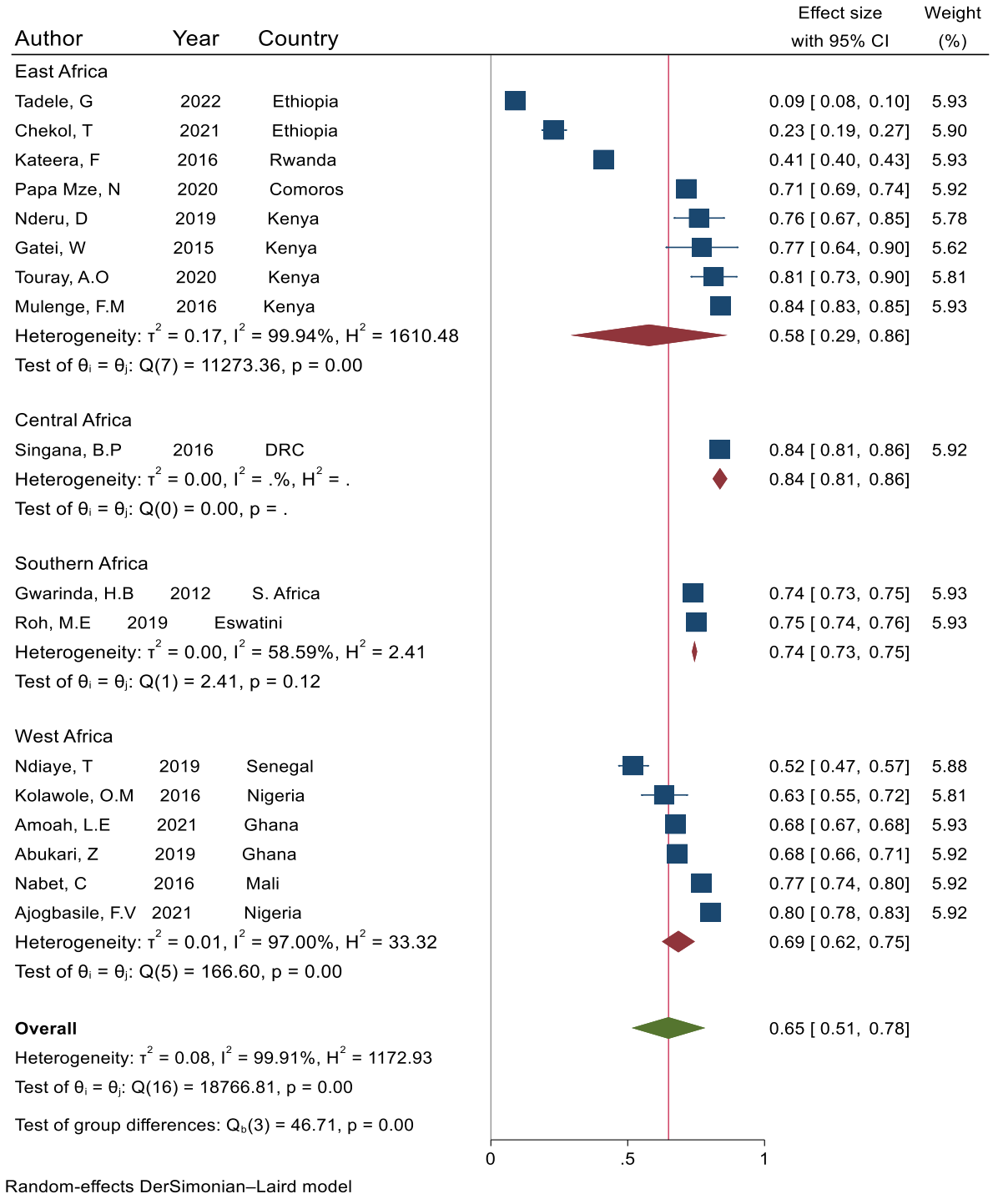


Figure 4 *Forest plot representing the pooled mean H_e of P. falciparum infection across 17 studies that reported measures of dispersion (CI and SD) for mean H_e in malaria-affected countries in SSA, sorted by region.*

P. falciparum MOI across malaria-affected countries in SSA

The mean MOI and prevalence of polyclonal infections varied across malaria-endemic regions in SSA. For example, the mean MOI ranged from 1.09 in Ethiopia (East Africa) (85) to 5.51 in Equatorial Guinea (West Africa) (91). Across all studies, the overall pooled mean MOI was 2.09 (95% CI: 1.88–2.30). When broken down by region, the pooled mean MOI was as follows: 2.05 (95% CI: 1.83–2.26) in East Africa, 2.37 (95% CI: 1.28–3.46) in Central Africa, 2.16 (95% CI: 2.09–2.23) in Southern Africa, and 1.96 (95% CI: 1.53–2.39) in West Africa (Figure 5).

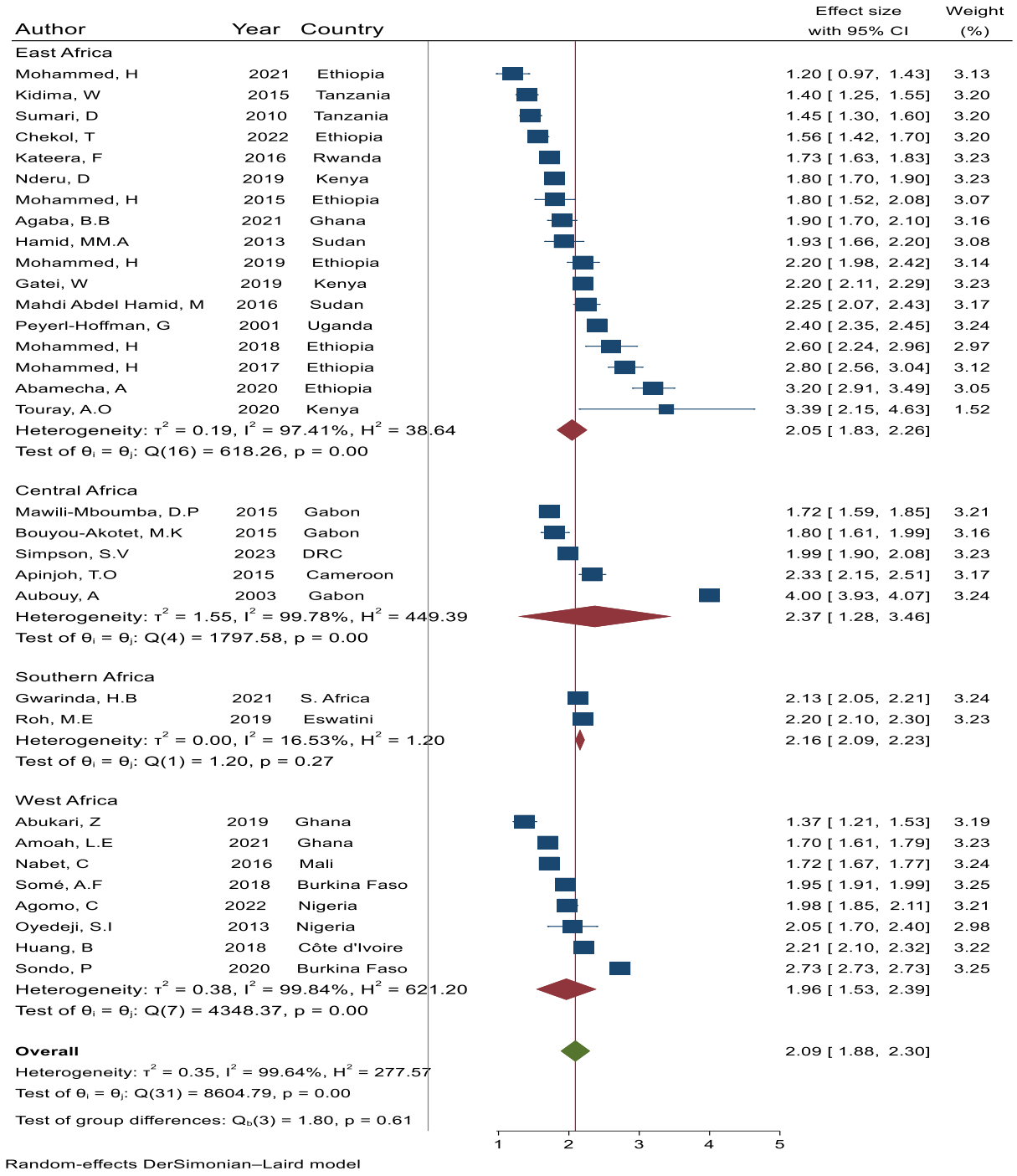


Figure. 5 *Forest plot representing the pooled mean MOI of P. falciparum infection across 32 studies that reported measures of dispersion (CI and SD) for mean MOI in malaria-affected countries in SSA, sorted by region.*

Similarly, the prevalence of *P. falciparum* polyclonal infections varied across malaria-endemic regions in SSA. In Ethiopia (East Africa), the prevalence was 16.3% (85), whereas in Equatorial Guinea (Central Africa), it was 98% (91). The overall pooled prevalence of polyclonal infections was 63% (95% CI: 56–70) across all studies. When broken down by region, the pooled prevalence of polyclonal infections was 62% (95% CI: 53–71) in East Africa, 71% (95% CI: 63–79) in Southern Africa, 65% (95% CI: 43–88) in Central Africa, and 61% (95% CI: 51–71) in West Africa (Figure. 6).

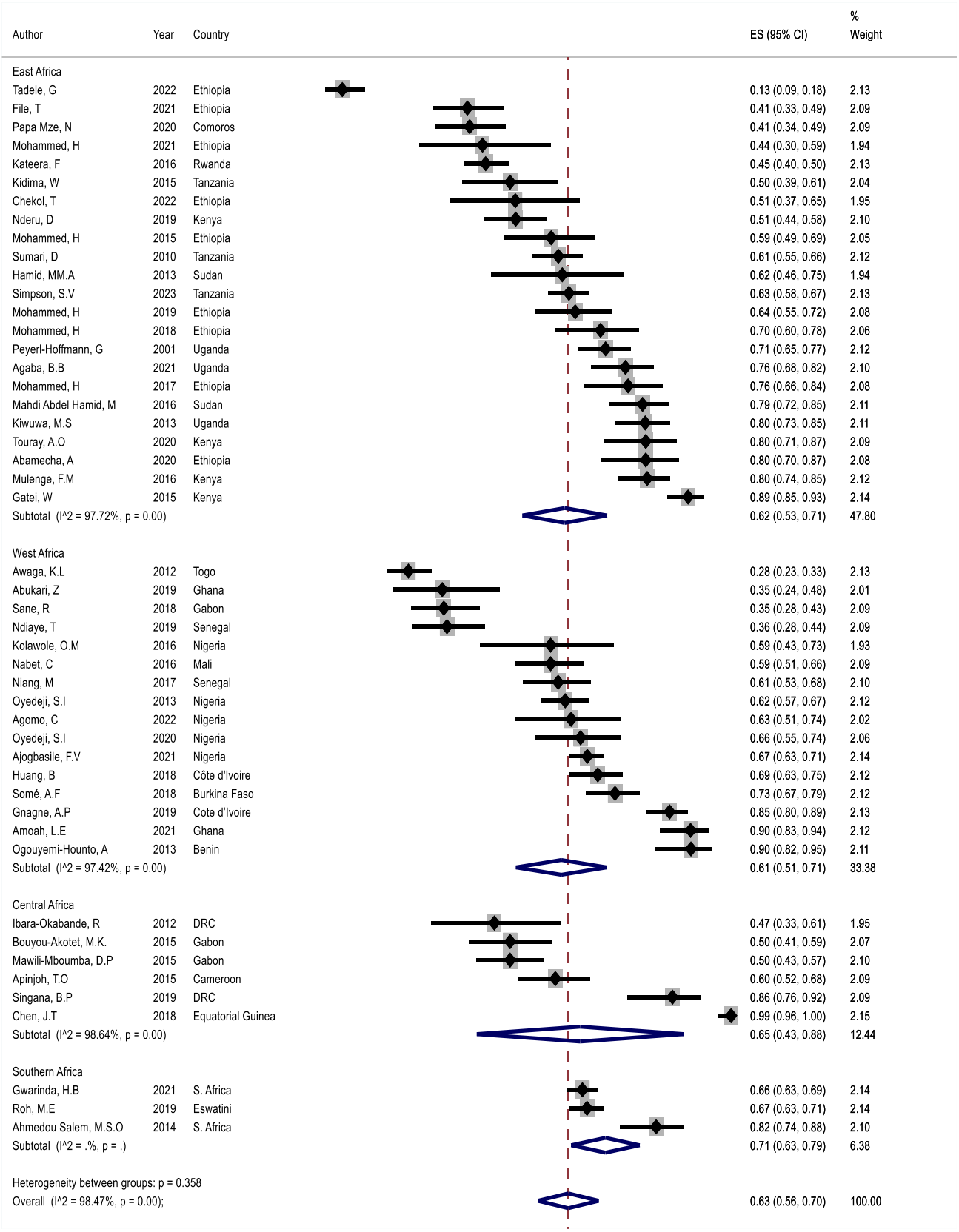


Figure. 6 *Forest plot representing the pooled prevalence P. falciparum polyclonal infections reported by 48 studies from malaria-affected countries in SSA, sorted by region.*

Factors associated with *P. falciparum* genetic diversity and MOI across SSA

Factors associated with *P. falciparum* genetic diversity and MOI across SSA include patient age, parasite density, chemotherapy use, and malaria transmission settings (102, 190, 191), although this relationship is not consistent across all studies (14, 107, 164). The use of ACT has notably decreased *P. falciparum* genetic diversity and MOI. For instance, Huang *et al.* (134) observed a decline in both over 10 years following ACT implementation, with a significant reduction in polyclonal infections for *msp-1* and *msp-2*. Rural areas generally show higher MOI than urban areas, with Tadele *et al.* (85) reporting a mean MOI of 1.88 in rural areas versus 1.55 in urban areas. However, in Uganda, urban areas exhibited higher MOI values (3.0 to 3.7 for severe and mild malaria cases, respectively). Both symptomatic (20, 191, 192) and asymptomatic cases (32, 118) have demonstrated high *P. falciparum* genetic diversity and MOI. Furthermore, malaria transmission settings correlate with genetic diversity and MOI, with higher values found in high transmission areas (2.13) compared to low transmission areas (1.29; $p < 0.001$), as well as higher expected heterozygosity in high transmission settings (0.49 to 0.62) (19).

4.2 Evaluation of *P. falciparum genetic* diversity and MOI in areas of low, medium, and high transmission intensities in Uganda

Evaluation of *P. falciparum* genetic diversity and MOI across areas with varying malaria transmission intensities was based on a total of 288 samples. Of these, 211 (73.3%) were successfully amplified at a minimum of five microsatellite loci and included in the genetic analysis. Among the successfully genotyped samples, 109 (51.7%) were from male participants, and the majority (54%) were from children under 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) (p=0.006), while participants from Jinja had significantly lower parasite densities than those from the other sites (p=0.001) (Table 1).

Table 1. Demographic and clinical characteristics of study participants from Jinja, Kanungu and Tororo included in the analysis of *P. falciparum* genetic diversity and MOI

Characteristic	Overall N=211	Jinja N=69	Kanungu N=71	Tororo N=71	P. value
Age in Years					
<5years		35 (50.7%)	43 (60.6%)	37 (52.1%)	
5-11 years		25 (36.2%)	28 (39.4%)	26 (36.6%)	
≥ 18 years		9 (13.0%)	0 (0.0%)	8 (11.3%)	
Gender (%)					
Male		37 (53.6%)	36 (50.7%)	36 (50.7%)	
Mean Axillary temperature, ^{0}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		11.34±1.89	10.36±2.33	11.37±1.84	

P. 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 had the highest number of alleles, ranging from 13 to 21 across the three study sites. 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 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. A Kruskal–

Wallis test showed no significant differences in genetic diversity indices across the sites ($p > 0.5$) (Table 2).

Table 2. *P. falciparum* genetic diversity among participants from Jinja, Kanungu and Tororo

Locus	Jinja			Kanungu			Tororo		
	N_a	N_e	H_e	N_a	N_e	H_e	N_a	N_e	H_e
2490	6	1.87	0.47	6	2.07	0.52	5	1.94	0.47
Poly-α	13	8.25	0.89	15	10.57	0.91	16	11.19	0.91
C2M34–313	18	11.57	0.92	20	12.90	0.93	21	13.09	0.93
TA1	13	7.27	0.87	17	7.19	0.88	16	6.64	0.86
TA109	10	4.92	0.80	10	5.44	0.82	8	4.74	0.80
C3M69–383	9	3.14	0.69	13	5.38	0.82	12	2.47	0.70
PfPK2	10	5.89	0.84	13	4.38	0.78	10	6.04	0.82
Mean	11.29	6.13	0.78	13.43	6.85	0.81	12.57	6.59	0.78
SD	3.82	3.27	0.16	4.58	3.73	0.14	5.47	4.20	0.16

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. The Kruskal–Wallis test showed no significant differences in the Ar values ($p > 0.05$).

***P. 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). Overall, Tororo had a significantly higher MOI compared to the other sites (p = 0.004) (Table 3).

Table 3. *P. falciparum* MOI among participants from Jinja, Kanungu and Tororo

Locus	Jinja (n= 69)			Kanungu (n= 71)			Tororo (n= 71)		
	Number genotyped	Percentage Polyclonal	MOI	Number genotyped	Percentage Polyclonal	MOI	Number genotyped	Percentage Polyclonal	MOI
2490	69	33.3	1.4	71	24.9	1.3	71	46.5	1.6
Poly-α	69	44.5	1.6	71	51.7	2.0	70	62.9	2.5
C2M34–313	64	73.2	2.1	69	31.9	1.4	55	54.5	2.1
TA1	68	69.1	2.2	71	81.7	2.4	57	80.7	2.6
TA109	69	47.6	1.9	70	43.8	1.8	71	74.6	2.7
C3M69–383	58	83.0	2.2	71	80.3	2.4	68	88.2	3.2
PfPK2	69	55.1	2.0	71	77.5	2.5	69	82.6	2.8
Mean	66.6	58.0	1.9	70.6	56.0	2.0	65.9	70.0	2.5
SD/*SE	4.2	*6.7	0.3	0.8	*9.0	0.5	6.8	*5.9	0.5

Across the sites, the microsatellites C3M69–383 and TA1 identified more polyclonal infections (number of clones ≥ 2), while 2490 identified the most monoclonal infections.

P. falciparum parasite population structure across study sites

P. falciparum genetic differentiation

The pairwise F_{ST} values between the three study sites showed low genetic differentiation across the sites, with all values being less than 0.05. Specifically, the F_{ST} value between Jinja and Tororo was 0.011, indicating the lowest genetic differentiation. The highest

differentiation was observed between Kanungu and Tororo, with an F_{ST} value of 0.021. The F_{ST} value between Jinja and Kanungu was 0.016.

The Analysis of molecular variance (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.

Linkage disequilibrium (LD)

Linkage disequilibrium (LD), assessed based on Multilocus Index of Association (I_A^S) values, ranged from 0.07 in Tororo to 0.14 in Jinja, with Kanungu showing an I_A^S value of 0.13. Significant multilocus LD was observed at all study sites, as indicated by the p-values (< 0.01) for each site. The variance (VD) values were 21.95 for Jinja, 19.28 for Kanungu, and 12.96 for Tororo, while the expected variance (VE) values were 4.77, 4.43, and 4.81, respectively. These results demonstrate significant non-random associations of alleles at all study sites, suggesting the presence of linkage disequilibrium in the *P. falciparum* populations from each location.

Principal Coordinates Analysis (PCoA) indicated no distinct clusters of parasites based on their geographic origins. 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 (Figure 7).

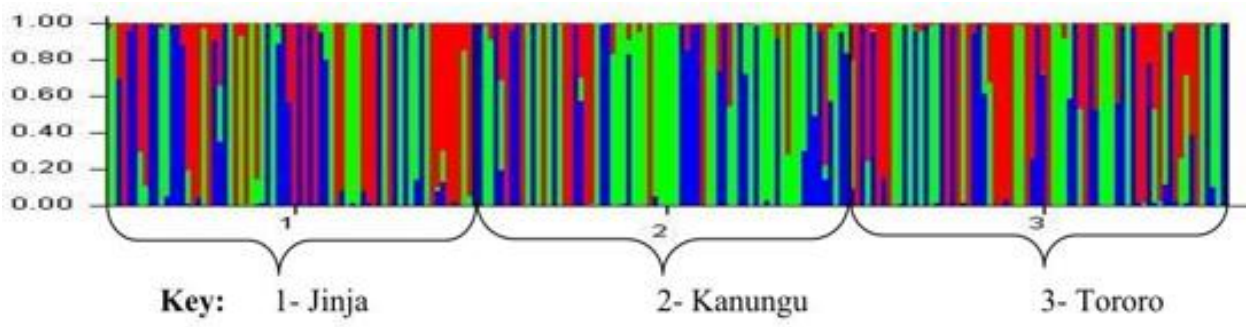


Figure 7. *The Bayesian bar plot illustrates the population structure of P. falciparum parasite isolates collected from Jinja, Kanungu and Tororo study sites. Each vertical bar in the plot represents an individual parasite isolate. Each bar is divided into colored segments, with colors (green, red, and blue) representing different genetic clusters of P. falciparum. The size of each colored segment within a bar corresponds to the proportion of that genetic cluster in the sample.*

4.3 Comparison of *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases

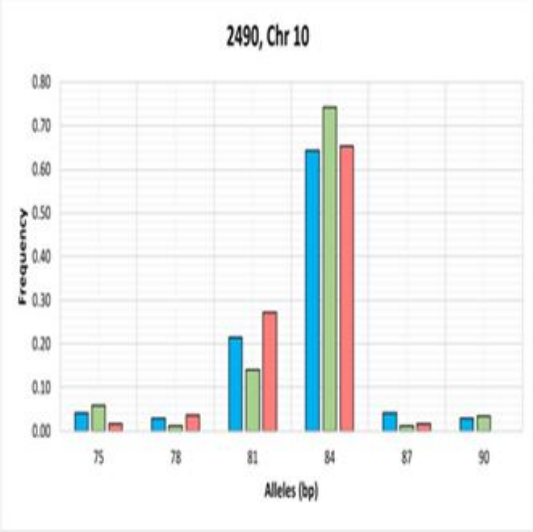
A comparison of *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria cases was conducted using 225 isolates. Of these, 211 (93.8%) were successfully genotyped at a minimum of five neutral microsatellite loci and were included in the final analysis. Among the successfully genotyped samples, 109 were obtained from male participants. The participants' ages ranged from 6 months to 53 years, with the majority being under 11 years old. Parasite densities ranged from 16 to 1,600,000 parasites/µL. Significant differences in axillary temperature, parasite density, and hemoglobin levels were detected among asymptomatic, symptomatic uncomplicated, and symptomatic complicated malaria individuals ($p < 0.001$, Kruskal-Wallis test) (Table 4).

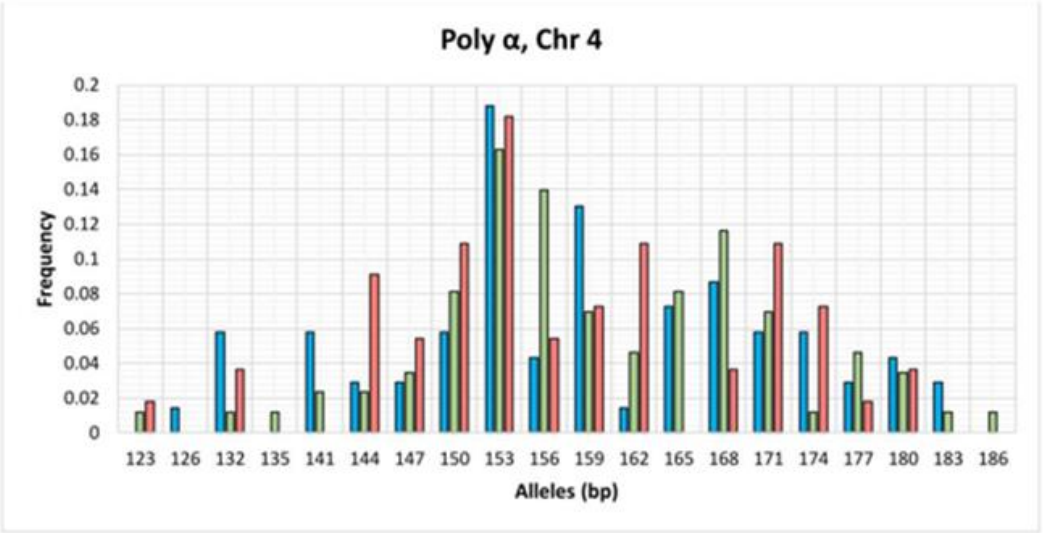
Table 4. Demographic and clinical characteristics of study participants included in the comparison of *P. falciparum* genetic diversity and MOI between asymptomatic and symptomatic malaria infections.

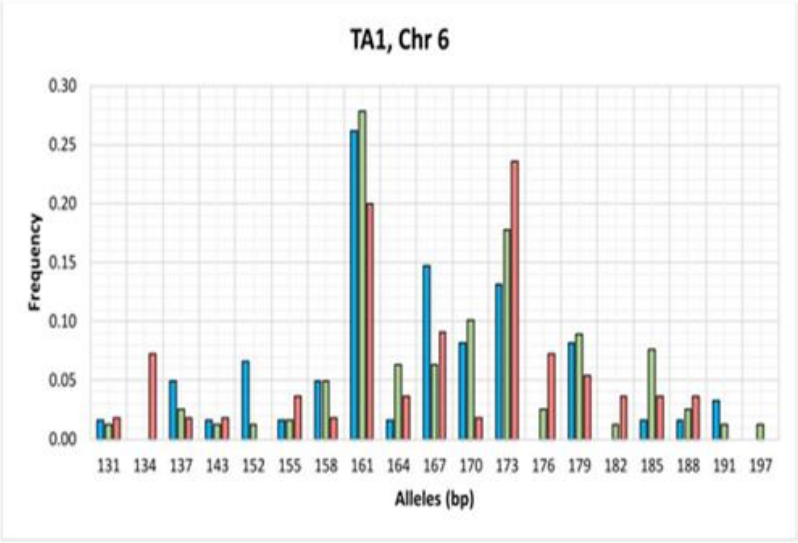
Characteristic	Asymptomatic malaria infection (n=70)	Uncomplicated malaria infection (n=86)	Complicated malaria infection (n=55)	P. value
Age (years): < 5	19 (27.1%)	39 (45.3%)	36 (65.5%)	
5-11	42 (60.0%)	40 (46.5%)	18 (32.7%)	
> 18	9 (12.9%)	7 (8.1%)	1 (1.8%)	
Gender ratio (male/female)	34/36	48/38	27/28	
Axillary temperature, ^{0}c (Mean±SD)	36.8±0.3	38.6±0.9	38.3±1.1	< 0.001
Mean parasite density/µL (Mean±SD)	5862.5±508.4	61036.4±671.9	446303.6±9136.5	< 0.001
Mean Hb g/dL (Mean±SD)	11.5±1.6	11.4±1.9	9.8±2.4	< 0.001

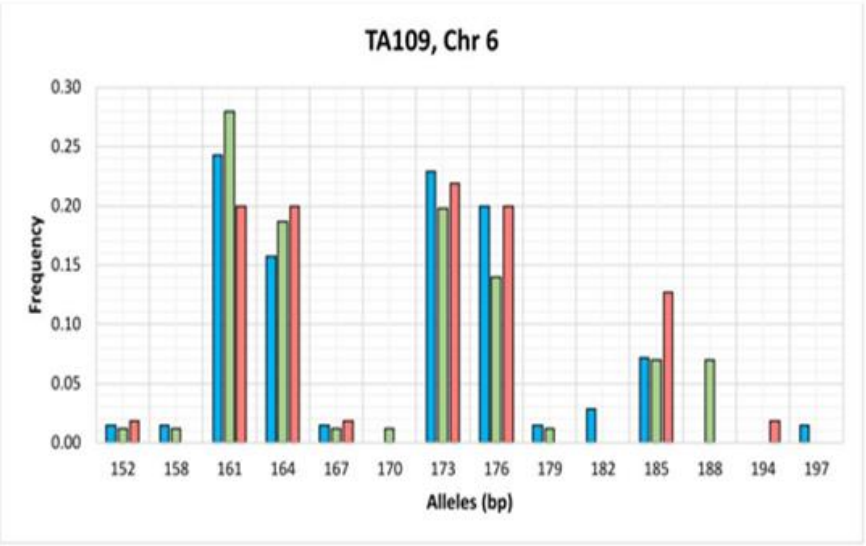
P. falciparum genetic diversity among asymptomatic and symptomatic uncomplicated and complicated study participants

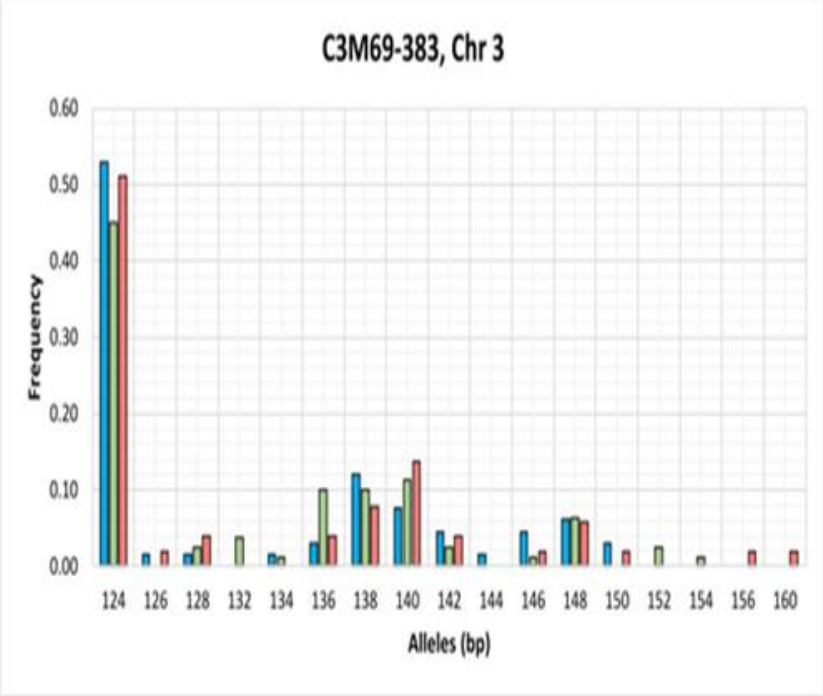
The genetic diversity of *P. falciparum*, as indicated by the N_a, allele frequencies, N_e, and H_e, was high across both symptomatic and asymptomatic malaria infections. The Kruskal-Wallis test showed no significant differences in the observed N_a, allele frequencies, N_e, and H_e between symptomatic and asymptomatic malaria infections ($p > 0.05$). The most polymorphic microsatellite loci were C2M34–313, TA1, and Poly-α, with 17-23, 15-16, and 14-19 distinct alleles, respectively. The least polymorphic microsatellite locus was 2490, with 5 to 6 distinct alleles (Figure 8 and Table 5).

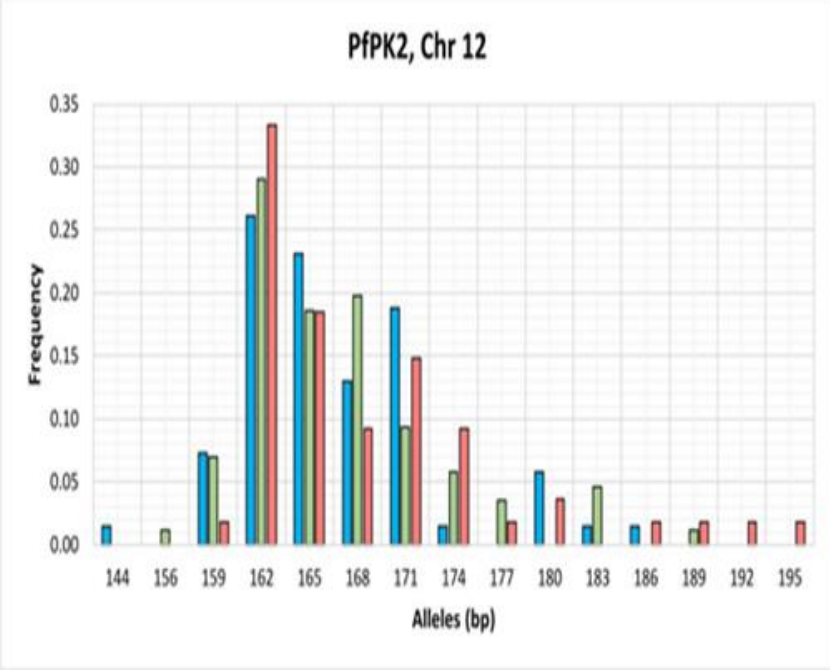

2490, Chr 10
Frequency
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
75
78
81
84
87
90
Alleles (bp)

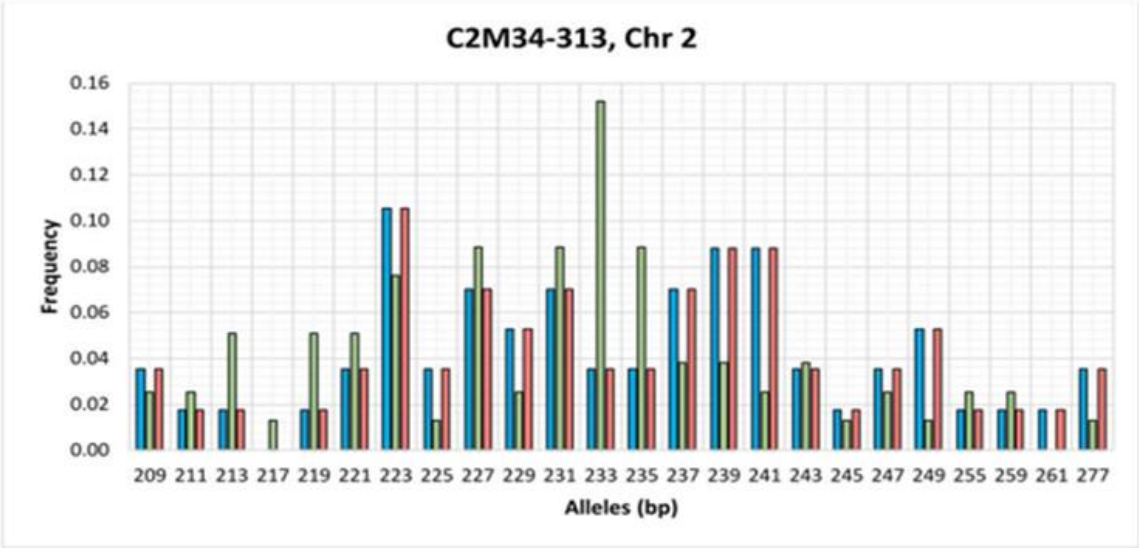

Poly α, Chr 4
Frequency
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
123 126 132 135 141 144 147 150 153 156 159 162 165 168 171 174 177 180 183 186
Alleles (bp)


TA1, Chr 6
Frequency
0.30
0.25
0.20
0.15
0.10
0.05
0.00
131 134 137 143 152 155 158 161 164 167 170 173 176 179 182 185 188 191 197
Alleles (bp)


TA109, Chr 6
Frequency
0.30
0.25
0.20
0.15
0.10
0.05
0.00
152 158 161 164 167 170 173 176 179 182 185 188 194 197
Alleles (bp)


C3M69-383, Chr 3
Frequency
0.60
0.50
0.40
0.30
0.20
0.10
0.00
124 126 128 132 134 136 138 140 142 144 146 148 150 152 154 156 160
Alleles (bp)


PfPK2, Chr 12
Frequency
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
144 156 159 162 165 168 171 174 177 180 183 186 189 192 195
Alleles (bp)



Abbreviations: Chr-*Chromosome,* ***bp****- base pair*

Figure 8. *The allele frequencies of the genotyped markers assessed among individuals with asymptomatic (blue), symptomatic uncomplicated (green) and complicated (red) malaria infections. The vertical axis represents the frequencies of individual alleles, whereas the horizontal axis represents the base pairs of each allele.*

Expected heterozygosity and number of effective alleles

The mean expected heterozygosity (H_e) ranged from 0.79 (± 0.17) in individuals with symptomatic uncomplicated malaria to 0.81 (± 0.15) in those with asymptomatic malaria. The number of effective alleles (N_e) ranged from 6.07 (± 3.65) among individuals with complicated malaria to 7.42 (± 5.15) in asymptomatic individuals (Table 5).

Table 5. The genetic diversity of *P. falciparum* in asymptomatic, symptomatic uncomplicated, and symptomatic complicated individuals

Locus	Asymptomatic malaria (n=70)			Symptomatic Uncomplicated malaria (n=86)			Symptomatic Complicated malaria (n=55)		
	N_a	N_e	H_e	N_a	N_e	H_e	N_a	N_e	H_e
2490	6	2.15	0.54	6	1.74	0.43	5	0.14	0.50
Poly α	17	11.15	0.92	19	10.97	0.92	14	10.19	0.92
C2M34–313	23	17.01	0.96	23	14.35	0.94	17	10.09	0.92
TA1	15	7.52	0.88	16	6.96	0.87	16	7.94	0.89
TA109	11	5.47	0.83	11	5.51	0.83	8	5.41	0.83
C3M69–383	12	3.20	0.69	14	4.11	0.77	12	3.39	0.72
PfPK2	10	5.44	0.83	10	5.59	0.83	12	5.32	0.83
Mean	13.43	7.42	0.81	14.14	7.03	0.79	12.29	6.07	0.80
SD	5.50	5.15	0.15	5.76	4.28	0.17	4.28	3.65	0.15

N_a = number of different alleles; N_e = number of effective alleles = 1/(∑pi^2); H_e = unbiased diversity/expected heterozygosity = [n/(n − 1)] [1 − ∑pi^2], and SD = standard deviation. N_a, N_e, and H_e were calculated from the predominant allele dataset. The 95% confidence interval (CI) was calculated as CI = $\bar{x}$ ± 1.96(SD/√n), where $\bar{x}$ is the sample mean, SD is the standard deviation of the sample, and n is the sample size.

***P. falciparum* MOI among asymptomatic, symptomatic uncomplicated and complicated malaria-infected individuals**

The mean MOI did not differ significantly across the various *P. falciparum* infection categories (p = 0.33). It ranged from 1.92 (± 0.42) in individuals with symptomatic

complicated malaria to 2.10 (± 0.36) among asymptomatic malaria individuals (Table 7). The percentages of polyclonal infections were generally consistent across the different *P. falciparum* infection types, ranging from 58.5% (95% CI: 44.60–72.39) among individuals with symptomatic uncomplicated malaria to 63% (95% CI: 51.22–74.78) among those with complicated malaria (Table 6).

Table 6. *P. falciparum* MOI in asymptomatic, symptomatic uncomplicated, and symptomatic complicated individuals

Locus	Asymptomatic malaria (n= 70)				Uncomplicated malaria (n= 86)				Complicated malaria (n= 55)			
	Number positive	Percentage monoclonal	Percentage polyclonal	Mean MOI	Number positive	Percentage monoclonal	Percentage polyclonal	Mean MOI	Number positive	Percentage monoclonal	Percentage polyclonal	Mean MOI
2490	70	64.3	35.7	1.5	85	64.7	35.3	1.4	55	63.6	36.4	1.4
Poly a	69	42.0	58.0	2.4	86	43.0	57.0	2.2	55	61.8	38.2	1.6
C2M34–313	57	43.9	56.1	1.7	79	48.1	51.9	1.8	52	50.0	50.0	1.7
TA1	62	29.0	71.0	2.1	79	25.3	74.7	2.3	55	10.9	89.1	2.6
TA109	70	47.1	52.9	2.2	85	38.8	61.2	2.2	55	50.9	49.1	2.0
C3M69–383	66	13.6	86.4	2.5	80	12.5	87.5	2.5	51	23.5	76.5	2.3
PfPK2	69	29.0	71.0	2.2	86	26.7	73.3	2.3	54	29.6	70.4	2.1
Mean		38.4	61.6	2.10		37.0	63.0	2.08		41.5	58.5	1.92
SD		16.24	16.24	0.36		17.19	17.19	0.37		20.26	20.26	0.42

The number positive represents the number of samples successfully amplified by each locus. The percentage of monoclonal infections is the proportion of isolates with only one allele score per locus. The percentage of polyclonal infections is the proportion of isolates with more than one allele score per locus.

Linkage Disequilibrium (LD) and Genetic differentiation

A multilocus index of association analysis was performed to assess the non-random association of all microsatellite loci in the dataset. Significant multilocus LD was observed in both symptomatic and asymptomatic malaria infections ($p < 0.01$). For the different infection categories, the variance (VD) was 19.05 in asymptomatic malaria, 19.38 in symptomatic complicated malaria, and 17.28 in symptomatic uncomplicated malaria. The expected variance (VE) was 4.95 for asymptomatic, 4.46 for symptomatic complicated, and 4.74 for symptomatic uncomplicated malaria. The standardized index of association (I_A^S) was 0.11 for asymptomatic malaria, 0.12 for symptomatic complicated malaria, and 0.09 for symptomatic uncomplicated malaria. The variance in VD (Var VD) was 0.25 for asymptomatic, 0.27 for symptomatic complicated, and 0.18 for symptomatic uncomplicated malaria. In all infection categories, the p-value was less than 0.01, indicating significant linkage disequilibrium.

The pairwise Wright's fixation index (F_{ST}) between the asymptomatic, symptomatic complicated, and symptomatic uncomplicated malaria infections revealed low genetic differentiation, with F_{ST} values ranging from 0.0034 to 0.0105. Specifically, the F_{ST} between asymptomatic and symptomatic complicated infections was 0.0034, between asymptomatic and symptomatic uncomplicated infections was 0.0059, and between symptomatic complicated and symptomatic uncomplicated infections was 0.0034.

4.4 Evaluation of temporal changes in *P. falciparum* genetic diversity and MOI across three sites with varying (low, medium and high) malaria transmission intensities

Evaluation of temporal changes in *P. falciparum* genetic diversity and MOI across areas with low, moderate, and high malaria transmission intensities in Uganda involved analysis of a total of 220 *P. falciparum*-positive samples. Of these, 180 (81.8%) were successfully amplified at a minimum of five microsatellite loci and included in the final analysis. Among the 180 samples, 91 (50.6%) were from male participants, and a significant proportion (46.7%) were from individuals aged 5–11 years. Overall, the mean parasite densities in the study areas decreased over time. During the 2011-2012 study period, the mean parasite counts were 17,034 parasites/µL in Jinja, 36,118 parasites/µL in Kanungu, and 68,240 parasites/µL in Tororo (Table 7). By the 2015-2016 period, these counts had declined to 14,882.4 parasites/µL in Jinja, 20,694 parasites/µL in Kanungu, and 23,586.2 parasites/µL in Tororo (Table 8). Regarding hemoglobin (Hb) levels among study participants, Jinja experienced an increase from 11.3 g/dL to 11.7 g/dL, and Tororo rose from 11.1 g/dL to 11.3 g/dL. In contrast, Kanungu saw a decrease in mean Hb levels from 10.9 g/dL to 10.7 g/dL during the same period (Table 7).

Table 7. Demographic and clinical characteristics of study participants included in the analysis of temporal changes in *P. falciparum* genetic diversity and MOI across areas with low, medium, and high malaria transmission intensities

Characteristic	Isolates collected 2011-2012			Isolates collected 2015-2016		
	Jinja (n=38)	Kanungu (n=27)	Tororo (n=24)	Jinja (n=26)	Kanungu (n=34)	Tororo (n=31)
Age in years						
<5years	21 (55.3%)	14 (51.9%)	14 (58.3%)	8 (30.8%)	12 (35.3%)	11 (35.5%)
5-11 years	11 (28.9%)	13 (48.1%)	9 (37.5%)	15 (57.7%)	22 (60.7%)	14 (45.2%)
≥ 18 years	6 (15.8%)	0 (0.0%)	1 (4.2%)	3 (11.5%)	0 (0.0%)	6 (19.4%)
Gender (%)						
Male	20 (52.6%)	15 (55.6%)	14 (58.3%)	13 (50.0%)	16 (47.1%)	13 (41.9%)
Mean Axillary temperature, ^{0}c (SD)	38.6(0.9)	38.4(1.8)	38.5(0.8)	36.8(0.5)	38.3(1.2)	37.3(0.9)
Mean Hb g/dL(SD)	11.3(1.9)	10.9(2)	11.1(1.5)	11.7(1.7)	10.7(2.4)	11.3(2.3)
Mean parasite density/µL (SD)	17,034 (2,736.1)	36,118 (4,157.9)	68,240 (8,491)	14,882.4 (1,181)	20,694 (2,197.1)	23,586.2 (1,927)

Temporal changes in *P. falciparum* genetic diversity between 2011-2012 and 2015-2016 study periods across sites

Overall, *P. falciparum* genetic diversity remained consistently high across all study sites over time, with mean H_e values consistently exceeding 0.7 (Figure 9). However, a slight decline in the mean values of N_a, N_e, and H_e was observed across all study sites (Table 8). Importantly, no significant differences were observed in mean N_a, N_e, or H_e between the study periods, nor were

there any significant temporal trends in these indices across the two study periods. These findings were supported by the non-significant results from both the Kruskal-Wallis and Jonckheere-Terpstra tests ($P > 0.05$).

Table 8. *P. falciparum* genetic diversity between 2011-2012 and 2015-2016 a cross-study sites

Locus	Site	Isolates collected 2011-2012			Isolates collected 2015-2016		
		N_a	N_e	H_e	N_a	N_e	H_e
2490	Jinja	5	1.87	0.48	5	2.06	0.54
	Kanungu	5	2.13	0.55	3	1.58	0.38
	Tororo	4	1.68	0.42	4	2.15	0.55
Poly α	Jinja	11	8.01	0.90	10	6.38	0.88
	Kanungu	12	9.23	0.93	12	7.92	0.90
	Tororo	11	6.68	0.89	11	8.04	0.91
C2M34–313	Jinja	14	9.09	0.92	14	11.26	0.95
	Kanungu	15	12.76	0.96	14	8.50	0.91
	Tororo	17	9.85	0.94	15	11.76	0.96
TA1	Jinja	10	7.76	0.89	9	5.95	0.87
	Kanungu	10	4.96	0.83	11	6.28	0.87
	Tororo	10	5.79	0.88	11	6.87	0.89
TA109	Jinja	6	3.30	0.71	6	4.69	0.72
	Kanungu	5	4.07	0.78	7	4.78	0.85
	Tororo	7	4.30	0.92	9	5.78	0.85
C3M69–383	Jinja	9	4.13	0.78	6	2.11	0.55
	Kanungu	7	3.13	0.71	7	3.35	0.52
	Tororo	9	4.96	0.83	10	4.35	0.57
PfPK2	Jinja	8	5.64	0.84	7	5.04	0.83
	Kanungu	8	4.64	0.81	9	3.12	0.70
	Tororo	7	5.45	0.85	6	5.06	0.83
Overall (Mean ± SD)	Jinja	9.0	5.68	0.79	8.14	5.36	0.76
	Kanungu	8.86	5.85	0.80	9.00	5.08	0.73
	Tororo	9.29	5.53	0.82	9.43	6.29	0.79

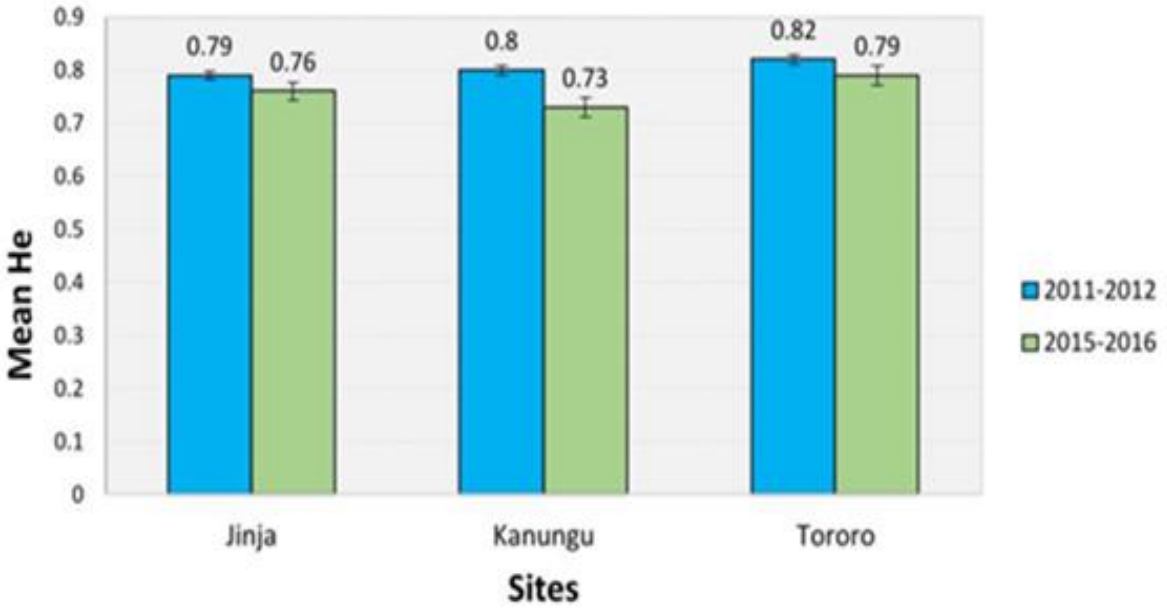


Figure 9. Changes in *P. falciparum* mean He values between 2011-2012 and 2015-2016 study periods across sites.

Temporal changes in *P. falciparum* MOI between the 2011-2012 and 2015-2016 study periods across sites

Overall, the percentage of *P. falciparum* polyclonal infections in the three study areas remained consistently high, exceeding 50%, which indicates a robust polyclonal parasite population over time. During the study periods, there was a significant temporal decrease in the percentage of polyclonal infections in Kanungu, declining from 56.1% to 52.8% overtime (P = 0.01). In contrast, Jinja and Tororo showed no significant temporal differences in the percentage of polyclonal infections (p > 0.05). Regarding *P. falciparum* mean MOI, there was a significant temporal reduction in Tororo, where it decreased from 2.2 to 2.0 overtime (p = 0.03) (Figure 9).

However, Jinja and Kanungu did not exhibit significant changes in mean MOI over time ($p > 0.05$) (Figure 10).

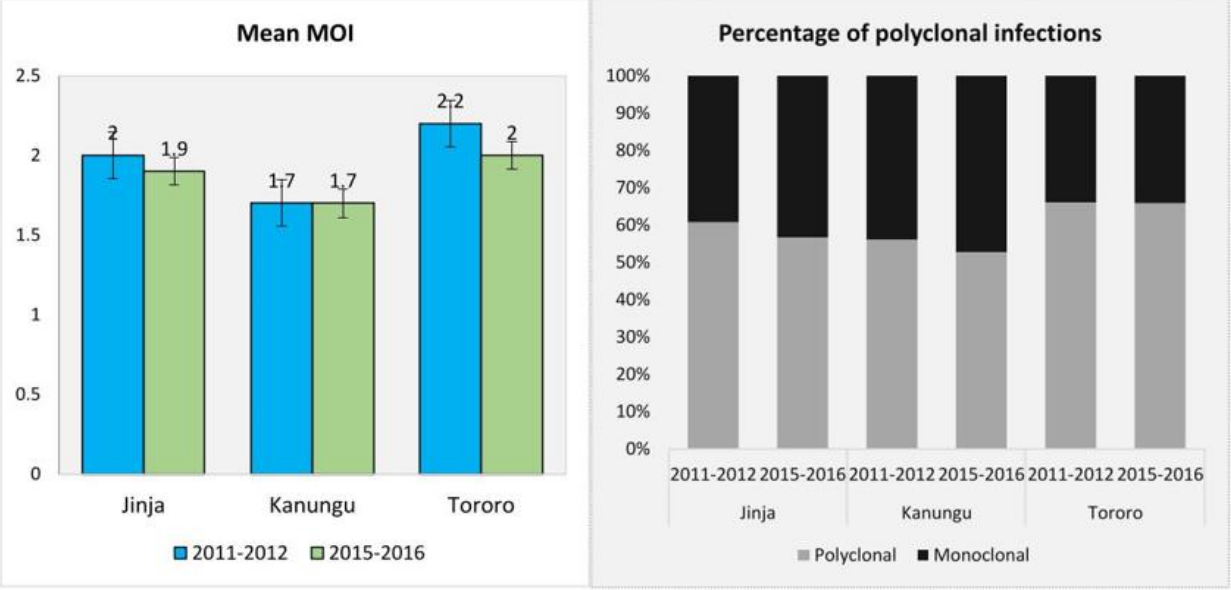


Figure 10. *Changes in P. falciparum mean MOI and the percentage of polyclonal infections between 2011-2012 and 2015-2016 study periods Jinja, Kanungu and Tororo.*

Temporal changes in Linkage disequilibrium (LD) among *P. falciparum* populations

Temporal changes in Linkage Disequilibrium (LD) among *P. falciparum* populations were assessed through a multilocus index of association analysis to evaluate the non-random associations of all microsatellite loci in the dataset. The statistical significance of LD was tested using 10,000 Monte Carlo simulations. Overall, there was a slight increase in LD, based on the standardized index of association (I_A^S), across the sites over time. During the 2011-2012 period, Kanungu recorded the lowest I_A^S at 0.0085 ($p = 0.31$), while Jinja had the highest significant I_A^S value at 0.0233 ($p = 0.06$). In the 2015-2016 study period, Kanungu's I_A^S rose to 0.0114 ($p =$

0.14), and in Tororo, it increased to 0.0168 (p = 0.18). Jinja also experienced a slight increase in I_A^S, from 0.0233 in 2011-2012 to 0.0239 in 2015-2016 (p = 0.01).

Temporal changes in genetic differentiation of *P. falciparum* populations

Overall, the genetic differentiation of the *P. falciparum* population among the sites was low, with a slight increase between the study periods. In the 2011-2012 period, the F_{ST} values were low: 0.0275 between Jinja and Kanungu, and 0.0145 between Jinja and Tororo, with the highest differentiation at 0.0503 between Kanungu and Tororo. In the 2015-2016 study period, F_{ST} values showed modest increases: 0.0384 between Jinja and Kanungu, and 0.0353 between Jinja and Tororo. The highest differentiation remained between Kanungu and Tororo, increasing to 0.0585

4.5 Performance of different genotyping markers and three algorithms (3/3 match-counting, ≥2/3 match-counting, and the Bayesian approach*)* for distinguishing *P. falciparum* recrudescence from reinfection

Assessing the performance of different genotyping markers and algorithms for distinguishing *P. falciparum* recrudescence from reinfection involved the analysis of 179 paired samples. Genotyping results showed that 174 (98.7%) of the paired samples were successfully amplified.

Diversity of the genotyped markers in Aduku, Arua and Masafu study sites

The genetic markers K1, MAD20, RO33, along with the 3D7 and FC27 allelic variants, correspond to the *msp-1* and *msp-2* genes, respectively. The overall mean expected heterozygosity (H_e) for *P. falciparum* was 0.84 ± 0.12 in Aduku, 0.85 ± 0.12 in Arua, and 0.87 ± 0.09 in Masafu. For *msp-1*, the K1 allelic family exhibited the highest H_e values, with 0.88 in

Aduku, 0.93 in Arua, and 0.92 in Masafu. For *msp-2*, the 3D7 allelic family displayed the highest H_e values, with 0.94 in both Aduku and Arua, and 0.95 in Masafu. The microsatellite marker 313 showed consistent H_e values of 0.95 across all sites. The Poly-α marker demonstrated mean expected H_e values of 0.92 in Aduku, 0.91 in Arua, and 0.94 in Masafu. Additionally, the overall mean effective number of alleles (N_e) was 7.61 ± 3.66 in Aduku, 8.77 ± 4.04 in Arua, and 9.24 ± 4.62 in Masafu (Table 9). These findings indicate a relatively high level of genetic diversity across the study sites.

Table 9. Genetic diversity of the *msp1*, *msp2, glurp* and microsatellite markers a cross Aduku, Arua and Masafu

Marker	Aduku		Arua		Masafu	
	H_e	N_e	H_e	N_e	H_e	N_e
K1	0.88	7.45	0.93	12	0.92	10.84
MAD20	0.89	6.67	0.93	11.59	0.9	8.29
RO33	0.63	2.56	0.56	2.21	0.68	2.99
IC/3D7	0.94	12.63	0.94	14.71	0.95	16.26
FC 27	0.87	6.92	0.87	6.92	0.85	6.23
Glurp	0.86	6.44	0.89	8.28	0.92	11.71
313	0.95	15.47	0.95	15.72	0.95	17.56
383	0.9	8.97	0.88	7.63	0.85	6.49
Poly-α	0.92	10.47	0.91	9.98	0.94	14.02
TA1	0.86	6.59	0.89	7.63	0.89	8.56
PfPK2	0.87	7.18	0.87	7.15	0.85	6.42
2490	0.56	2.2	0.6	2.47	0.68	3.03
TA109	0.83	5.39	0.88	7.7	0.88	7.78
Mean	0.84	7.61	0.85	8.77	0.87	9.24
SD	0.12	3.66	0.12	4.04	0.09	4.62

The mean allele richness (Ar) across the study sites was 10.6 ± 3.9 in Aduku, 12.5 ± 4.4 in Arua, and 11.9 ± 4.5 in Masafu. The number of genotypes for the K1 allelic family ranged from 14 to 18 in Aduku and Arua, respectively, while for I/C 3D7, it ranged from 19 to 25 in Aduku and Masafu. The most polymorphic microsatellite, 313, exhibited 22 to 28 genotypes in Aduku and Arua. Poly-α genotypes ranged from 15 to 24 in Aduku and Masafu. Microsatellite 383 showed

14 to 20 genotypes in Aduku and Arua. The less polymorphic microsatellite 2490 had 5 genotypes in Aduku and 6 genotypes in Arua and Masafu. Markers such as TA1, TA109, PfPK2, 2490, and *glurp* exhibited 3 genotypes with allele frequencies greater than 10%, with 2490 showing a single genotype with a frequency exceeding 50%. The Kruskal-Wallis test revealed no significant differences in the observed number of genotypes, allele richness (Ar), allele frequencies, number of effective alleles (N_e), or expected heterozygosity (H_e) across the sites ($p > 0.05$). These results suggest that there is no significant variation in genetic diversity across the three sites, despite the observed differences in allele richness and genotype distribution.

P. falciparum MOI in Aduku, Arua and Masafu

Across all study sites, *msp-1* and the microsatellites PfPK2 and Poly-α exhibited the highest average MOIs, while microsatellite 313, the most diverse marker, generally showed lower MOIs. In Aduku, the highest average MOIs for microsatellites were observed for PfPK2, TA1, and Poly-α, with values of 1.77, 1.68, and 1.59, respectively. In Arua, PfPK2 had the highest average MOI of 1.71, followed by TA1 and 383, each with an MOI of 1.59. In Masafu, Poly-α, TA1, and TA109 showed the highest MOIs, with average values of 1.79, 1.68, and 1.58, respectively (Figure 11). Across all three sites, *glurp* consistently exhibited one of the lowest MOIs. The Kruskal-Wallis test revealed significant differences in MOIs: Poly-α had significantly higher MOIs in Masafu, PfPK2 exhibited significantly higher MOIs in Arua and Aduku, and *msp-1* showed significantly higher MOIs in Arua and Masafu ($P < 0.05$).

Spearman's rank correlation analysis showed no significant correlation between mean H_e and mean MOI ($p > 0.05$).

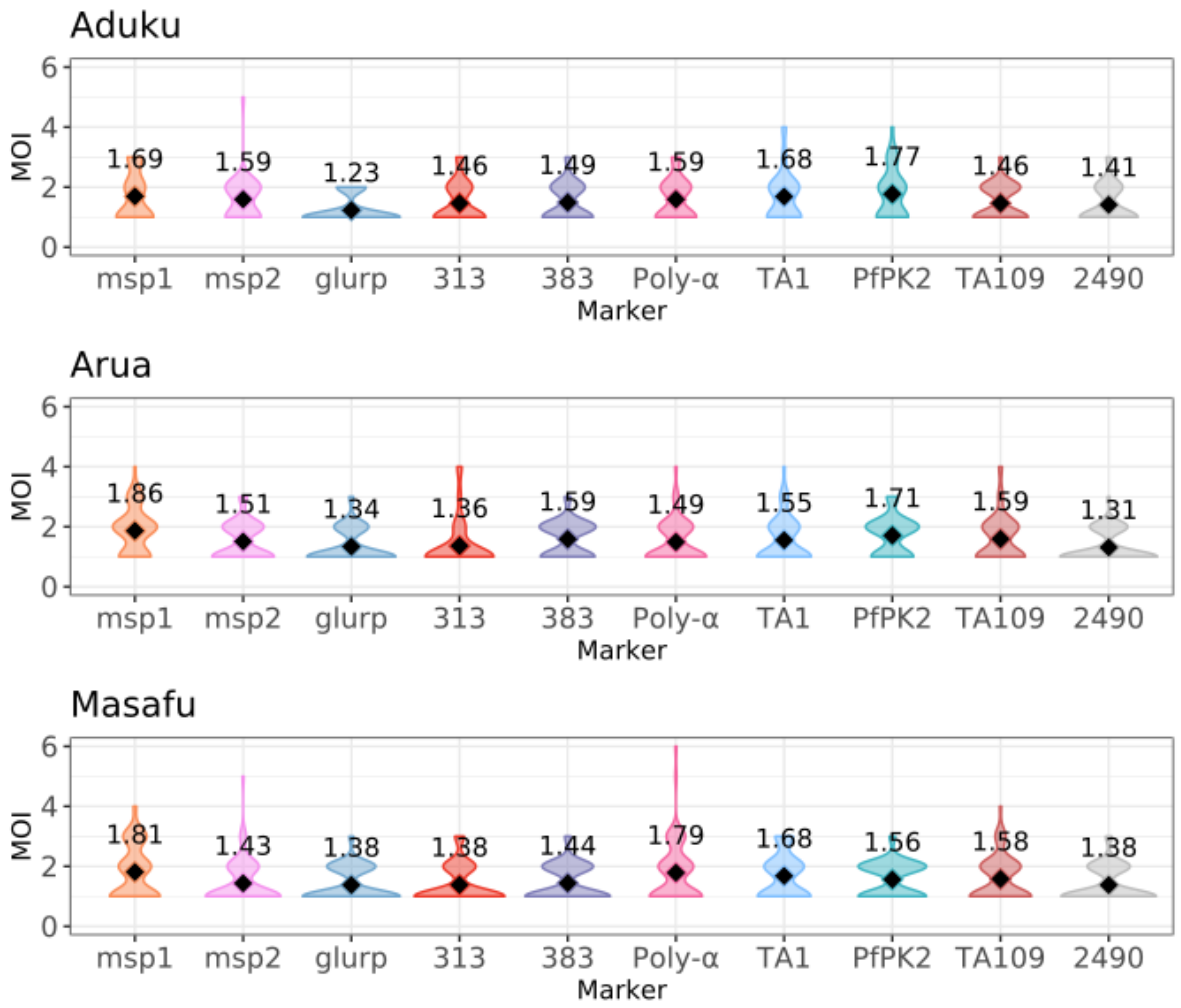


Figure 11: **Distribution of the *P. falciparum* MOI across Aduku, Arua and Masfu study sites.** *Each violin plot shows, for a given site, the distribution of the observed MOIs in the samples collected at Day 0. The diamond from each violin plot represents the average MOI whose corresponding value is displayed above.*

Comparison of ≥2/3, 3/3 and Bayesian algorithm to classify recrudescence

The 3/3 algorithm was the most conservative, identifying significantly fewer recrudescences than both the ≥2/3 and Bayesian algorithms at probability cutoffs of ≥ 0.7 and ≥ 0.8 ($P < 0.05$). For

the AL arm, this ranged from two recrudescences with *msp-1*, *msp-2* paired with microsatellites 383 and Poly-α, to seven recrudescences using microsatellite 2490 as third marker. Similarly, for the DP arm, the 3/3 algorithm identified two recrudescences when using microsatellites TA1 or TA104 as the third marker, compared to four recrudescences when using *glurp*, 383, Poly-α, or PfPK2. In contrast, the ≥2/3 algorithm identified significantly more recrudescences. For the AL arm, the number ranged from minimum ten recrudescences with microsatellite TA1 to fourteen with PfPK2 and 2490. For the DP arm, it ranged from seven recrudescences with microsatellite 313 to ten with *glurp* (Figure 12). The number of recrudescences classified by the Bayesian algorithm varied significantly depending on the probability cutoff used. For the AL arm, on average, nearly twice as many recrudescences were identified with a 0.7 cutoff compared to 0.8, while for the DP arm, the 0.7 cutoff resulted in over 62% more recrudescences than with the 0.8 cutoff. The marker combinations yielding the highest and lowest numbers of recrudescences also varied with the cutoff and drug arm. Specifically, microsatellites 313 and 2490 produced the highest number of recrudescences for both thresholds in the AL arm, while microsatellites TA109 and 383 resulted in the lowest number at the 0.7 and 0.8 thresholds, respectively. In the DP arm, microsatellite 383 led to the most recrudescences at the 0.7 cutoff, while TA109 produced the highest number at 0.8 cutoff. For the same drug arm, the fewest recrudescences were identified using Poly-α and TA1 at the 0.7 cutoff, and Poly-α again at the 0.8 cutoff (Figure 12).

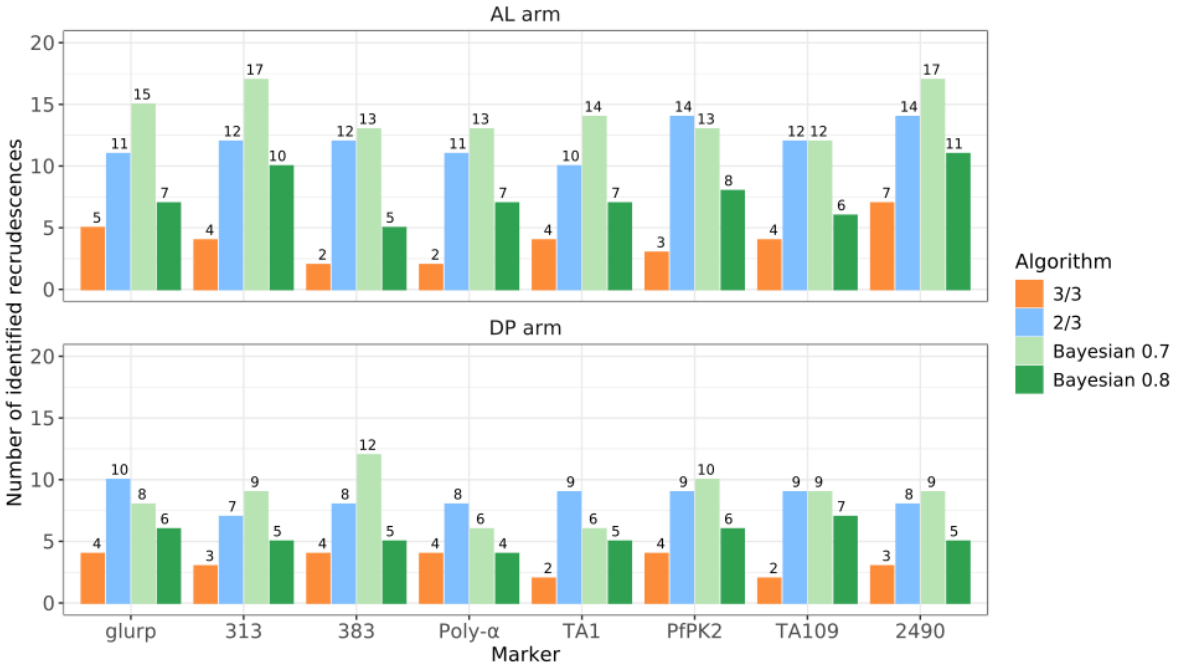


Figure 12: Comparison of the number of recrudescences classified by ≥2/3, 3/3, and Bayesian algorithm. *Results are presented for the ≥2/3, 3/3, and Bayesian algorithm using cutoffs of 0.7 and 0.8 for the probability of recrudescence, across each marker combination (x-axis). The number of recrudescences identified by each algorithm (y-axis) is illustrated with the colored bars.*

CHAPTER FIVE: DISCUSSION

5.1 Discussion

The overarching goal of this study was to assess the status of *P. falciparum* genetic diversity and MOI across SSA, with specific focus on areas with varying malaria transmission intensities in Uganda, and to evaluate the performance of different genetic markers and algorithms in distinguishing recrudescence from reinfection. The findings showed significant variation in genetic diversity and MOI across SSA, with high genetic diversity and minimal population structure observed in Uganda, regardless of transmission intensity. This suggests extensive gene flow and parasite diversity across diverse transmission settings. Notably, *P. falciparum* infections exhibited high genetic diversity in both asymptomatic and symptomatic individuals, with asymptomatic individuals showing slightly higher genetic diversity. While the study observed a modest temporal decline in both genetic diversity and MOI, these metrics remained high, indicating a slow reduction in malaria transmission in Uganda's three study sites with differing malaria endemicity levels. The study also highlighted the potential of microsatellite markers such as PfPK2 and Poly-α, which exhibited higher MOIs, as promising alternatives to *glurp* for classifying recrudescence particularly in high-transmission areas. These findings enhance our understanding of parasite gene flow dynamics and are critical for refining malaria control strategies, strengthening surveillance, and optimizing interventions in regions with varying transmission patterns.

The systematic review and meta-analysis showed high *P. falciparum* genetic diversity and MOI across SSA, with regional variations likely influenced by differences in malaria transmission intensity. These findings align with previous reports of high *P. falciparum* genetic diversity

observed in Uganda and in other high (36, 193) and low (87) malaria transmission areas within SSA. In such regions, the abundance of highly efficient mosquito vectors, particularly female mosquitoes of the *Anopheles gambiae* and *Anopheles funestus* complexes, which are well adapted to human environments (2, 3), promotes frequent genetic recombination, thereby contributing to the observed genetic variation (11, 194). Despite intensifying malaria control efforts, regions with high genetic diversity continue to present concerns due to ongoing transmission, while regions with lower genetic diversity may reflect the success of malaria control measures (132). The prevalence of polyclonal infections, indicative of high transmission was notably high across all the study sites, with a pooled mean of 63%. This high occurrence of polyclonal infections can be attributed to increased vector populations, which may promote superinfections or the concurrent transmission of unrelated parasite genotypes (195). While 28 potentially relevant publications were not accessible due to restricted access. The screening of titles and abstracts suggested these studies likely covered settings as those included, their omission may have introduced a potential for missing evidence bias, which could slightly refine pooled estimates but is unlikely to alter the overall conclusions regarding high diversity and MOI across SSA. By incorporating data from SSA and study sites across Uganda, our review provides important insights into regional variations in *P. falciparum* genetic diversity, expanding our understanding of how ecological and epidemiological factors influence parasite populations in different settings.

Further review of markers used to estimate *P. falciparum* MOI found that antigen-coding loci, such as *msp-1* and *msp-2*, typically report higher mean MOI compared to microsatellites. Microsatellites offer greater polymorphism and are less influenced by immune selection. The lower polymorphism and fewer alleles in *msp-1, msp-2*, and *glurp* limit their effectiveness in

accurately estimating MOI (196). While these traditional markers remain widely used and are recommended by WHO for molecular correction in therapeutic efficacy studies, they have lower resolution than SNP-based or whole genome sequencing approaches, particularly for detecting minority clones and fine-scale population structure. Deep amplicon sequencing or whole-genome sequencing, though beyond the scope of this work due to resource and feasibility constraints, offer enhanced discrimination of parasite haplotypes and structural variation. Our findings are therefore interpreted within the capabilities and constraints of commonly used molecular markers. These findings emphasize the importance of using more advanced molecular tools, such as microsatellites and whole-genome sequencing, for precise assessments of parasite genetic diversity and MOI.

P. falciparum genetic diversity and MOI were found to be influenced by several factors, including parasite density, clinical malaria category, patient age, malaria control interventions, and transmission intensity (17, 19, 85, 118, 190-192). Consistent with previous research, the current study confirmed that higher parasite densities correlate with increased genetic diversity and MOI (19, 197), as higher parasite densities increase the likelihood of distinct parasite genotypes. Immunity development with age was inversely related to *P. falciparum* genetic diversity (198), while rural areas exhibited higher genetic diversity compared to urban areas (199). Regions with well-implemented malaria control interventions, such as Ethiopia (85), exhibited lower diversity and MOI, suggesting that long-term interventions like LLIN distribution, IRS, and ACT deployment may indirectly shape parasite population dynamics. However, persistent high diversity in Uganda despite these interventions indicates ongoing transmission pressure.

The analysis of *P. falciparum* genetic diversity and population structure indicated high genetic diversity and MOI, with limited population structure across Uganda's varying malaria transmission settings. The mean number of alleles (N_a) ranged from 11.29 to 13.34, which aligns with high-transmission areas in SSA. In contrast, low-transmission regions, such as Djibouti, reported fewer alleles (N_a = 5.3 to 13.5) (200). The observed high mean H_e, ranging from 0.78 to 0.81, suggests large effective parasite populations that promote genetic recombination and diversity (82, 201), a characteristic shared with other high-transmission regions, such as Madagascar (202). Genetic diversity persists in low-transmission areas due to the influx of parasites from high-transmission regions (87), which complicates immunity development, facilitates immune evasion, and fosters the emergence of drug-resistant strains (9).

Significant variations in *P. falciparum* MOI were observed across study sites ($p < 0.001$). Tororo, a high-transmission area, exhibited the highest mean MOI (2.5), followed by Kanungu (2.1), and Jinja (1.9), a low-transmission region. These findings are consistent with studies from Rwanda, where higher MOI values were observed in high-transmission areas like Ruhaha (mean MOI = 2.13, polyclonal infection = 62%) and lower values in Mubuga (mean MOI = 1.29, polyclonal infection = 26.9%) (19). Increased MOI indicates frequent exposure to multiple parasite strains, leading to genetic recombination and a higher risk of immune evasion and drug resistance (40). Conversely, low-transmission areas tend to have more monoclonal infections, reflecting fewer mosquito bites and limited genetic recombination (203, 204). These findings also provide a temporal baseline based on samples collected in 2011. While parasite populations may evolve over time, microsatellite-based neutral genetic diversity patterns are relatively stable in high-transmission settings, supporting the relevance of historical comparisons while emphasizing the need for ongoing surveillance to capture current dynamics.

Genetic differentiation (F_{ST}) analysis showed minimal variation, with F_{ST} values ranging from 0.011 to 0.021, consistent with findings from other malaria-endemic regions, such as Kenya (F_{ST} = 0.072) (88). Principal coordinates analysis confirmed this low genetic differentiation, indicating minimal geographic structuring of *P. falciparum* populations. Linkage disequilibrium (LD) values were also low (ranging from 0.07 to 0.14), suggesting that alleles at different loci are largely independent within populations. These patterns align with studies from high-transmission areas, including Mali and Nigeria, where low LD values are attributed to high genetic recombination (205, 206). The low F_{ST} and LD values suggest significant gene flow between regions, likely facilitated by human migration, which supports parasite exchange across transmission settings (80, 205, 206). In Uganda, where internal migration is common, gene flow enhances *P. falciparum* genetic diversity and complicates local malaria control efforts. These findings underscore the need for regional malaria control strategies that account for gene flow across populations.

Our study found that both asymptomatic and symptomatic *P. falciparum* infections exhibited high *P. falciparum* genetic diversity, with asymptomatic individuals showing slightly higher diversity (H_e = 0.81 ± 0.15) compared to those with symptomatic uncomplicated malaria (H_e = 0.79 ± 0.17) and complicated malaria cases (H_e = 0.80 ± 0.15). This suggests that asymptomatic individuals may serve as reservoirs for parasite transmission, contributing to ongoing genetic evolution. Similar results have been reported in Côte d'Ivoire (207) and Kenya (118), where asymptomatic individuals harbored a broader range of parasite genotypes. Despite some studies reporting no significant difference in *P. falciparum* genetic diversity between symptomatic and asymptomatic cases (136, 208), our findings highlight the potential role of asymptomatic

infections in sustaining transmission and the importance of interventions targeting these reservoirs.

Temporal analysis showed slight decreases in *P. falciparum* genetic diversity and MOI, suggesting reduced malaria transmission intensity. However, genetic diversity remained high, with mean H_e values exceeding 0.7 across all study sites. These results align with studies from Eswatini and Kenya, where high parasite genetic diversity persisted despite interventions, likely due to parasite importation from neighboring regions (133, 209). This indicates that human migration plays a crucial role in maintaining genetic diversity across regions, irrespective of transmission intensity. The slight decreases in *P. falciparum* MOI over time suggest a gradual decline in transmission intensity, a trend observed in Tororo following vector control measures (210). Studies from the Democratic Republic of the Congo (DRC) (135) and Kenya (136) showed similar patterns, suggesting regional variability in the relationship between transmission intensity and MOI. Our analysis found polyclonal infections in over 50% of cases, typical in mesoendemic and holoendemic areas (136, 211). The decline in both metrics suggests that control measures may reduce polyclonal infections, which could influence the genetic structure of *P. falciparum* populations (134).

The study further indicated that although *glurp* exhibited high genetic diversity (mean H_e = 0.86–0.92), it showed a lower mean MOI (1.23–1.38), leading to fewer recrudescence cases being classified. This lower MOI suggests that *glurp* is less sensitive in detecting minority clones, which may result in an underestimation of recrudescence and an overestimation of drug efficacy (181). Previous studies have reported similar findings, indicating that *glurp* has moderate diversity but lower sensitivity for detecting minority clones (212). However, some studies found

no significant differences in recrudescence classification when *glurp* was used in place of microsatellites (213), though its reduced sensitivity remains a limitation (181). Markers such as PfPK2 and Poly-α, which exhibit higher MOIs and greater sensitivity to minority clones, are therefore better suited for recrudescence classification, particularly in high-transmission areas. Combining microsatellites with SNP-rich markers or deep amplicon sequencing improves minority clone detection. While such approaches were beyond the scope of this study, our results are consistent with emerging evidence supporting higher-resolution genotyping for accurate PCR correction in therapeutic efficacy studies.

Regarding the classification algorithms, the 3/3 approach identified fewer recrudescences, enhancing specificity but potentially misclassifying true recrudescences as reinfections in high-transmission areas. In contrast, the ≥2/3 approach classified more recrudescences than the 3/3 algorithm, particularly with markers such as 383 and PfPK2, suggesting it may be better suited for low to moderate-transmission areas. However, this approach could overestimate recrudescence and inflate drug efficacy in high-transmission regions (146). The Bayesian algorithm, on the other hand, identified more recrudescences than both the ≥2/3 and 3/3 algorithms and showed a more uniform distribution of likelihoods for recrudescence classification, differing from previous studies from Angola, which reported a bimodal distribution between recrudescences and reinfections (148). Direct comparison with the primary TES study was not possible due to data access limitations; thus, our discussion focuses on comparative performance metrics rather than exact recrudescence counts.

Overall, the study findings indicate high *P. falciparum* genetic diversity, MOI, and significant gene flow, presenting ongoing challenges for malaria transmission. Microsatellites PfPK2 and

Poly-α, which are sensitive to minority clones, show promise as alternatives to *glurp*. The 3/3 match-counting algorithm classified fewer recrudescences compared to the ≥2/3 and Bayesian algorithms. These findings highlight the importance of region-specific strategies that account for asymptomatic carriers, human migration, evolving environmental pressures such as drug resistance and insecticide use, and the potential adoption of higher-resolution genotyping methods to improve malaria surveillance and therapeutic efficacy assessment.

5.2 Strengths and limitations of the study

Strengths of the study

The systematic review and meta-analysis were conducted following the PRISMA-P guidelines, ensuring methodological rigor, transparency, and reproducibility throughout the process. By focusing on peer-reviewed articles published over an extended period, the study captured a comprehensive range of data on *P. falciparum* genetic diversity and MOI across SSA.

To address potential limitations and enhance the robustness of the systematic review and meta-analysis findings, a subgroup meta-analysis was conducted based on geographic regions, improving coverage and reducing regional bias. This approach provided a more detailed understanding of the *P. falciparum* genetic diversity and MOI patterns across different parts of SSA.

The inclusion of three study sites with varying malaria transmission intensities increased the generalizability of the results, allowing for a more nuanced understanding of regional variation in parasite genetic diversity and MOI.

Finally, the use of advanced molecular techniques, including nested PCR and capillary electrophoresis, enabled high-resolution genotyping of *P. falciparum*. Additionally, statistical tools such as GENALEX and Arlequin were used to analyze genetic markers including seven neutral microsatellites and key genes (*msp-1*, *msp-2*, and *glurp*), thereby enhancing the accuracy and depth of genetic diversity and MOI assessments.

Limitations of the study

The systematic review and meta-analysis geographic coverage were limited, with relatively few studies from Southern Africa included (87, 214). While a subgroup analysis addressed this to some extent, the underrepresentation of certain regions could affect the broader applicability of the findings.

The inclusion of only articles published in English may have introduced selection bias, potentially excluding relevant studies published in other languages.

Although neutral microsatellite markers were used to assess genetic diversity, these markers may not fully capture the entirety of *P. falciparum's* genetic variation. To address this, markers were selected across six of the parasite's 14 chromosomes to increase coverage. However, whole-genome sequencing would provide a more comprehensive view of the genetic landscape and is recommended for future research. Furthermore, microsatellites have lower resolution compared to SNP-based genotyping or whole-genome sequencing approaches, which may limit detection of fine-scale population structure and minority clones.

Technical challenges, including incomplete amplification rates and unequal sample sizes across study sites, may have contributed to random error and impacted statistical precision. Stringent quality control protocols were implemented to minimize these issues.

The use of the QIAxcel system, which has lower resolution and discriminatory power compared to more advanced genotyping platforms (e.g., capillary electrophoresis), may have led to allelic misclassification**,** particularly in distinguishing between alleles with small size differences. This limitation could have resulted in an underestimation of the multiplicity of infection (MOI) or inaccurate classification of treatment outcomes, especially in differentiating recrudescence from new infections. To minimize variability and maintain consistency, standardized sample processing protocols were implemented across all study sites.

The field component of this study was conducted in three sub-counties within Uganda, While these sites were purposively selected to reflect varying transmission intensities, they cannot fully represent the ecological and epidemiological diversity of parasite populations across the country. Therefore, findings should be interpreted as within Uganda rather than across the Uganda.

Finally, parasite samples analyzed in Sub-studies I and II were originally collected between 2011 and 2016, and TES samples for Sub-study III were collected in 2019. Although neutral genetic diversity patterns in high-transmission settings may remain relatively stable over moderate time periods, parasite populations are dynamic and influenced by evolving intervention pressures. The findings therefore provide important baseline insights in genetic status of *P. falciparum* populations in Uganda.

CHAPTER SIX: CONCLUSIONS, RECOMMENDATIONS AND IMPLICATIONS

6.1 Conclusions and recommendations

This thesis addressed five objectives structured under three sub-studies, focusing on (i) regional patterns of *P. falciparum* genetic diversity and MOI across SSA, (ii) parasite population structure and transmission dynamics in Uganda, and (iii) performance of genotyping markers and classification algorithms for distinguishing recrudescence from reinfection. The conclusions below are drawn directly from the findings of each objective.

The systematic review and meta-analysis demonstrated high *P. falciparum* genetic diversity and MOI with in SSA, with significant regional variation. A high prevalence of polyclonal infections (pooled mean 63%) indicates sustained transmission intensity in many endemic settings. These findings confirm that parasite populations across SSA remain genetically diverse despite ongoing malaria control efforts.

High genetic diversity (H_e = 0.78–0.81) and moderate to high MOI were observed across study sites in Uganda, irrespective of transmission intensity. Low genetic differentiation (F_{ST} = 0.011–0.021) and low linkage disequilibrium indicate substantial gene flow and limited population structuring across sites. Asymptomatic and symptomatic infections both harbored high parasite diversity, with slightly higher diversity observed among asymptomatic individuals, supporting their role as transmission reservoirs. Although slight temporal declines in MOI and genetic diversity were observed, overall levels remained high, suggesting persistent transmission pressure.

Microsatellite markers PfPK2 and Poly-α demonstrated higher MOI and improved minority clone detection compared to glurp, supporting their suitability for PCR correction in high-transmission settings. The 3/3 match-counting algorithm classified fewer recrudescences compared to the ≥2/3 and Bayesian approaches, while the Bayesian algorithm identified the highest number of recrudescences. These findings demonstrate that both marker selection and algorithm choice significantly influence classification outcomes in therapeutic efficacy studies.

Overall, high parasite genetic diversity, persistent polyclonal infections, and substantial gene flow characterize *P. falciparum* populations in the studied settings. Marker resolution and algorithm selection materially affect recrudescence classification, underscoring the importance of methodological standardization in molecular surveillance and drug efficacy monitoring.

In light of the findings demonstrating high parasite genetic diversity, persistent polyclonal infections, low population structure, marker-dependent variation in MOI, and algorithm-dependent differences in recrudescence classification, the following recommendations are proposed:

- National Malaria Control Programmes (NMCPs) and Ministries of Health in SSA countries, including Uganda, should develop and implement region-specific malaria control strategies informed by local parasite genetic diversity and transmission patterns. This recommendation is supported by the observed high genetic diversity and substantial gene flow across transmission settings, which indicate that uniform intervention approaches may not sufficiently address localized transmission dynamics.

• In Uganda, NMCPs, district health authorities, and community health workers must prioritize the identification and treatment of asymptomatic carriers. This is directly supported by the finding that asymptomatic individuals harbored high parasite genetic diversity and may serve as reservoirs sustaining transmission**.** Active case detection strategies, such as targeted screening in high-transmission areas, will be vital to reduce hidden parasite reservoirs.

• Uganda Ministry of Health, in collaboration with NMCPs and surveillance teams, should strengthen adaptive molecular surveillance systems. Given the persistent high H_e, MOI, and low F_{ST} observed across sites, continuous monitoring of parasite population structure and genetic diversity is necessary to track transmission dynamics and intervention impact**.**

• Research institutions and academic partners should further validate and optimize highly polymorphic microsatellite markers such as PfPK2 and Poly-α for therapeutic efficacy studies (TES). This recommendation arises directly from the demonstrated higher MOI and improved minority clone detection of these markers compared to *glurp* in Sub-study III**.** Further evaluation of algorithm performance, including Bayesian approaches, is also warranted to improve classification accuracy.

• Global health agencies and technical bodies, such as WHO and the Global Malaria Programme, should promote standardized PCR correction guidelines and harmonized adoption of validated genotyping markers. This recommendation is justified by the observed discrepancies between match-counting and Bayesian algorithms, which significantly influenced recrudescence classification.

• Investment in molecular and genomic surveillance capacity within endemic countries should be strengthened. This recommendation is grounded in the methodological limitations identified in this study, including marker resolution constraints and technical amplification challenges, which highlight the need for improved laboratory and analytical infrastructure**.**

6.2 Study implications for future research and policy

This study highlights the essential role of continuous genomic surveillance led by national malaria control programs and public health authorities in tracking *P. falciparum* genetic diversity and MOI dynamics. The high parasite genetic diversity, persistent polyclonal infections, and low population structure observed across Ugandan sites indicate that surveillance must be sensitive to local transmission heterogeneity.

Incorporating advanced molecular tools such as targeted deep sequencing and whole-genome sequencing is recommended. Although not applied in this study due to resource constraints, these tools would enhance resolution beyond microsatellites, enabling detection of minority clones and structural variants, which are critical for monitoring parasite evolution and transmission.

The observed high parasite genetic diversity and MOI have direct implications for immunity acquisition, vaccine efficacy, and treatment outcomes. Specifically, the data suggest that asymptomatic carriers contribute to ongoing transmission and that gene flow across regions may facilitate immune escape, highlighting the need to integrate molecular surveillance into vaccine impact evaluations, particularly for RTS,S/AS01 deployment in Uganda.

Further research is warranted to understand the ecological and evolutionary factors maintaining low linkage disequilibrium in *P. falciparum* populations. This insight is crucial to anticipate the emergence of drug-resistant or vaccine-escape variants, which could undermine control efforts.

Continued validation and optimization of highly polymorphic markers (PfPK2, Poly-α) is recommended. Our study demonstrated that these markers better detect minority clones than *glurp*, making them more reliable for differentiating recrudescence from reinfection in TES. Broad field validation will enable standardized use in diverse transmission settings.

Bioinformatics and public health agencies should refine genotyping algorithms, including Bayesian models, to improve sensitivity and specificity. This refinement addresses the discrepancies observed between match-counting and Bayesian approaches in Sub-study III, ensuring that PCR correction frameworks are context-specific and reliable.

REFERENCES

1. Organization WH. World malaria report 2024: World Health Organization; 2024.

2. Kahamba NF, Finda M, Ngowo HS, Msugupakulya BJ, Baldini F, Koekemoer LL, et al. Using ecological observations to improve malaria control in areas where Anopheles funestus is the dominant vector. Malaria Journal. 2022;21(1):158.

3. Takken W, Charlwood D, Lindsay SW. The behaviour of adult Anopheles gambiae, sub-Saharan Africa's principal malaria vector, and its relevance to malaria control: a review. Malaria Journal. 2024;23(1):161.

4. WHO. Uganda MPs Move to End Malaria https://www.afro.who.int/countries/uganda/news/uganda-mps-move-end-malaria. 2023.

5. Okia M, Hoel DF, Kirunda J, Rwakimari JB, Mpeka B, Ambayo D, et al. Insecticide resistance status of the malaria mosquitoes: Anopheles gambiae and Anopheles funestus in eastern and northern Uganda. Malaria journal. 2018;17:1-12.

6. Conrad MD, Asua V, Garg S, Giesbrecht D, Niaré K, Smith S, et al. Evolution of partial resistance to artemisinins in malaria parasites in Uganda. New England Journal of Medicine. 2023;389(8):722-32.

7. Thiam LG, Mangou K, Ba A, Mbengue A, Bei AK. Leveraging genome editing to functionally evaluate Plasmodium diversity. Trends in Parasitology. 2022;38(7):558-71.

8. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature. 2002;419(6906):498-511.

9. 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. Clinical microbiology reviews. 2019;32(4):10.1128/cmr. 00019-19.

10. Obi OA, Obiezue RN, Eze D, Adebote DA. Evasive mechanisms of human VSG and PfEMP1 antigens with link to Vaccine scenario: a review. Journal of Parasitic Diseases. 2024:1-16.

11. 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 biology. 2011;12(4):1-15.

12. 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. Infection, Genetics and Evolution. 2006;6(5):417-24.

13. Lapp Z, Obala AA, Abel L, Rasmussen DA, Sumner KM, Freedman E, et al. Plasmodium falciparum genetic diversity in coincident human and mosquito hosts. Mbio. 2022;13(5):e02277-22.

14. 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. Malaria journal. 2020;19:1-9.

15. 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. Molecular biology and evolution. 2000;17(10):1467-82.

16. Wang M, Jia T, Jiang N, Wang L, Hu X, Luo Z. Inferring linkage disequilibrium from non-random samples. BMC genomics. 2010;11:1-12.

17. Abukari Z, Okonu R, Nyarko SB, Lo AC, Dieng CC, Salifu SP, et al. The diversity, multiplicity of infection and population structure of P. falciparum parasites circulating in asymptomatic carriers living in high and low malaria transmission settings of Ghana. Genes. 2019;10(6):434.

18. Mwesigwa A, Ocan M, Musinguzi B, Nante RW, Nankabirwa JI, Kiwuwa SM, et al. P lasmodium 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. Malaria Journal. 2024;23(1):97.

19. 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. Malaria journal. 2016;15:1-10.

20. 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. Parasitology research. 2013;112:1691-700.

21. Pinkevych M, Petravic J, Bereczky S, Rooth I, Färnert A, Davenport MP. Understanding the relationship between Plasmodium falciparum growth rate and multiplicity of infection. The Journal of infectious diseases. 2015;211(7):1121-7.

22. Zhong D, Koepfli C, Cui L, Yan G. Molecular approaches to determine the multiplicity of Plasmodium infections. Malaria journal. 2018;17(1):1-9.

23. Alizon S. Parasite co-transmission and the evolutionary epidemiology of virulence. Evolution. 2013;67(4):921-33.

24. Nkhoma SC, Nair S, Cheeseman IH, Rohr-Allegrini C, Singlam S, Nosten F, et al. Close kinship within multiple-genotype malaria parasite infections. Proceedings of the Royal Society B: Biological Sciences. 2012;279(1738):2589-98.

25. 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 Medicine. 2017;9:1-12.

26. 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. Immunological reviews. 2020;293(1):230-52.

27. 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. The Journal of Infectious Diseases. 2020;222(10):1692-701.

28. 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 infectious diseases. 2020;20:1-10.

29. Hasugian A, Purba H, Kenangalem E, Wuwung R, Ebsworth E, Maristela R, et al. Dihydroartemisinin-piperaquine versus artesunate-amodiaquine: superior efficacy and posttreatment prophylaxis against multidrug-resistant Plasmodium falciparum and Plasmodium vivax malaria. Clinical Infectious Diseases. 2007;44(8):1067-74.

30. White NJ. Malaria parasite clearance. Malaria journal. 2017;16(1):1-14.

31. Mita T, Jombart T. Patterns and dynamics of genetic diversity in Plasmodium falciparum: what past human migrations tell us about malaria. Parasitology international. 2015;64(3):238-43.

32. 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 Infectious Diseases. 2018;18(1):1-10.

33. Niang M, Thiam LG, Loucoubar C, Sow A, Sadio BD, Diallo M, et al. Spatio-temporal analysis of the genetic diversity and complexity of Plasmodium falciparum infections in Kedougou, southeastern Senegal. Parasites & vectors. 2017;10(1):1-9.

34. Landier J, Parker DM, Thu AM, Lwin KM, Delmas G, Nosten FH, et al. Effect of generalised access to early diagnosis and treatment and targeted mass drug administration on Plasmodium falciparum malaria in Eastern Myanmar: an observational study of a regional elimination programme. The Lancet. 2018;391(10133):1916-26.

35. Carter TE, Malloy H, Existe A, Memnon G, Victor YS, Okech BA, et al. Genetic diversity of Plasmodium falciparum in Haiti: insights from microsatellite markers. PLoS One. 2015;10(10):e0140416.

36. 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. Malaria journal. 2016;15(1):1-10.

37. Taylor AR, Schaffner SF, Cerqueira GC, Nkhoma SC, Anderson TJ, Sriprawat K, et al. Quantifying connectivity between local Plasmodium falciparum malaria parasite populations using identity by descent. PLoS genetics. 2017;13(10):e1007065.

38. Wesolowski A, Taylor AR, Chang H-H, Verity R, Tessema S, Bailey JA, et al. Mapping malaria by combining parasite genomic and epidemiologic data. BMC medicine. 2018;16(1):1-8.

39. Funwei RI, Thomas BN, Falade CO, Ojurongbe O. Extensive diversity in the allelic frequency of Plasmodium falciparum merozoite surface proteins and glutamate-rich protein in rural and urban settings of southwestern Nigeria. Malaria journal. 2018;17:1-8.

40. 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. Scientific Reports. 2019;9(1):13704.

41. Amambua-Ngwa A, Tetteh KK, Manske M, Gomez-Escobar N, Stewart LB, Deerhake ME, et al. Population genomic scan for candidate signatures of balancing selection to guide antigen characterization in malaria parasites. PLoS genetics. 2012;8(11):e1002992.

42. Carter TE, Malloy H, Existe A, Memnon G, St. Victor Y, Okech BA, et al. Genetic diversity of Plasmodium falciparum in Haiti: insights from microsatellite markers. PLoS One. 2015;10(10):e0140416.

43. 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. Malaria Journal. 2021;20(1):242.

44. Su X-z, Wellems TE. Toward a High-ResolutionPlasmodium falciparumLinkage Map: Polymorphic Markers from Hundreds of Simple Sequence Repeats. Genomics. 1996;33(3):430-44.

45. Apinjoh TO, Ouattara A, Titanji VP, Djimde A, Amambua-Ngwa A. Genetic diversity and drug resistance surveillance of Plasmodium falciparum for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? Malaria journal. 2019;18(1):217.

46. Kyabayinze DJ, Karamagi C, Kiggundu M, Kamya MR, Wabwire-Mangen F, Kironde F, et al. Multiplicity of Plasmodium falciparum infection predicts antimalarial treatment outcome in Ugandan children. African health sciences. 2008;8(4):200-5.

47. 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. Parasitology research. 2013;112(4):1691-700.

48. Childs LM, Prosper OF. Simulating within-vector generation of the malaria parasite diversity. PLoS One. 2017;12(5):e0177941.

49. 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. Malaria journal. 2009;8(1):1-11.

50. Bousema T, Drakeley C. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clinical microbiology reviews. 2011;24(2):377-410.

51. Andolina C, Rek J, Briggs J, Okoth J, Musiime A, Ramjith J, et al. Persistent malaria transmission from asymptomatic children despite highly effective malaria control in eastern Uganda. medRxiv. 2021:2021.05. 04.21255999.

52. Castro MC. Malaria transmission and prospects for malaria eradication: the role of the environment. Cold Spring Harbor perspectives in medicine. 2017;7(10):a025601.

53. Rek J, Musiime A, Zedi M, Otto G, Kyagamba P, Asiimwe Rwatooro J, et al. Non-adherence to long-lasting insecticide treated bednet use following successful malaria control in Tororo, Uganda. PloS one. 2020;15(12):e0243303.

54. Asua V, Tukwasibwe S, Conrad M, Walakira A, Nankabirwa JI, Mugenyi L, et al. Plasmodium species infecting children presenting with malaria in Uganda. The American journal of tropical medicine and hygiene. 2017;97(3):753-7.

55. Das S, Muleba M, Stevenson JC, Pringle JC, Norris DE. Beyond the entomological inoculation rate: characterizing multiple blood feeding behavior and Plasmodium falciparum multiplicity of infection in Anopheles mosquitoes in northern Zambia. Parasites & vectors. 2017;10(1):1-13.

56. Tadesse FG, Slater HC, Chali W, Teelen K, Lanke K, Belachew M, et al. The relative contribution of symptomatic and asymptomatic Plasmodium vivax and Plasmodium falciparum infections to the infectious reservoir in a low-endemic setting in Ethiopia. Clinical infectious diseases. 2018;66(12):1883-91.

57. Sato S. Plasmodium—a brief introduction to the parasites causing human malaria and their basic biology. Journal of physiological anthropology. 2021;40(1):1.

58. 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. The American journal of tropical medicine and hygiene. 2015;92(5):903-12.

59. Lynd A, Gonahasa S, Staedke SG, Oruni A, Maiteki-Sebuguzi C, Dorsey G, et al. LLIN Evaluation in Uganda Project (LLINEUP): a cross-sectional survey of species diversity and insecticide resistance in 48 districts of Uganda. Parasites & vectors. 2019;12(1):1-10.

60. WHO. World Malaria Report 2018 (World Health Organization, Geneva, Switzerland, 2018). 2018.

61. Cockburn IA, Seder RA. Malaria prevention: from immunological concepts to effective vaccines and protective antibodies. Nature immunology. 2018;19(11):1199-211.

62. White N, Pukrittayakamee S, Hien T, Faiz M, Mokuolu O, Dondorp A. Erratum: Malaria (The Lancet (2014) 383 (723-735)). The Lancet. 2014;383(9918).

63. Vaughan AM, Aly AS, Kappe SH. Malaria parasite pre-erythrocytic stage infection: gliding and hiding. Cell host & microbe. 2008;4(3):209-18.

64. Joice R, Nilsson SK, Montgomery J, Dankwa S, Egan E, Morahan B, et al. Plasmodium falciparum transmission stages accumulate in the human bone marrow. Science translational medicine. 2014;6(244):244re5-re5.

65. Tibúrcio M, Sauerwein R, Lavazec C, Alano P. Erythrocyte remodeling by Plasmodium falciparum gametocytes in the human host interplay. Trends in parasitology. 2015;31(6):270-8.

66. Brancucci NM, Goldowitz I, Buchholz K, Werling K, Marti M. An assay to probe Plasmodium falciparum growth, transmission stage formation and early gametocyte development. Nature protocols. 2015;10(8):1131-42.

67. Kori LD, Valecha N, Anvikar AR. Insights into the early liver stage biology of Plasmodium. Journal of vector borne diseases. 2018;55(1):9.

68. Felger I, Smith T, Edoh D, Kitua A, Alonso P, Tanner M, et al. 6. multiple Plasmodium falciparum infections in Tanzanian infants. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1999;93:29-34.

69. Conway DJ. Molecular epidemiology of malaria. Clinical Microbiology Reviews. 2007;20(1):188-204.

70. Mobegi VA, Loua KM, Ahouidi AD, Satoguina J, Nwakanma DC, Amambua-Ngwa A, et al. Population genetic structure of Plasmodium falciparum across a region of diverse endemicity in West Africa. Malaria journal. 2012;11:1-9.

71. Scherf A, Lopez-Rubio JJ, Riviere L. Antigenic variation in Plasmodium falciparum. Annu Rev Microbiol. 2008;62(1):445-70.

72. Miles A, Iqbal Z, Vauterin P, Pearson R, Campino S, Theron M, et al. Indels, structural variation, and recombination drive genomic diversity in Plasmodium falciparum. Genome research. 2016;26(9):1288-99.

73. Neafsey DE, Taylor AR, MacInnis BL. Advances and opportunities in malaria population genomics. Nature Reviews Genetics. 2021;22(8):502-17.

74. 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. Frontiers in Epidemiology. 2023;3:1227071.

75. 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. Pathogens and global health. 2022;116(7):428-37.

76. 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. The Journal of infectious diseases. 2018;217(4):622-7.

77. Mohd Abd Razak 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(3):e0152415.

78. 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. Parasites & vectors. 2020;13:1-12.

79. 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. Nature communications. 2019;10(1):2665.

80. 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. The American Journal of Tropical Medicine and Hygiene. 2016;95(5):1077.

81. 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. Malaria journal. 2009;8:1-8.

82. 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 tropica. 2013;125(1):53-9.

83. Eldh M, Hammar U, Arnot D, Beck H-P, Garcia A, Liljander A, et al. Multiplicity of asymptomatic Plasmodium falciparum infections and risk of clinical malaria: a systematic review and pooled analysis of individual participant data. The Journal of infectious diseases. 2020;221(5):775-85.

84. Zhang C-L, Zhou H-N, Liu Q, Yang Y-M. Genetic polymorphism of merozoite surface proteins 1 and 2 of Plasmodium falciparum in the China–Myanmar border region. Malaria journal. 2019;18(1):1-10.

85. Tadele G, Jaiteh FK, Oboh M, Oriero E, Dugassa S, Amambua-Ngwa A, et al. Low genetic diversity of Plasmodium falciparum merozoite surface protein 1 and 2 and multiplicity of infections in western Ethiopia following effective malaria interventions. Malaria Journal. 2022;21(1):1-9.

86. Sane R, Talla C, Diouf B, Sarr FD, Diagne N, Faye J, et al. Low genetic diversity and complexity of submicroscopic Plasmodium falciparum infections among febrile patients in low transmission areas in Senegal. Plos one. 2019;14(4):e0215755.

87. 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. The Journal of infectious diseases. 2019;220(8):1346-54.

88. 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. Infection, Genetics and Evolution. 2016;39:372-80.

89. 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. Malaria journal. 2016;15(1):1-10.

90. Mohammed H, Kassa M, Mekete K, Assefa A, Taye G, Commons RJ. Genetic diversity of the msp-1, msp-2, and glurp genes of Plasmodium falciparum isolates in Northwest Ethiopia. Malaria journal. 2018;17(1):1-8.

91. Chen J-T, Li J, Zha G-C, Huang G, Huang Z-X, Xie D-D, et al. Genetic diversity and allele frequencies of Plasmodium falciparum msp1 and msp2 in parasite isolates from Bioko Island, Equatorial Guinea. Malaria Journal. 2018;17(1):1-9.

92. Yakubu B, Longdet IY, Tony HJ, Davou DT, Obishakin E. High-complexity Plasmodium falciparum infections, north Central Nigeria, 2015–2018. Emerging infectious diseases. 2019;25(7):1330.

93. 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. The American journal of tropical medicine and hygiene. 2016;95(5):1077-85.

94. Touray AO, Mobegi VA, Wamunyokoli F, Herren JK. Diversity and Multiplicity of P. falciparum infections among asymptomatic school children in Mbita, Western Kenya. Scientific reports. 2020;10(1):1-8.

95. Mara SE, Silué KD, Raso G, N'Guetta SP, N'Goran EK, Tanner M, et al. Genetic diversity of Plasmodium falciparum among school-aged children from the Man region, western Côte d'Ivoire. Malaria journal. 2013;12(1):1-10.

96. Agyeman-Budu A, Brown C, Adjei G, Adams M, Dosoo D, Dery D, et al. Trends in multiplicity of Plasmodium falciparum infections among asymptomatic residents in the middle belt of Ghana. Malaria journal. 2013;12(1):1-6.

97. Nkhoma SC, Nair S, Al-Saai S, Ashley E, McGready R, Phyo AP, et al. Population genetic correlates of declining transmission in a human pathogen. Molecular ecology. 2013;22(2):273-85.

98. Daniels R, Chang H-H, Séne PD, Park DC, Neafsey DE, Schaffner SF, et al. Genetic surveillance detects both clonal and epidemic transmission of malaria following enhanced intervention in Senegal. PloS one. 2013;8(4):e60780.

99. Yeka A, Gasasira A, Mpimbaza A, Achan J, Nankabirwa J, Nsobya S, et al. Malaria in Uganda: challenges to control on the long road to elimination: I. Epidemiology and current control efforts. Acta tropica. 2012;121(3):184-95.

100. Nankabirwa JI, Arinaitwe E, Rek J, Kilama M, Kizza T, Staedke SG, et al. Malaria transmission, infection, and disease following sustained indoor residual spraying of insecticide in Tororo, Uganda. The American journal of tropical medicine and hygiene. 2020;103(4):1525.

101. 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. The American journal of tropical medicine and hygiene. 2015;92(5):903.

102. Peyerl-Hoffmann G, Jelinek T, Kilian A, Kabagambe G, Metzger W, Von Sonnenburg F. Genetic diversity of Plasmodium falciparum and its relationship to parasite density in an area with different malaria endemicities in West Uganda. Tropical medicine & international health. 2001;6(8):607-13.

103. Bartoloni A, Zammarchi L. Clinical aspects of uncomplicated and severe malaria. Mediterranean journal of hematology and infectious diseases. 2012;4(1).

104. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malaria journal. 2012;11:1-15.

105. Bendixen M, Msangeni H, Pedersen B, Shayo D, Bedker R. Diversity of Plasmodium falciparum populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2001;95(2):143-8.

106. Gray K-A, Dowd S, Bain L, Bobogare A, Wini L, Shanks GD, et al. Population genetics of Plasmodium falciparum and Plasmodium vivax and asymptomatic malaria in Temotu Province, Solomon Islands. Malaria journal. 2013;12:1-10.

107. Mahdi Abdel Hamid M, Elamin AF, Albsheer MMA, Abdalla AA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria in Gezira State, Sudan. Parasites & vectors. 2016;9:1-8.

108. Hamid MMA, Elamin AF, Albsheer MMA, Abdalla AA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria in Gezira State, Sudan. Parasites & vectors. 2016;9(1):1-8.

109. Mandala WL, Msefula CL, Gondwe EN, Drayson MT, Molyneux ME, MacLennan CA. Cytokine profiles in Malawian children presenting with uncomplicated malaria, severe malarial anemia, and cerebral malaria. Clinical and vaccine immunology. 2017;24(4):e00533-16.

110. Popa GL, Popa MI. Recent advances in understanding the inflammatory response in malaria: a review of the dual role of cytokines. Journal of immunology research. 2021;2021(1):7785180.

111. Kimenyi KM, Wamae K, Ochola-Oyier LI. Understanding P. falciparum asymptomatic infections: a proposition for a transcriptomic approach. Frontiers in immunology. 2019;10:2398.

112. Cham GK, Turner L, Kurtis JD, Mutabingwa T, Fried M, Jensen AT, et al. Hierarchical, domain type-specific acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 in Tanzanian children. Infection and immunity. 2010;78(11):4653-9.

113. Su X-z, Zhang C, Joy DA. Host-malaria parasite interactions and impacts on mutual evolution. Frontiers in Cellular and Infection Microbiology. 2020;10:587933.

114. Buchwald AG, Sixpence A, Chimenya M, Damson M, Sorkin JD, Wilson ML, et al. Clinical implications of asymptomatic Plasmodium falciparum infections in Malawi. Clinical Infectious Diseases. 2019;68(1):106-12.

115. Somé AF, Bazié T, Zongo I, Yerbanga RS, Nikiéma F, Neya C, et al. Plasmodium falciparum msp 1 and msp 2 genetic diversity and allele frequencies in parasites isolated from symptomatic malaria patients in Bobo-Dioulasso, Burkina Faso. Parasites & vectors. 2018;11:1-8.

116. Agonhossou R, Akoton R, Lagnika H, Djihinto OY, Sovegnon PM, Saizonou HD, et al. P. falciparum msp1 and msp2 genetic diversity in P. falciparum single and mixed infection with P. malariae among the asymptomatic population in Southern Benin. Parasitology International. 2022;89:102590.

117. Sarah-Matio EM, Guillochon E, Nsango SE, Abate L, Ngou CM, Bouopda GA, et al. Genetic diversity of Plasmodium falciparum and distribution of antimalarial drug resistance mutations in symptomatic and asymptomatic infections. Antimicrobial Agents and Chemotherapy. 2022;66(8):e00188-22.

118. Touray AO, Mobegi VA, Wamunyokoli F, Herren JK. Diversity and Multiplicity of P. falciparum infections among asymptomatic school children in Mbita, Western Kenya. Scientific reports. 2020;10(1):5924.

119. Sumner KM, Freedman E, Mangeni JN, Obala AA, Abel L, Edwards JK, et al. Exposure to diverse Plasmodium falciparum genotypes shapes the risk of symptomatic malaria in incident

and persistent infections: a longitudinal molecular epidemiologic study in Kenya. Clinical Infectious Diseases. 2021;73(7):1176-84.

120. Lagnika HO, Moussiliou A, Agonhossou R, Sovegnon P, Djihinto OY, Medjigbodo AA, et al. Plasmodium falciparum msp1 and msp2 genetic diversity in parasites isolated from symptomatic and asymptomatic malaria subjects in the South of Benin. Parasitology Research. 2022;121(1):167-75.

121. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nature Reviews Microbiology. 2014;12(12):833-40.

122. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. Expert review of anti-infective therapy. 2013;11(6):623-39.

123. De Roode JC, Helinski ME, Anwar MA, Read AF. Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. The American Naturalist. 2005;166(5):531-42.

124. Ofosu-Okyere A, Mackinnon M, Sowa M, Koram K, Nkrumah F, Osei Y, et al. Novel Plasmodium falciparum clones and rising clone multiplicities are associated with the increase in malaria morbidity in Ghanaian children during the transition into the high transmission season. Parasitology. 2001;123(2):113-23.

125. Mueller I, Schoepflin S, Smith TA, Benton KL, Bretscher MT, Lin E, et al. Force of infection is key to understanding the epidemiology of Plasmodium falciparum malaria in Papua New Guinean children. Proceedings of the National Academy of Sciences. 2012;109(25):10030-5.

126. Soulama I, Nébié I, Ouédraogo A, Gansane A, Diarra A, Tiono AB, et al. Plasmodium falciparum genotypes diversity in symptomatic malaria of children living in an urban and a rural setting in Burkina Faso. Malaria journal. 2009;8:1-8.

127. Tessema S, Wesolowski A, Chen A, Murphy M, Wilheim J, Mupiri A-R, et al. Using parasite genetic and human mobility data to infer local and cross-border malaria connectivity in Southern Africa. Elife. 2019;8:e43510.

128. Daniels RF, Schaffner SF, Wenger EA, Proctor JL, Chang H-H, Wong W, et al. Modeling malaria genomics reveals transmission decline and rebound in Senegal. Proceedings of the National Academy of Sciences. 2015;112(22):7067-72.

129. Escalante AA, Ferreira MU, Vinetz JM, Volkman SK, Cui L, Gamboa D, et al. Malaria molecular epidemiology: lessons from the International Centers of Excellence for Malaria Research Network. The American journal of tropical medicine and hygiene. 2015;93(3_Suppl):79-86.

130. 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 Infectious Diseases. 2018;18:1-10.

131. 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. Malaria journal. 2009;8:1-11.

132. Gatei W, Gimnig JE, Hawley W, Ter Kuile F, Odero C, Iriemenam NC, et al. Genetic diversity of Plasmodium falciparum parasite by microsatellite markers after scale-up of insecticide-treated bed nets in western Kenya. Malaria Journal. 2015;14:1-12.

133. 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(8):1346-54.

134. Huang B, Tuo F, Liang Y, Wu W, Wu G, Huang S, et al. Temporal changes in genetic diversity of msp-1, msp-2, and msp-3 in Plasmodium falciparum isolates from Grande Comore Island after introduction of ACT. Malaria Journal. 2018;17:1-14.

135. Pringle JC, Wesolowski A, Berube S, Kobayashi T, Gebhardt ME, Mulenga M, et al. High Plasmodium falciparum genetic diversity and temporal stability despite control efforts in high transmission settings along the international border between Zambia and the Democratic Republic of the Congo. Malaria journal. 2019;18:1-13.

136. Kimenyi KM, Wamae K, Ngoi JM, de Laurent ZR, Ndwiga L, Osoti V, et al. Maintenance of high temporal Plasmodium falciparum genetic diversity and complexity of infection in asymptomatic and symptomatic infections in Kilifi, Kenya from 2007 to 2018. Malaria Journal. 2022;21(1):192.

137. 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(5):903-12.

138. WWARN. Artemether-lumefantrine treatment of uncomplicated Plasmodium falciparum malaria: a systematic review and meta-analysis of day 7 lumefantrine concentrations and therapeutic response using individual patient data. BMC medicine. 2015;13:1-19.

139. Shibeshi MA, Kifle ZD, Atnafie SA. Antimalarial drug resistance and novel targets for antimalarial drug discovery. Infection and drug resistance. 2020:4047-60.

140. Zhong A, Zhang H, Li J. Insight into molecular diagnosis for antimalarial drug resistance of Plasmodium falciparum parasites: A review. Acta Tropica. 2023;241:106870.

141. WHO. Global report on antimalarial drug efficacy and drug resistance: 2000-2010. 2010.

142. WHO. Report on antimalarial drug efficacy, resistance and response: 10 years of surveillance (2010-2019): World Health Organization; 2020.

143. Plucinski MM, Hastings IM, Moriarty LF, Venkatesan M, Felger I, Halsey ES. Variation in calculating and reporting antimalarial efficacy against Plasmodium falciparum in sub-Saharan Africa: a systematic review of published reports. The American Journal of Tropical Medicine and Hygiene. 2021;104(5):1820.

144. Slater M, Kiggundu M, Dokomajilar C, Kamya MR, Bakyaita N, Talisuna A, et al. Distinguishing recrudescences from new infections in antimalarial clinical trials: major impact of interpretation of genotyping results on estimates of drug efficacy. American Journal of Tropical Medicine and Hygiene. 2005;73(2):256-62.

145. Organization WH. Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations: informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29-31 May 2007, Amsterdam, The Netherlands: World Health Organization; 2008.

146. Organization WH. Informal consultation on methodology to distinguish reinfection from recrudescence in high malaria transmission areas: report of a virtual meeting, 17–18 May 2021. 2021.

147. Ishengoma DS, Mandara CI, Madebe RA, Warsame M, Ngasala B, Kabanywanyi AM, et al. Microsatellites reveal high polymorphism and high potential for use in anti-malarial efficacy

studies in areas with different transmission intensities in mainland Tanzania. Malaria Journal. 2024;23(1):79.

148. Plucinski MM, Morton L, Bushman M, Dimbu PR, Udhayakumar V. Robust algorithm for systematic classification of malaria late treatment failures as recrudescence or reinfection using microsatellite genotyping. Antimicrobial agents and chemotherapy. 2015;59(10):6096-100.

149. Apinjoh TO, Ouattara A, Titanji VP, Djimde A, Amambua-Ngwa A. Genetic diversity and drug resistance surveillance of Plasmodium falciparum for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? Malaria journal. 2019;18(1):1-12.

150. Sondo P, Derra K, Diallo-Nakanabo S, Tarnagda Z, Zampa O, Kazienga A, et al. Effectiveness and safety of artemether–lumefantrine versus artesunate–amodiaquine for unsupervised treatment of uncomplicated falciparum malaria in patients of all age groups in Nanoro, Burkina Faso: a randomized open label trial. Malaria journal. 2015;14(1):1-8.

151. Mayengue PI, Ndounga M, Malonga FV, Bitemo M, Ntoumi F. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in Plasmodium falciparum isolates from Brazzaville, Republic of Congo. Malaria journal. 2011;10(1):1-7.

152. WHO. Methods and techniques for clinical trials on antimalarial drug efficacy: genotyping to identify parasite populations: informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29-31 May 2007, Amsterdam, The Netherlands: World Health Organization; 2008.

153. Kiwanuka GN. Genetic diversity in Plasmodium falciparum merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997-2007. Journal of vector borne diseases. 2009;46(1):1.

154. Zhong D, Afrane Y, Githeko A, Yang Z, Cui L, Menge DM, et al. Plasmodium falciparum genetic diversity in western Kenya highlands. The American journal of tropical medicine and hygiene. 2007;77(6):1043-50.

155. Mathema VB, Nakeesathit S, Pagornrat W, Smithuis F, White NJ, Dondorp AM, et al. Polymorphic markers for identification of parasite population in Plasmodium malariae. Malaria journal. 2020;19(1):48.

156. Nyachieo A, Van Overmeir C, Laurent T, Dujardin J-C, D'ALESSANDRO U. Plasmodium falciparum genotyping by microsatellites as a method to distinguish between recrudescent and new infections. The American journal of tropical medicine and hygiene. 2005;73(1):210-3.

157. Campos M, Conn JE, Alonso DP, Vinetz JM, Emerson KJ, Ribolla PEM. Microgeographical structure in the major Neotropical malaria vector Anopheles darlingi using microsatellites and SNP markers. Parasites & vectors. 2017;10(1):1-8.

158. Chenet SM, Schneider KA, Villegas L, Escalante AA. Local population structure of Plasmodium: impact on malaria control and elimination. Malaria journal. 2012;11(1):1-13.

159. Orjuela-Sánchez P, Sá JM, Brandi MC, Rodrigues PT, Bastos MS, Amaratunga C, et al. Higher microsatellite diversity in Plasmodium vivax than in sympatric Plasmodium falciparum populations in Pursat, Western Cambodia. Experimental parasitology. 2013;134(3):318-26.

160. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. International journal of surgery. 2021;88:105906.

161. WHO. World malaria report 2022: World Health Organization; 2022.

162. Barry AE, Schultz L, Senn N, Nale J, Kiniboro B, Siba PM, et al. High levels of genetic diversity of Plasmodium falciparum populations in Papua New Guinea despite variable infection prevalence. The American journal of tropical medicine and hygiene. 2013;88(4):718.

163. Mobegi VA, Loua KM, Ahouidi AD, Satoguina J, Nwakanma DC, Amambua-Ngwa A, et al. Population genetic structure of Plasmodium falciparum across a region of diverse endemicity in West Africa. Malaria journal. 2012;11(1):1-9.

164. Ogouyèmi-Hounto A, Gazard DK, Ndam N, Topanou E, Garba O, Elegbe P, et al. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in Plasmodium falciparum isolates from children in South of Benin. Parasite. 2013;20.

165. Oyebola MK, Idowu ET, Olukosi YA, Iwalokun BA, Agomo CO, Ajibaye OO, et al. Genetic diversity and complexity of Plasmodium falciparum infections in Lagos, Nigeria. Asian Pacific journal of tropical biomedicine. 2014;4:S87-S91.

166. Cooper H, Hedges LV, Valentine JC. The handbook of research synthesis and meta-analysis: Russell Sage Foundation; 2019.

167. Bowden J, Tierney JF, Copas AJ, Burdett SJBmrm. Quantifying, displaying and accounting for heterogeneity in the meta-analysis of RCTs using standard and generalised Qstatistics. 2011;11(1):1-12.

168. Huedo-Medina TB, Sánchez-Meca J, Marín-Martínez F, Botella J. Assessing heterogeneity in meta-analysis: Q statistic or I^2 index? Psychological methods. 2006;11(2):193.

169. Tools CA. JBI. URL: https://jbi global/critical-appraisal-tools [accessed 2021-03-03]. 2020.

170. Schunemann H, Brozek J, Guyatt G, Oxman A. Handbook for grading the quality of evidence and the strength of recommendations using the GRADE approach,(updated October

2013). GRADE Working Group Available from gdt guidelinedevelopment org/app/handbook/handbook html. 2013.

171. Simmonds M. Quantifying the risk of error when interpreting funnel plots. Systematic reviews. 2015;4:1-7.

172. Sterne JA, Egger M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. Journal of clinical epidemiology. 2001;54(10):1046-55.

173. Mohammed H, Hassen K, Assefa A, Mekete K, Tadesse G, Taye G, et al. Genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria based on msp-1 and msp-2 genes in Gublak, North West Ethiopia. Malaria journal. 2019;18(1):1-8.

174. Reda AG, Messele A, Mohammed H, Assefa A, Golassa L, Mamo H. Temporal dynamics of Plasmodium falciparum population in Metehara, east-central Ethiopia. Malaria Journal. 2022;21(1):267.

175. MoH. Malaria indicator survey 2018–19. Kampala: Ministry of Health,

National Malaria Control Division Kampala, Uganda; 2020 https://www.dhsprogram.com/pubs/pdf/MIS34/MIS34.pdf. 2020.

176. Organization WH. Basic malaria microscopy: World Health Organization; 2010.

177. Musapa M, Kumwenda T, Mkulama M, Chishimba S, Norris DE, Thuma PE, et al. A simple Chelex protocol for DNA extraction from Anopheles spp. JoVE (Journal of Visualized Experiments). 2013(71):e3281.

178. 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. 1999.

179. Congpuong K, Sukaram R, Prompan Y, Dornae A. Genetic diversity of the msp-1, msp-2, and glurp genes of P lasmodium falciparum isolates along the Thai-Myanmar borders. Asian Pacific journal of tropical biomedicine. 2014;4(8):598-602.

180. Baina MT, Lissom A, Assioro Doulamo NV, Djontu JC, Umuhoza DM, Mbama-Ntabi JD, et al. Comparative study of Plasmodium falciparum msp-1 and msp-2 Genetic Diversity in Isolates from Rural and Urban Areas in the South of Brazzaville, Republic of Congo. Pathogens. 2023;12(5):742.

181. Messerli C, Hofmann NE, Beck H-P, Felger I. Critical evaluation of molecular monitoring in malaria drug efficacy trials and pitfalls of length-polymorphic markers. Antimicrobial agents and chemotherapy. 2017;61(1):10.1128/aac. 01500-16.

182. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular ecology notes. 2006;6(1):288-95.

183. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary bioinformatics. 2005;1:117693430500100003.

184. Balloux F, Lugon-Moulin N. The estimation of population differentiation with microsatellite markers. Molecular ecology. 2002;11(2):155-65.

185. Haubold B, Hudson RR. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics. 2000;16(9):847-9.

186. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155(2):945-59.

187. Mohammed H, Mindaye T, Belayneh M, Kassa M, Assefa A, Tadesse M, et al. Genetic diversity of Plasmodium falciparum isolates based on MSP-1 and MSP-2 genes from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia. Malaria journal. 2015;14:1-7.

188. Kolawole OM, Mokuolu OA, Olukosi YA, Oloyede TO. Population genomics diversity of Plasmodium falciparum in malaria patients attending Okelele Health Centre, Okelele, Ilorin, Kwara State, Nigeria. African Health Sciences. 2016;16(3):704-11.

189. Singana BP, Mayengue PI, Niama RF, Ndounga M. Genetic diversity of Plasmodium falciparum infection among children with uncomplicated malaria living in Pointe-Noire, Republic of Congo. Pan African Medical Journal. 2019;32(1).

190. 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. Parasites & vectors. 2020;13(1):1-12.

191. Bouyou-Akotet MK, M'Bondoukwé NP, Mawili-Mboumba DP. Genetic polymorphism of merozoite surface protein-1 in Plasmodium falciparum isolates from patients with mild to severe malaria in Libreville, Gabon. Parasite. 2015;22.

192. Metoh TN, Chen J-H, Fon-Gah P, Zhou X, Moyou-Somo R, Zhou X-N. Genetic diversity of Plasmodium falciparum and genetic profile in children affected by uncomplicated malaria in Cameroon. Malaria Journal. 2020;19:1-15.

193. Mwesigwa A, Tukwasibwe S, Cummings B, Kawalya H, Kiyaga S, Okoboi S, et al. Genetic diversity and population structure of Plasmodium falciparum across areas of varied malaria transmission intensities in Uganda. Malaria Journal. 2025;24(1):97.

194. Mu J, Awadalla P, Duan J, McGee KM, Joy DA, McVean GAT, et al. Recombination hotspots and population structure in Plasmodium falciparum. PLoS biology. 2005;3(10):e335.

195. Nkhoma SC, Banda RL, Khoswe S, Dzoole-Mwale TJ, Ward SA. Intra-host dynamics of co-infecting parasite genotypes in asymptomatic malaria patients. Infection, Genetics and Evolution. 2018;65:414-24.

196. Zhong D, Koepfli C, Cui L, Yan G. Molecular approaches to determine the multiplicity of Plasmodium infections. Malaria journal. 2018;17:1-9.

197. Hamid MMA, Mohammed SB, El Hassan IM. Genetic diversity of Plasmodium falciparum field isolates in Central Sudan inferred by PCR genotyping of merozoite surface protein 1 and 2. North American journal of medical sciences. 2013;5(2):95.

198. Hviid L. Clinical disease, immunity and protection against Plasmodium falciparum malaria in populations living in endemic areas. Expert reviews in molecular medicine. 1998;1(4):1-10.

199. Mawili-Mboumba DP, Mbondoukwe N, Adande E, Bouyou-Akotet MK. Allelic diversity of msp1 gene in Plasmodium falciparum from rural and urban areas of Gabon. The Korean Journal of Parasitology. 2015;53(4):413.

200. 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. The American journal of tropical medicine and hygiene. 2006;74(6):953-9.

201. 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 biology. 2011;12:1-15.

202. 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. Malaria Journal. 2021;20(1):239.

203. 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. Parasites & vectors. 2011;4(1):1-9.

204. 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. Malaria journal. 2010;9(1):1-6.

205. 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. Malaria journal. 2016;15:1-10.

206. 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. Malaria Journal. 2021;20(1):236.

207. Gnagne AP, Konate A, Bedia-Tanoh AV, Amiah-Droh M, Menan HIE, N'Guetta AS-P, et al. Dynamics of Plasmodium falciparum genetic diversity among asymptomatic and symptomatic children in three epidemiological areas in Cote d'Ivoire. Pathogens and Global Health. 2019;113(3):133-42.

208. Sorontou Y, Pakpahan A. Genetic diversity in MSP-1 gene of Plasmodium falciparum and its association with malaria severity, parasite density, and host factors of asymptomatic and symptomatic patients in Papua, Indonesia. Int J Med Sci Public Health. 2015;4(11):1584-93.

209. Nkya TE, Fillinger U, Dlamini M, Sangoro OP, Marubu R, Zulu Z, et al. Malaria in Eswatini, 2012–2019: a case study of the elimination effort. Malaria Journal. 2021;20:1-13.

210. Oguttu DW, Matovu JK, Okumu DC, Ario AR, Okullo AE, Opigo J, et al. Rapid reduction of malaria following introduction of vector control interventions in Tororo District, Uganda: a descriptive study. Malaria journal. 2017;16:1-8.

211. Yakubu B, Longdet IY, Horsefield T, Davou DT, Obishakin E. High-complexity Plasmodium falciparum infections, north Central Nigeria, 2015–2018. Emerging infectious diseases. 2019;25(7):1330-8.

212. Mwingira F, Nkwengulila G, Schoepflin S, Sumari D, Beck H-P, Snounou G, et al. Plasmodium falciparum msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa. Malaria journal. 2011;10:1-10.

213. Schnoz A, Beuret C, Concu M, Hosch S, Rutaihwa LK, Golumbeanu M, et al. Comparison of different genotyping techniques to distinguish recrudescence from new infection in studies assessing the efficacy of antimalarial drugs against Plasmodium falciparum. bioRxiv. 2023:2023.04. 24.538072.

214. 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. Malaria journal. 2021;20(1):1-13.

APPENDICES

APPENDIX 1: Publication from study objective 1

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Malaria Journal

REVIEW Open Access

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

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Abstract

Background In sub-Saharan Africa (SSA), *Plasmodium falciparum* causes most of the malaria cases. Despite its crucial roles in disease severity and drug resistance, comprehensive data on *Plasmodium falciparum* genetic diversity and multiplicity of infection (MOI) are sparse in SSA. This study summarizes available information on genetic diversity and MOI, focusing on key markers (*msp-1*, *msp-2*, *glurp*, and microsatellites). The systematic review aimed to evaluate their influence on malaria transmission dynamics and offer insights for enhancing malaria control measures in SSA.

Methods The review was conducted following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines. Two reviewers conducted article screening, assessed the risk of bias (RoB), and performed data abstraction. Meta-analysis was performed using the random-effects model in STATA version 17.

Results The review included 52 articles: 39 cross-sectional studies and 13 Randomized Controlled Trial (RCT)/cohort studies, involving 11,640 genotyped parasite isolates from 23 SSA countries. The overall pooled mean expected heterozygosity was 0.65 (95% CI: 0.51–0.78). Regionally, values varied: East (0.58), Central (0.84), Southern (0.74), and West Africa (0.69). Overall pooled allele frequencies of *msp-1* alleles K1, MAD20, and RO33 were 61%, 44%, and 40%, respectively, while *msp-2* I/C 3D7 and FC27 alleles were 61% and 55%. Central Africa reported higher frequencies (K1: 74%, MAD20: 51%, RO33: 48%) than East Africa (K1: 46%, MAD20: 42%, RO33: 31%). For *msp-2*, East Africa had 60% and 55% for I/C 3D7 and FC27 alleles, while West Africa had 62% and 50%, respectively. The pooled allele frequency for *glurp* was 66%. The overall pooled mean MOI was 2.09 (95% CI: 1.88–2.30), with regional variations: East (2.05), Central (2.37), Southern (2.16), and West Africa (1.96). The overall prevalence of polyclonal *Plasmodium falciparum* infections was 63% (95% CI: 56–70), with regional prevalences as follows: East (62%), West (61%), Central (65%), and South Africa (71%).

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Conclusion The study shows substantial regional variation in *Plasmodium falciparum* parasite genetic diversity and MOI in SSA. These findings suggest a need for malaria control strategies and surveillance efforts considering regional-specific factors underlying *Plasmodium falciparum* infection.

Keywords *Plasmodium falciparum*, Genetic diversity and multiplicity of infection, Sub-Saharan Africa

Background

Plasmodium falciparum presents a significant public health challenge in sub-Saharan Africa (SSA), constituting the majority of reported malaria cases. In 2022, out of the 249 million malaria cases recorded globally, 233 million occurred in SSA, contributing to an estimated 580,000 out of the 608,000 malaria-related deaths worldwide [1]. While the development of a robust immune response is necessary for controlling *Plasmodium falciparum* infection [2], timely diagnosis and the administration of effective treatments [3] are required to control symptomatic infection and reduce transmission.

The control of *Plasmodium falciparum* is hindered by the high propensity for genetic diversity of parasites infecting individuals and the frequency of multiplicity of infection (MOI) within individual infections. These factors favour immune evasion, may contribute to malaria pathology, and could promote the emergence of variants resistant to anti-malarial drugs [4]. Moreover, genetic diversity, particularly involving protein-coding genes targeted by diagnostic tests such as histidine-rich protein 2/3 (HRP2/3) [5], which have become important tools for malaria diagnosis and surveillance, could have significant implications for malaria surveillance and control.

Genetic diversity and MOI are emerging as relevant biomarkers of *Plasmodium falciparum* transmission. *Plasmodium falciparum* genetic diversity arises from genetic recombination during the parasite lifecycle in the mosquito [6], while MOI results from infection by multiple distinct parasite genotypes [7]. Infection by distinct parasite genotypes occurs either when an individual is bitten by different mosquitoes carrying unique parasite strains (superinfection) or when bitten by a single mosquito carrying multiple distinct genotypes (co-transmission) [8, 9].

The genetic diversity and MOI of *Plasmodium falciparum* may be assessed by targeted genotyping of markers such as *msp-1*, *msp-2*, and *glurp*, which are coding and therefore targets for immune evasion [10] or microsatellite markers, which are not targets for immune evasion [11]. High-throughput methods, including molecular (DNA) barcodes, targeted deep sequencing, and genome-wide variation analysis, have also been utilized [12, 13], but these are expensive. Although more labour-intensive and subject to some biases, such as amplification efficiency bias due to size differences between *msp-1*, *msp-2*, and *glurp* alleles [14, 15], genotyping of these markers is cheaper and more readily available in resource-limited settings in SSA [12].

The mean values of parasite genetic diversity and MOI are higher in areas with high malaria transmission intensity [16, 17] and lower in those with low transmission intensity [18]. Additionally, mean *Plasmodium falciparum* genetic diversity and MOI apparently decreased following the suppression of *Plasmodium falciparum* transmission intensity in areas of Ethiopia [19] and Senegal [20]. In other studies, mean values of *Plasmodium falciparum* genetic diversity were higher among individuals with symptomatic infections [21, 22] and lower in those with asymptomatic infections [23], and were inversely correlated with parasite density and patient age [24].

Data on *Plasmodium falciparum* genetic diversity and MOI are relatively sparse, making it difficult to easily identify relevant patterns in SSA. Some studies have focused solely on MOI but not genetic diversity [25, 26], while others have been conducted within a single country [27], or utilized a single genetic marker [28]. This study collated published data on *Plasmodium falciparum* genetic diversity and MOI in SSA and summarized this data for symptomatic and asymptomatic individuals using a few genetic markers that are widely utilised for parasite genotyping in SSA. The aim of the study was to generate a systematic summary that can inform public health initiatives for malaria control in different regions of SSA.

Methods

Study design and protocol registration

The systematic review was conducted using the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines [29]. The review protocol is registered in PROSPERO (#CRD42021267661).

Review question

The study reviewed data on *Plasmodium falciparum* genetic diversity and MOI in SSA based on *msp-1*, *msp-2*, *glurp*, and microsatellite genetic markers from articles published from January 2000 through May 2023. This period was chosen because access to malaria genomic technologies was reasonably high, yielding representative data in the regions sampled [30]. Also, the period coincides with rapid decline in malaria incidence in SSA

[31]. The objectives of the review were to: a) characterize the geographical distribution of *Plasmodium falciparum* genetic diversity in SSA; b) determine the prevalence of *Plasmodium falciparum* polyclonal infections in SSA; and c) identify factors associated with *Plasmodium falciparum* genetic diversity and MOI in SSA.

Search strategy and information sources

A systematic search, conducted by an experienced librarian (AAK), utilized PubMed, EMBASE, EBSCOhost, Web of Science, and the first 50 pages of Google Scholar after searching several pages and found no more relevant studies Additionally, citation lists of the identified articles were searched for additional relevant articles [32–37]. The search terms included keywords such as '*Plasmodium falciparum*,' '*P. falciparum* genetic diversity,' '*P. falciparum* multiplicity of infection,' and 'sub-Saharan Africa' (Additional file 1).

Eligibility

Inclusion criteria

The review considered:

- Articles published in English,
- Study design, i.e., observational (cross-sectional/survey, case control, and cohort) or randomized clinical trials (RCTs),
- Minimum required data elements: country, sample size, calendar year(s) when the study was conducted, and detailed laboratory methods used to genotype markers for genetic diversity or MOI,
- Detailed methods for determining *Plasmodium falciparum* genetic diversity and MOI, including mean expected heterozygosity, allele frequencies, and mean MOI or percentage of multiple infections.

Exclusion criteria

- Absence of key terms '*Plasmodium falciparum* genetic diversity and or MOI' in the title and or abstract,
- Studies using experimental animals,
- Review articles, case reports, case series, or editorials
- Use of inappropriate laboratory molecular methods (DNA extraction, PCR and then fragment analysis).

Article screening and data extraction

The articles were deduplicated using Endnote software *version* X9. Subsequently, the unique articles underwent screening by two independent reviewers (AM and RWN), who also performed data abstraction using predetermined review criteria (Additional file 2). Abstracted data were compared, and any disagreements were resolved through discussion.

Harmonized extracted data included:

- Study characteristics (author name, article title, publication year, country, malaria transmission setting, and study design); participant characteristics (sample size, age group, and malaria clinical category),
- Malaria diagnosis and genotyping (malaria diagnosis method, name of genotyped markers, and PCR fragment analysis method),
- Outcome results on *Plasmodium falciparum* genetic diversity and MOI based on mean expected heterozygosity, allele frequencies of selected genotyped genetic markers, and the prevalence of polyclonal infections or mean MOI, respectively. Data on factors associated with Plasmodium falciparum genetic diversity and MOI were also extracted.

Data analysis

Meta-analysis was performed using the random effects model (DerSimonian and Laird approach) in STATA (version 17, Stata Corporation, College Station, TX). Forest plots included only studies that reported measures of dispersion such as SD or CI for the respective effect sizes (Mean MOI and or Mean He) to enable computation of standard error for use in meta-analysis using STATA. Pooled estimates for *Plasmodium falciparum* genetic diversity and MOI were generated, sorted by region. Additionally, patterns of *Plasmodium falciparum* genetic diversity and MOI were assessed according to malaria clinical categories (asymptomatic and symptomatic malaria infection) [38] and specific genetic marker(s) used to evaluate *Plasmodium falciparum* genetic diversity and MOI [12].

Heterogeneity analysis

Heterogeneity across the studies was assessed using the chi-squared test and Cochran's Q statistic, with a 5% level of statistical significance [39], and the I-squared (I^2) statistic [40]. An I^2 statistic of 25% indicates low heterogeneity, 50% indicates moderate heterogeneity, and > 75% indicates high heterogeneity [41].

Risk of bias and quality of evidence assessment

Risk of bias (RoB) in the selected articles was independently evaluated by two reviewers (AM, RWN) using an RoB assessment tool adapted from Joanna Briggs Institute's (JBI) critical appraisal tools [42]. The quality of evidence was determined by two independent reviewers using the Grading of Recommendations, Assessment,

Development, and Evaluations (GRADE) guidelines [43]. RoB assessment covered five domains: study design and limitations, inconsistency in selected articles, indirectness of the evidence, imprecision, and publication bias. Studies scoring 0 to 1, 2 to 3, 4 to 5, and at least 6 were judged to be very low, low, moderate, and high-quality studies, respectively.

Publication bias

Publication bias was assessed by visualizing the asymmetry of the funnel plot and examining the presence and distribution of dots in the plot [44]. Egger's statistical test was performed to assess the asymmetry of the funnel plot. A statistically significant result ($p < 0.05$) in Egger's test indicates that the funnel plot asymmetry is due to small-study effects [45]. All data analysis was conducted using STATA *version* 17 software package (Stata Corporation, College Station, TX).

Missing data

Variables that were missing from included articles were recorded as not reported (NR). Authors of articles with missing data were contacted for additional information, but only a small number (5/12: 41.67%) responded.

Ethics considerations

The study used already published literature with no direct human subject contact and posed no risk to the participants who participated in the primary studies as determined by the Makerere University School of Medicine Institutional Review Board (# Mak-SOMREC-2021-152) and Uganda National Council for Science and Technology (# HS2744ES) (Table 1).

Results

A total of 1,718 articles were retrieved from the literature search, and an additional 6 articles were found through a search of the bibliographies of the identified articles.

Table 1 The PECOST framework

Element	Description
Population	Individuals infected with *P. falciparum*
Exposure	*P. falciparum* infection
Comparator	None
Outcomes	Primary outcomes: 1) Distribution of *P. falciparum* genetic diversity in SSA and 2) Prevalence of *P. falciparum* polyclonal in SSA Secondary outcomes: Factors associated with *P. falciparum* genetic diversity and MOI in SSA
Settings	All SSA malaria-affected countries
Time	2000-to-May 2023

Of these, 52 articles met the inclusion criteria and were included in the review analysis (Fig. 1). The articles were from 23 of the 54 countries in SSA, covering a total of 11,640 genotyped parasite isolates from 9,062 symptomatic and 2,578 asymptomatic *Plasmodium falciparum* infections. Among the 52 articles, 39 (75%) employed cross-sectional study design while 13 (25%) utilized RCT/cohort study designs. A total of 23 studies enrolled both children and adults, while 22 studies enrolled only children, 2 studies enrolled only adults, and 5 studies did not specify the age group of their study population. The predominant genetic markers used to genotype parasites were the antigen-coding loci, especially *msp-1* and/or *msp-2*, in 76.9% (40/52) of the studies, followed by microsatellites markers only in 19.2% (10/52). In one study (1.92%; 1/52), both microsatellites and *msp-1* and/or *msp-2)* were used, while in another one study (1.92%; 1/52), genotyping of *Plasmodium falciparum* parasites involved the use of both *msp-1*, *msp-2*, and single nucleotide polymorphisms (SNPs) (Table 2).

Plasmodium falciparum genetic diversity in SSA

Across studies, *P. falciparum* genetic diversity, primarily assessed using antigen-coding loci (*msp-1*, *msp-2*, and *glurp*), and microsatellites, was reported using either allele frequency, mean expected heterozygosity, or both. The frequencies of *msp-1* alleles (K1, MAD20, and RO33) were 20.8%, 4.2%, and 4.2%, respectively, in Ethiopia, a country with moderate malaria transmission in East Africa [79]. In high malaria transmission areas of Equatorial Guinea in West Africa, these same alleles had substantially higher frequencies of 96.07%, 96.09%, and 70.78%, respectively [22]. The frequency of the *msp-2* gene I/C 3D7 allele ranged from 15.9% to 98.3% in Ethiopia [19, 79], while the FC27 allele frequency ranged from 10.3% in Ethiopia [84] to 98.9% in high malaria transmission areas in Benin [36]. Meanwhile, the frequency of *glurp* ranged from 39.53% among symptomatic individuals in a high malaria transmission setting in Nigeria [68] to 97.6% among severe malaria cases living in malaria-moderate areas in Uganda [74] (Table 2).

The overall pooled allele frequencies of *msp-1* alleles K1, MAD20, and RO33 were 61%, 44%, and 40%, respectively, while the overall polled allele frequencies of *msp-2* I/C 3D7 and FC27 alleles were 61% and 55%, respectively, across reviewed studies. Across regions, the pooled allele frequencies of *msp-1* alleles K1, MAD20, and RO33 were 46%, 42%, and 31%, respectively, in East Africa; 74%, 51%, and 48%, respectively, in Central Africa; and 67%, 43%, and 44%, respectively, in West Africa. In comparison, the pooled allele frequencies of the *msp-2* I/C 3D7 and FC27 alleles were 60% and 55%, respectively, in East Africa, 67% in Central Africa, and 62% and 50%, respectively, in

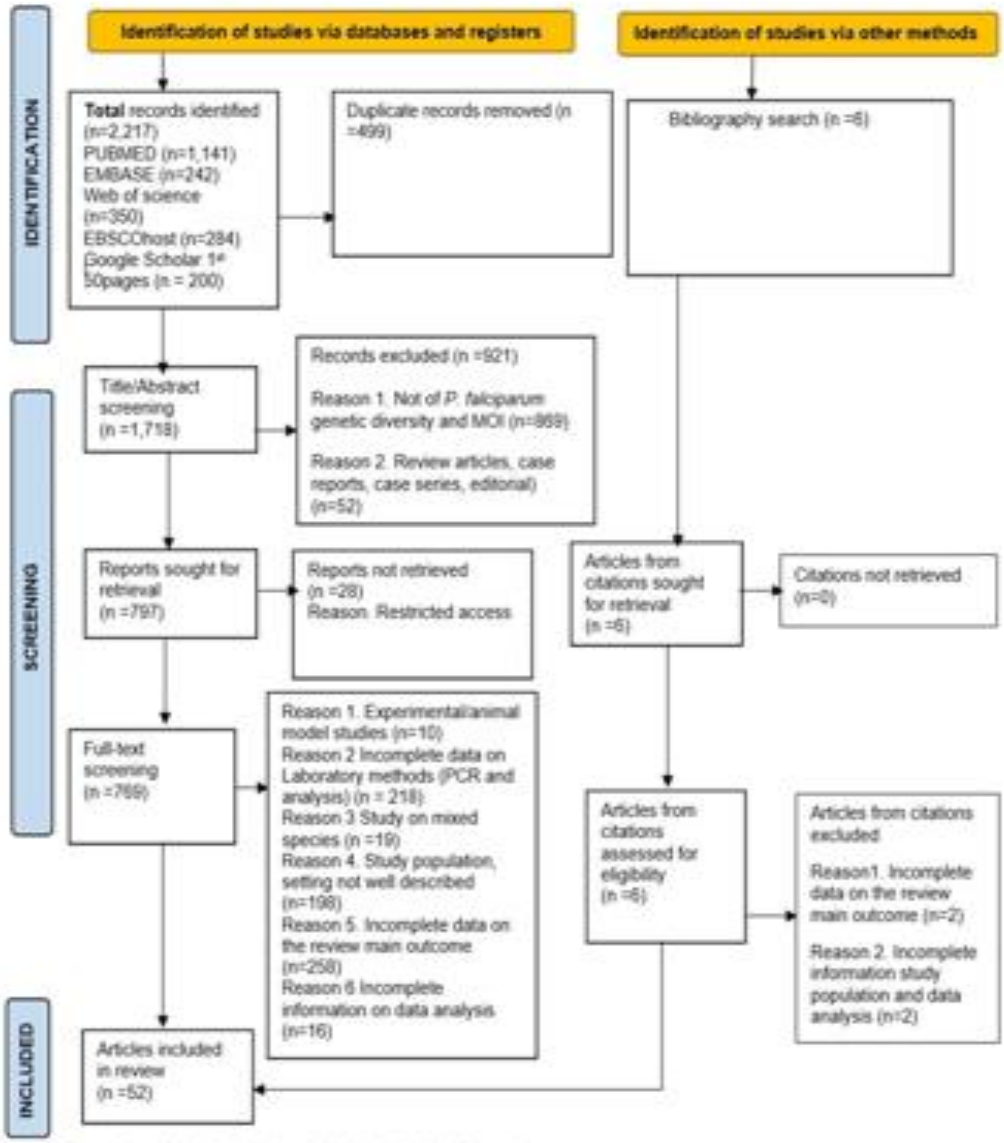


Fig. 1 PRISMA Flow diagram for identification of articles included in the review

Table 2 Summary of *P falciparum* genetic diversity and MOI

First author and year	Country	Malaria clinical category	Age group	Number genotyped	Genotyped marker (s)	Genetic diversity								MOI	
						msp-1 allelic frequencies				msp-2 allelic frequencies		glurp			
						Mean He	%K1	%MAD20	%RO33	%IC/3D7	% FC27	Number of haplotypes	Prevance of polyclonal infections		Mean MOI
Cross sectional studies															
Chekol et al. 2021 [46]	Ethiopia	Asymptomatic	Children and adults	30	msp-1 and msp-2	0.23	46.5	65	37.2	80.7	62.8		30		1.56
Amoah, et al 2021 [47]	Ghana	Asymptomatic	Children	119	Microsatellites	0.56–0.8							89.9		1.7
Tsuma et al. 2020 [10]	Kenya	Asymptomatic	Children	95	Microsatellites	0.81							79.99		3.39
Abukari et al. 2019 [48]	Ghana	Asymptomatic	Children and adults	160	msp-2 and Microsatellites	0.67–0.99				66	34		45.48		1.37
Mulenga et al. 2014 [49]	Kenya	Asymptomatic	NR	198	Microsatellites	0.84							88		2.8
Simpson et al. 2023 [17]	DRC	Asymptomatic and Symptomatic	Children	438	msp-1 and msp-2		78	52	44	84	66		63		1.99
Gnagne et al. 2019 [50]	Cote d'Ivoire	Asymptomatic and Symptomatic	Children	282	msp-1 and msp-2		81.6	53.4	57	84.3	72.2		85		3.16
Yalcouye et al. 2016 [51]	Mali	Asymptomatic and Symptomatic	Children and adults	156	Microsatellites	0.77							58.9		1.72
Ayanyah et al. 2015 [52]	Cameroon	Asymptomatic and symtomatic	Children and adults	151	msp-1		80.2(65.7/14.6)(91)		52.3 (43) 55.6 (91)	42.6 (45) 48.8(91)			60		2.33
Galen et al. 2015 [53]	Kenya	Asymptomatic and Symptomatic	Children	235	Microsatellites	0.77							89		2.66
Oyeniyi et al. 2015 [54]	Nigeria	Asymptomatic and Symptomatic	Children	330	msp-2					89 (45) 51 (91)	99(45) 49(91)		62(45) 66(91)		2.5 (91) 2.1 (45)

Table 2 (continued)

First author and year	Country	Malaria clinical category	Age group	Number genotyped	Genotyped marker (s)	Genetic diversity							MOI		
						msp-1 allelic frequencies				msp-2 allelic frequencies		p/vp			
						Mean He	%K1	%MAD20	%RO33	%IC/3D7	% FC27	Number of haplotypes	Prevance of polyclonal infections	Mean MOI
Agomo-et al. 2022 [59]	Nigeria	Symptomatic	Children	83	msp-1	0.64	52	35	87				63.1	1.98
Tadele et al. 2022 [19]	Ethiopia	Symptomatic	Children and adults	225	msp-1 and msp-2	0.66	25.5 (47/5); 14.5(67)	87.2(475); 12.7 (67)	5 (4/5); 5.45 (3/5)	61.8 (47/5); 98.3 (5/5)	61.8 (47/5); 98.3 (5/5)		16.1	1.09
Ale et al. 2022 [16]	Ethiopia	Symptomatic	Children and adults	148	msp-2	0.49				31.8	27.7		40.5	1.4
Alaribeole et al. 2021 [57]	Nigeria	Symptomatic	Children	633	Microsatellites	0.604							67.1	1.68
Gwamda et al. 2021 [58]	S. Africa	Symptomatic	Adults	747	Microsatellites	0.74							66	2.13
Ajadia et al. 2021 [18]	Uganda	Symptomatic	Children	85	Microsatellites								75.5	1.9
Oyedeji et al. 2020 [60]	Nigeria	Symptomatic	Children	91	msp-2					39.5	60.5		69.8	2.31
Papa Moi et al. 2020 [61]	Cameroon	Symptomatic	NR	111	msp-1, msp-2 and SNPs	0.715	55	15.2	3.2	36.7	47.4		80.8	1.43
Ndiaye et al. 2019 [62]	Senegal	Symptomatic	Children and adults	139	msp-1 and msp-2	0.784–0.837	71	51	38	61	48		36	2.56
Mohammed et al. 2019 [63]	Ethiopia	Symptomatic	Adults	119	msp-1 and msp-2		43.8	22	27.1	49.2	58.5		64.4	2.2
Sabiu et al. 2019 [64]	Kenya	Symptomatic	NR	201	Microsatellites	0.76							51	1.8
Ruh et al. 2019 [65]	Eswatini	Symptomatic	Children and adults	666	Microsatellites	0.71							67	2.2
Sane et al. 2019 [20]	Senegal	Symptomatic	Children and adults	71	msp-1 and msp-2		Mono 9 (54; Submicro 87.5	Mono (60; Submicro 43.6	Mono 44.8 41 (61; Sub micro 22.5	Mono 61 (47; Submicro 32.5	Mono 41 (61; Submicro 10		35.48	msp-1 1.7–2.12 msp-2 1.03–1.60
Huang et al. 2014 [66]	Cameroon	Symptomatic	Children	232	msp-1 and msp-2		11.8 (2006–2007); 41.8(2013–2014)	42.9 (2006–2007); 23.6 (2013–2014)	84.8 (2006–2007; 2008–2009; 2013–2014)	80.8(2006–2007; 37.1(2013–2014)	71.6(2006–2007); 91.1(2013–2014)		76.7(2006–2007); 28.3(2013–2014)	2.2
Chen et al. 2018 [22]	Equatorial Guinea	Symptomatic	Children and adults	181	msp-1 and msp-2		36.07	16.48	70.79	72.25	67.69		48.88	5.51

Table 2 (continued)

First author and year	Country	Malaria clinical category	Age group	Number genotyped	Genotyped marker (s)	Genetic diversity							MOI		
						msp-1 allelic frequencies				msp-2 allelic frequencies		glurp			
						Mean He	%K1	%MAD20	%RO33	%IC/3D7	% FC27	Number of haplotypes	Prevance of polyclonal infections	Mean MOI
Tiono et al. 2017 [57]	Senegal	Symptomatic	Children and adults	100	msp-1 and msp-2		89.07	87.5	62.5	96.25	29.37		msp-1 92.5; msp-2 28.75	2.21
Kolasoye et al. 2016 [66]	Nigeria	Symptomatic	NR	50	msp-1 msp-2 and glurp	0.29–0.86	56	48	42	27.1	43.8	3–7	59	1.85
Mahdi Abdel Hamid et al. 2016 [68]	Sudan	Symptomatic	Children and adults	140	msp-1, msp-2 and glurp		38 (MW), 46 (SW)	53.5 (MAD42 OM)	11 (MW), 100 (M	76(MW), 62(MA	77(MW), 68 (SW)		81	2.25
Kelmou et al. 2018 [70]	Rwanda	Symptomatic	Children and adults	388	msp-2	0.413				70	68		44.8	1.75
Mawili Mboumba et al. 2015 [71]	Gabon	Symptomatic	Children	108	msp-1		65.5	20.8	53.9				50	1.72
Bouyou Akotet et al. 2015 [72]	Gabon	Symptomatic	Children	172	msp-1		63.4	33.9	57.1				30	1.8
Ahmedou Salem et al. 2014 [73]	Mauritania	Symptomatic	Children and adults	115	msp-1		90	84.1	65.5				82.3	3.2
Oyebola et al. 2014 [37]	Nigeria	Symptomatic	Children and adults	536	msp-1 and msp-2		60	50	45	55	62		50	1.4
Kiwuwa et al. 2013 [74]	Uganda	Symptomatic	Children	164	msp-1, msp-2 and glurp		82.9 (SM), 85.6 (MW)	50 both SM and MM	48.8(SM), 37.8(MW)	62.7 Both SM and MM	86.8(SM) and 78(MW)	3 (360–1,293 bp)	80.4	3.7 (SM) and 3.6 (MW)
Hamid et al. 2013 [75]	Sudan	Symptomatic	Children and adults	34	msp-1 and msp-2		31	38	41	59	64		62	1.93
Ogoyemi-Inoumo et al. 2013 [34]	Benin	Symptomatic	Children	93	msp-1 and msp-2		85.2	67	82.9	81.5	98.9		89.4	3.6
Olasehinde et al. 2012 [76]	Nigeria	Symptomatic	NR	100	msp-1, msp-2 and glurp		68	40	20	76	56	3 (700–900 bp)	98	1.87
Awaga et al. 2012 [77]	Togo	Symptomatic	Children	309	msp-1 and msp-2		54.98	25	20.42	48.29	11.71		26 (MW)58.67 (SM)	3

RCT, Cohort studies

Table 2 (continued)

First author and year	Country	Malaria clinical category	Age group	Number genotyped	Genotyped marker (s)	Genetic diversity							MOI	
						msp-1 allelic frequencies				msp-2 allelic frequencies		glurp		
						Mean He	%K1	%MAD20	%RO33	%IC/3D7	% FC27	Number of haplotypes	Prevance of polyclonal infections	Mean MOI
Mohammed et al. 2021 [78]	Ethiopia	Symptomatic	Children and adults	41	msp-2	0.5				34.1	22		40.4	1.2
Abamecha et al. 2020 [79]	Ethiopia	Symptomatic	Children and adults	80	msp-1 and msp-2	0.43–0.85	20.8	4.2	4.2	13.9	26.1		80	3.2
Sondo et al. 2020 [24]	Burkina Faso	Symptomatic	Children and adults	724	msp-1 and msp-2		51	27	22	58	42		NR	2.71
Singana et al.2019 [80]	DRC	Symptomatic	Children	71	msp-1 and msp-2	0.68–0.93	41	33	24	49.6	48.8		86	2.64
Mohammed et al. 2018 [81]	Ethiopia	Symptomatic	Children and adults	90	msp-1, msp-2 and glurp	0.2–0.82	41.1	47.7	35.7	76	77	9 (301–800 bp)	70	2.6
Sorré et al. 2018 [21]	Burkina Faso	Symptomatic	Children	228	msp-1 and msp-2		77.4	41.3	36	93.1	41.3		61.9	1.95
Mohammed et al. 2017 [82]	Ethiopia	Symptomatic	Children and adults	92	msp-2	0.66				51	49		76	2.8
Nsobya, W., et al.2015 [83]	Tanzania	Symptomatic	Children	82	msp-2					48.1	27.3		50	1.4
Mohammed, H., et al.2015 [84]	Ethiopia	Symptomatic	Children and adults	88	msp-1 and msp-2	0.54–0.79	33.9	8.3	19.2	21.5	10.3		60	1.8
Ibara-Okabande 2012 [85]	DRC	Symptomatic	Children	52	msp-2					45	57		54	1.78
Sumari et al. 2010 [86]	Tanzania	Symptomatic	Children	300	msp-2					52.5	47.5		60.6	1.45
Aubouy et al. 2003 [87]	Gabon	Symptomatic	Children	52	msp-1 and msp-2		90.4	63.5	36.5	82.7	65.4		NR	4
Peyerl-Hoffmann et al. 2001[88]	Uganda	Symptomatic	Children and adults	225	msp-1 and msp-2		81.1	41.3	35.5	66.7	55.6		71	2.4

AS-Asymptomatic, DRC – Democratic Republic of Congo, glurp-Glutamate rich protein, He- Expected heterozygosity, HTS-High malaria transmission setting, LTS- Low malaria transmission setting, Micro-Microscopic parasitemia, MM-Mild malaria, MOI- multiplicity of infection, msp-Merozoite surface protein, NR-Not reported, Ref-Reference, RCT-Randomized controlled trial, Sub-micro-submicroscopic parasitemia, SM-severe malaria, SY-Symptomatic

West Africa. For *glurp*, the overall pooled allele frequency was 66%, with a pooled frequency of 90% and 70% in East and West African regions, respectively. The only two reviewed studies from Southern Africa [58, 65] studied parasite genetic diversity and MOI using only microsatellites and not *msp-1*, *msp-2*, or *glurp* (Table 3).

Based on expected heterozygosity, the mean expected heterozygosity was 0.09 in Ethiopia [19], a country with moderate malaria transmission in East Africa, and 0.93 in the DRC [80], a country with high malaria transmission in Central Africa (Table 2). The overall pooled mean expected heterozygosity across all studies was 0.65 (95% CI: 0.51–0.78). Across regions, the pooled mean expected heterozygosity was 0.58 (95% CI: 0.29–0.86), 0.84 (95% CI: 0.81–0.86), 0.74 (95% CI: 0.73–0.75), and 0.69 (95% CI: 0.62–0.75) in East, Central, Southern, and West African regions, respectively (Fig. 2).

Each blue square bar indicates the estimated mean expected heterozygosity in one study, and the lines through the square represent the confidence interval around the estimate. The red diamond symbols represent the pooled mean expected heterozygosity in each region, while the green diamond symbol represents the overall pooled mean expected heterozygosity across all regions. The x-axis represents the scale for mean expected heterozygosity which ranges between 0 to 1.

Plasmodium falciparum MOI across malaria-affected countries in SSA

Plasmodium falciparum MOI, defined as the number of distinct parasite genotypes co-existing within a given infection. *Plasmodium falciparum* MOI was reported using mean MOI and or prevalence of polyclonal infection. The mean MOI ranged from 1.09 in Ethiopia in East Africa [19] to 5.51 in Equatorial Guinea in West Africa [22] (Table 2). The overall pooled mean MOI across studies was 2.09 (95% CI: 1.88–2.30). Across regions, the pooled mean MOI was 2.05 (95% CI: 1.83–2.26), 2.37 (95% CI: 1.28–3.46), 2.16 (95% CI: 2.09–2.23), and 1.96 (95% CI: 1.53–2.39) in East, Central, Southern, and West African regions, respectively (Fig. 3).

The prevalence of polyclonal infections ranged from 16.3% in Ethiopia in East Africa [19] to 98% in Equatorial Guinea in Central Africa [22] to 98% in Equatorial Guinea in Central Africa [27] (Table 2). The overall pooled prevalence of *Plasmodium falciparum* polyclonal infections was 63% (95% CI 56–70) across all studies. Across the regions, the pooled prevalence of polyclonal infections was 62% (95% CI: 53–71), 61% (95% CI: 51–71), 65% (95% CI: 43–88), and 71% (95% CI: 63–79) in East, West, Central, and Southern Africa regions, respectively (Fig. 4).

Factors associated with *Plasmodium falciparum* genetic diversity and MOI across SSA

In three studies [24, 72, 88], a positive association between patient age and parasite density with *Plasmodium falciparum* genetic diversity and MOI was observed; however, this association was not consistent across other studies [36, 69, 79]. Some studies indicated an association between *Plasmodium falciparum* genetic diversity and MOI with the use of chemotherapy to suppress malaria infections. For instance, in a study by Huang, B et al. [66], a decrease in genetic diversity was found over a 10-year period following the introduction of artemisinin-based combination therapy (ACT) in an island population, with a 28% decrease for *msp-1* (from 32 to 23) and *msp-2* (from 29 to 21). MOI declined from 3.11 to 1.63 for *msp-1* and from 2.75 to 1.35 for *msp-2*. The prevalence of polyclonal infection for *msp-1* declined from 76.7% to 29.1% ($P < 0.01$), and for *msp-2*, it declined from 62.4% to 28.3% ($P < 0.01$).

Similarly, a study by Tadele et al. [19] reported a decline in *Plasmodium falciparum* genetic diversity and MOI. Variations in *Plasmodium falciparum* genetic diversity and MOI were found in both rural and urban settings. MOI was higher in rural than in urban settings; for instance, the mean MOI for rural versus urban areas was 1.88 versus 1.55, while the prevalence of polyclonal infection was 42.2% versus 57.7% ($p = 0.04$) [71]. However, in a

Table 3 Pooled proportions of K1, MAD20, RO33, IC/3D7, FC27 and *glurp* alleles across studies

Gene family	Alleles	East Africa		Central Africa		West Africa		All regions	
		Freq (%)	95% CI	Freq (%)	95% CI	Freq (%)	95% CI	Freq (%)	95% CI
msp-1	K1	46	31–60	74	60–88	67	56–78	61	53–69
	MAD20	42	23–60	51	22–81	43	34–51	44	32–56
	RO33	31	17–45	48	35–60	44	33–58	40	32–48
msp-2	IC 3D7	60	48–72	67	52–82	62	51–72	61	54–69
	FC 27	55	42–68	67	45–90	50	33–68	55	46–64
glurp		90	86–93	–	–	70	63–77	66	40–92

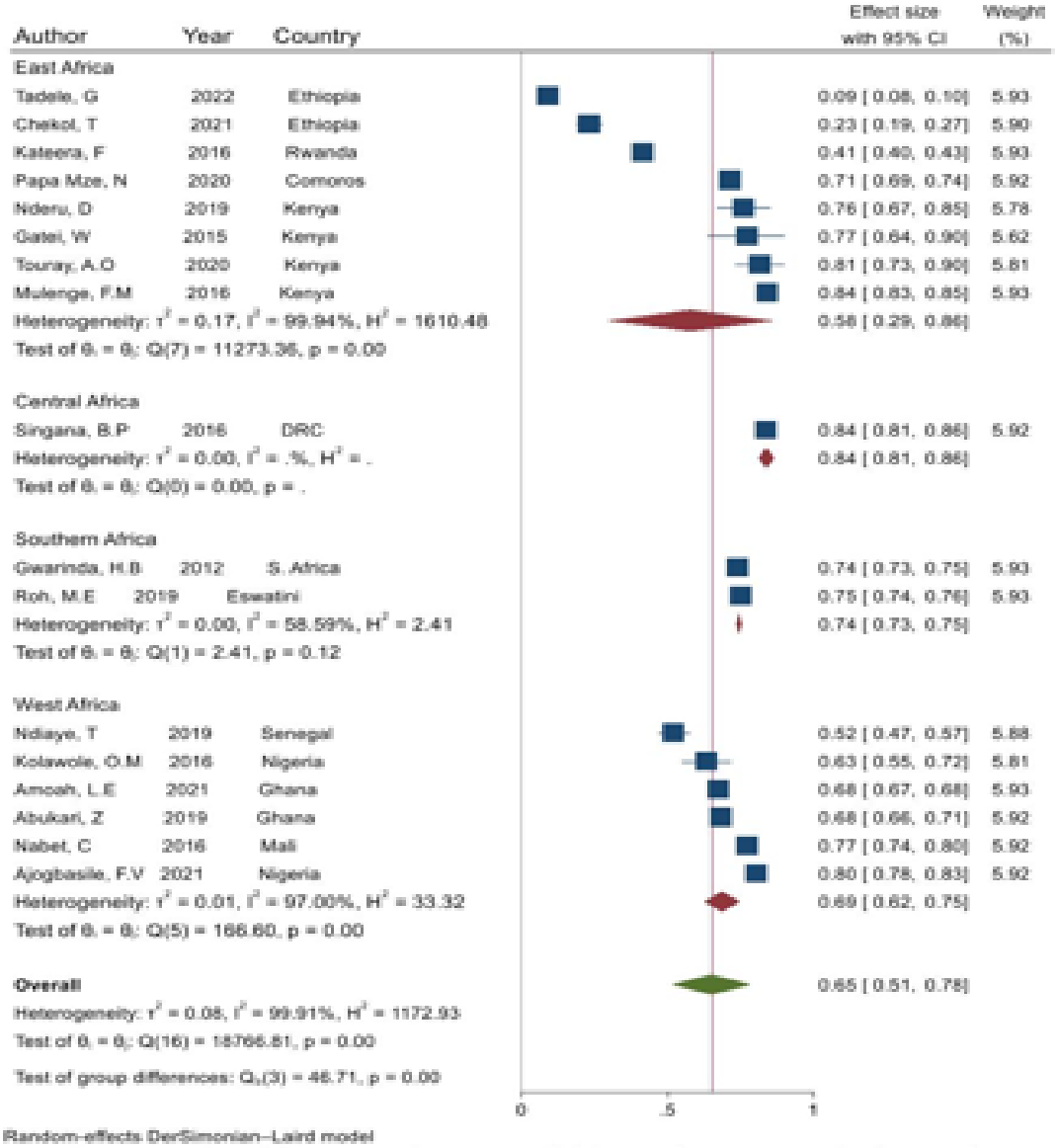


Fig. 2 Forest plot representing the pooled mean expected heterozygosity of *P. falciparum* infection across 17 studies that reported measures of dispersion (CI and SD) for mean expected heterozygosity in malaria-affected countries in SSA, sorted by region

study conducted in an urban setting in Uganda [74], the mean MOI values were even higher (3.0 to 3.7 for severe and mild malaria cases, respectively p = 0.002) than those observed in rural areas elsewhere. High *Plasmodium falciparum* genetic diversity and MOI were also reported among both symptomatic [72, 74, 89] and asymptomatic malaria cases [16, 90]. Furthermore, a positive association between the genetic diversity and MOI of

Plasmodium falciparum with malaria transmission settings, showing higher values in areas with high malaria transmission and lower values in those with low malaria transmission (2.13 in high and 1.29 in low malaria transmission; $p < 0.0001$) has been reported [70]. Meanwhile, the expected heterozygosity was high (0.49 to 0.62) and low (0.26 to 0.28) in high and low malaria transmission settings, respectively. However, this relationship was not observed elsewhere reported [37].

Subgroup analysis of *Plasmodium falciparum* genetic diversity and MOI based on malaria clinical category and the genotyped markers

Subgroup analysis of genetic diversity and MOI was conducted using mean expected heterozygosity and mean MOI, respectively. Considering patient phenotype, the pooled mean expected heterozygosity was 0.64 (95% CI 0.515–0.78) in studies that enrolled only individuals with asymptomatic infection and 0.63 (0.42–0.83) in those that enrolled only individuals with symptomatic infection. However, the pooled mean expected heterozygosity was 0.77 (0.75–0.79) in studies that enrolled individuals with either asymptomatic or symptomatic *Plasmodium falciparum* infections.

Based on antigen-coding loci, *msp-1*, and/or *msp-2* genotypes, the pooled *Plasmodium falciparum* mean expected heterozygosity was 0.49 (95% CI 0.24–0.74). In comparison, it was 0.76 (95% CI 0.72–0.79) based on microsatellite markers. The pooled mean MOI was 1.90 (95% CI 1.50–2.30) in studies enrolling asymptomatic individuals, 2.16 (95% CI 1.92–2.41) in studies enrolling symptomatic individuals, and 1.85 (95% CI 1.59–2.11) in studies enrolling both asymptomatic and symptomatic cases. In studies using antigen-coding loci *msp-1* and/or *msp-2* only, the pooled mean MOI was 2.14 (95% CI 1.91–2.38), while in studies using microsatellites, it was 1.63 (95% CI 1.11–2.15).

Plasmodium falciparum heterogeneity in the included studies

Studies were combined and assessed for heterogeneity. Based on mean expected heterozygosity, there was high heterogeneity among studies enrolling asymptomatic individuals ($I^2 = 99.61\%$, $P < 0.001$) and among studies enrolling individuals with symptomatic malaria ($I^2 = 99.94\%$, $P < 0.001$). Similarly, a high level of heterogeneity was observed among studies using antigen-coding loci, namely *msp-1* and *msp-2* ($I^2 = 99.87\%$, $P < 0.001$), as well as microsatellites ($I^2 = 98.28\%$, $P < 0.001$). Regarding mean MOI, heterogeneity was high in studies enrolling individuals with asymptomatic malaria ($I^2 = 98.64\%$, $P < 0.001$), symptomatic malaria ($I^2 = 99.60\%$, $P < 0.001$), and both asymptomatic and symptomatic cases ($I^2 = 96.20\%$, $P < 0.001$). A high level of heterogeneity was also observed across the genotyped markers, including antigen coding loci, *msp-1*, and *msp-2* ($I^2 = 99.619\%$, $P < 0.001$), and microsatellites ($I^2 = 96.52\%$, $P < 0.001$) (Additional file 3).

Risk of bias in the included studies

Methodological quality and repoeting bias were identified as high in 28.8% (15/52) of the studies. There was a potential for selection bias, as successful genotyping was reported to be < 90% in 11.5% (6/52) of the reviewed articles. Additionally, detection bias related to the assessment of confounding factors was noted in 36.5% (19/52) of the included articles (Additional file 4).

Publication bias assessment

Visual inspection of the funnel plots obtained using mean expected heterozygosity and mean MOI revealed an asymmetrical distribution of estimates from the middle line (Additional file 5). Egger's statistical test showed a coefficient of 2.16, $z = 0.54$, and $P = 0.59$ for mean expected heterozygosity, and a coefficient of 1.65, $z = 1.28$, and $P = 0.2$ for mean MOI.

Discussion

The systematic review covered studies investigating *Plasmodium falciparum* genetic diversity and MOI in malaria-affected countries in SSA. Study findings indicate substantial genetic diversity and MOI among parasites circulating in SSA. The substantial regional variation in parasite genetic diversity and MOI identified in this current study likely reflects differences in regional malaria transmission intensity. This suggests that these markers may be useful in evaluating malaria transmission patterns and the effectiveness of control interventions.

Parasite genetic diversity exhibited variations across regions, with *msp-1* (K1, MAD20, and RO33) and *msp-2*

(See figure on next page.)

Fig. 3 Forest plot representing the pooled mean MOI of *P. falciparum* infection across 32 studies that reported measures of dispersion (CI and SD) for mean MOI in malaria-affected countries in SSA, sorted by region. Each blue square bar indicates the estimated mean *P. falciparum* MOI in one study, and the lines through the square represent the confidence interval around the estimate. The red diamond symbols represent the pooled mean *P. falciparum* MOI in each region, while the green diamond symbol represents the overall pooled *P. falciparum* mean MOI across all regions. The x-axis represents the scale for mean MOI

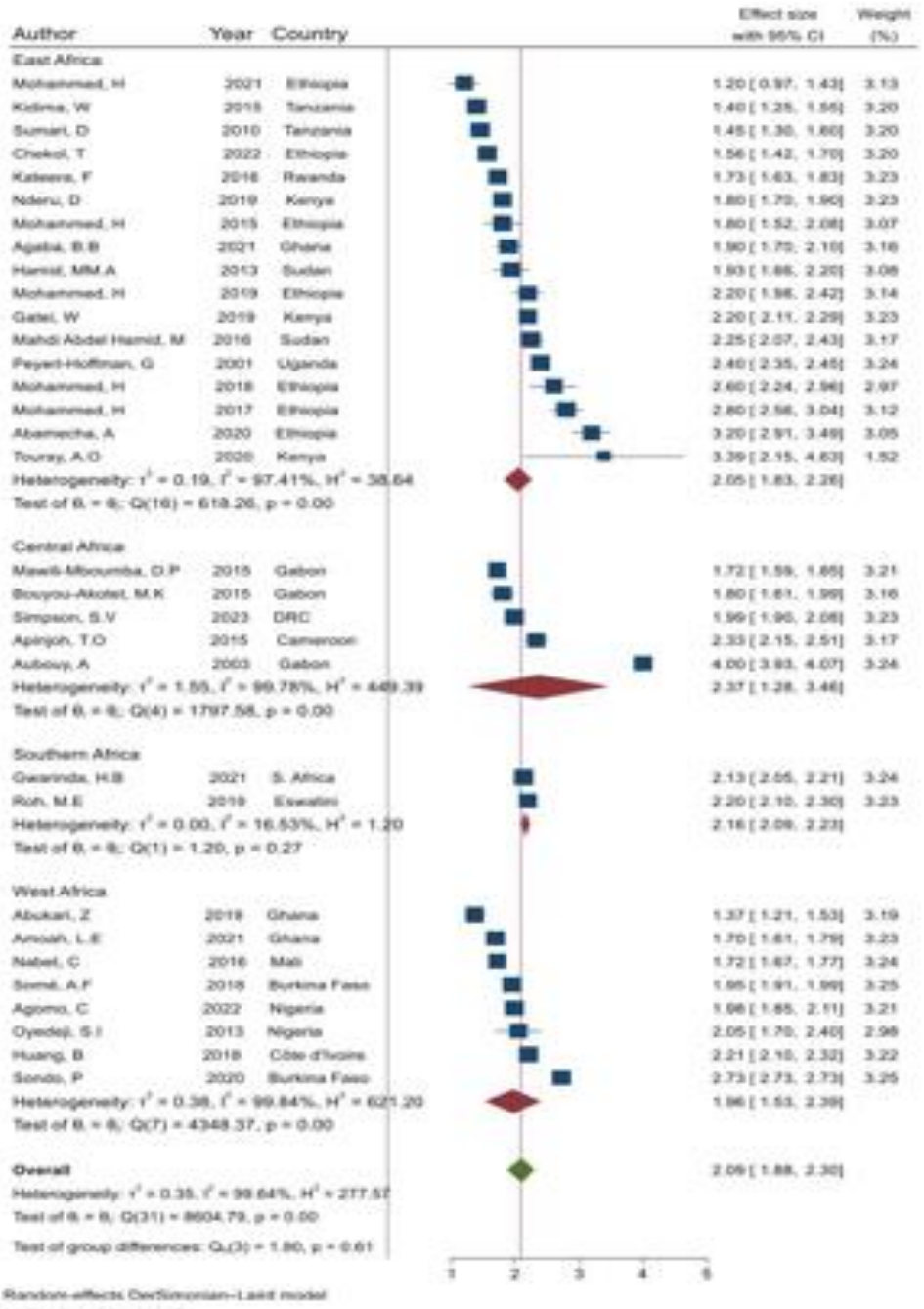


Fig. 3 (See legend on previous page.)

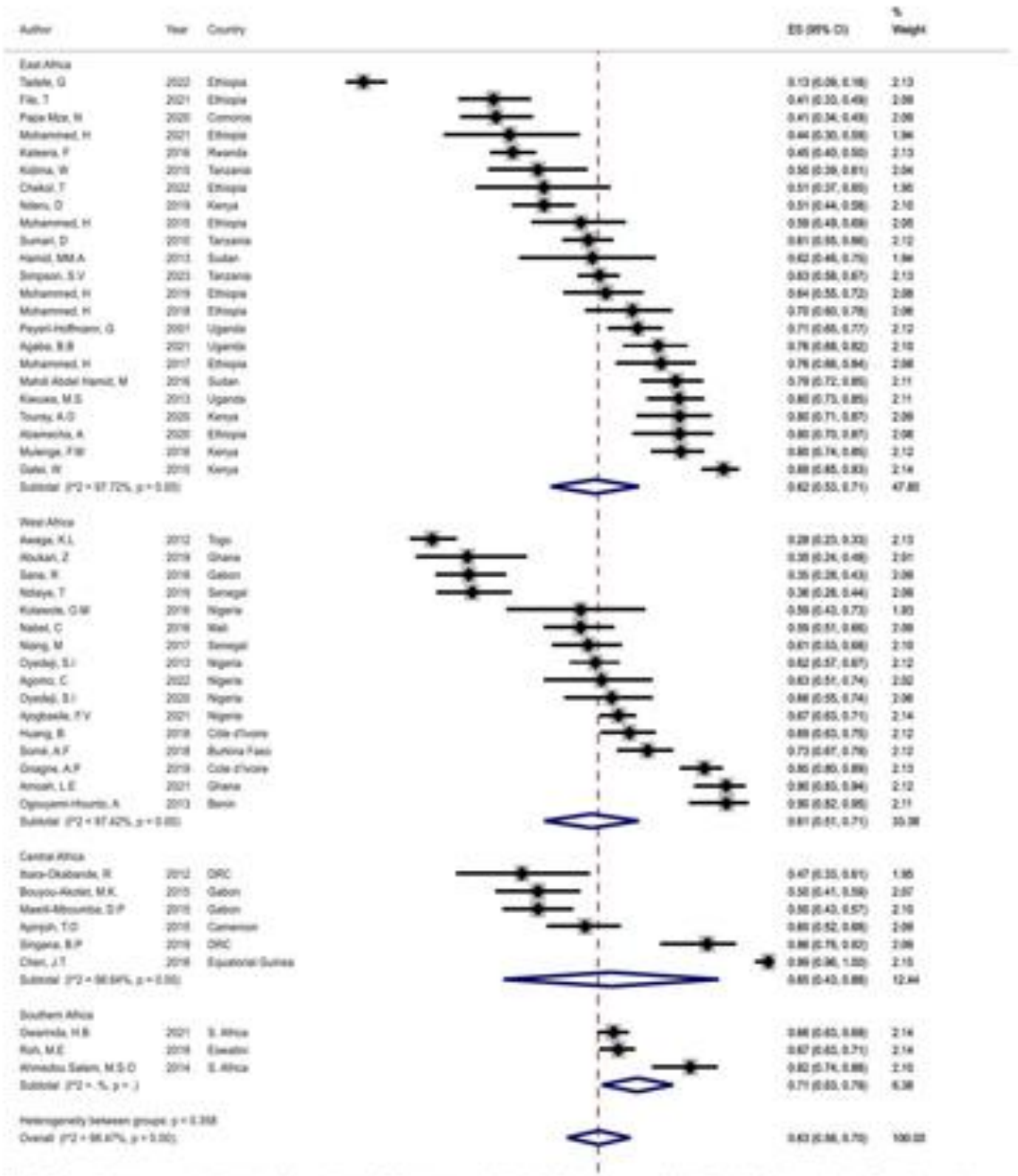


Fig. 4 Forest plot representing the pooled prevalence *P. falciparum* polyclonal infections reported by 48 studies from malaria affected countries in SSA, sorted by region. Each gray square bar with a black dot indicates the estimated prevalence of *P. falciparum* polyclonal infections in one study, and the lines through the square represent the confidence interval around the estimate. The diamond symbol represents the pooled prevalence of *P. falciparum* polyclonal infections

(I/C3D7 and FC27) alleles showing different frequencies in different regions. The finding that high *Plasmodium falciparum* genetic diversity was reported in both high [51] and low [65] malaria transmission areas in SSA is interesting. Parasite genetic diversity results from genetic recombination in the mosquito [6, 91], and is more likely in areas with high local malaria transmission intensity. The high parasite genetic diversity in some areas is therefore a cause for concern because it may indicate ongoing transmission despite the intensification of malaria control measures [53]. Nonetheless, there are areas with low *Plasmodium falciparum* genetic diversity, indicating that malaria control and surveillance efforts should be tailored accordingly.

Plasmodium falciparum mean MOI also exhibited wide variations across regions, ranging from 1.09 to 5.51, with an overall pooled mean MOI of 2.09. Meanwhile, the prevalence of polyclonal infection also varied significantly, ranging from 16.3% to 98%, with an overall pooled prevalence of 63% across studies. Previous reports have documented wide variations in *Plasmodium falciparum* mean MOI (ranging from 1 to 6.1) and the percentage prevalence of polyclonal infections (ranging from 0 to 96%) [25]. High mean MOI and the presence of polyclonal infections serve as key indicators of high malaria transmission intensity [16, 70]. These factors are influenced by increased vector populations, promoting either superinfection or the concurrent transmission of unrelated parasite genotypes [92]. The variations across regions suggest differences in malaria transmission patterns across SSA, emphasizing the need for modifications in malaria vector control and the implementation of customized regional malaria control measures.

The review identified several factors associated with *Plasmodium falciparum* genetic diversity and MOI, including parasite density, the clinical category of malaria infection, patient age, malaria control interventions, and malaria transmission intensity [16, 19, 24, 48, 70, 72, 89]. These findings extend those from previous studies that reported a positive correlation between parasite density and parasite genetic diversity/MOI [70, 75]. Higher parasite density increases the likelihood of carrying distinct parasite genotypes [70], while an increase in age enhances immunity to malaria [93] Additionally, *Plasmodium falciparum* genetic diversity and MOI were found to be higher in rural settings [71], although other reports indicated higher genetic diversity in urban settings [74]. Low parasite genetic diversity and MOI have been reported in areas of Ethiopia, suggesting the effectiveness of malaria control interventions [19].

High *Plasmodium falciparum* genetic diversity and MOI were reported among both symptomatic [72, 74, 89] and asymptomatic malaria cases [16, 48, 90]. The occurrence of multiple *Plasmodium falciparum* infections could pose a challenge to parasite elimination efforts [16] due to its positive association with antimalarial drug failure [94]. Asymptomatic infection is typically characterized by low parasitaemia [95] and high MOI [38]. Asymptomatic individuals with low parasitaemia often remain undetected, thus forming a reservoir for malaria transmission and its spread [96]. The variability in parasite genetic diversity infection profiles has implications for treatment strategies, as well as the efficacy of antimalarial drugs.

msp-1 and *msp-2* are commonly used genetic markers for assessing *Plasmodium falciparum* genetic diversity and MOI. Studies exclusively employing antigen-coding loci (*msp-1* and/or *msp-2*) reported a higher pooled mean MOI (2.14), while those utilizing microsatellites showed a lower pooled mean MOI (1.63). The abundance and high polymorphism of microsatellites make them more suitable for estimating MOI compared to *msp-1*, *msp-2*, and *glurp*, which are relatively fewer and exhibit lower levels of polymorphism [13]. Moreover, *msp-1*, *msp-2*, and *glurp* are susceptible to immune selection [97]. Another limitation arises from significant size variations among *msp-1*, *msp-2*, and *glurp* alleles, potentially introducing bias in amplification efficiency. In cases of multiclonal infections, this bias may result in the preferential amplification of shorter fragments, leading to the loss of longer alleles [14, 15]. This emphasizes the importance of employing advanced tools such as microsatellite analysis and whole-genome sequencing for accurately assessing *Plasmodium falciparum* genetic diversity.

Implications for future research and policy

The substantial variations in *Plasmodium falciparum* genetic diversity and MOI in SSA necessitates continuous genomic surveillance in different malaria transmission settings. Current research predominantly focuses on symptomatic malaria infections in children utilising *msp-1* and *msp-2* genetic markers for assessing genetic diversity and MOI. Future studies should broaden their focus to include both adults and children across different malaria transmission contexts. Incorporation of advanced tools like microsatellite and whole-genome sequencing, is crucial for accurate assessments of parasite genetic diversity.

Strengths of the study

The review focused on peer-reviewed articles published over an extended period of time to adequately appreciate the genetic diversity and MOI of *Plasmodium falciparum* parasites circulation in SSA., an area which contributes over 95% of global malaria cases. The review focused on genetic markers (*msp-1*, *msp-2*, *glurp*, and

microsatellites) that are more common and readily available in resource-limited settings in SSA. The review was conducted following standard PRISMA-P review guidelines to enhance the reliability of the findings.

Limitations of the study

The present study has several limitations. Firstly, reliance on peer-reviewed published articles may have introduced potential publication bias. Secondly, studies that did not explicitly mention *Plasmodium falciparum* genetic diversity and/or MOI in the title may have been missed. Additionally, the geographical coverage of articles was not comprehensive, as they did not encompass all countries in SSA, thereby impacting the generalizability of findings within the region. For instance, only two of the reviewed studies originated from Southern Africa [58, 65], focusing solely on microsatellites rather than *msp-1*, *msp-2*, and *glurp*, which limits inferences about marker distribution in this region. Furthermore, the exclusion of studies lacking measures of dispersion (CI and SD) affected the meta-analysis of mean expected heterozygosity and mean MOI.

Conclusion

This systematic review reveals considerable variations in *Plasmodium falciparum* genetic diversity and MOI across malaria-affected countries in SSA. Despite control efforts, the high observed parasite genetic diversity and MOI emphasize the necessity for customized, region-specific malaria control strategies, and continuous surveillance.

Abbreviations

GRADE	Grading of Recommendations, Assessment and Development and Evaluations
HRP2	Histidine rich protein 2
JBI	Joanna Briggs Institute's
MOI	Multiplicity of infection
MSP	Merozoite surface protein
NR	Not reported
PCR	Polymerase chain reaction
PRISMA-P	Reporting Items for Systematic Review and Meta-Analysis for Protocols
RoB	Risk of bias
STREGA	STrengthening the REporting of Genetic Association Studies
STROBE	Strengthening the Reporting of Observational Studies in Epidemiology

Supplementary Information

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Additional file 1. Search strategy.

Additional file 2. Screening criteria.

Additional file 3. Subgroup and Heterogeneity analysis.

Additional file 4. RoB assessment.

Additional file 5. Publication bias assessment.

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Author contributions

AM, MO, SLN, JN, CK, BM, SMK EAO and PBK conceived the idea, planned, and designed the study protocol. AAK and AM designed the search strategy. AM and RWN screened articles and abstracted data. AM performed the analysis and wrote the first draft of the manuscript. PBK, SMM, BC, SLN and EAO advised on the analysis and critically edited the manuscript. AM, PBK and SMM wrote the final draft of the manuscript.

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Availability of data and materials

The datasets generated and/or analysed in this review are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The study utilized already published literature with no direct human subject contact, posing no risk to the participants involved in the primary studies, as determined by the Makerere University School of Medicine Institutional Review Board (# Mak-SOMREC-2021–153) and the Uganda National Council for Science and Technology (# HS2194ES).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. WHO. World Malaria Report 2023. Geneva: World Health Organization; 2023.

2. Hu W-C. Human immune responses to *Plasmodium falciparum* infection: molecular evidence for a suboptimal Th1/β and Th17 bias over ideal and effective traditional TH1 immune response. Malar J. 2013;12:392.
3. White NJ. The role of anti-malarial drugs in eliminating malaria. Malar J. 2008;7(Suppl 1):S8.
4. 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;32:00019–19.
5. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, et al. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis. 2005;192:870–7.
6. Mu J, Awadalla P, Duan J, McGee KM, Joy DA, McVean GA, et al. Recombination hotspots and population structure in *Plasmodium falciparum*. PLoS Biol. 2005;3: e335.
7. Pinkevych M. Understanding the relationship between *Plasmodium falciparum* growth rate and multiplicity of infection. J Infect Dis. 2015;211:1121–7.
8. Alizon S. Parasite co-transmission and the evolutionary epidemiology of virulence. Evolution. 2013;67:921–33.
9. 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.
10. Patel P, Bharti PK, Bansal D, Ransan RK, Mohapatra PK, Sehgal R, et al. Genetic diversity and antibody responses against *Plasmodium falciparum* vaccine candidate genes from Chhattisgarh, Central India: implication for vaccine development. PLoS Biol. 2017;12: e0182674.
11. Ghansah A, Tiedje KE, Argyropoulos DC, Onwona CO, Deed SL, Labbé F, et al. Comparison of molecular surveillance methods to assess changes in the population genetics of *Plasmodium falciparum* in high transmission. Front Parasitol. 2023;2:1067966.
12. Apinjoh TO, Ouattara A, Titanji VPK, Djimde A, Amambua-Ngwa A. Genetic diversity and drug resistance surveillance of *Plasmodium falciparum* for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? Malar J. 2019;18:217.
13. Zhong D, Koepfli C, Cui L, Yan G. Molecular approaches to determine the multiplicity of *Plasmodium* infections. Malar J. 2018;17:172.
14. Messerli C, Hofmann NE, Beck HP, Felger I. Critical evaluation of molecular monitoring in malaria drug efficacy trials and pitfalls of length-polymorphic markers. Antimicrob Agents Chemother. 2016;61:e01500-e1516.
15. Porter KA, Burch CL, Poole C, Juliano JJ, Cole SR, Meshnick SR. Uncertain outcomes: adjusting for misclassification in antimalarial efficacy studies. Epidemiol Infect. 2011;139:544–51.
16. Touray AO, Mobegi VA, Wamunyokoli F, Herren JK. Diversity and multiplicity of *Plasmodium falciparum* infections among asymptomatic school children in Mbita, Western Kenya. Sci Rep. 2020;10:5924.
17. Simpson SV, Nundu SS, Arima H, Kaneko O, Mita T, Culleton R, Yamamoto T. The diversity of *Plasmodium falciparum* isolates from asymptomatic and symptomatic school-age children in Kinshasa province, Democratic Republic of Congo. Malar J. 2023;22:102.
18. Mohd Abd Razak 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 Biol. 2016;11:e0152413.
19. Tadele G, Jaleta FK, Oboh M, Diarra E, Dugassa S, Amambua-Ngwa A, et al. Low genetic diversity of *Plasmodium falciparum* merozoite surface protein 1 and 2 and multiplicity of infections in western Ethiopia following effective malaria interventions. Malar J. 2022;21:383.
20. Sane R, Talla C, Diouf B, Sarr FD, Diagne N, Faye J, et al. Low genetic diversity and complexity of submicroscopic *Plasmodium falciparum* infections among febrile patients in low transmission areas in Senegal. PLoS Biol. 2019;14: e0215155.
21. Somé AF, Badolo T, Zongo I, Yerbanga RS, Nikiéma F, Neya C, et al. *Plasmodium falciparum msp1* and *msp2* genetic diversity and allele frequencies in parasites isolated from symptomatic malaria patients in Bobo-Dioulasso, Burkina Faso. Parasit Vectors. 2018;11:323.
22. Chen JT, Li J, Zha GC, Huang G, Huang ZX, Xie DD, et al. Genetic diversity and allele frequencies of *Plasmodium falciparum msp1* and *msp2* in parasite isolates from Bioko Island, Equatorial Guinea. Malar J. 2018;17:458.
23. Agonhossou R, Akoton R, Lagnika H, Djihinto OY, Sovegnon PM, Saizonou HD, et al. *Plasmodium falciparum msp1* and *msp2* genetic diversity in *Plasmodium falciparum* single and mixed infection with *Plasmodium malariae* among the asymptomatic population in Southern Benin. Parasitol Int. 2022;89: 102590.
24. 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.
25. Lopez L, Koepfli C. Systematic review of *Plasmodium falciparum* and *Plasmodium vivax* polyclonal infections: impact of prevalence, study population characteristics, and laboratory procedures. PLoS Biol. 2021;16: e0249382.
26. Eldh M, Hamrmar U, Arnot D, Beck HP, Garcia A, Liljander A, et al. Multiplicity of asymptomatic *Plasmodium falciparum* infections and risk of clinical malaria: a Systematic review and pooled analysis of individual participant data. J Infect Dis. 2020;221:775–85.
27. Opute AO, Akinkunmi IA, Funsho AO, Obanyi AK, Anifowoshe AT. Genetic diversity of Plasmodium falciparum isolates in Nigeria. A review. Egypt J Med Hum Genetics. 2022;23:129.
28. Chaudhry S, Singh V. A systematic review on genetic diversity of var gene DBL1α domain from different geographical regions in *Plasmodium falciparum* isolates. Infect Genet Evol. 2021;95: 105049.
29. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. Syst Rev. 2021;10:89.
30. Neafsey DE, Taylor AR, MacInnis BL. Advances and opportunities in malaria population genomics. Nat Rev Genet. 2021;22:502–17.
31. WHO. World Malaria Report 2022. Geneva: World Health Organization; 2022.
32. Bendixen M, Msangeni HA, Pedersen BV, Shayo D, Bødker R. Diversity of *Plasmodium falciparum* populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. Trans R Soc Trop Med Hyg. 2001;95:143–8.
33. Barry AE, Schultz L, Senn N, Nale J, Kiniboro B, Siba PM, et al. High levels of genetic diversity of *Plasmodium falciparum* populations in Papua New Guinea despite variable infection prevalence. Am J Trop Med Hyg. 2013;88:718–25.
34. 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.
35. Mobegi VA, Loua KM, Ahouidi AD, Satoguina J, Nwakanma DC, Amambua-Ngwa A, et al. Population genetic structure of *Plasmodium falciparum* across a region of diverse endemicity in West Africa. Malar J. 2012;11:223.
36. Ogouyèmi-Hounto A, Gazard DK, Ndam N, Topanou E, Garba O, Elegbe P, et al. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from children in South of Benin. Parasite. 2013;20:37.
37. Oyebola MK, Idowu ET, Olukosi YA, Iwalokun BA, Agomo CO, Ajibaye OO, et al. Genetic diversity and complexity of *Plasmodium falciparum* infections in Lagos, Nigeria. Asian Pac J Trop Biomed. 2014;4(Suppl 1):S87–91.
38. Sarah-Matio EM, Guillochon E, Nsango SE, Abate L, Ngou CM, Bouopda GA, et al. Genetic diversity of *Plasmodium falciparum* and distribution of antimalarial drug resistance mutations in symptomatic and asymptomatic infections. Antimicrob Agents Chemother. 2022;66: e0018822.
39. Cooper H, Hedges LV, Valentine JC. The handbook of research synthesis and meta-analysis 2nd edition. 2009. Russell Sage Foundation
40. Bowden J, Tierney JF, Copas AJ, Burdett S. Quantifying, displaying and accounting for heterogeneity in the meta-analysis of RCTs using standard and generalised Q statistics. BMC Med Res Methodol. 2011;11:41.
41. Huedo-Medina TB, Sánchez-Meca J, Marín-Martínez F, Botella J. Assessing heterogeneity in meta-analysis: Q statistic or I2 index? Psychol Methods. 2006;11:193–206.
42. Joanna Briggs Institute -Critical appraisal tools. https://jbi.global/critical-appraisal-tools
43. Schunemann HJ, Brożek J, Guyatt G, Oxman A. Handbook for grading the quality of evidence and the strength of recommendations using the GRADE approach. 2013;15. https://gdc.gradepro.org/app/handbook/handbook.html
44. Simmonds M. Quantifying the risk of error when interpreting funnel plots. Syst Rev. 2015;4:24.

45. Sterne JA, Egger M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. J Clin Epidemiol. 2001;54:1046–55.
46. Chekol T, Alemayehu GS, Tafesse W, Legesse G, Zerfu B, File T, et al. Genetic diversity of Merozoite Surface Protein-1 and -2 Genes in Plasmodium falciparum isolates among asymptomatic population in Boset and Badewacho districts, Southern Ethiopia. a J Parasitol Res. 2022;2022:7728975.
47. Amoah LE, Abukari Z, Dawson-Amoah ME, Dieng CC, Lo E, Afrane YA. Population structure and diversity of Plasmodium falciparum in children with asymptomatic malaria living in different ecological zones of Ghana. BMC Infect Dis. 2021;21:439.
48. Abukari Z, Okoriu R, Nyarko SB, Lo AC, Dieng CC, Salifu SP, et al. The diversity, multiplicity of infection and population structure of Plasmodium falciparum parasites circulating in asymptomatic carriers living in high and low malaria transmission settings of Ghana. Genes (Basel). 2019;10:434.
49. Mulenga 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.
50. Gnagne AP, Konate A, Bedia-Tanoh AV, Amsih-Droh M, Menan HE, N'Guetta AS, et al. Dynamics of Plasmodium falciparum genetic diversity among asymptomatic and symptomatic children in three epidemiological areas in Cote d'Ivoire. Pathog Glob Health. 2019;113:133–42.
51. Nabet C, Doumbo S, Jeddi F, Konaté S, Mancuilli T, Fofana B, et al. Genetic diversity of Plasmodium falciparum in human malaria cases in Mali. Malar J. 2016;15:353.
52. Apinjoh TO, Tata RB, Anchang-Kimbi JK, Chi HF, Fon EM, Mugri RN, et al. Plasmodium falciparum merozoite surface protein 1 block 2 gene polymorphism in field isolates along the slope of mount Cameroon: a cross - sectional study. BMC Infect Dis. 2015;15:309.
53. Gatei W, Ginny JE, Hawley W, Ter Kuile F, Odero C, Inieremam NC, et al. Genetic diversity of Plasmodium falciparum parasite by microsatellite markers after scale-up of insecticide-treated bed nets in western Kenya. Malar J. 2015;1:XSuppl 1):495.
54. Oyedeji SI, Awobode HO, Anumudu C, Kun J. Genetic diversity of Plasmodium falciparum isolates from naturally infected children in north-central Nigeria using the merozoite surface protein-2 as molecular marker. Asian Pac J Trop Med. 2013;6:589–94.
55. Agomo CO, Lameed O, Ajibaye O. Genetic diversity in merozoite surface protein 1 of Plasmodium falciparum isolates from Igbogbo-Bayeku, Lagos, Nigeria Ife J Sci. 2022;24:025–34.
56. File T, Golassa L, Dinka H. Plasmodium falciparum clinical isolates reveal analogous circulation of 3D7 and FC27 allelic variants and multiplicity of infection in urban and rural settings: the case of Adama and its surroundings, Oromia, Ethiopia. J Parasitol Res. 2022;2022:5773593.
57. 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.
58. Gwarinda HB, Tessema SK, Raman J, Greenhouse B, Birkholtz LM. 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.
59. 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.
60. Oyedeji SI, Bassi PU, Oyedeji SA, Ojurongbe O, Awobode HO. Genetic diversity and complexity of Plasmodium falciparum infections in the microenvironment among siblings of the same household in North-Central Nigeria. Malar J. 2020;19:338.
61. Papa Mze N, Bogreau H, Diedhiou CK, Herdell V, Rahamatou S, Bei AK, et al. Genetic diversity of Plasmodium falciparum in Grande Comore Island. Malar J. 2020;19:320.
62. Ndiaye T, Sy M, Gaye A, Ndiaye D. Genetic polymorphism of Merozoite Surface Protein 1 (msp1) and 2 (msp2) genes and multiplicity of Plasmodium falciparum infection across various endemic areas in Senegal. Afr Health Sci. 2019;19:2446–56.
63. Mohammed H, Hassen K, Assefa A, Mekete K, Tadesse G, Taye G, et al. Genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria based on msp-1 and msp-2 genes in Gublak, North West Ethiopia. Malar J. 2019;18:413.
64. Nderu D, Kimani F, Karanja E, Thiongo K, Akinyi M, Too E, et al. Genetic diversity and population structure of Plasmodium falciparum in Kenyan-Ugandan border areas. Trop Med Int Health. 2019;24:647–56.
65. 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;220:1946–54.
66. Huang B, Tuo F, Liang Y, Wu W, Wu G, Huang S, et al. Temporal changes in genetic diversity of msp-1, msp-2, and msp-3 in Plasmodium falciparum isolates from Grande Comore Island after introduction of ACT. Malar J. 2018;17:83.
67. Niang M, Thiam LG, Loucoubar C, Sow A, Sadio BD, Diallo M, et al. Spatio-temporal analysis of the genetic diversity and complexity of Plasmodium falciparum infections in Kedougou, southeastern Senegal. Parasit Vectors. 2017;10:33.
68. Kolawole OM, Mokuolu OA, Olukosi YA, Oloyede TO. Population genomics diversity of Plasmodium falciparum in malaria patients attending Okelele Health Centre, Okelele, Ilorin, Kwara State Nigeria Afr Health Sci. 2016;16:704–11.
69. Mahdi Abdel Hamid M, Elamin AF, Albsheer MM, Abdalla AA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria in Gezira State, Sudan Parasit Vectors. 2016;9:362.
70. Kateera F, Nkolboya 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.
71. Mawili-Mboumba DP, Mbondoukwe N, Adande E, Bouyou-Akotet MK. Allelic diversity of MSP1 gene in Plasmodium falciparum from rural and urban areas of Gabon. Korean J Parasitol. 2015;53:413–9.
72. Bouyou-Akotet MK, M'Bondoukwé NP, Mawili-Mboumba DP. Genetic polymorphism of merozoite surface protein 1 in Plasmodium falciparum isolates from patients with mild to severe malaria in Libreville, Gabon Parasite. 2015;22:12.
73. Ahmedou Salem MS, Ndiaye M, OuldAbdallahi M, Lekweiry KM, Bogreau H, Konaté L, et al. Polymorphism of the merozoite surface protein-1 block 2 region in Plasmodium falciparum isolates from Mauritania. Malar J. 2014;13:26.
74. Kiwuwa MS, Ribacke U, Mull 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.
75. Hamid MM, Mohammed SB, El Hassan IM. Genetic diversity of Plasmodium falciparum field isolates in central Sudan inferred by PCR genotyping of Merozoite Surface Protein 1 and 2. N Am J Med Sci. 2013;5:95–101.
76. Olaleinde GI, Yah CS, Singh R, Ojuronbge OO, Ajayi AA, Valecha N, et al. Genetic diversity of Plasmodium falciparum field isolates from south western Nigeria. Afr Health Sci. 2012;12:355–61.
77. Awaga KL, Missihoun TD, Karou SD, Djadou KE, Chabi NW, Akati A, et al. Genetic diversity and genotype multiplicity of Plasmodium falciparum infections in symptomatic individuals in the maritime region of Togo. Trop Med Int Health. 2012;17:153–60.
78. Mohammed H, Assefa A, Chernet M, Wuletaw Y, Commons RJ. Genetic polymorphisms of Plasmodium falciparum isolates from Melka-Wewe, North East Ethiopia based on the merozoite surface protein 2 (msp-2) gene as a molecular marker. Malar J. 2021;20:85.
79. 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.
80. Singana BP, Mayengue PI, Niama RF, Ndounga M. Genetic diversity of Plasmodium falciparum infection among children with uncomplicated malaria living in Pointe-Noire, Republic of Congo. Pan Afr Med J. 2019;32:183.
81. Mohammed H, Kassa M, Mekete K, Assefa A, Taye G, Commons RJ. Genetic diversity of the msp-1, msp-2, and glurp genes of Plasmodium falciparum isolates in Northwest Ethiopia. Malar J. 2018;17:386.
82. Mohammed H, Kassa M, Assefa A, Tadesse M, Kebede A. Genetic polymorphism of Merozoite Surface Protein-2 (MSP-2) in Plasmodium

falciparum isolates from Pawe District, North West Ethiopia. PLoS Biol. 2017;12:e0177559.
83. Kidima W, Nkwengulila G. *Plasmodium falciparum* msp-2 genotypes and multiplicity of infections among children under five years with uncomplicated malaria in Kibaha, Tanzania. J Parasitol Res. 2015;2015:721201.
84. Mohammed H, Mindaye T, Belayneh M, Kassa M, Assefa A, Tadesse M, et al. Genetic diversity of *Plasmodium falciparum* isolates based on msp-1 and msp-2 genes from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia. Malar J. 2015;14:73.
85. Ibara-Okabande R, Koukouikila-Koussounda F, Ndounga M, Vouvoungui J, Malonga V, Casimiro PN, et al. Reduction of multiplicity of infections but no change in msp2 genetic diversity in *Plasmodium falciparum* isolates from Congolese children after introduction of artemisinin-combination therapy. Malar J. 2012;11:410.
86. Sumari D, Hosea KM, Mugasa JP, Abdulla S. Genetic diversity of *Plasmodium falciparum* strains in children under five years of age in Southeastern Tanzania. Open Trop Med J. 2010;3.
87. Aubouy A, Migot-Nabias F, Deloron P. Polymorphism in two merozoite surface proteins of *Plasmodium falciparum* isolates from Gabon. Malar J. 2003;2:12.
88. Peyerl-Hoffmann G, Jelinek T, Kilian A, Kabagambe G, Metzger WG, von Sonnenburg F. Genetic diversity of *Plasmodium falciparum* and its relationship to parasite density in an area with different malaria endemicities in West Uganda. Trop Med Int Health. 2001;6:607–13.
89. Metuh TN, Chen JH, Fon-Gah P, Zhou X, Moyou-Somo R, Zhou XN. Genetic diversity of *Plasmodium falciparum* and genetic profile in children affected by uncomplicated malaria in Cameroon. Malar J. 2020;19:115.
90. 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.
91. 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.
92. Nkhoma SC, Banda RL, Khoswe S, Dzoole-Mwale TJ, Ward SA. Intra-host dynamics of co-infecting parasite genotypes in asymptomatic malaria patients. Infect Genet Evol. 2018;65:414–24.
93. Hviid L. Clinical disease, immunity and protection against *Plasmodium falciparum* malaria in populations living in endemic areas. Expert Rev Mol Med. 1998;1998:1–10.
94. Kyabayinze DJ, Karamagi C, Kiggundu M, Kamya MR, Wabwire-Mangen F, Kironde F, et al. Multiplicity of *Plasmodium falciparum* infection predicts antimalarial treatment outcome in Ugandan children. Afr Health Sci. 2008;8:200–5.
95. Babiker HA, Gadalla AA, Ranford-Cartwright LC. The role of asymptomatic *Plasmodium falciparum* parasitaemia in the evolution of antimalarial drug resistance in areas of seasonal transmission. Drug Resist Updat. 2013;16:1–9.
96. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malar J. 2012;11:29.
97. Ferreira MU, da Silva NM, Wunderlich G. Antigenic diversity and immune evasion by malaria parasites. Clin Diagn Lab Immunol. 2004;11:987–95.

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APPENDIX II. Publication from study objective 2.

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

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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

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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

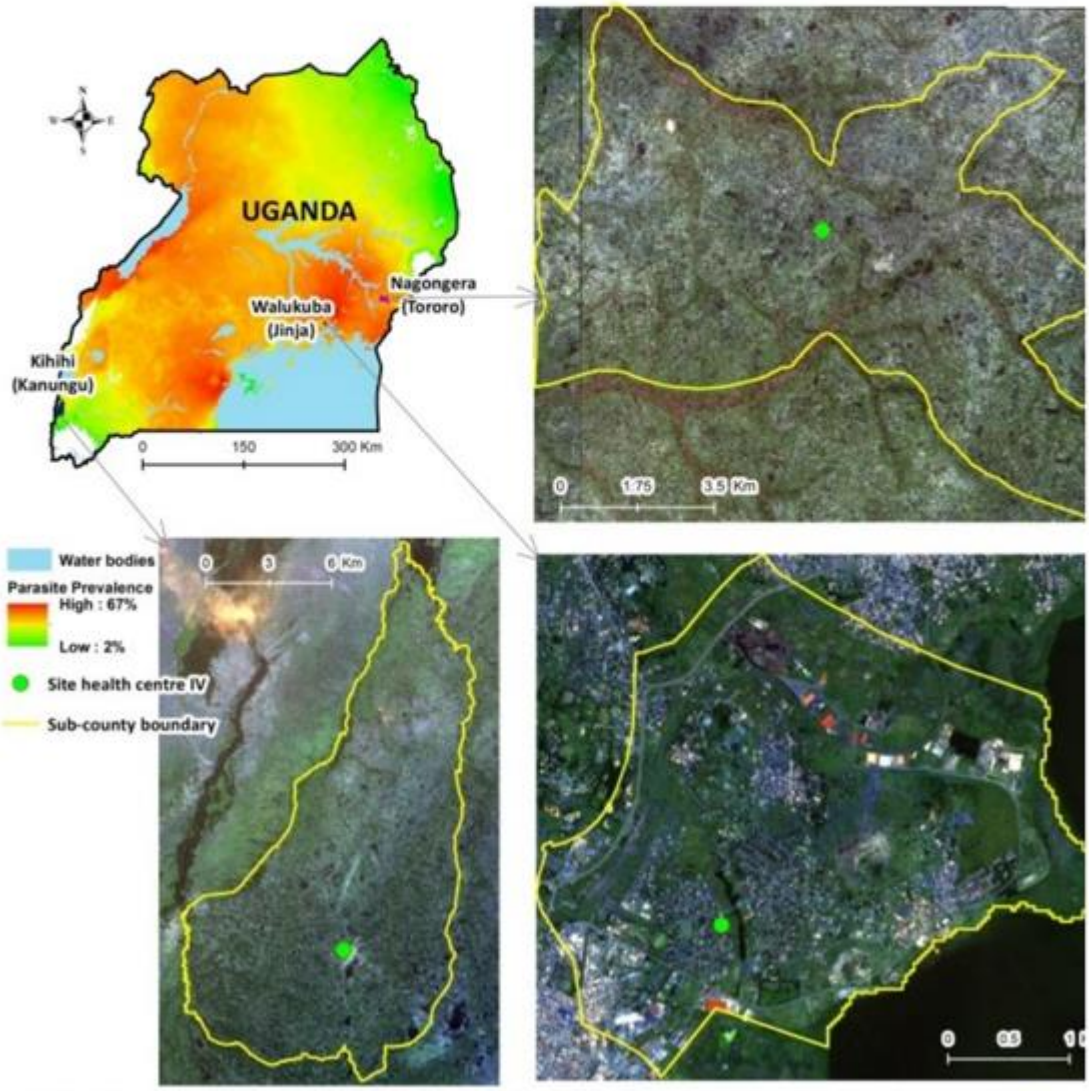


Fig. 1 Map of Uganda showing the malaria endemicity of the study sites at the time of sample collection. Adapted from Kamya et al., 2015 [40]

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

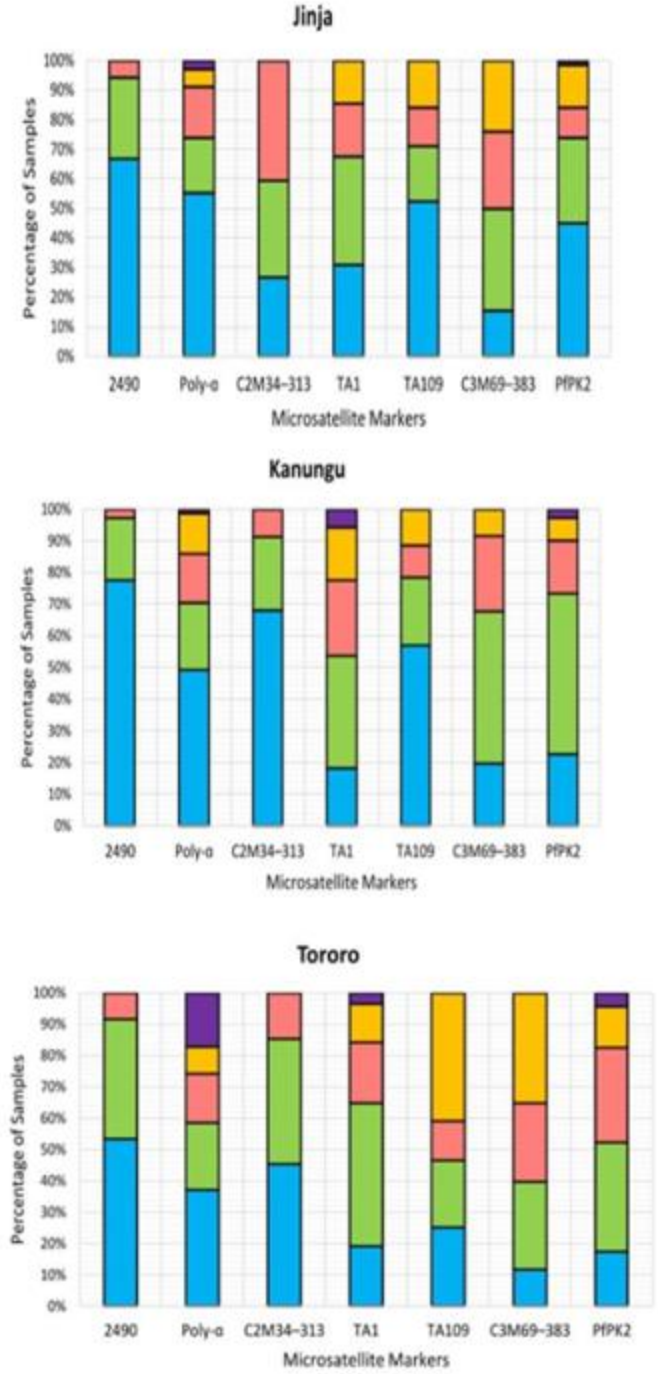


Fig. 2 Percentage of clones within each microsatellite marker with 1 clone (blue), 2 clones (green), 3 clones (red), 4 clones (yellow), and 5 clones (purple) in Jinja, Kanungu and Tororo. The vertical axis represents the percentage of samples while the horizontal axis represents the genetic markers

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.

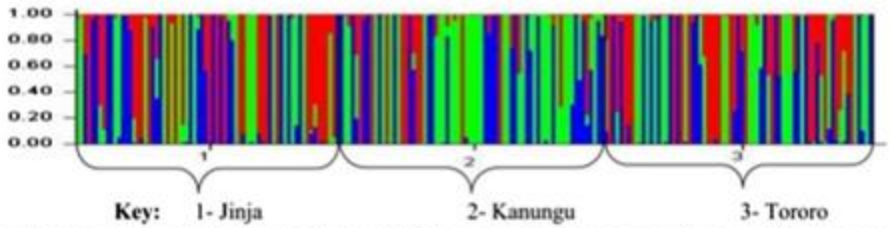


Fig. 3 The Bayesian bar plot illustrates the population structure of *P. falciparum* parasite isolates collected from three different regions with varying levels of malaria transmission: Jinja (low malaria transmission), Kanungu (medium malaria transmission), and Tororo (high malaria transmission). Each 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

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Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.

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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.

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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.

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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-Hasharni 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.

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APPENDIX III. Publication from study objective 3.

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RESEARCH Open Access

Plasmodium falciparum genetic diversity and multiplicity of infection among asymptomatic and symptomatic malaria-infected individuals in Uganda

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Abstract

Background *Plasmodium falciparum* (*P. falciparum*) remains a significant public health challenge globally, especially in sub-Saharan Africa (SSA), where it accounts for 99% of all malaria infections. The outcomes of *P. falciparum* infection vary, ranging from asymptomatic to severe, and are associated with factors such as host immunity, parasite genetic diversity, and multiplicity of infection (MOI). Using seven neutral microsatellite markers, the current study investigated *P. falciparum* genetic diversity and MOI in both asymptomatic and symptomatic malaria individuals in Uganda.

Methods This cross-sectional study analyzed 225 *P. falciparum* isolates from both asymptomatic and symptomatic malaria patients, ranging in age from 6 months to ≥ 18 years. *P. falciparum* genetic diversity, MOI, and multi-locus linkage disequilibrium (LD) were assessed through genotyping of seven neutral microsatellite markers: Poly-α, TA1, TA109, PfPK2, 2490, C2M34–313, and C3M69–383. Genetic data analysis was performed using appropriate genetic analysis software.

Results *P. falciparum* infections exhibited high genetic diversity in both asymptomatic and symptomatic individuals. The mean expected heterozygosity (He) ranged from 0.79 in symptomatic uncomplicated malaria cases to 0.81 in asymptomatic individuals. There was no significant difference ($p = 0.33$) in MOI between individuals with asymptomatic and symptomatic infections, with the mean MOI ranging from 1.92 in symptomatic complicated cases to 2.10 in asymptomatic individuals. Polyclonal infections were prevalent, varying from 58.5% in symptomatic complicated malaria to 63% in symptomatic uncomplicated malaria cases. A significant linkage disequilibrium (LD) was observed between asymptomatic and symptomatic uncomplicated/complicated infections ($p < 0.01$). Genetic differentiation was low, with F_{ST} values ranging from 0.0034 to 0.0105 among *P. falciparum* parasite populations in asymptomatic and symptomatic uncomplicated/complicated infections.

Conclusion There is a high level of *P. falciparum* genetic diversity and MOI among both symptomatic and asymptomatic individuals in Uganda. Asymptomatic carriers harbor a diverse range of parasites, which poses challenges for malaria control and necessitates targeted interventions to develop effective strategies.

Keywords *P. falciparum*, Genetic diversity, Multiplicity of infection, Malaria, Uganda

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Background

Plasmodium falciparum (*P. falciparum*) malaria poses a significant global health challenge. Among the five parasite species causing human malaria, *P. falciparum* is responsible for the greatest burden of morbidity and mortality, particularly in sub-Saharan Africa (SSA), where it accounts for 99% of the cases [1]. *P. falciparum* infection manifests in a range of clinical outcomes, from asymptomatic parasitemia to symptomatic uncomplicated (mild) and complicated (severe) disease [2, 3]. These manifestations are influenced by various factors, including host factors such as age and immunity, transmission intensity, and parasite-related factors such as genetic diversity, multiplicity of infection (MOI), and parasite density [4–6].

P. falciparum possesses a haploid genome spread across 14 chromosomes, totaling 23 megabases (Mb). The genome encodes multigene families, such as var, rif, and stevor genes, which are crucial for invading host red blood cells [7, 8]. These genes encode hypervariable antigens and virulence factors that are exported to the surface of the infected erythrocyte and are targeted by naturally acquired immunity [8, 9]. They are expressed haplotypically, with only one copy per infection, allowing parasites to maintain fitness through gene switching.

Novel *P. falciparum* genotypes arise from genetic recombination or haplotype switching during the parasite lifecycle in the female Anopheles mosquito vector [10, 11]. In humans, genetically complex infections may occur due to exposure to multiple parasite strains from different mosquito bites (superinfection) or from a single mosquito bite carrying multiple strains (co-transmission) [12, 13]. Multiple *P. falciparum* genotypes result in a higher MOI [14] and may lead to an increased release of proinflammatory cytokines such as IL-6 and IL-8, contributing to the development of severe malaria [15, 16]. Conversely, immunity to a specific parasite clone can protect against clinical malaria caused by that clone [17]. For example, immunity to *P. falciparum* var gene-encoded *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is thought to protect individuals against severe malaria infections [18, 19]. Increased host immunity to infections genetically similar to previous infections may protect against symptomatic malaria [20]. Conversely, *P. falciparum* infection following recovery from previous polygenic infections is less likely to progress to clinical disease [21, 22], whereas infection with new parasite strains increases the risk of symptomatic infections [23] and may favor gametocyte development [24, 25], enhancing parasite transmissibility [26].

The evaluation of *P. falciparum* genetic diversity and MOI in resource-limited settings typically relies on targeted genotyping of antigen-coding loci such as *msp-1*, *msp-2*, and *glurp* [27, 28]. Although genotyping these antigen-coding loci is readily available, these markers are subject to immune selection [29]. Advanced approaches, such as targeted deep sequencing, are sensitive methods for assessing parasite diversity but remain inaccessible for most laboratories in SSA due to their high development and maintenance costs [30]. Microsatellite markers, considered selection-neutral and not targets for immune evasion, provide an unbiased view of parasite genetic diversity [31]. Neutral polymorphic microsatellites, including Poly-α, TA1, TA109, and PfPK2, offer valuable and cost-effective tools for evaluating *P. falciparum* genetic diversity [32–34]. In the *P. falciparum* genome, these microsatellite loci are abundant, existing as [TA]n, [T]n, and [TAA]n repeats [35]. Their high variability allows them to distinguish between different parasite strains, which is crucial for assessing MOI, an indicator of the number of distinct strains infecting a host. Additionally, their broad genomic distribution provides a comprehensive understanding of genetic diversity across different regions of the genome.

In areas with low malaria transmission, parasite strains exhibit strong linkage disequilibrium (LD), low genetic diversity, and significant population differentiation. Conversely, in high transmission areas, parasite strains show weak LD, high genetic diversity, and minimal population differentiation [33]. Analyzing the *P. falciparum* genetic profile associated with malaria infection enhances understanding of infection severity, risk for antimalarial resistance, and potential effects on treatment outcomes [36, 37]. The genetic diversity and MOI of *P. falciparum* are correlated with malaria severity and are greater in symptomatic infections [38–40] than in asymptomatic infections [41]. However, other studies have reported high genetic diversity and MOI in both asymptomatic and symptomatic malaria infections [23, 42–44], although some have found no significant differences [45]. The diversity of *P. falciparum* in asymptomatic malaria poses challenges for parasite elimination, as asymptomatic infections are often untreated and serve as reservoirs of transmission [46, 47].

In Uganda, studies on *P. falciparum* genetic diversity and MOI have primarily focused on symptomatic children, who often experience high clinical disease rates [34, 48, 49], with little attention given to asymptomatic and adults individuals. This study aimed to evaluate the genetic diversity and MOI of *P. falciparum* infections among both asymptomatic and symptomatic individuals by genotyping seven neutral microsatellite markers.

Methods

Study design and population

This was a cross-sectional study. *P. falciparum* genotyping was conducted using dried blood spot (DBS) filter paper samples collected from participants enrolled in cohort studies under the Program for Resistance, Immunology, and Modeling of Malaria (PRISM) project. Details of these cohort studies are described elsewhere [50]. Briefly, each cohort included participants aged 6 months to ≥ 18 years from 100 randomly selected households in three sub-counties: Walukuba (Jinja district), Kihihi (Kanungu district), and Nagongera (Tororo district) from 2011 to 2016. Although malaria transmission may have changed recently, at the time of the study, Walukuba was a relatively low-transmission peri-urban area near Lake Victoria in the south-central part of the country. Kihihi, on the other hand, is a rural area with moderate transmission intensity, bordering Bwindi Impenetrable National Park in the southwestern part of the country. Nagongera is a rural area with high transmission intensity in the southeastern part of the country, near the border with Kenya (Additional file 1, Figure S1). The DBS samples analyzed in the current study were collected from participants on the day of enrollment for parasite detection.

Participants' demographics and clinical data

Demographic and clinical data, including age, sex, axillary temperature, hemoglobin levels, and parasite density, were extracted from the PRISM cohort database and managed in an Excel spreadsheet. Participants' hemoglobin levels were systematically measured using a portable spectrophotometer (Hemocue).

Laboratory methods

Laboratory assays, including malaria microscopy and parasite genotyping, were conducted at the Molecular Research Laboratory at the Infectious Disease Research Collaboration in Kampala-Uganda.

P. falciparum parasite density determination

Microscopy slides for parasite density and species detection were prepared using a 10% Giemsa solution and stained for 30 min, with thick and thin blood films used, respectively. Experienced microscopists examined the stained slides under a light microscope at 100× oil immersion. Parasite density of *P. falciparum* was assessed 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 [51]. For quality control, each smear was independently read by two microscopists. Discrepancies, defined as differences in species diagnosis, parasite density > 50%, or presence of parasites, prompted a review by a third microscopist. Final parasitemia was determined by averaging the readings of the two microscopists or, in cases of disagreement, by averaging the third microscopist's reading with the closest of the initial two. The third microscopist's reading was used as the final determination for parasite species.

P. falciparum DNA extraction and detection

The genomic DNA of *P. falciparum* was extracted from DBS filter paper samples using Chelex 100 Resin (Sigma–Aldrich, USA) following the method described by Bereczky et al. [52]. Briefly, each filter paper punch (6 mm disc) was incubated overnight at 4 °C in 1 mL of 0.5% saponin in phosphate-buffered saline (PBS). The discs were washed for 30 min in PBS at 4 °C, transferred to new tubes containing 25 µL of stock solution (20% Chelex-100 and 75 µL of distilled water), and vortexed for 30 s. The tubes were then heated at 99 °C for 15 min to elute the DNA, vortexed again, and centrifuged at 10,000×*g* for 2 min. The extracted DNA was stored at − 20 °C until further use. Detection and confirmation of *P. falciparum* were performed through genotyping of *P. falciparum* 18S rRNA using nested PCR [53].

Microsatellite genotyping

Parasite genotyping utilized seven neutral polymorphic microsatellites distributed across six chromosomes of *P. falciparum*: Poly-α on chromosome 4, TA1 and TA109 on chromosome 6, PfPK2 on chromosome 12, 2490 on chromosome 10, C2M34–313 on chromosome 2, and C3M69–383 on chromosome 3, following established methods. The PCR reactions for each marker were conducted in a total volume of 15 µL. Poly-α, TA1, TA109, PfPK2, and 2490 microsatellites were nested, whereas C2M34–313 and C3M69–383 microsatellites were unnested [54, 55]. For the nested PCR reactions, the primary reaction for each marker was carried out in a 15 µL solution 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: 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 sample 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 microsatellites 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), 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 (Additional file 2, Table S2). A 2 µL sample of the PCR product was then electrophoresed on a 2% agarose gel to confirm amplification. 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

An ABI 3730xl (Thermo Fisher/Hitachi) genetic analyzer was used to determine the lengths of the microsatellite fluorescence-labeled PCR products. GeneMarker HID V2.9.5 software was used to score the peaks. For samples producing more than one peak, the highest peak was defined as the dominant allele, while other peaks were defined as minor alleles if their peak heights were > 200 relative fluorescence units (RFU) and > 20% of the highest peak.

Data analysis

The participants' demographic and clinical data, including age, gender, parasite density, and hemoglobin levels, were managed in Excel and exported to STATA version 17.0 (Stata Corp., College Station, TX, USA) for analysis. Statistical comparisons of axillary temperature, hemoglobin levels, and parasite density among asymptomatic, symptomatic uncomplicated, and symptomatic complicated cases were performed using the Kruskal–Wallis test. The statistical significance threshold was set at $p < 0.05$. Microsatellite data were retrieved from the ABI 3730xl genetic analyzer (Thermo Fisher/Hitachi). Subsequent genetic analysis was conducted on a total of 211 samples, where at least five microsatellite markers were successfully amplified. Among these 211 successfully amplified samples, the majority (129, 61%) were polyclonal infections. Therefore, all genetic analyses were conducted using only the predominant alleles to minimize bias associated with analyzing samples with multiple infections.

P. falciparum genetic diversity

Genetic diversity of *P. falciparum*, reflecting variation due to recombination or mutation [56], was assessed by calculating the mean number of alleles (A), the number of effective alleles (Ne), and the mean expected heterozygosity across each locus using GENALEX 6.5 software [57]. Expected heterozygosity (He), defined as the probability that two randomly selected individuals carry distinct alleles at a marker locus, was calculated using the following formula:

$$\mathrm{He} = [\mathrm{n}/(\mathrm{n}-1)]\left[1 - \sum \mathrm{pi}\hat{}2\right],$$

where 'n' is the number of isolates analyzed and 'pi' is the frequency of the ith allele in the population [33].

P. falciparum multiplicity of infection

P. falciparum MOI was defined as the number of distinct parasite genotypes coexisting within a given infection [58]. Isolates with one allele were considered monoclonal infections, whereas those with more than one allele were considered polyclonal [59].

Analysis of multi-locus linkage disequilibrium and genetic differentiation

Multi-locus linkage disequilibrium (LD) measured as the standardized index of association (I_A^S) was calculated using the program LIAN version 3.5 [60] for the whole dataset. This index was calculated using the formula:

$$I_A^S - = (1/\mathrm{n} - 1((\mathrm{VD}/(\mathrm{VE}) - 1),$$

where VE is the expected variance of the nth number of loci for which two individuals differ. VD is the observed variance. The significance of the I_A^S values was tested using the Monte Carlo method. Genetic differentiation was assessed using Wrights fixation index (F_{ST}) calculated using Arlequin 3.11 [61]. The F_{ST} values ranging from 0 to 0.05 indicates low genetic variability, 0.05–0.15 indicates moderate genetic variability, 0.15–0.25 indicated high great genetic differentiation and > 0.25 indicates substantial genetic differentiation [62].

Results

Study population

A total of 225 isolates were genotyped, of which 211 (93.8%) were successfully genotyped on at least five neutral microsatellites and included in the final analysis. Among the successfully genotyped samples, 109 were from males. The age of the participants ranged from 6 months to 53 years, with the majority being less than 11 years old. Parasite density ranged from 16 to 1,600,000 parasites/µL. Significant differences in axillary temperature, parasite density, and hemoglobin levels were detected among asymptomatic, symptomatic uncomplicated, and symptomatic complicated malaria individuals ($p < 0.05$, Kruskal–Wallis test). Symptomatic complicated individuals had the highest parasite density and the lowest hemoglobin levels (Table 1).

Table 1 Demographic and clinical characteristics of the study participants

Characteristic	Asymptomatic malaria infection ($n=70$)	Uncomplicated malaria infection ($n=86$)	Complicated malaria infection ($n=55$)	*P*. value
Age (years): < 5	19	39	36	
5–11	42	40	18	
> 18	9	7	1	
Gender ratio (male/female)	34/36	48/38	27/28	
Axillary temperature, ^{0}c (mean ± SD)	36.8 ± 0.3	38.6 ± 0.9	38.3 ± 1.1	< 0.001
Mean parasite density/μL (mean ± SD)	5862.5 ± 508.4	61,036.4 ± 671.9	446,303.6 ± 9136.5	< 0.001
Mean Hb g/dL (mean ± SD)	11.5 ± 1.6	11.4 ± 1.9	9.8 ± 2.4	< 0.001

P. falciparum genetic diversity among asymptomatic and symptomatic uncomplicated and complicated study participants

The genetic diversity of *P. falciparum*, as indicated by the number of alleles, allele frequencies, number of effective alleles, and expected heterozygosity, was high across both symptomatic and asymptomatic malaria infections. The Kruskal–Wallis test showed no significant differences in the observed number of alleles, allele frequencies, number of effective alleles, and expected heterozygosity between symptomatic and asymptomatic malaria infections ($p > 0.05$). The most polymorphic microsatellite loci were C2M34–313, TA1, and Poly-α, with 17–23, 15–16, and 14–19 distinct alleles, respectively. The least polymorphic microsatellite locus was 2490, with 5 to 6 distinct alleles (Fig. 1 and Table 2).

Expected heterozygosity and number of effective alleles

The mean expected heterozygosity (He) ranged from 0.79 (± 0.17) in individuals with symptomatic uncomplicated malaria to 0.81 (± 0.15) in those with asymptomatic malaria. The number of effective alleles (Ne) ranged from 6.07 (± 3.65) among individuals with complicated malaria to 7.42 (± 5.15) in asymptomatic individuals (Table 2).

P. falciparum MOI among asymptomatic, symptomatic uncomplicated and complicated malaria-infected individuals

The mean MOI was not significantly different ($p = 0.33$) across the various *P. falciparum* infection categories. The mean MOI ranged from 1.92 (± 0.42) in individuals with symptomatic complicated malaria to 2.10 (± 0.36) among asymptomatic malaria individuals (Table 3). The percentages of polyclonal infections were generally consistent across the different *P. falciparum* infection types, ranging from 58.5% (95% CI 44.60–72.39) among individuals with symptomatic uncomplicated malaria to 63% (95% CI 51.22–74.78) among those with complicated malaria.

Linkage disequilibrium (LD) and genetic differentiation

A multi-locus index of association analysis was performed to assess the non-random association of all microsatellite loci in the dataset. The statistical significance of linkage disequilibria (LD) was tested using 10,000 Monte Carlo simulations. Significant multi-locus LD was observed in both symptomatic and asymptomatic malaria infections ($p < 0.01$) (Table 4). The LD ranged from 0.09 in symptomatic uncomplicated malaria to 0.12 in symptomatic complicated malaria infections.

Discussion

Genetically diverse and multiple *P. falciparum* malaria infections occur in both symptomatic and asymptomatic individuals within a population [42, 44]. The presence of diverse *P. falciparum* strains in asymptomatic individuals presents a major challenge to malaria elimination [46]. This is because asymptomatic infections often go undetected by conventional screening programs and remain untreated, persisting for over a year in an individual and thus serving as reservoirs for malaria parasites [47, 63]. Analyzing the genetic profiles of *P. falciparum* provides valuable information on malaria infection outcomes [64], helping to elucidate why some patients develop severe disease while others experience a milder form. It also offers essential parasite characteristics required for designing effective intervention strategies to control or prevent the disease [65].

Compared with other microsatellites, C2M34–313, Poly-α, and TA1 were found to be genetically more diverse, supporting the findings of a previous study by Ishengoma et al. [55] in Tanzania. This suggests that in malaria-endemic countries, C2M34–313, Poly-α, and TA1 may circulate at higher frequencies. The high polymorphism of these microsatellites reflects the high genetic diversity of *P. falciparum* parasites in the population [54, 55].

In this study, *P. falciparum* genetic diversity was high in both asymptomatic and symptomatic malaria-infected individuals, although it was slightly greater in asymptomatic individuals (He = 0.81 ± 0.15; Ne = 7.42 ± 5.15)

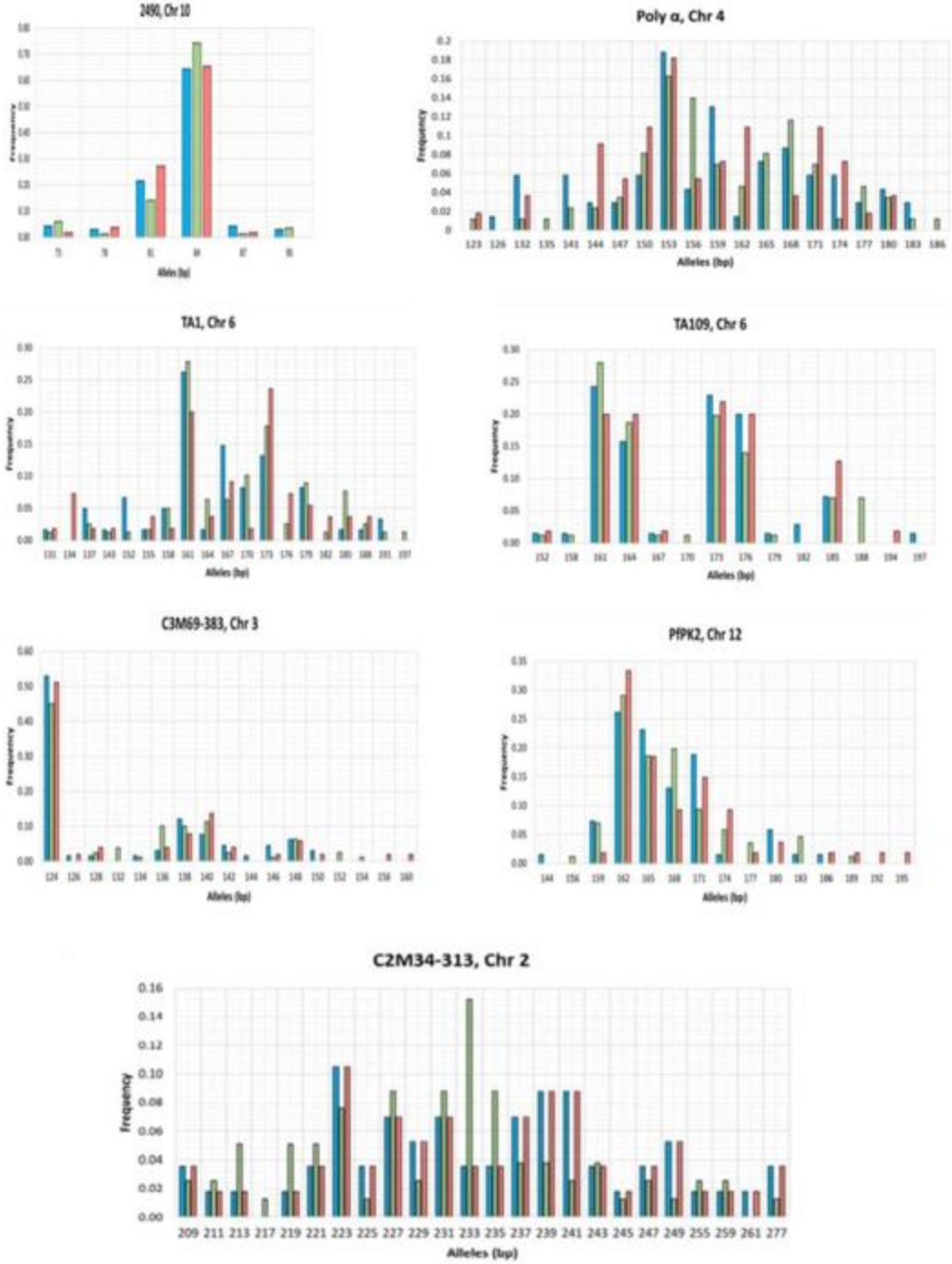


Fig. 1 The allele frequencies of the genotyped markers assessed among individuals with asymptomatic (blue), symptomatic uncomplicated (green) and complicated (red) malaria infections. The vertical axis represents the frequencies of individual alleles, whereas the horizontal axis represents the base pairs of each allele. *Chr* chromosome, *bp* base pair

Table 2 The genetic diversity of *P. falciparum* isolates based on seven neutral microsatellites markers

Locus	Asymptomatic malaria ($n=70$)			Symptomatic uncomplicated malaria ($n=86$)			Symptomatic complicated malaria ($n=55$)		
	Na	Ne	He	Na	Ne	He	Na	Ne	He
2490	6	2.15	0.54	6	1.74	0.43	5	0.14	0.50
Poly α	17	11.15	0.92	19	10.97	0.92	14	10.19	0.92
C2M34–313	23	17.01	0.96	23	14.35	0.94	17	10.09	0.92
TA1	15	7.52	0.88	16	6.96	0.87	16	7.94	0.89
TA109	11	5.47	0.83	11	5.51	0.83	8	5.41	0.83
C3M69–383	12	3.20	0.69	14	4.11	0.77	12	3.39	0.72
PfPK2	10	5.44	0.83	10	5.59	0.83	12	5.32	0.83
Mean	13.43	7.42	0.81	14.14	7.03	0.79	12.29	6.07	0.80
SD	5.50	5.15	0.15	5.76	4.28	0.17	4.28	3.65	0.15

Na = number of different alleles; Ne = number of effective alleles = 1/(∑pi^2); He = unbiased diversity/expected heterozygosity = [n/(n − 1)] [1 − ∑pi^2], and SD = standard deviation. Na, Ne, and He were calculated from the predominant allele dataset. The 95% confidence interval (CI) was calculated as $CI = \bar{x} \pm 1.96(SD/\sqrt{n})$, where $\bar{x}$ is the sample mean, SD is the standard deviation of the sample, and n is the sample size

compared to those with symptomatic uncomplicated malaria (He = 0.79 ± 0.17; Ne = 7.03 ± 4.28) and symptomatic complicated malaria (He = 0.80 ± 0.15; Ne = 6.07 ± 3.65). This finding suggests that asymptomatic malaria patients are likely to present a broader range of parasite genotypes than symptomatic malaria patients. Similar observations of high *P. falciparum* genetic diversity, characterized by high allele frequencies, have been reported in asymptomatic compared with symptomatic malaria individuals in Côte d'Ivoire [66]. Additionally, high *P. falciparum* genetic diversity, as indicated by a mean expected heterozygosity (He) of 0.81 (range: 0.57–0.95), has previously been noted among children with asymptomatic malaria infections in Kenya [43]. Other studies have indicated that symptomatic malaria infections can also harbor highly diverse *P. falciparum* infections [48, 67, 68]. Conversely, some studies have not reported a difference in *P. falciparum* genetic diversity between symptomatic and asymptomatic malaria infections [69, 70].

In symptomatic uncomplicated malaria infections, the high genetic diversity of *P. falciparum* may significantly affect disease severity and contribute to within-host competition among diverse strains [71]. These competitive interactions can lead to the selection of resistant genotypes [72]. The occurrence of high *P. falciparum* genetic diversity in asymptomatic infections may stem from two causes. First, in areas with moderate to high malaria transmission, the development of strain-specific immunity may favor asymptomatic carriers as reservoirs for parasite transmission, allowing the continuous introduction of new strains into the host population and thereby promoting genetic diversity and increased parasite fitness [3]. Second, asymptomatic infections often persist as low-density chronic infections, which can sustain diverse parasite strains within the host [73, 74].

The *P. falciparum* mean MOI did not significantly differ between asymptomatic and symptomatic infections, although it was slightly higher among asymptomatic individuals. This finding aligns with several previous studies reporting no significant differences in mean MOI between symptomatic and asymptomatic malaria individuals [45, 66] or between uncomplicated and complicated malaria cases [64]. Conversely, other studies have reported higher MOIs in asymptomatic malaria individuals [75]. For example, a recent study by Sarah-Matio, Elangwe et al. [42], conducted in a high malaria transmission area in Cameroon, revealed a greater MOI in asymptomatic individuals compared to symptomatic individuals (MOI = 5 in asymptomatic individuals versus median MOI = 2 in symptomatic individuals; $p < 0.001$). However, Simpson et al. [40] reported a contrasting result, with a higher MOI in symptomatic individuals than in asymptomatic malaria-infected individuals (2.24 versus 1.69; 95% CI 0.01–0.72; $p = 0.046$). The high mean MOI in symptomatic *P. falciparum* individuals may be partly due to the fact that symptomatic malaria infections often involve higher parasite densities [76], which can facilitate the presence of multiple strains. Infection with multiple strains in symptomatic individuals can lead to increased severity, complicating clinical management and treatment strategies [49]. Asymptomatic malaria cases often occur in areas with relatively high transmission intensities [77, 78], where multigenotype infections result from exposure to multiple parasite strains from different mosquito bites (superinfection) or from a single mosquito carrying multiple strains (co-transmission)

Table 3 *P. falciparum* MOI in asymptomatic, symptomatic uncomplicated, and symptomatic complicated individuals

Locus	Asymptomatic malaria ($n = 70$)				Uncomplicated malaria ($n = 86$)				Complicated malaria ($n = 55$)			
	Number positive	Percentage monoclonal	Percentage polyclonal	Mean MOI	Number positive	Percentage monoclonal	Percentage polyclonal	Mean MOI	Number positive	Percentage monoclonal	Percentage polyclonal	Mean MOI
2490	70	64.3	35.7	1.5	85	64.7	35.3	1.4	55	63.6	36.4	1.4
Poly a	69	42.0	58.0	2.4	86	43.0	57.0	2.2	55	61.8	38.2	1.6
C2M34–313	57	43.9	56.1	1.7	79	48.1	51.9	1.8	52	50.0	50.0	1.7
TA1	62	29.0	71.0	2.1	79	25.3	74.7	2.3	55	10.9	89.1	2.6
TA109	70	47.1	52.9	2.2	85	38.8	61.2	2.2	55	50.9	49.1	2.0
C3M69–383	66	13.6	86.4	2.5	80	12.5	87.5	2.5	51	23.5	76.5	2.3
PfPK2	69	29.0	71.0	2.2	86	26.7	73.3	2.3	54	29.6	70.4	2.1
Mean		38.4	61.6	2.10		37.0	63.0	2.08		41.5	58.5	1.92
SD		16.24	16.24	0.36		17.19	17.19	0.37		20.26	20.26	0.42

The number positive represents the number of samples successfully amplified by each locus. The percentage of monoclonal infections is the proportion of isolates with only one allele score per locus. The percentage of polyclonal infections is the proportion of isolates with more than one allele score per locus

Table 4 Linkage disequilibrium analysis for *P. falciparum* populations obtained in each infection category

Test factor	Asymptomatic	Symptomatic complicated	Symptomatic uncomplicated
V_O	19.05	19.38	17.28
V_E	4.95	4.46	4.74
I^A_S	0.11	0.12	0.09
Var (V_O)	0.25	0.27	0.18
P. value	< 0.01	< 0.01	< 0.01

The pairwise Wright's fixation index (F_{ST}) between the asymptomatic, symptomatic complicated and uncomplicated malaria infections revealed low genetic differentiation with F_{ST} values ranging between 0.0034 and 0.0105 (Table 5)

V_O variance, V_E expected variance if linkage equilibrium exists, I^A_S standardized index of association

[12, 13]. Additionally, asymptomatic malaria infections may be associated with partial immunity [63, 79], allowing a wider range of parasite strains to establish infection simultaneously. The presence of multiple parasite strains in asymptomatic malaria infections is thought to pose a risk for developing symptomatic malaria [75, 80], in addition to providing a reservoir of genetically diverse parasites [42, 70].

Our study revealed low linkage disequilibrium (LD) values (0.09 to 0.12), suggesting that alleles at different loci within the populations are largely independent. High malaria transmission areas favor high genetic recombination rates, which leads to low LD [81]. Additionally, the study observed similar allele frequencies in both asymptomatic and symptomatic malaria infections, as evidenced by low F_{ST} values ranging from 0.0034 to 0.0105. The low F_{ST} observed in our study is consistent with findings of low *P. falciparum* population genetic differentiation reported in other malaria-endemic countries [82, 83]. This indicates low genetic differentiation and, hence, relatively low overall genetic variation between the parasite populations. These results suggest relatively free gene flow across the study areas. In addition to reducing the occurrence of clinical disease, increased genetic diversity within a parasite population raises the risk of antimalarial drug resistance and could lower the efficacy of malaria vaccines [28, 84, 85].

Significant differences were observed in parasite density and hemoglobin (Hb) levels across individuals with asymptomatic and symptomatic malaria ($p < 0.001$). Symptomatic uncomplicated and complicated malaria patients had higher parasite densities and lower Hb levels compared to asymptomatic malaria patients. Increased *P. falciparum* parasite density has been reported in complicated malaria cases in previous studies [86, 87], whereas low parasitemia has been observed in asymptomatic individuals [88]. However, some studies have not reported significant differences in parasite density between asymptomatic and symptomatic malaria infections [89]. Asymptomatic individuals tend to have partial immunity through repeated exposure, allowing them to control parasite levels without experiencing symptoms [17]. The reduced parasite density among asymptomatic individuals enables them to maintain higher Hb levels compared to symptomatic malaria-infected individuals, where high parasite density leads to increased hemolysis [90]. High parasite density associated with inadequate dosing poses a risk for the development and emergence of antimalarial resistance [91]. Additionally, individuals with asymptomatic malaria often do not seek antimalarial treatment, allowing their infections to persist for extended periods and serve as reservoirs for malaria transmission [92–94]. Thus, malaria control programs should design strategies to eliminate asymptomatic malaria infections.

This current study provides new insights into the genetic diversity of *P. falciparum* among asymptomatic and symptomatic individuals, shedding light on aspects that had not been previously described. The findings highlight the complexity of malaria infections and their implications for disease management and control strategies, including the risk of antimalarial drug resistance. The high genetic diversity and MOI observed may challenge malaria vaccine development by potentially reducing vaccine efficacy, emphasizing the need for region-specific considerations.

However, there are some limitations to this study. Genotyping based on a limited set of microsatellite markers may have led to an overestimation of *P. falciparum* genetic diversity and MOI, as these markers may not fully

Table 5 Pairwise genetic differentiation (F_{ST}) among malaria infection categories

	Asymptomatic	Symptomatic complicated	Symptomatic uncomplicated
Asymptomatic	0	0.0034	0.0059
Symptomatic complicated	0.0034	0	0.0034
Symptomatic uncomplicated	0.0059	0.0105	0

represent the genome-wide genetic diversity. Employing a broader array of genetic markers or whole-genome sequencing could offer a more comprehensive evaluation of genetic diversity. Additionally, the study included relatively few complicated malaria cases, and the majority of participants were children (< 11 years), which may have influenced the accuracy of the assessments of genetic diversity and MOI in this group.

Conclusion

This study found significant genetic diversity among *P. falciparum* parasites associated with different infection categories with asymptomatic individuals exhibiting greater genetic diversity. The high genetic diversity and evolving parasite populations highlight the need for strategies to maximize the effectiveness of malaria vaccines. Future studies should employ a longitudinal study to assess temporal and seasonality changes in *P. falciparum* genetic diversity and MOI.

Abbreviations

DBS	Dried blood spot
Hb	Hemoglobin
He	Expected heterozygosity
LD	Linkage disequilibrium
MOI	Multiplicity of infection
MSP	Merozoite surface protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PfEMP1	*P. falciparum* Erythrocyte membrane protein 1
PRISM	Program for Resistance, Immunology and Modeling of Malaria
RFU	Relative fluorescence units

Supplementary Information

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Additional file 1.

Additional file 2.

Additional file 3.

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Author contributions

AM, SLN, JIN, CK, JNK and PBK conceived the idea, planned, and designed the study. AM, BC, and SK performed the analysis. AM wrote the first draft of the manuscript. MO, SO, EMB, BC, BM and PB revised the manuscript. AM wrote the final draft of the manuscript.

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Availability of data and materials

The detailed datasets used during the current study are available from the corresponding author upon request. Genotyping raw data are provided in Additional File 3 (Table S3).

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 that they have no competing interests.

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References

1. WHO. World malaria report 2022. Geneva: World Health Organization; 2022.
2. Bartoloni A, Zammarchi L. Clinical aspects of uncomplicated and severe malaria. Mediterr J Hematol Infect Dis. 2012;4(1): e2012026.
3. Laishram DD, Sutton PL, Nanda N, Sharma VL, Sobti RC, Carlton JM, et al. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malar J. 2012;11:1–15.
4. Bendixen M, Msangeni H, Pedersen B, Shayo D, Bedker R. Diversity of *Plasmodium falciparum* populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. Trans R Soc Trop Med Hyg. 2001;95(2):143–8.
5. Gray K-A, Dowd S, Bain L, Bobogare A, Wini L, Shanks GD, et al. Population genetics of *Plasmodium falciparum* and *Plasmodium vivax* and asymptomatic malaria in Temotu Province Solomon Islands. Malar J. 2013;12:1–10.
6. Mahdi Abdel Hamid M, Elamin AF, Albsheer MMA, Abdalla AA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria in Gezira State Sudan. Parasit Vectors. 2016;9:1–8.
7. 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(4): e00019-19.
8. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature. 2002;419(6906):498–511.
9. Scherf A, Lopez-Rubio JJ, Riviere L. Antigenic variation in *Plasmodium falciparum*. Annu Rev Microbiol. 2008;62(1):445–70.

10. Lapp Z, Obala AA, Abel L, Rasmussen DA, Sumner KM, Freedman E, et al. *Plasmodium falciparum* genetic diversity in coincident human and mosquito hosts. MBio. 2022;13(5):e02277-e2322.
11. Li X, Kumar S, McDew-White M, Haile M, Cheeseman IH, Emrich S, et al. Genetic mapping of fitness determinants across the malaria parasite *Plasmodium falciparum* life cycle. PLoS Genet. 2019;15(10): e1008453.
12. Alizon S. Parasite co-transmission and the evolutionary epidemiology of virulence. Evolution. 2013;67(4):921–33.
13. 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;9:1–12.
14. 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(7):1121–7.
15. Mandala WL, Msefula CL, Gondwe EN, Drayson MT, Molyneux ME, MacLennan CA. Cytokine profiles in Malawian children presenting with uncomplicated malaria, severe malarial anemia, and cerebral malaria. Clin Vaccine Immunol. 2017;24(4):e00533-e616.
16. Popa GL, Popa MI. Recent advances in understanding the inflammatory response in malaria: a review of the dual role of cytokines. J Immunol Res. 2021;2021:7785180.
17. Kimenyi KM, Wamae K, Ochola-Oyier LI. Understanding *P. falciparum* asymptomatic infections: a proposition for a transcriptomic approach. Front Immunol. 2019;10:2398.
18. Cham GK, Turner L, Kurtis JD, Mutabingwa T, Fried M, Jensen AT, et al. Hierarchical, domain type-specific acquisition of antibodies to *Plasmodium falciparum* erythrocyte membrane protein 1 in Tanzanian children. Infect Immun. 2010;78(11):4653–9.
19. Su X-Z, Zhang C, Joy DA. Host–malaria parasite interactions and impacts on mutual evolution. Front Cell Infect Microbiol. 2020;10:587933.
20. Buchwald AG, Sixpence A, Chimenya M, Damson M, Sorkin JD, Wilson ML, et al. Clinical implications of asymptomatic *Plasmodium falciparum* infections in Malawi. Clin Infect Dis. 2019;68(1):106–12.
21. 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(4):608–16.
22. Liljander A, Bejon P, Mwacharo J, Kai O, Ogada E, Peshu N, et al. Clearance of asymptomatic *P. falciparum* infections interacts with the number of clones to predict the risk of subsequent malaria in Kenyan children. PLoS ONE. 2011;6(2): e16940.
23. Sumner KM, Freedman E, Mangeni JN, Obala AA, Abel L, Edwards JK, et al. Exposure to diverse *Plasmodium falciparum* genotypes shapes the risk of symptomatic malaria in incident and persistent infections: a longitudinal molecular epidemiologic study in Kenya. Clin Infect Dis. 2021;73(7):1176–84.
24. Lamptey H, Ofori MF, Kusi KA, Adu B, Owusu-Yeboa E, Kyei-Baafour E, et al. The prevalence of submicroscopic *Plasmodium falciparum* gametocyte carriage and multiplicity of infection in children, pregnant women and adults in a low malaria transmission area in Southern Ghana. Malar J. 2018;17:1–12.
25. Nassir E, Abdel-Muhsin A-MA, Suliaman S, Kenyon F, Kheir A, Geha H, et al. Impact of genetic complexity on longevity and gametocytogenesis of *Plasmodium falciparum* during the dry and transmission-free season of eastern Sudan. Int J Parasitol. 2005;35(1):49–55.
26. Looareesuwan P, Krudsood S, Lawpoolsri S, Tangpukdee N, Matsee W, Nguitragool W, et al. Gametocyte prevalence and risk factors of *P. falciparum* malaria patients admitted at the Hospital for Tropical Diseases, Thailand: a 20-year retrospective study. Malar J. 2023;22(1):321.
27. Funwei RI, Thomas BN, Falade CO, Ojurongbe O. Extensive diversity in the allelic frequency of *Plasmodium falciparum* merozoite surface proteins and glutamate-rich protein in rural and urban settings of southwestern Nigeria. Malar J. 2018;17:1–8.
28. 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(1):13704.
29. Amambua-Ngwa A, Tetteh KK, Manske M, Gomez-Escobar N, Stewart LB, Deerhake ME, et al. Population genomic scan for candidate signatures of balancing selection to guide antigen characterization in malaria parasites. PLoS Genet. 2012;8(11): e1002992.
30. Apinjoh TO, Ouattara A, Titanji VP, Djimde A, Amambua-Ngwa A. Genetic diversity and drug resistance surveillance of *Plasmodium falciparum* for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? Malar J. 2019;18(1):217.
31. Ghansah A, Tiedje KE, Argyropoulos DC, Onwona CO, Deed SL, Labbé F, et al. Comparison of molecular surveillance methods to assess changes in the population genetics of *Plasmodium falciparum* in high transmission. Front Parasitol. 2023;2:1067966.
32. Carter TE, Malloy H, Existe A, Memnon G, St. Victor Y, Okech BA, et al. Genetic diversity of *Plasmodium falciparum* in Haiti: insights from microsatellite markers. PLoS ONE. 2015;10(10): e0140416.
33. 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(10):1467–82.
34. 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(1):242.
35. Su X-Z, Wellems TE. Toward a high-resolution *Plasmodium falciparum* linkage map: polymorphic markers from hundreds of simple sequence repeats. Genomics. 1996;33(3):430–44.
36. Gosi P, Lanteri CA, Tyner SD, Se Y, Lon C, Spring M, et al. Evaluation of parasite subpopulations and genetic diversity of the *msp1*, *msp2* and *glurp* genes during and following artesunate monotherapy treatment of *Plasmodium falciparum* malaria in Western Cambodia. Malar J. 2013;12(1):1–13.
37. Mohammed H, Hassen K, Assefa A, Mekete K, Tadesse G, Taye G, et al. Genetic diversity of *Plasmodium falciparum* isolates from patients with uncomplicated and severe malaria based on *msp-1* and *msp-2* genes in Gublak North West Ethiopia. Malar J. 2019;18:1–8.
38. Somé AF, Bazié T, Zongo I, Yerbanga RS, Nikiéma F, Neya C, et al. *Plasmodium falciparum msp 1* and *msp 2* genetic diversity and allele frequencies in parasites isolated from symptomatic malaria patients in Bobo-Dioulasso Burkina Faso. Parasit Vectors. 2018;11:1–8.
39. Chen J-T, Li J, Zha G-C, Huang G, Huang Z-X, Xie D-D, et al. Genetic diversity and allele frequencies of *Plasmodium falciparum* msp1 and msp2 in parasite isolates from Bioko Island Equatorial Guinea. Malar J. 2018;17(1):1–9.
40. Simpson SV, Nundu SS, Arima H, Kaneko O, Mita T, Culleton R, et al. The diversity of *Plasmodium falciparum* isolates from asymptomatic and symptomatic school-age children in Kinshasa Province, Democratic Republic of Congo. Malar J. 2023;22(1):102.
41. Agonhossou R, Akoton R, Lagnika H, Djihinto OY, Sovegnon PM, Saizonou HD, et al. *P. falciparum* msp1 and msp2 genetic diversity in *P. falciparum* single and mixed infection with *P. malariae* among the asymptomatic population in Southern Benin. Parasitol Int. 2022;89:102590.
42. Sarah-Matio EM, Guillochon E, Nsango SE, Abate L, Ngou CM, Bouopda GA, et al. Genetic diversity of *Plasmodium falciparum* and distribution of antimalarial drug resistance mutations in symptomatic and asymptomatic infections. Antimicrob Agents Chemother. 2022;66(8): e0018822.
43. 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(1):5924.
44. 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(1):97.
45. Lagnika HO, Moussiliou A, Agonhossou R, Sovegnon P, Djihinto OY, Medjigbodo AA, et al. *Plasmodium falciparum msp1* and *msp2* genetic diversity in parasites isolated from symptomatic and asymptomatic malaria subjects in the South of Benin. Parasitol Res. 2022;121(1):167–75.
46. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. Nat Rev Microbiol. 2014;12(12):833–40.

47. Prusty D, Gupta N, Upadhyay A, Dar A, Naik B, Kumar N, et al. Asymptomatic malaria infection prevailing risks for human health and malaria elimination. Infect Genet Evol. 2021;93:104987.
48. 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(4):1691–700.
49. Kyabayinze DJ, Karamagi C, Kiggundu M, Kamya MR, Wabwire-Mangen F, Kironde F, et al. Multiplicity of *Plasmodium falciparum* infection predicts antimalarial treatment outcome in Ugandan children. Afr Health Sci. 2008;8(4):200–5.
50. 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(5):903–12.
51. WHO. Basic malaria microscopy. Geneva: World Health Organization; 2010.
52. Bereczky S, Martensson A, Gil JP, Farnert A. Rapid DNA extraction from archive blood spots on filter paper for genotyping of *Plasmodium falciparum*. Am J Trop Med Hyg. 2005;72(3):249–51.
53. 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(4):687–92.
54. Anderson T, Su X-Z, Bockarie M, Lagog M, Day K. Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. Parasitol. 1999;119(2):113–25.
55. Ishengoma DS, Mandara CI, Madebe RA, Warsame M, Ngasala B, Kabanywanyi AM, et al. Microsatellites reveal high polymorphism and high potential for use in anti-malarial efficacy studies in areas with different transmission intensities in mainland Tanzania. Malar J. 2024;23(1):79.
56. Meyer CG, May J, Arez AP, Gil JP, Do RV. Genetic diversity of *Plasmodium falciparum*: asexual stages. Trop Med Int Health. 2002;7(5):395–408.
57. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Bioinformatics. 2012;28(19):2537–9.
58. 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;13:1–12.
59. Mohd Abd Razak 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(3): e0152415.
60. Haubold B, Hudson RR. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics. 2000;16(9):847–8.
61. 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.
62. Balloux F, Lugon-Moulin N. The estimation of population differentiation with microsatellite markers. Mol Ecol. 2000;11(2):155–65.
63. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. Expert Rev Anti Infect Ther. 2013;11(6):623–39.
64. Hamid MMA, Elamin AF, Albsheer MMA, Abdalla AA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria in Gezira State Sudan. Parasit Vectors. 2016;9(1):1–8.
65. 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(1):239.
66. Gnagne AP, Konate A, Bedia-Tanoh AV, Amiah-Droh M, Menan HIE, N'Guetta AS-P, et al. Dynamics of *Plasmodium falciparum* genetic diversity among asymptomatic and symptomatic children in three epidemiological areas in Cote d'Ivoire. Pathog Glob Health. 2019;113(3):133–42.
67. Bouyou-Akotet MK, M'Bondoukwé NP, Mawili-Mboumba DP. Genetic polymorphism of merozoite surface protein-1 in *Plasmodium falciparum* isolates from patients with mild to severe malaria in Libreville Gabon. Parasite. 2015;22:12.
68. Metoh TN, Chen J-H, Fon-Gah P, Zhou X, Moyou-Somo R, Zhou X-N. Genetic diversity of *Plasmodium falciparum* and genetic profile in children affected by uncomplicated malaria in Cameroon. Malar J. 2020;19:1–15.
69. Sorontou Y, Pakpahan A. Genetic diversity in MSP-1 gene of *Plasmodium falciparum* and its association with malaria severity, parasite density, and host factors of asymptomatic and symptomatic patients in Papua, Indonesia. Int J Med Sci Public Health. 2015;4(11):1584–93.
70. Kimenyi KM, Wamae K, Ngoi JM, de Laurent ZR, Ndwiga L, Osoti V, et al. Maintenance of high temporal *Plasmodium falciparum* genetic diversity and complexity of infection in asymptomatic and symptomatic infections in Kilifi, Kenya from 2007 to 2018. Malar J. 2022;21(1):192.
71. Sondo P, Derra K, Lefevre T, Diallo-Nakanabo S, Tarnagda Z, Zampa O, et al. Genetically diverse *Plasmodium falciparum* infections, within-host competition and symptomatic malaria in humans. Sci Rep. 2019;9(1):127.
72. Bushman M, Morton L, Duah N, Quashie N, Abuaku B, Koram KA, Dimbu PR, Plucinski M, Gutman J, Lyaruu P, Kachur SP, de Roode JC, Udhayakumar V. Within-host competition and drug resistance in the human malaria parasite *Plasmodium falciparum*. Proc Biol Sci. 1826;2016(283):20153038.
73. Nyarko PB, Claessens A. Understanding host–pathogen–vector interactions with chronic asymptomatic malaria infections. Trends Parasitol. 2021;37(3):195–204.
74. Galatas B, Bassat Q, Mayor A. Malaria parasites in the asymptomatic: looking for the hay in the haystack. Trends Parasitol. 2016;32(4):296–308.
75. Fogang B, Biabi MF, Megnekou R, Maloba FM, Essangui E, Donkeu C, et al. High prevalence of asymptomatic malarial anemia and association with early conversion from asymptomatic to symptomatic infection in a *Plasmodium falciparum* hyperendemic setting in Cameroon. The Am J Trop Med Hyg. 2022;106(1):293.
76. Prah DA, Amoah LE, Gibbins MP, Bediako Y, Cunnington AJ, Awandare GA, et al. Comparison of leucocyte profiles between healthy children and those with asymptomatic and symptomatic *Plasmodium falciparum* infections. Malar J. 2020;19:1–12.
77. Heinemann M, Phillips RO, Vinnemeier CD, Rolling CC, Tannich E, Rolling T. High prevalence of asymptomatic malaria infections in adults, Ashanti Region, Ghana, 2018. Malar J. 2020;19:1–7.
78. Mbah CE, Ambe LA, Ngwewondo A, Kidzeru EB, Akwah L, Mountchissi C, et al. A comparative study of asymptomatic malaria in a forest rural and depleted forest urban setting during a low malaria transmission and COVID-19 pandemic period. Biomed Res Int. 2022;2022:2545830.
79. Mendonça VRd, Barral-Netto M. Immunoregulation in human malaria: the challenge of understanding asymptomatic infection. Mem Inst Oswaldo Cruz. 2015;110(8):945–55.
80. Eldh M, Hammar U, Arnot D, Beck H-P, Garcia A, Liljander A, et al. Multiplicity of asymptomatic *Plasmodium falciparum* infections and risk of clinical malaria: a systematic review and pooled analysis of individual participant data. J Infect Dis. 2020;221(5):775–85.
81. Mobegi VA, Loua KM, Ahouidi AD, Satoguina J, Nwakanma DC, Amambua-Ngwa A, et al. Population genetic structure of Plasmodium falciparum across a region of diverse endemicity in West Africa. Malar J. 2012;11:1–9.
82. 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;15:1–10.
83. 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(1):236.
84. Chen Y-A, Shiu T-J, Tseng L-F, Cheng C-F, Shih W-L, de Assunção Carvalho AV, et al. Dynamic changes in genetic diversity, drug resistance mutations, and treatment outcomes of falciparum malaria from the low-transmission to the pre-elimination phase on the islands of São Tomé and Príncipe. Malar J. 2021;20:1–15.
85. 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(5):417–24.
86. Earland D, Buchwald AG, Sixpence A, Chimenya M, Damson M, Seydel KB, et al. Impact of multiplicity of *Plasmodium falciparum* infection on clinical disease in Malawi. Am J Trop Med Hyg. 2019;101(2):412–5.

87. Tangpukdee N, Krudsood S, Kano S, Wilairatana P. *Falciparum* malaria parasitemia index for predicting severe malaria. Int J Lab Hematol. 2012;34(3):320–7.
88. Harris I, Sharrock WW, Bain LM, Gray K-A, Bobogare A, Boaz L, et al. A large proportion of asymptomatic Plasmodium infections with low and sub-microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in an elimination setting. Malar J. 2010;9:254.
89. Ugwu OC, Onah CE, Ugwu GO, Ameh J, Ogefere HO, Ibeh IN. *Plasmodium falciparum* parasite density in symptomatic and asymptomatic malaria among residents of Abuja Nigeria. J Health Sci Res. 2023;8(2):45–50.
90. White NJ. Anaemia and malaria. Malar J. 2018;17(1):371.
91. White NJ, Pongtavornpinyo W, Maude RJ, Saralamba S, Aguas R, Stepniewska K, et al. Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. Malar J. 2009;8:1–18.
92. Drakeley C, Gonçalves B, Okell L, Slater H. Understanding the importance of asymptomatic and low-density infections for malaria elimination. Towards Malaria Elimination-A Leap Forward. 2018;18:1–20.
93. Tadesse FG, Slater HC, Chali W, Teelen K, Lanke K, Belachew M, et al. The relative contribution of symptomatic and asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections to the infectious reservoir in a low-endemic setting in Ethiopia. Clin Infect Dis. 2018;66(12):1883–91.
94. Kotepui M, Kotepui KU, Masangkay FR, Mahittikorn A, Wilairatana P. Prevalence and proportion estimate of asymptomatic Plasmodium infection in Asia: a systematic review and meta-analysis. Sci Rep. 2023;13(1):10379.

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APPENDIX IV. Publication from study objective 4.

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RESEARCH Open Access

Temporal changes in *Plasmodium falciparum* genetic diversity and multiplicity of infection across three areas of varying malaria transmission intensities in Uganda

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Abstract

Background Malaria is a significant public health challenge in Uganda, with *Plasmodium falciparum* (*P. falciparum*) responsible for most of malaria infections. The high genetic diversity and multiplicity of infection (MOI) associated with *P. falciparum* complicate treatment and prevention efforts. This study investigated temporal changes in *P. falciparum* genetic diversity and MOI across three sites with varying malaria transmission intensities. Understanding these changes is essential for informing effective malaria control strategies for the different malaria transmission settings.

Methods A total of 220 *P. falciparum*-positive dried blood spot (DBS) filter paper samples from participants in a study conducted during 2011–2012 and 2015–2016 were analyzed. Genotyping utilized seven polymorphic markers: Poly-α, TA1, TA109, PfPK2, 2490, C2M34–313, and C3M69–383. Genetic diversity metrics, including the number of alleles and expected heterozygosity, were calculated using GENALEX and ARLEQUIN software. MOI was assessed by counting distinct genotypes. Multi-locus linkage disequilibrium (LD) and genetic differentiation were evaluated using the standardized index of association (I_A^S) and Wright's fixation index (F_{ST}), respectively. Statistical comparisons were made using the Kruskal–Wallis test, and temporal trends were analyzed using the Jonckheere–Terpstra test, with statistical significance set at $p < 0.05$.

Results Of the 220 samples, 180 were successfully amplified. The majority of participants were males (50.6%) and children aged 5–11 years (46.7%). Genetic diversity remained high, with mean expected heterozygosity (H_e) showing a slight decrease over time (range: 0.73–0.82). Polyclonal infections exceeded 50% at all sites, and mean MOI ranged from 1.7 to 2.2, with a significant reduction in Tororo (from 2.2 to 2.0, $p = 0.03$). Linkage disequilibrium showed a slight increase, with Kanungu exhibiting the lowest I_A^S in 2011–2012 (0.0085) and Jinja the highest (0.0239) in 2015–2016. Overall genetic differentiation remained low, with slight increases in pairwise F_{ST} values over time, notably between Jinja and Tororo (from 0.0145 to 0.0353).

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Conclusions This study highlights the genetic diversity and MOI of *P. falciparum* in Uganda's malaria transmission settings, noting a slight decrease in both genetic diversity and MOI overtime. Continued surveillance and targeted control strategies are essential for monitoring the impact of malaria control efforts in Uganda.

Keywords *P. falciparum*, Genetic diversity, Multiplicity of infection, Transmission intensity

Background

Although the malaria burden in Uganda steadily declined in the last two decades [1], malaria remains a significant public health concern [2]. According to the World Health Organization (WHO) Malaria Report 2023, there were 249 million malaria cases recorded globally, with 233 million occurring in sub-Saharan Africa (SSA), contributing to an estimated 580,000 out of 608,000 malaria-related deaths worldwide in 2022 alone. Uganda accounted for 5% of global malaria cases and ranked as the third-highest contributor to malaria cases [3]. More than 90% of these malaria cases are caused by *Plasmodium falciparum* [4], which poses high morbidity and mortality compared to other species [5].

Malaria control is hindered by several factors, including the high genetic diversity of *P. falciparum* parasites, the frequent occurrence of multiplicity of infection (MOI). MOI, which is the presence of multiple genetic variants or genotypes within a single infection [6], complicates efforts to control the disease. It usually occurs in two ways: when an individual is bitten by different mosquitoes carrying unique parasite strains (superinfection), or when a single mosquito transmits multiple distinct genotypes in a single bite (co-transmission) [7, 8]. The genetic diversity of *P. falciparum* arises primarily from genetic recombination during the parasite's lifecycle in the mosquito [9]. Increased genetic recombination leads to low linkage disequilibrium (LD) within parasite populations, meaning that alleles at different loci become more randomly associated. As a result, it becomes less likely for specific genetic variants to be inherited together [10–12].

The presence of diverse and multiple *P. falciparum* strains within an individual enhances parasite virulence and contributes to the pathology of malaria [13, 14]. Parasite diversity also plays a significant role in the development of drug resistance [15], posing major challenges to malaria control and elimination efforts, such as the use of artemisinin-based combination therapies (ACTs) and long-lasting insecticide-treated bed nets (LLINs) [16]. Furthermore, the extensive genetic variability of *P. falciparum* vaccine targets complicates the development of an effective vaccine [17, 18]. Therefore, understanding *P. falciparum* genetic diversity and MOI at the population level, as well as the dynamics of this diversity, is crucial for informing malaria control strategies [19]. In addition, longitudinal analysis of LD offers valuable insights into the temporal changes in the genetic structure of *P. falciparum* parasite populations [20]. Advanced techniques such as whole genome sequencing (WGS) and targeted deep sequencing are sensitive methods for assessing parasite genetic diversity. However, these methods remain costly and inaccessible in many SSA regions [21]. In contrast, microsatellite markers, including Poly-α, TA1, TA109, and PfPK2, offer cost-effective and unbiased alternatives for evaluating *P. falciparum* genetic diversity [10, 11, 22]. These markers, which are neutral polymorphic loci abundant in the *P. falciparum* genome, consist of repeat motifs like [TA]n, [T]n, and [TAA]n [23]. Due to their high variability, they allow for differentiation of parasite strains and the assessment of MOI across various genomic regions.

Previous studies have shown that the genetic diversity of *P. falciparum* varies across individuals, populations, transmission settings, as well as with fluctuations in parasite prevalence [24–27]. This variability makes *P. falciparum* genetic diversity and MOI effective tools for tracking changes in malaria transmission intensity [28–30], and assessing the impact of interventions on malaria transmission patterns [31–33]. In areas with intense malaria transmission, such as Kenya [34] and Senegal [35], high genetic diversity and the presence of multiple parasite genotypes are consistently observed. In these regions, *P. falciparum* parasites are characterized by weak LD, high genetic diversity, and minimal population differentiation, which are key indicators of high malaria transmission intensity [10]. Conversely, parasites circulating in low malaria transmission areas, such as São Tomé, exhibit reduced genetic diversity, strong LD, and significant population differentiation [36]. While these trends reflect the influence of malaria transmission intensity on the genetic structure of *P. falciparum* populations, similar patterns have not always been observed in other low transmission regions [37]. In addition, *P. falciparum* MOI tends to decrease as malaria transmission intensity declines over time [38]. These patterns are particularly relevant for Uganda, where malaria transmission intensity varies considerably across regions with some areas experiencing high transmission, while others have low transmission [39]. Understanding the dynamics of *P. falciparum* genetic

diversity and MOI within Uganda is critical for effective malaria control and elimination strategies.

Despite the importance of genetic diversity and MOI as essential tools for monitoring malaria transmission, there is limited data on the temporal changes in *P. falciparum* genetic diversity and MOI in Uganda, particularly in relation to fluctuating transmission intensities. Most studies have primarily focused on malaria case numbers from high transmission areas [40–42], which may not offer a comprehensive understanding of malaria transmission dynamics over time. In addition, research on *P. falciparum* genetic diversity and MOI in Uganda has mostly been cross-sectional, examining single timepoints [14, 22, 43]. Consequently, there is a gap in knowledge regarding how these factors evolve over time within the country. This study addressed these gaps by investigating the dynamics of *P. falciparum* genetic diversity and MOI using isolates from participants enrolled at three sites with varying transmission intensities, over two distinct time periods: 2011–2012 and 2015–2016. The aim was to better understand how transmission intensity influences parasite genetic diversity and MOI in Uganda.

Methods

Study settings

This study utilized dried blood spot (DBS) samples collected from participants enrolled in the Program for Resistance, Immunology, Surveillance, and Modeling of Malaria (PRISM) study at two distinct timepoints: 2011–2012 and 2015–2016. Participants were recruited from three sub-counties with varying malaria transmission intensities, determined by the number of malaria cases: Walukuba in Jinja District, Kihihi in Kanungu District, and Nagongera in Tororo District. Walukuba is a relatively low-transmission, peri-urban area near Lake Victoria in the south-central region of Uganda. Kihihi, a rural area near Bwindi Impenetrable National Park in the southwest, has moderate transmission intensity. In contrast, Nagongera, a rural area near the border with Kenya in the southeast, experiences high transmission intensity (Fig. 1). A total of 220 DBS samples were retrieved, with the distribution across study sites and time periods as follows: 40 samples from Walukuba (Jinja District), 35 from Kihihi (Kanungu District), and 33 from Nagongera (Tororo District) during the 2011-2012 study period. In the 2015–2016 period, 35 samples were collected from Walukuba, 39 from Kihihi, and 38 from Nagongera.

Study population

Participants were enrolled in the PRISM study at two distinct timepoints: from August 2011 to December 2012 and from July 2015 to December 2016. Detailed information about the study population has been described previously [39]. Briefly, all households in the three sub-counties were enumerated and mapped, and 100 households were randomly selected for participation. Children aged 0.5–10 years, along with one primary adult caregiver from each household, were enrolled. This participant recruitment approach was designed to provide a comprehensive understanding of factors influencing malaria infection and transmission patterns across different age groups within households. Participants were encouraged to visit a clinic open 7 days a week for medical care. Routine visits were conducted every 3 months, during which standardized evaluations were performed. Blood samples were collected by finger prick for thick blood smears, hemoglobin measurements, and DBS filter paper samples for future molecular studies. At each visit, participants with a fever (tympanic temperature > 38.0 °C) or a history of fever within the previous 24 h had a thick blood smear read immediately. If the smear was positive, the patient was diagnosed with malaria and treated according to national guidelines.

Laboratory methods

Laboratory assays, including malaria microscopy and parasite genotyping, were conducted at the Molecular Biology Research Laboratory at the Infectious Diseases Research Collaboration (IDRC) in Kamapala-Uganda.

Determination of parasite density

Microscopy slides for parasite density and species detection were prepared using a 10% Giemsa solution and stained for 30 minutes, 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 8,000 leukocytes per µL of blood [44]. 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 microscopist. 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. Final parasitemia 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,

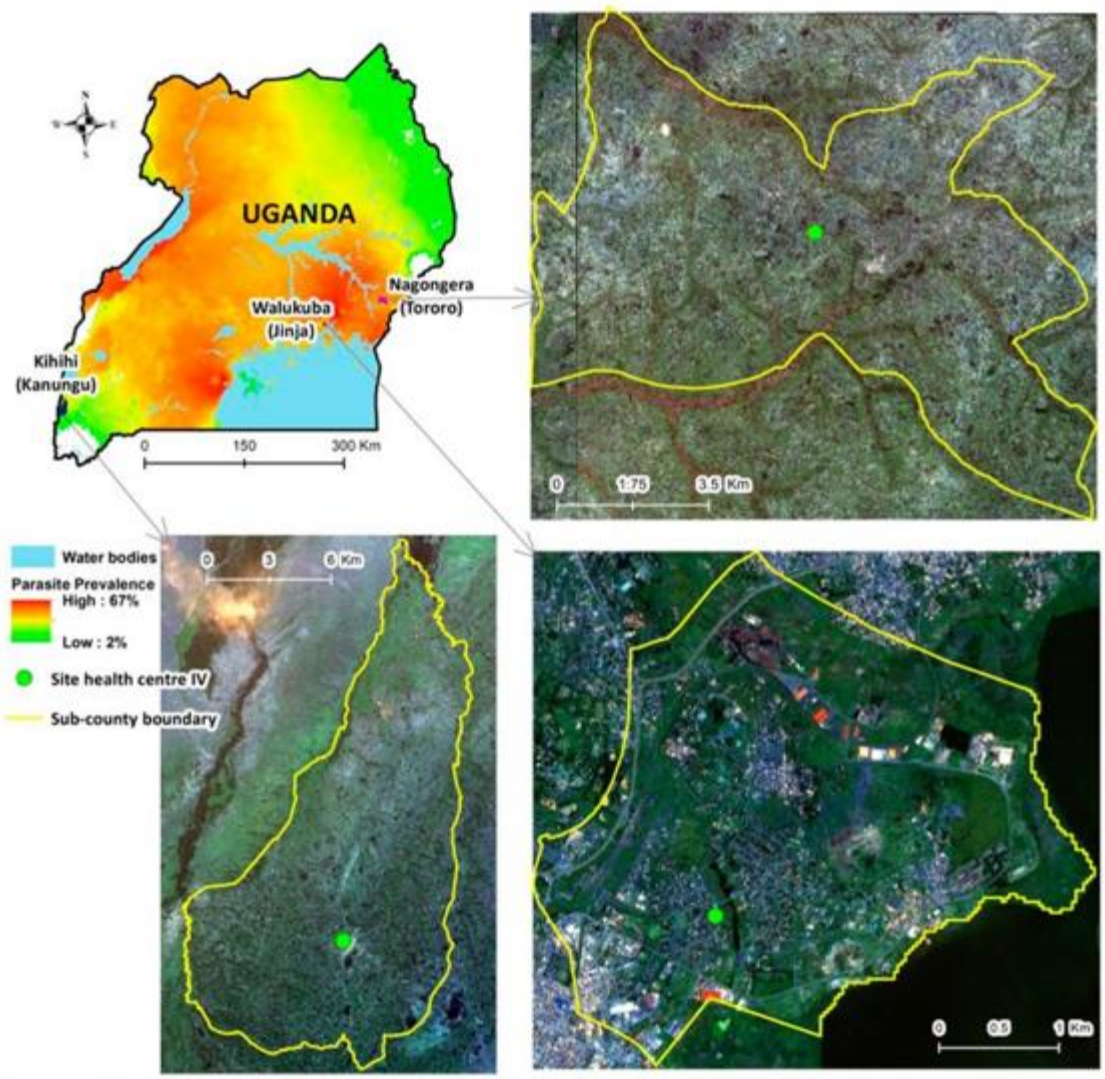


Fig. 1 Map of Uganda showing the malaria endemicity of the study sites at the time of sample collection. Adapted from Kamya et al. [39]

it was used as the final determination for parasite density and or species.

Selection of the samples for molecular analysis

A stratified random sampling approach was employed to ensure that the molecular analysis included participants from diverse demographic and clinical groups, reflecting the broader study population. A total of 220 DBS filter paper samples were selected from two study periods: 2011–2012 and 2015–2016. The selection criteria included availability of DBS filter paper sample, *P. falciparum* mono-infection positivity, and sufficient demographic and clinical data for each participant. To achieve a representative sample, the selection was stratified based on key factors, including age, gender, geographical location, and study period. The selected DBS filter paper samples were then linked to participants' demographic and clinical information via cohort ID, as well as the date and year of sample collection.

DNA extraction

Genomic DNA of *P. falciparum* was extracted from dried blood spots (DBS) using Chelex 100 Resin (Sigma-Aldrich, USA), following the method described by Musapa et al.[45]. 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. Detection and confirmation of *P. falciparum* was performed through genotyping of *P. falciparum* 18S rRNA using nested PCR [46]. 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

A panel of seven neutral polymorphic microsatellites of *P. falciparum* was genotyped, including TA1 (Chr6), Poly-α (Chr4), PfPK2 (Chr12), TA109 (Chr6), 2490 (Chr10), C2M34–313 (Chr2), and C3M69–383 (Chr3). Primers labeled with HEX or 6-FAM were used for genotyping at the Infectious Diseases Research Collaboration (IDRC) Molecular Biology Laboratory in Kampala, Uganda (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, and 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, and 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, and 60 °C for 30 s), then 40 cycles of (94 °C for 30 s, 45 °C for 30 s, and 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 analyzed 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-labeled PCR products were analyzed using an Applied Biosystems ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) to determine their length. The peaks were scored using GeneMarker HID V2.9.5 software. For samples that produced more than one peak, the highest peak was defined as the dominant allele. Additional peaks were classified as minor alleles if their peak heights exceeded 200 relative fluorescence units (RFU) and were > 20% of the height of the dominant peak. This threshold was used to identify minor alleles, which may represent clones present at lower frequencies but still contribute to the genetic diversity of the infection. The identification of these additional minor alleles, including third and fourth alleles, was based on the relative peak heights at each microsatellite locus. Peaks that met these criteria were recorded as distinct alleles.

P. falciparum genetic diversity

The genetic diversity of *P. falciparum*, usually resulting from genetic recombination [47], was assessed in each parasite population from each study site by calculating the mean number of alleles (N_a), and the number of effective alleles (N_e) across each locus. These metrics were calculated from the predominant allele data set using GENALEX 6.5 software [48]. Expected heterozygosity (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:

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

where '*n*' represents the number of isolates analyzed and '*pi*' is the frequency of the i^{th} allele in a given population. H_e values range between 0 (no genetic diversity) and 1 (high genetic diversity) [10]. The mean N_a, N_e, and H_e values for each study site were computed as the mean of the values from each locus.

P. falciparum MOI

P. falciparum MOI was defined as the number of distinct parasite genotypes co-existing within a given infection [50]. Isolates with only one allele were considered monoclonal infections, while those with more than one allele were classified as polyclonal infections [51]. The MOI for each infection was determined by identifying the highest number of alleles observed across any of the microsatellite markers used in the analysis. This maximum allele count was considered the MOI for that particular infection. To assess the parentage of polyclonal infections, we counted the number of isolates with more than one allele for each microsatellite marker that successfully amplified. The results were then summarized across the different study sites and time periods.

Analysis of multi-locus linkage disequilibrium and genetic differentiation

Multi-locus linkage disequilibrium (LD) measured as the standardized index of association (I_A^S) was calculated using the program LIAN version 3.5 [52] for the whole data set. This index was calculated using the formula:

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

where VE is the expected variance of the n^{th} number of loci for which two individuals differ. VD is the observed variance. The significance of the I_A^S values was tested using the Monte Carlo method. Genetic diferentiation was assessed using Wrights fixation index (F_{ST}) calculated using ARLEQUIN software version 3.11 [49]. The F_{ST} values ranging from 0 to 0.05 indicates low genetic variability, 0.05–0.15 indicates moderate genetic variability, 0.15–0.25 indicated high great genetic differentiation and > 0.25 indicates substantial genetic differentiation [53].

Data analysis

Participants' demographic and clinical data, including age, gender, parasite density, and hemoglobin levels, were extracted from the primary PRISM cohort database and exported to STATA version 17 (Stata Corp., College Station, TX, USA) for analysis. These data were summarized using descriptive statistics, such as means and proportions. Microsatellite data were retrieved from the ABI 3730xl Genetic Analyzer. Genetic analysis was performed only on samples, where at least five microsatellite markers were successfully amplified. To minimize bias associated with multiple infections, only the predominant alleles were included in the analysis. Samples with incomplete or poor-quality amplification, or failure to amplify on at least 3 markers, were excluded from further analysis to ensure the accuracy and reliability of the data. Statistical comparisons of *P. falciparum* genetic diversity (including the mean N_a, N_e and H_e) and MOI, as measured by mean MOI and the percentage of polyclonal infections, were performed using Kruskal–Wallis test. Temporal trends in these indices were assessed using the Jonckheere–Terpstra test. Statistical significance was set at $p < 0.05$.

Results

Characteristics of the study population

Of the 220 *P. falciparum* positive samples selected, 180 (81.8%) successfully amplified on at least five microsatellites and were included in the final analysis. Of the 180 samples, 91 (50.6%) were from male participants, and many (46.7%) were from participants aged 5–11 years of age. Overall, the mean parasite densities in the study areas decreased over time. In the 2011–2012 study period, the mean parasite counts were 17,034 parasites/μL in Walukuba Subcounty (Jinja District), 36,118 parasites/μL in Kihihi Subcounty (Kanungu District), and 68,240 parasites/μL in Nongongera Subcounty (Tororo District) (Table 1). By the 2015–2016 period, these counts had declined to 14,882.4 parasites/μL in Walukuba, 20,694 parasites/μL in Kihihi, and 23,586.2 parasites/μL in Nongongera (Table 1). Regarding hemoglobin (Hb) levels among study participants, Walukuba Subcounty experienced an increase from 11.3 to 11.7 g/dL, and Nongongera Subcounty rose from 11.1 to 11.3 g/dL. In contrast, Kihihi Subcounty noted a decrease in mean Hb levels from 10.9 to 10.7 g/dL during the same period (Table 1).

Temporal changes in *P. falciparum* genetic diversity between 2011–2012 and 2015–2016 study periods across sites

Overall, *P. falciparum* genetic diversity remained consistently high across all study sites over time, with mean H_e values consistently exceeding 0.7 (Fig. 2). However, a slight decline in the mean values of N_a, N_e, and H_e was observed across all study sites. Importantly, no significant differences were observed in mean N_a, N_e, or H_e between the study periods, nor were there any significant temporal trends in these indices across the two study periods. These findings were supported by the non-significant results from both the Kruskal–Wallis and

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

Characteristic	Isolates collected 2011–2012			Isolates collected 2015–2016			Overall
	Jinja ($n=38$)	Kanungu ($n=27$)	Tororo ($n=24$)	Jinja ($n=26$)	Kanungu ($n=34$)	Tororo ($n=31$)	
Age in years							
< 5 years	21	14	14	8	12	11	80
5–11 years	11	13	9	15	22	14	84
≥ 18 years	6	0	1	3	0	6	16
Gender (%)							
Male	52.6	55.6	58.3	50	47.1	41.9	50.9
Mean Axillary temperature, ^{0}c (SD)	38.6(0.9)	38.4(1.8)	38.5(0.8)	36.8(0.5)	38.3(1.2)	37.3(0.9)	37(1.2)
Mean Hb g/dL(SD)	11.3(1.9)	10.9(2)	11.1(1.5)	11.7(1.7)	10.7(2.4)	11.3(2.3)	11.1(2.1)
Mean parasite density/μL (SD)	17,034 (2,736.1)	36,118 (4,157.9)	68,240 (8,491)	14,882.4 (1,181)	20,694 (2,197.1)	23,586.2 (1,927)	30,092 (2,750)

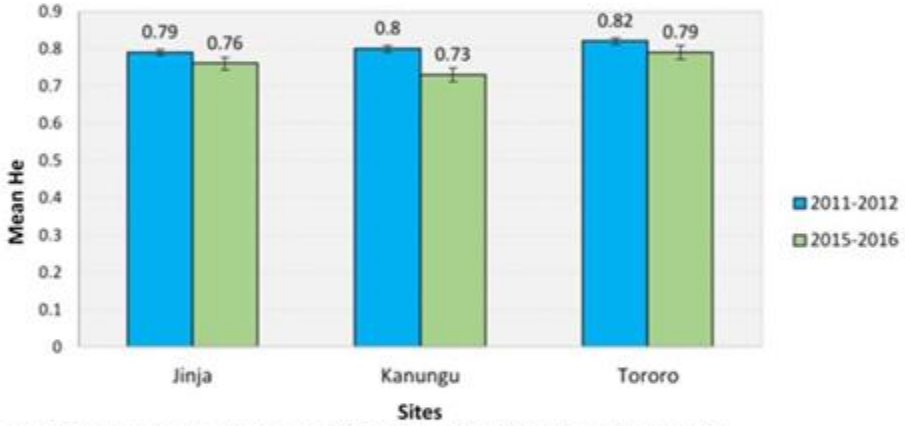


Fig. 2 Changes in *P. falciparum* mean H_e values between 2011–2012 and 2015–2016 study periods across sites

Jonckheere–Terpstra tests ($p>0.05$) (Fig. 2; Additional file 2, Table S2).

Temporal changes in *P. falciparum* MOI between the 2011–2012 and 2015–2016 study periods across sites

Overall, the percentage of *P. falciparum* polyclonal infections in the three study areas remained consistently high, exceeding 50%, which indicates a robust polyclonal parasite population over time. During the study periods, there was a significant temporal decrease in the percentage of polyclonal infections in Kanungu, declining from 56.1 to 52.8% overtime ($p=0.01$). In contrast, Jinja and Tororo showed no significant temporal differences in the percentage of polyclonal infections ($p>0.05$). Regarding *P. falciparum* mean MOI, there was a significant temporal reduction in Tororo, where it decreased from 2.2 to 2.0 overtime ($p=0.03$) (Fig. 3; Additional file 3, Table S3). However, Jinja and Kanungu did not exhibit significant changes in mean MOI over time ($p>0.05$) (Fig. 3).

Temporal changes in *P. falciparum* population multi-locus linkage disequilibrium and genetic differentiation between the 2011–2012 and 2015–2016 study periods across study sites

Temporal changes in linkage disequilibrium (LD) among P. falciparum populations

A multilocus index of association analysis was performed to assess the non-random associations of all microsatellite loci in the data set. The statistical significance of LD was tested using 10,000 Monte Carlo simulations. Overall, there was a slight increase in LD (based on the standardized index of association (I_A^S)) across the study sites over time. During the 2011–2012 period, Kanungu recorded the lowest I_A^S at 0.0085, while Jinja had the highest significant I_A^S value at 0.0233 ($p=0.01$). In the

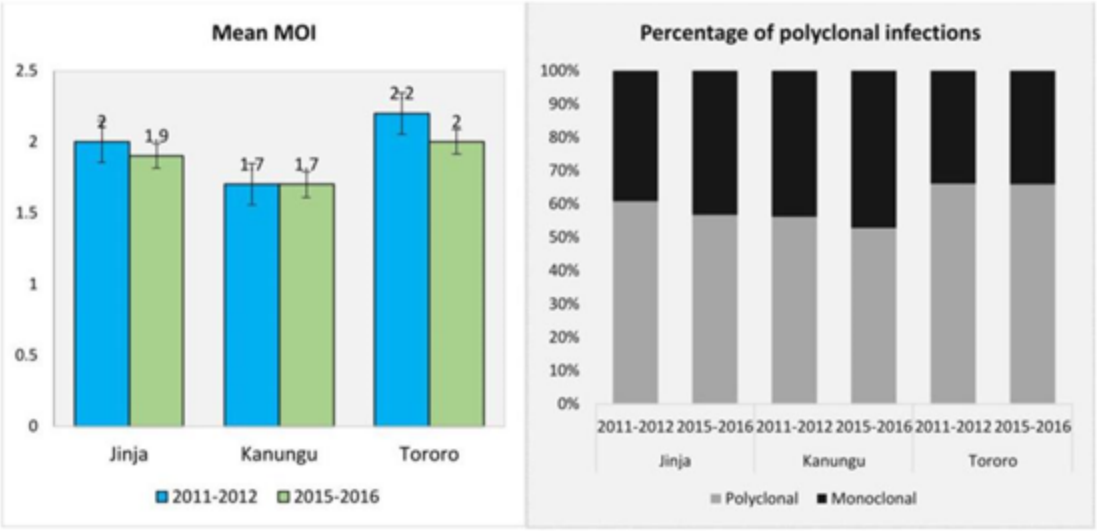


Fig. 3 Changes in *P. falciparum* mean MOI and the percentage of polyclonal infections between 2011–2012 and 2015–2016 study periods across sites

Table 2 Changes in *P. falciparum* parasites' linkage disequilibrium between the 2011–2012 and 2015–2016 study periods across study sites

Site/population	2011–2012		2015–2016	
	I_A^S	*P* value	I_A^S	*P* value
Jinja	0.0233	0.06	0.0239	0.01
Kanungu	0.0085	0.31	0.0114	0.14
Tororo	0.0097	0.26	0.0168	0.18

2015–2016 study period, Kanungu's I_A^S rose to 0.0114, and in Tororo it increased to 0.0168. Jinja also experienced a slight increase in I_A^S, from 0.0233 to 0.0239 (Table 2).

Temporal changes in genetic differentiation of P. falciparum populations

Overall, the genetic differentiation of the *P. falciparum* population among the study sites was low, with a slight increase between the study periods. In the 2011–2012 period, the F_{ST} values were low: 0.0275 between Jinja and Kanungu, and 0.0145 between Jinja and Tororo, with the highest differentiation at 0.0503 between Kanungu and Tororo. In the 2015–2016 study period, F_{ST} values showed modest increases: 0.0384 between Jinja and Kanungu, and 0.0353 between Jinja and Tororo. The highest differentiation remained between Kanungu and Tororo, increasing to 0.0585 (Table 3).

Discussion

P. falciparum genetic diversity and MOI are influenced by several factors, including malaria transmission intensity and the effectiveness of control interventions [31, 54]. To our knowledge, no studies have assessed temporal changes in *P. falciparum* genetic diversity and MOI across different malaria transmission areas in Uganda. This study examined temporal changes in *P. falciparum* genetic diversity and MOI of malaria parasites in regions with varying transmission intensities. The findings revealed slight decreases in both genetic diversity

Table 3 Changes in pairwise genetic differentiation (F_{ST}) between the 2011–2012 and 2015–2016 study periods among study sites

2011–2012				2015–2016			
	Jinja	Kanungu	Tororo		Jinja	Kanungu	Tororo
Jinja	0.0000	0.0275	0.0145	Jinja	0.0000	0.0384	0.0353
Kanungu	0.0275	0.0000	0.0503	Kanungu	0.0384	0.0000	0.0585
Tororo	0.0145	0.0503	0.0000	Tororo	0.0353	0.0585	0.0000

and MOI between 2011–2012 and 2015–2016. This suggests a relatively stable maintenance of high parasite genetic diversity and MOI across the three study areas, underscoring the importance of continued surveillance and monitoring of malaria control efforts.

Overall, the genetic diversity of *P. falciparum* remained generally high, with mean expected heterozygosity (H_e) values exceeding 0.7 across all study areas over time. This likely reflects significant malaria transmission intensity, even in regions like Jinja, which is classified as having low transmission. These findings align with reports from other regions, including Eswatini, where malaria transmission has decreased due to intensive control measures (e.g., insecticide-treated nets, indoor residual spraying, and access to diagnostics and treatment), yet *P. falciparum* genetic diversity remains high due to the importation of malaria parasites from neighboring areas with high transmission [55, 56]. This highlights that even in areas with reduced malaria transmission, regional transmission dynamics and ongoing malaria parasites importation continue to maintain high levels of genetic diversity. Similarly, in Kenya, regions with high malaria transmission intensity still exhibit high *P. falciparum* genetic diversity (mean $H_e \sim 0.78$), despite substantial control interventions, such as insecticide-treated nets, indoor residual spraying [34], and the use of artemisinin-based combination therapies [57], which have been implemented. The persistence of high genetic diversity in both low- and high-malaria transmission settings suggests that gene flow, driven by human migration and cross-border transmission, plays a key role in sustaining genetic diversity. This further supports the idea that malaria transmission intensity is not the sole determinant of *P. falciparum* genetic diversity.

Our findings suggest that *P. falciparum* populations possess substantial capacity to adapt to environmental pressures, such as antimalarial drugs and insecticides [21, 58, 59]. The high genetic diversity, along with low genetic differentiation between study sites, implies that these populations form interconnected reproductive units, likely shaped by ongoing gene flow [60]. This is consistent with a recent study by Arinaitwe et al. [61] which suggested that human mobility is a driver of malaria transmission and the maintenance of large parasite reservoirs in Uganda. Our study also underscores the complexity of inferring malaria transmission intensity from genetic data alone. External factors like human migration and malaria importation from neighboring high-transmission areas complicate the relationship between genetic diversity and local transmission. This is evident in regions like Eswatini and Kenya, where local transmission and regional dynamics influence genetic diversity, demonstrating the importance of considering broader transmission networks when evaluating malaria transmission intensity.

Previous studies have indicated that high *P. falciparum* MOI values are commonly found in regions with intense malaria transmission [62, 63] and are directly linked to transmission intensity [64]. Our study also observed a slight temporal decrease in MOI, with values in Jinja and Tororo dropping from 2.0 to 1.9 and from 2.2 to 2.0, respectively, in line with a gradual decline in transmission intensity in these areas (Table 1). These decreases align with findings from Tororo [65], which indicated reduced transmission following the implementation of vector control measures. Research from Grande Comore Island also demonstrated a decrease in *P. falciparum* mean MOI values—declining from 3.11 to 1.63 for *msp-1* and from 2.75 to 1.35 for *msp-2*—after the introduction of artemisinin-based combination therapy (ACT) [66]. These trends contrast with studies from the Democratic Republic of the Congo (DRC), where MOI increased from 3.78 in to 4.64 [67], and in Kenya, where MOI increased from 1.7 to 3.0 [68] despite ongoing interventions. This suggests that the relationship between transmission intensity and MOI is not uniform and can vary by region and intervention effectiveness. Moreover, our study found that polyclonal infections, constituting more than 50% of infections across the three study areas, were common, in line with observations from mesoendemic and holoendemic regions [68, 69]. However, some studies have reported a decrease in polyclonal infections following control interventions, suggesting that control measures may also influence the genetic structure of the parasite population [66].

The longitudinal analysis of LD provided additional insights into the genetic structure of *P. falciparum* populations. In regions with high transmission intensity, where multiple genetically diverse *P. falciparum* strains circulate, low LD typically reflects ongoing genetic recombination between these diverse strains during the sexual phase of the parasite's lifecycle [70]. Conversely, in areas with lower transmission intensity, lower LD can sometimes reflect the persistence of clonal populations of *P. falciparum*, especially in cases of clonal expansion following a bottleneck [71]. Our study revealed a slight increase in LD between 2011–2012 and 2015–2016, which may reflect reduced genetic recombination, potentially due to a decrease in malaria transmission intensity. This finding aligns with previous studies that noted increased LD in areas with reduced malaria transmission [51]. The significantly high LD values observed in Jinja during 2011–2012 are suggestive of a clonal population structure, which is typical of low-transmission areas [26, 72]. In contrast, study sites like Kanungu and Tororo

exhibited lower LD levels, reflecting the high transmission intensity in these regions.

As malaria transmission dynamics evolve, continuous monitoring of parasite genetic diversity and MOI is crucial for adapting control strategies. Our study offers several strengths. It includes diverse sampling locations with varying malaria transmission intensities, providing a comprehensive comparison of genetic diversity and MOI across different transmission settings. The longitudinal design, spanning 2011–2016, tracks changes in parasite genetics over time, offering valuable insights into the impact of malaria control interventions. In addition, the use of microsatellite genotyping provides detailed insights into *P. falciparum* genetic diversity and population dynamics.

However, there are some limitations to our study. The reliance on neutral microsatellite markers may not fully capture the complexity of *P. falciparum* populations, particularly in the context of evolving control strategies. While microsatellites remain a cost-effective and accessible tool for assessing genetic diversity and MOI, especially in regions like Uganda, where advanced genomic technologies are less accessible, they have certain limitations. Furthermore, challenges such as sample amplification issues, unequal sample sizes across sites and timepoints, and potential selection bias due to participant mobility may have influenced our results. To address these limitations, we recommend future studies to incorporate newer technologies, such as whole-genome sequencing (WGS) or deep amplicon sequencing, and expand recruitment across multiple locations to better capture transmission dynamics and minimize potential biases.

Conclusion

This study provides valuable insights into *P. falciparum* genetic diversity and MOI across Uganda's diverse malaria transmission settings. Despite slight decreases in both genetic diversity and MOI between the 2011–2012 and 2015–2016 study periods, overall genetic diversity remained high, reflecting the parasite's robust transmission dynamics and ability to adapt to environmental pressures. The findings highlight the need for continued surveillance and adaptive malaria control strategies, ensuring that interventions remain effective across regions with varying transmission intensities. By considering regional transmission dynamics, human mobility, and the impact of control measures, future strategies can better target malaria control efforts and address the evolving challenges of malaria management in Uganda.

Abbreviations

ACT	Artemisinin based combination therapy
DBS	Dried blood spot
DRC	Democratic Republic of Congo
Hb	Hemoglobin
H_e	Expected heterozygosity
IDRC	Infectious diseases research collaboration
LD	Linkage disequilibrium
LLIN	Long-lasting insecticide-treated bed nets
MOI	Multiplicity of infection
MSP	Merozoite surface protein
N_a	Number of alleles
N_e	Number of effective alleles
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRISM	Program for resistance, immunology and modeling of malaria
RFU	Relative fluorescence units
SSA	Sub-Saharan Africa
WGS	Whole genome sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41182-024-00672-7.

Supplementary material 1.

Supplementary material 2.

Supplementary material 3.

Supplementary material 4.

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Author contributions

AM.SLN. JIN.CK.JNK and PBK conceived the idea, planned, and designed the study. AM. BM performed the laboratory assays AM, and HK performed the analysis AM wrote the first draft of the manuscript SMK. JDK. AB. IMM. JIN and IAA revised the manuscript All authors reviewed and agreed to the content of the manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

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.

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References

1. Kigozi SP, Kigozi RN, Sebuguzi CM, Cano J, Rutazaana D, Opigo J, et al. Spatial-temporal patterns of malaria incidence in Uganda using HMIS data from 2015 to 2019. BMC Public Health. 2020;20(1):1–14.
2. Nankabirwa JI, Briggs J, Rek J, Arinaitwe E, Nayebare P, Katrak S, et al. Persistent parasitemia despite dramatic reduction in malaria incidence after 3 rounds of indoor residual spraying in Tororo, Uganda J Infect Dis. 2019;219(7):1104–11.
3. WHO. World malaria report 2023. Geneva: World Health Organization; 2023.
4. Asua V, Tukwasibwe S, Conrad M, Walakira A, Nankabirwa JI, Mugenyi L, et al. Plasmodium species infecting children presenting with malaria in Uganda. Am J Trop Med Hyg. 2017;97(3):753–7.
5. WHO. World malaria report 2019. Geneva: World Health Organisation; 2019.
6. 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(7):1121–7.
7. Alizon S. Parasite co-transmission and the evolutionary epidemiology of virulence. Evol. 2013;67(4):921–33.
8. 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(1):5.
9. Mu J, Awadalla P, Duan J, McGee KM, Joy DA, McVean GAT, et al. Recombination hotspots and population structure in *Plasmodium falciparum*. PLoS Biol. 2005;3(10):e335.
10. 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(10):1467–82.
11. Carter TE, Malloy H, Existe A, Memnon G, St. Victor Y, Okech BA, et al. Genetic diversity of *Plasmodium falciparum* in Haiti: insights from microsatellite markers. PLoS One. 2015;10(10):e0140416.
12. Su X, Hayton K, Wellems TE. Genetic linkage and association analyses for trait mapping in *Plasmodium falciparum*. Nat Rev Genet. 2007;8(7):497–506.
13. 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(1):230–52.
14. 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(4):1691–700.
15. 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(10):1692–701.
16. Ghosh SK, Ghosh C. New challenges in malaria elimination. In: Rodriguez-Morales AJ, editor. Current topics and emerging issues in malaria elimination. London: Intechopen; 2021. p. 133.
17. Healer J, Murphy V, Hodder AN, Masciantonio R, Gemmill AW, Anders RF, et al. Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. Mol Microbiol. 2004;52(1):159–68.
18. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A. A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1–2b trial in Papua New Guinea. J Infect Dis. 2002;185(6):820–7.
19. 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;20:1–13.
20. Chenet SM, Taylor JE, Blair S, Zuluaga L, Escalante AA. Longitudinal analysis of Plasmodium falciparum genetic variation in Turbo, Colombia: implications for malaria control and elimination. Malar J. 2015;14:363.
21. Apinjoh TO, Ouattara A, Titanji VP, Djimde A, Amambua-Ngwa A. Genetic diversity and drug resistance surveillance of Plasmodium falciparum for malaria elimination: is there an ideal tool for resource-limited sub-Saharan Africa? Malar J. 2019;18(1):217.
22. 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(1):242.
23. Su X-z, Wellems TE. Toward a high-resolution *Plasmodium falciparum* Linkage map: polymorphic markers from hundreds of simple sequence repeats. Genomics. 1996;33(3):430–44.
24. Abukari Z, Okonu R, Nyarko SB, Lo AC, Dieng CC, Salifu SP, et al. The diversity, multiplicity of infection and population structure of *P. falciparum* parasites circulating in asymptomatic carriers living in high and low malaria transmission settings of Ghana. Genes. 2019;10(6):434.
25. 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;18:1–10.
26. 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;8:1–11.
27. Childs LM, Prosper OF. Simulating within-vector generation of the malaria parasite diversity. PLoS ONE. 2017;12(5):e0177941.
28. Neafsey DE, Volkman SK. Malaria genomics in the era of eradication. Cold Spring Harb Perspect Med. 2017;7(8):a025544.
29. Pringle JC, Tessema S, Wesolowski A, Chen A, Murphy M, Carpi G, et al. Genetic evidence of focal *Plasmodium falciparum* transmission in a pre-elimination setting in Southern Province, Zambia. J Infect Dis. 2019;219(8):1254–63.
30. Auburn S, Barry AE. Dissecting malaria biology and epidemiology using population genetics and genomics. Int J Parasitol. 2017;47(2–3):77–85.
31. Daniels R, Chang H-H, Séne PD, Park DC, Neafsey DE, Schaffner SF, et al. Genetic surveillance detects both clonal and epidemic transmission of malaria following enhanced intervention in Senegal. PLoS ONE. 2013;8(4):e60780.
32. Daniels RF, Schaffner SF, Wenger EA, Proctor JL, Chang H-H, Wong W, et al. Modeling malaria genomics reveals transmission decline and rebound in Senegal. Proc Natl Acad Sci U S A. 2015;112(22):7067–72.
33. Escalante AA, Ferreira MU, Vinetz JM, Volkman SK, Cui L, Gamboa D, et al. Malaria molecular epidemiology: lessons from the International Centers of Excellence for Malaria Research Network. Am J Trop Med Hyg. 2015;93(3 Suppl):79–86.
34. Gatei W, Gimnig JE, Hawley W, Ter Kuile F, Odero C, Iriemenam NC, et al. Genetic diversity of *Plasmodium falciparum* parasite by microsatellite markers after scale-up of insecticide-treated bed nets in western Kenya. Malar J. 2015;14:1–12.
35. Niang M, Thiam LG, Loucoubar C, Sow A, Sadio BD, Diallo M, et al. Spatiotemporal analysis of the genetic diversity and complexity of *Plasmodium falciparum* infections in Kedougou, southeastern Senegal. Parasit Vectors. 2017;10(1):33.
36. Chen Y-A, Shiu T-J, Tseng L-F, Cheng C-F, Shih W-L, de Assunção Carvalho AV, et al. Dynamic changes in genetic diversity, drug resistance mutations, and treatment outcomes of falciparum malaria from the low-transmission to the pre-elimination phase on the islands of São Tomé and Príncipe. Malar J. 2021;20:1–15.
37. Branch OH, Sutton PL, Barnes C, Castro JC, Hussin J, Awadalla P, et al. *Plasmodium falciparum* genetic diversity maintained and amplified over 5 years of a low transmission endemic in the Peruvian Amazon. Mol Biol Evol. 2011;28(7):1973–86.

38. Niang M, Thiam LG, Loucoubar C, Sow A, Sadio BD, Diallo M, et al. Spatio-temporal analysis of the genetic diversity and complexity of *Plasmodium falciparum* infections in Kedougou, southeastern Senegal. Parasit Vectors. 2017;10(1):1–9.
39. 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(5):903–12.
40. Kigozi SP, Kigozi RN, Sebuguzi CM, Cano J, Rutazaana D, Opigo J, et al. Spatial-temporal patterns of malaria incidence in Uganda using HMIS data from 2015 to 2019. BMC Public Health. 2020;20:1–14.
41. Nankabirwa JI, Arinaitwe E, Rek J, Kilama M, Kizza T, Staedke SG, et al. Malaria transmission, infection, and disease following sustained indoor residual spraying of insecticide in Tororo, Uganda. Am J Trop Med Hyg. 2020;103(4):1525.
42. Siya A, Kalule BJ, Ssentongo B, Lukwa AT, Egeru A. Malaria patterns across altitudinal zones of Mount Elgon following intensified control and prevention programs in Uganda. BMC Infect Dis. 2020;20:1–16.
43. Kyabayinze DJ, Karamagi C, Kiggundu M, Kamya MR, Wabwire-Mangen F, Kironde F, et al. Multiplicity of *Plasmodium falciparum* infection predicts antimalarial treatment outcome in Ugandan children. Afri Health Sc. 2008;8(4):200–5.
44. WHO. Basic malaria microscopy. Geneva: World Health Organization; 2010.
45. 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.
46. 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(4):687–92.
47. Miles A, Iqbal Z, Vauterin P, Pearson R, Campino S, Theron M, et al. Indels, structural variation, and recombination drive genomic diversity in *Plasmodium falciparum*. Genome Res. 2016;26(9):1288–99.
48. Peakall R, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Bioinformatics. 2012;28(19):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. 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;13:1–12.
51. Mohd Abd Razak 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(3):e0152415.
52. Haubold B, Hudson RR. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics. 2000;16(9):847–9.
53. Balloux F, Lugon-Moulin N. The estimation of population differentiation with microsatellite markers. Mol Ecol. 2002;11(2):155–65.
54. Karl S, White MT, Milne GJ, Gurarie D, Hay SI, Barry AE, et al. Spatial effects on the multiplicity of *Plasmodium falciparum* infections. PLoS ONE. 2016;11(10):e0164054.
55. 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(8):1346–54.
56. Nkya TE, Fillinger U, Dlamini M, Sangoro OP, Marubu R, Zulu Z, et al. Malaria in Eswatini, 2012–2019: a case study of the elimination effort. Malar J. 2021;20:1–13.
57. Musuva A, Ejersa W, Kiptui R, Memusi D, Abwao E. The malaria testing and treatment landscape in Kenya: results from a nationally representative survey among the public and private sector in 2016. Malar J. 2017;16:1–13.
58. Lefevre T, Ohm J, Dabiré KR, Cohuet A, Choisy M, Thomas MB, et al. Transmission traits of malaria parasites within the mosquito: genetic variation, phenotypic plasticity, and consequences for control. Evol Appl. 2017;11(4):456–69.
59. Vardo-Zalik AM, Zhou G, Zhong D, Afrane YA, Githeko AK, Yan G. Alterations in *Plasmodium falciparum* genetic structure two years after increased malaria control efforts in western Kenya. Am J Trop Med Hyg. 2013;88(1):29.
60. 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(1):236.
61. Arinaitwe E, Dorsey G, Nankabirwa JI, Kigozi SP, Katureebe A, Kakande E, et al. Association between recent overnight travel and risk of malaria: a prospective cohort study at 3 sites in Uganda. Clin Infect Dis. 2019;68(2):313–20.
62. 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;9:1–6.
63. Akoniyon OP, Akiibinu M, Adeleke MA, Maharaj R, Okpeku M. A comparative study of genetic diversity and multiplicity of infection in uncomplicated *Plasmodium falciparum* infections in selected regions of pre-elimination and high transmission settings using MSP1 and MSP2 genes. Pathogens. 2024;13(2):172.
64. Agyeman-Budu A, Brown C, Adjei G, Adams M, Dosoo D, Dery D, et al. Trends in multiplicity of *Plasmodium falciparum* infections among asymptomatic residents in the middle belt of Ghana. Malar J. 2013;12:1–6.
65. Oguttu DW, Matovu JK, Okumu DC, Ario AR, Okullo AE, Opigo J, et al. Rapid reduction of malaria following introduction of vector control interventions in Tororo District, Uganda: a descriptive study. Malar J. 2017;16:1–8.
66. Huang B, Tuo F, Liang Y, Wu W, Wu G, Huang S, et al. Temporal changes in genetic diversity of *msp-1*, *msp-2*, and *msp-3* in *Plasmodium falciparum* isolates from Grande Comore Island after introduction of ACT. Malar J. 2018;17:1–14.
67. Pringle JC, Wesolowski A, Berube S, Kobayashi T, Gebhardt ME, Mulenga M, et al. High *Plasmodium falciparum* genetic diversity and temporal stability despite control efforts in high transmission settings along the international border between Zambia and the Democratic Republic of the Congo. Malar J. 2019;18:1–13.
68. Kimenyi KM, Wamae K, Ngoi JM, de Laurent ZR, Ndwiga L, Osoti V, et al. Maintenance of high temporal *Plasmodium falciparum* genetic diversity and complexity of infection in asymptomatic and symptomatic infections in Kilifi, Kenya from 2007 to 2018. Malar J. 2022;21(1):192.
69. Yakubu B, Longdet IY, Horsefield T, Davou DT, Obishakin E. High-complexity *Plasmodium falciparum* infections, north Central Nigeria, 2015–2018. Emerg Infect Dis. 2019;25(7):1330–8.
70. 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(5):1077.
71. Griffing SM, Mixson-Hayden T, Sridaran S, Alam MT, McCollum AM, Cabezas C, et al. South American *Plasmodium falciparum* after the malaria eradication era: clonal population expansion and survival of the fittest hybrids. PLoS ONE. 2011;6(9):e23486.
72. Nkhoma SC, Nair S, Al-Saai S, Ashley E, McGready R, Phyo AP, et al. Population genetic correlates of declining transmission in a human pathogen. Mol Ecol. 2013;22(2):273–85.

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APPENDIX V. Publication from study objective 5.

scientific reports

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Assessment of different genotyping markers and algorithms for distinguishing *Plasmodium falciparum* recrudescence from reinfection in Uganda

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Antimalarial therapeutic efficacy studies are vital for monitoring drug efficacy in malaria-endemic regions. The WHO recommends genotyping polymorphic markers including *msp-1*, *msp-2*, and *glurp* for distinguishing recrudescences from reinfections. Recently, WHO proposed replacing *glurp* with microsatellites (Poly-α, PfPK2, TA1). However, suitable combinations with *msp-1* and *msp-2*, as well as the performance of different algorithms for classifying recrudescence, have not been systematically assessed. This study investigated various microsatellites alongside *msp-1* and *msp-2* for molecular correction and compared different genotyping algorithms across three sites in Uganda. Microsatellites 313, Poly-α, and 383 exhibited the highest diversity, while PfPK2 and Poly-α revealed elevated multiplicity of infection (MOI) across all sites. The 3/3 match-counting algorithm classified significantly fewer recrudescences than both the ≥2/3 and Bayesian algorithms at probability cutoffs of ≥0.7 and ≥0.8 ($P < 0.05$). The *msp-1*/*msp-2*/2490 combination identified more recrudescences using the ≥2/3 and 3/3 algorithms in the artemether-lumefantrine (AL) treatment arm, while *msp-1*/*msp-2*/*glurp* combination classified more cases of recrudescence using the ≥2/3 in the dihydroartemisinin-piperaquine (DP) arm. Microsatellites PfPK2 and Poly-α, potentially sensitive to detecting minority clones, are promising replacements for *glurp*. Discrepancies in recrudescence classification between match-counting and Bayesian algorithms highlight the need for standardized PCR correction practices.

Keywords *Plasmodium falciparum*, Recrudescence, Reinfection, Microsatellites, *msp-1*, *msp-2*, Antimalarial drug

Malaria remains a major global health challenge, with *Plasmodium falciparum* (*P. falciparum*) responsible for the majority of malaria-related deaths worldwide[1,2]. Antimalarial drug resistance, especially in *P. falciparum*, poses a significant threat to malaria control efforts, making rigorous monitoring of drug efficacy crucial through clinical trials[3,4]. Consequently, malaria-endemic countries are advised to conduct therapeutic efficacy studies (TES) at least every two years[5,6].

A key challenge in TES is distinguishing recrudescence (the recurrence of asexual parasitemia caused by the same genotype(s) that caused the original infection) from reinfection (a new infection after the primary one) in

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patients with recurrent malaria[6–8]. The WHO recommends using genotyping to distinguish recrudescence from reinfection through molecular correction, and suggests changing first-line antimalarial therapy if the molecular-corrected failure rate exceeds 10%[9]. The ability to accurately distinguish recrudescence from reinfection depends on the sensitivity of the genotyping methods, the genetic diversity of the markers used, and the classification algorithms applied[10]. High-throughput methods like amplicon sequencing can overcome the limitations of length-polymorphic markers, such as glutamate-rich protein (*glurp*), which may fail to detect minority clones, resulting in more accurate differentiation between recrudescence and reinfection[11].

The WHO 2008 guidelines recommended the use of three polymorphic genetic markers for molecular correction: merozoite surface protein 1 (*msp-1*), merozoite surface protein 2 (*msp-2*), and *glurp*[12]. However, *glurp* has been found to have limitations in detecting minority clones in polyclonal infections[13]. Recently, the WHO recommended replacing *glurp* with polymorphic microsatellites, including Poly-α, PfPK2, and TA1, to address these limitations[10]. These highly polymorphic markers are identified as cost-effective and unbiased tools for use in TES in malaria-endemic areas[14]. The WHO also recommended the use of a 3/3 match-counting algorithm, which classifies a recurrent malaria infection as recrudescence if one or more alleles are present at all three genotyped markers on both day 0 (the day of the primary infection) and the day of the recurrent infection[10]. Nevertheless, the WHO has emphasized the importance of evaluating additional algorithms, such as the ≥ 2/3 match-counting algorithm, which classifies a recurrent infection as recrudescence if one or more alleles are found in at least two of the three genotyped markers on both day 0 and the recurrent infection day[10]. Another approach is a probabilistic method using Bayesian statistics to quantify the probability that an infection is either a recrudescence or a reinfection[10,15].

The choice of genotyping methods for molecular correction is influenced by malaria transmission intensity. In high-transmission areas, robust microsatellite markers, including Poly-α, TA1, TA109, and PfPK2, are preferred due to their cost-effective sensitivity and ability to detect greater genetic diversity within parasite populations. These markers can provide more accurate results and reduce the need for follow-up studies or interventions[14]. In contrast, regions with lower transmission intensities may benefit from simpler markers like *msp-1*, *msp-2*, and *glurp*[10]. According to the 2018–2019 malaria indicator survey, Uganda experiences perennial, high-intensity malaria transmission, with the highest parasite prevalence rates observed in the Karamoja, West Nile, and Busoga subregions, which have prevalence rates of 34%, 22%, 21%, and 13%, respectively, among children aged 0–59 months[16]. This high transmission intensity significantly influences the selection of genotyping markers, with complex markers such as microsatellites being more suitable for high-transmission settings, where greater parasite genetic diversity is observed.

To our knowledge, microsatellites suitable for molecular correction in combination with *msp-1* and *msp-2* have not been systematically evaluated across Uganda's malaria-endemic regions. Additionally, limited evidence exists regarding the performance of match-counting and Bayesian algorithms in distinguishing recrudescence from reinfection in regions with high *P. falciparum* transmission intensity. Therefore, this study focused specifically on high-transmission settings in Uganda, aiming to identify microsatellite markers that could replace *glurp* and, using various alternative marker combinations, assessed the performance of three algorithms (the 3/3 match-counting, ≥ 2/3 match-counting, and Bayesian approaches) in distinguishing *P. falciparum* recrudescence from reinfection.

Results

Diversity of the genotyped markers across three malaria-endemic sites in Uganda

The genotyping results revealed that 174 (98.7%) of the 179 paired samples were successfully processed (Supplementary Table S1). The genetic markers K1, MAD20, RO33, along with the 3D7 and FC27 allelic variants, correspond to the *msp-1* and *msp-2* genes, respectively. The overall mean expected heterozygosity (H_e) for *P. falciparum* was 0.84 ± 0.12 in Aduku, 0.85 ± 0.12 in Arua, and 0.87 ± 0.09 in Masafu. For *msp-1*, the K1 allelic family exhibited the highest H_e values, with 0.88 in Aduku, 0.93 in Arua, and 0.92 in Masafu. For *msp-2*, the 3D7 allelic family displayed the highest H_e values, with 0.94 in both Aduku and Arua, and 0.95 in Masafu. The microsatellite marker 313 showed consistent H_e values of 0.95 across all sites. The Poly-α marker demonstrated mean expected H_e values of 0.92 in Aduku, 0.91 in Arua, and 0.94 in Masafu. Additionally, the overall mean effective number of alleles (N_e) was 7.61 ± 3.66 in Aduku, 8.77 ± 4.04 in Arua, and 9.24 ± 4.62 in Masafu (Table 1). These findings indicate a relatively high level of genetic diversity across the study sites.

The mean allele richness (Ar) across the study sites was 10.6 ± 3.9 in Aduku, 12.5 ± 4.4 in Arua, and 11.9 ± 4.5 in Masafu (Supplementary Table S2). The number of genotypes for the K1 allelic family ranged from 14 to 18 in Aduku and Arua, respectively, while for I/C 3D7, it ranged from 19 to 25 in Aduku and Masafu. The most polymorphic microsatellite, 313, exhibited 22 to 28 genotypes in Aduku and Arua. Poly-α genotypes ranged from 15 to 24 in Aduku and Masafu. Microsatellite 383 showed 14 to 20 genotypes in Aduku and Arua. The less polymorphic microsatellite 2490 had 5 genotypes in Aduku and 6 genotypes in Arua and Masafu. Markers such as TA1, TA109, PfPK2, 2490, and *glurp* exhibited 3 genotypes with allele frequencies greater than 10%, with 2490 showing a single genotype with a frequency exceeding 50% (Supplementary Figure S1). The Kruskal-Wallis test revealed no significant differences in the observed number of genotypes, allele richness (Ar), allele frequencies, number of effective alleles (N_e), or expected heterozygosity (H_e) across the sites ($p > 0.05$). These results suggest that there is no significant variation in genetic diversity across the three sites, despite the observed differences in allele richness and genotype distribution.

P. falciparum multiplicity of infection (MOI) across three malaria-endemic sites in Uganda

Across all study sites, *msp-1* and the microsatellites PfPK2 and Poly-α exhibited the highest average MOIs, while microsatellite 313, the most diverse marker, generally showed lower MOI. In Aduku, the highest average MOIs for microsatellites were observed in PfPK2, TA1, and Poly-α, with values of 1.77, 1.68, and 1.59, respectively.

	Aduku		Arua		Masafu	
Marker	H_e	N_e	H_e	N_e	H_e	N_e
K1	0.88	7.45	0.93	12	0.92	10.84
MAD20	0.89	6.67	0.93	11.59	0.9	8.29
RO33	0.63	2.56	0.56	2.21	0.68	2.99
IC/3D7	0.94	12.63	0.94	14.71	0.95	16.26
FC 27	0.87	6.92	0.87	6.92	0.85	6.23
glurp	0.86	6.44	0.89	8.28	0.92	11.71
313	0.95	15.47	0.95	15.72	0.95	17.56
383	0.9	8.97	0.88	7.63	0.85	6.49
Poly-α	0.92	10.47	0.91	9.98	0.94	14.02
TA1	0.86	6.59	0.89	7.63	0.89	8.56
PfPK2	0.87	7.18	0.87	7.15	0.85	6.42
2490	0.56	2.2	0.6	2.47	0.68	3.03
TA109	0.83	5.39	0.88	7.7	0.88	7.78
Mean	0.84	7.61	0.85	8.77	0.87	9.24
SD	0.12	3.66	0.12	4.04	0.09	4.62

Table 1. Mean expected heterozygosity (H_e) and number of effective alleles (N_e) of the genotyped markers.

In Arua, PfPK2 had the highest average MOI of 1.71, followed by TA1 and 383, each with an MOI of 1.59. In Masafu, Poly-α, TA1, and TA109 showed the highest MOIs, with average values of 1.79, 1.68, and 1.58, respectively (Fig. 2). Across all three sites, *glurp* consistently exhibited one of the lowest MOIs. The Kruskal-Wallis test revealed significant differences in MOIs: Poly-α had significantly higher MOIs in Masafu, PfPK2 exhibited significantly higher MOIs in Arua and Aduku, and *msp-1* showed significantly higher MOIs in Arua and Masafu ($P<0.05$).

Spearman's rank correlation analysis showed no significant correlation between mean H_e and mean MOI ($p>0.05$).

Comparison of three algorithms to classify recrudescence across three malaria-endemic sites in Uganda

To classify *P. falciparum* malaria recrudescences, we generated all possible genetic marker combinations by pairing *glurp* and each microsatellite with *msp-1* and *msp-2*, resulting in eight distinct marker combinations. These datasets were then analyzed using three classification algorithms: two match-counting algorithms (≥2/3 and 3/3) and a Bayesian algorithm. The goal was to classify each infection as either a recrudescence or a reinfection. Table 2 details the number of recrudescences classified by each marker combination across the different treatment arms. The Wilcoxon signed-rank test revealed that the ≥2/3 match-counting algorithm classified significantly more recrudescences than the 3/3 algorithm across both treatment arms (AL and DP) ($P\leq0.05$).

The Bayesian algorithm was applied to estimate the probability of recrudescence for all samples across eight marker combinations (Supplementary Table S3). Upon visual inspection of the probability distributions, we observed that while there was no clear, definitive cutoff to distinguish recrudescence from reinfection, certain marker combinations exhibited relatively distinct thresholds. Specifically, in the AL arm, combinations of *msp-1* and *msp-2* with *glurp*, *msp-1* and *msp-2* with microsatellite 313, *msp-1* and *msp-2* with microsatellite 383, and *msp-1* and *msp-2* with microsatellite 2490 showed a relatively clear separation in probability distributions. For the DP arm, combinations of *msp-1* and *msp-2* with *glurp*, *msp-1* and *msp-2* with microsatellite Poly-α, *msp-1* and *msp-2* with microsatellite TA109, *msp-1* and *msp-2* with microsatellite PfPK2, and *msp-1* and *msp-2* with microsatellite 2490 also exhibited more discernible probability distributions.

Based on visual inspection of these distributions, we identified a threshold range between 0.7 and 0.8 as a potential cutoff for classifying recrudescence. These observations suggest that while a clear, universally applicable cutoff was not evident across all combinations, certain marker combinations provided a relatively good distinction, particularly in the AL and DP drug arms (Fig. 2).

A probability cutoff was applied to classify infections as either recrudescence or reinfection. Infections with a probability above the cutoff were classified as recrudescences. The Wilcoxon signed-rank test revealed that the number of recrudescences classified using a 0.7 cutoff was significantly higher than those classified using a 0.8 cutoff ($P\leq0.05$) (Table 3).

We compared the total number of recrudescences classified by each algorithm and marker combination. Results varied widely (Fig. 2), with no clear pattern where a specific marker combination consistently yielded the lowest or highest number of recrudescences.

In general, the 3/3 algorithm was the most conservative, identifying the fewest recrudescences across all marker combinations. For the AL arm, this ranged from two recrudescences with *msp-1*, *msp-2* paired with microsatellites 383 and Poly-α, to seven recrudescences using microsatellite 2490 as third marker. Similarly, for the DP arm, the 3/3 algorithm identified two recrudescences when using microsatellites TA1 or TA104 as the third marker, compared to four recrudescences when using *glurp*, 383, Poly-α, or PfPK2. In contrast, the ≥2/3

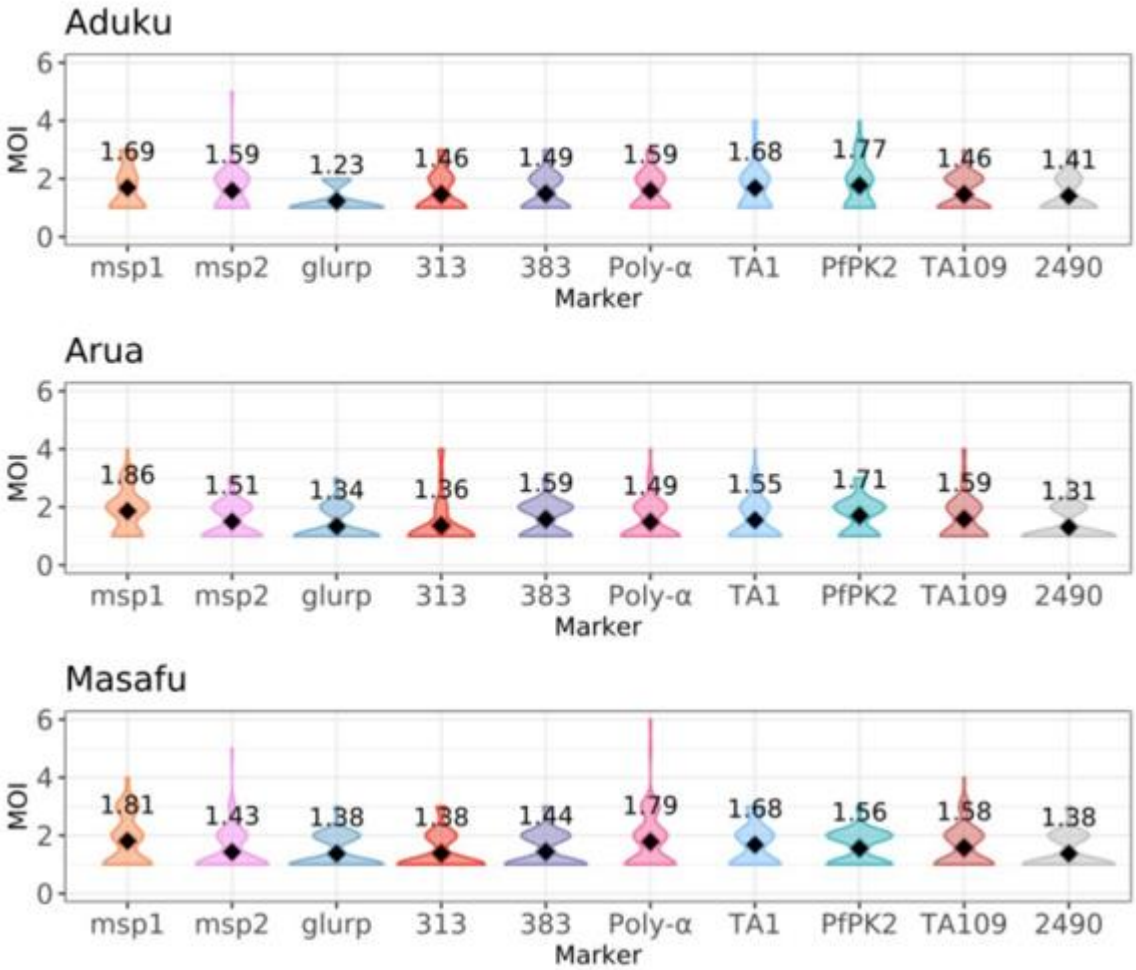


Fig. 1. Distribution of the P. falciparum MOI across the three study sites. Each violin plot shows, for a given site, the distribution of the observed MOIs in the samples collected at Day 0. The diamond from each violin plot represents the average MOI whose corresponding value is displayed above.

algorithm identified significantly more recrudescences. For the AL arm, the number ranged from minimum ten recrudescences with microsatellite TA1 to fourteen with PfPK2 and 2490. For the DP arm, it ranged from seven recrudescences with microsatellite 313 to ten with *glurp* (Fig. 3).

The number of recrudescences classified by the Bayesian algorithm varied significantly depending on the probability cutoff used. For the AL arm, on average, nearly twice as many recrudescences were identified with a 0.7 cutoff compared to 0.8, while for the DP arm, the 0.7 cutoff resulted in over 62% more recrudescences than with the 0.8 cutoff. The marker combinations yielding the highest and lowest numbers of recrudescences also varied with the cutoff and drug arm. Specifically, microsatellites 313 and 2490 produced the highest number of recrudescences for both thresholds in the AL arm, while microsatellites TA109 and 383 resulted in the lowest number at the 0.7 and 0.8 thresholds, respectively. In the DP arm, microsatellite 383 led to the most recrudescences at the 0.7 cutoff, while TA109 produced the highest number at 0.8 cutoff. For the same drug arm, the fewest recrudescences were identified using Poly-α and TA1 at the 0.7 cutoff, and Poly-α again at the 0.8 cutoff (Fig. 3).

Discussion

Accurately distinguishing *P. falciparum* recrudescence from reinfection is critical for evaluating antimalarial drug efficacy. The WHO recommends genotyping markers such as *msp-1*, *msp-2*, and highly polymorphic microsatellites like Poly-α, PfPK2, and TA1 for this purpose[10]. However, studies comparing these markers for recrudescence classification, especially in high-transmission areas, are limited. Our study aimed to address this gap by evaluating eight marker combinations and three classification algorithms in recurrent *P. falciparum* malaria infections from a TES assessing AL and DP.

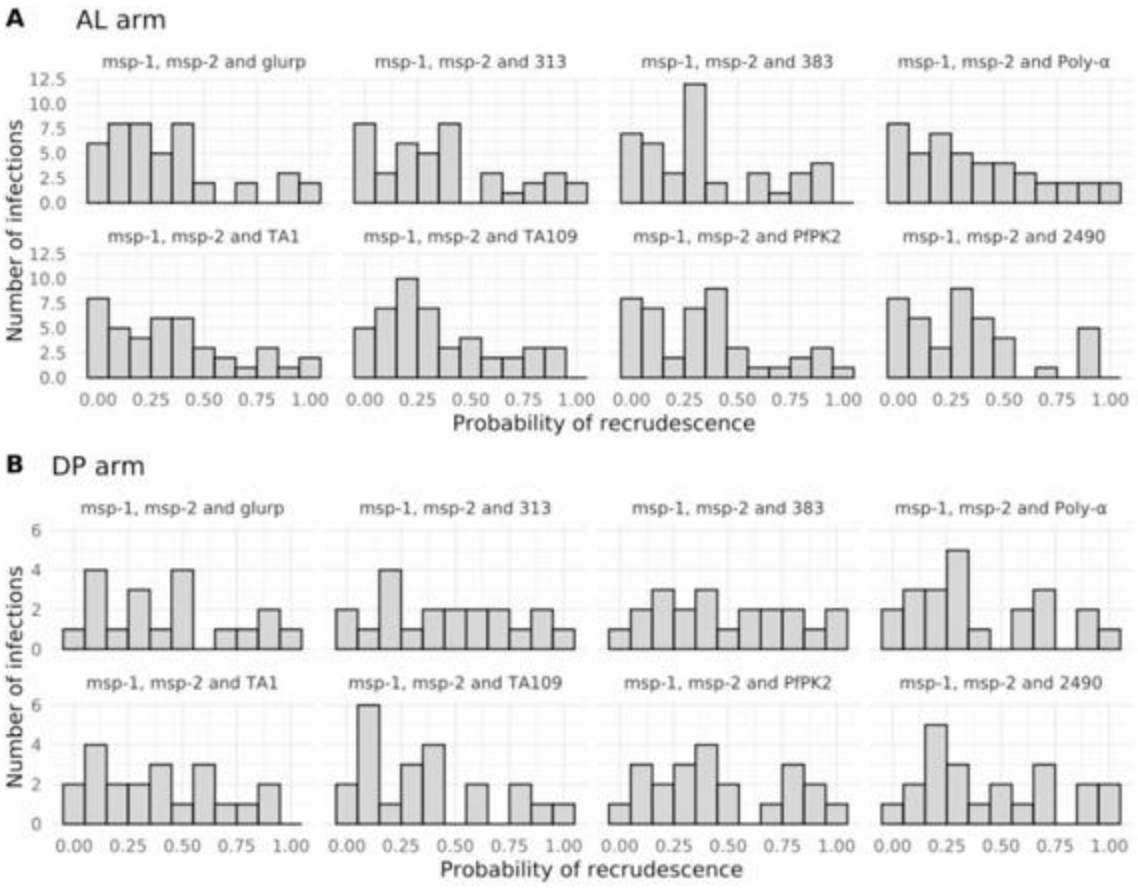


Fig. 2. Distribution of the probability of recrudescence estimated with the Bayesian algorithm. Results are presented for the infections in all three study sites in the AL drug arm (**A**) and the DP drug arm (**B**). Each sub-panel displays, for each marker combination, the distribution of probabilities of recrudescence estimated using the algorithm. The heights of the bars correspond to the number of samples with the respective probability.

Our study, conducted in areas with high malaria transmission, revealed considerable genetic diversity (mean $H_e > 0.8$) and a mean MOI ranging from 1.23 to 1.86 among *P. falciparum* isolates, consistent with findings from other malaria-endemic regions[17]. This high diversity, shaped by host immunity, ecological factors, and malaria control efforts, complicates malaria control and facilitates the emergence of drug-resistant strains[18–20].

Microsatellite markers, particularly those with high diversity and MOI, are sensitive in detecting minority clones, making them valuable for distinguishing recrudescence from reinfection[21,22]. Our results showed that while *glurp* exhibited high diversity ($H_e = 0.86–0.92$), its lower MOI (1.23–1.38) resulted in fewer cases of recrudescence being classified. This aligns with previous studies that noted *glurp*'s moderate diversity but lower sensitivity for detecting minority clones[23]. The low MOI of *glurp* suggests reduced sensitivity in identifying minority clones compared to other markers, potentially underestimating recrudescence consequently overestimating drug efficacy[13]. However, some studies have found no significant differences when *glurp* was used instead of microsatellites[24], though its reduced sensitivity remains a limitation.

Interestingly, markers like *PfPK2* exhibited higher MOIs than expected, contradicting earlier reports of low diversity for this marker[14]. Conversely, markers like 313 and *glurp*, which are more diverse, showed lower MOIs, indicating that high genetic diversity in microsatellites can hinder their ability to detect minority clones, complicating recrudescence classification. While MOI often serves as a proxy for diversity, it does not fully capture a marker's sensitivity, highlighting the need for further studies to evaluate the utility of diverse microsatellites.

Regarding classification algorithms, the WHO recommends the 3/3 match-counting approach for classifying recrudescence, with the alternative ≥ 2/3 approach suggested for specific settings[10]. Our findings indicate that the 3/3 approach classified significantly fewer recrudescences compared to other algorithms. While this stricter approach increases specificity, it may misclassify true recrudescences as reinfections, particularly in high-transmission areas where genetic diversity and multiple clones are common. In contrast, the ≥ 2/3 approach classified significantly more recrudescences, especially when using microsatellite markers such as 383 or PfPK2

	AL treatment arm		DP treatment arm	
Third marker	≥ 2/3	3/3	≥ 2/3	3/3
Poly-α	11	2	8	4
PfPK2	14	3	9	4
TA1	10	4	9	2
TA109	12	4	9	2
2490	14	7	8	3
313	12	3	7	3
383	12	2	8	4
glurp	11	5	10	4

Table 2. Number of recrudescences classified by the match-counting algorithms. Each row represents one of the eight possible combinations defined by the third marker indicated in the leftmost column. The columns report the number of recrudescences classified by the ≥ 2/3 and 3/3 algorithms across all three sites for each drug arm. AL = Artemether-Lumefantrine. DP = Dihydroartemisinin-piperaquine.

	AL treatment arm		DP treatment arm	
Third marker	0.8	0.7	0.8	0.7
Poly-α	7	13	4	6
PfPK2	8	13	6	10
TA1	7	14	5	6
TA109	6	12	7	9
2490	11	17	5	9
313	10	17	5	9
383	5	13	5	12
glurp	7	15	6	8

Table 3. Number of recrudescences identified by the Bayesian algorithm. Each row represents one of the eight possible marker combinations defined by the third marker indicated in the leftmost column. The remaining columns report the number of recrudescences identified per site using a 0.7 and a 0.8 cutoff for the probability of recrudescence, as well as the total number of recrudescences across all three sites for each drug arm. AL = Artemether-Lumefantrine. DP = Dihydroartemisinin-piperaquine.

in the AL treatment arm. This suggests that the ≥ 2/3 approach may be more suitable for low- to moderate-transmission areas, although it risks overestimating recrudescence and inflating drug efficacy estimates in high-transmission regions[10].

We observed variability in the number of recrudescences classified by different marker combinations and algorithms. Combinations of *msp-1*, *msp-2*, and *glurp*, or *msp-1*, *msp-2*, and 2490, identified more recrudescences with both the 3/3 and ≥ 2/3 match-counting algorithms. The low diversity and MOI of 2490 likely account for its higher recrudescence count, while the increased count for *msp-1*, *msp-2*, and *glurp* may be due to the 50 bp bin size used for genotyping, which could group similar genotypes together, leading to overestimation. Additionally, the higher recrudescence count for *glurp* may result from PCR amplification bias, where shorter fragments are preferentially amplified, limiting the detection of minority clones[13,25].

The Bayesian algorithm performed poorly in our study, with a flat distribution of likelihoods for recrudescence classification. Unlike previous studies from Angola, which reported a bimodal distribution between recrudescences and reinfections[15], our analysis found that the Bayesian algorithm did not provide useful thresholds for distinguishing between recrudescence and reinfection. This suggests that the genotyped markers lacked sufficient discriminatory power in this context.

In high-transmission areas, microsatellites like Poly-α and 313 have been suggested as useful for distinguishing recrudescence from reinfection[14]. Our study found that markers like PfPK2 and Poly-α, which exhibited higher MOIs, performed better in classifying recrudescence compared to *glurp*. These markers may therefore offer more reliable results in high-transmission areas, supporting their use in such settings. In contrast, markers like *glurp* may still perform adequately in low-transmission regions, where a more lenient algorithm like ≥ 2/3 match-counting could help reduce misclassification of recrudescences as reinfections.

While a standardized approach for PCR correction could improve consistency across studies[10], a more flexible, adaptive framework that considers local transmission intensity and genetic diversity would enhance recrudescence detection accuracy. Our study suggests that a one-size-fits-all approach is not feasible across diverse malaria transmission environments. Future research should explore adapting PCR correction techniques to different regional settings to improve recrudescence classification and drug efficacy assessments.

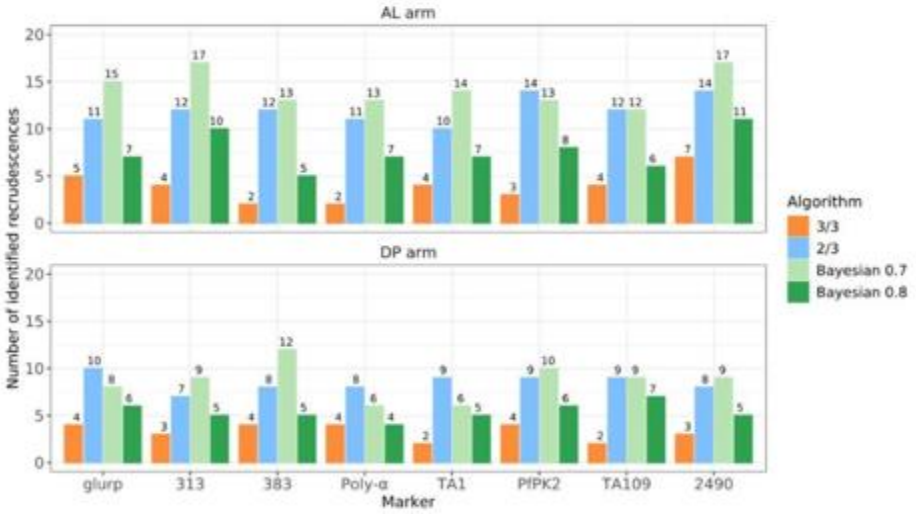


Fig. 3. Comparison of the number of recrudescences identified by each algorithm and marker combination. Results are presented for the ≥ 2/3, 3/3, and Bayesian algorithm using cutoffs of 0.7 and 0.8 for the probability of recrudescence, across each marker combination (x-axis). The number of recrudescences identified by each algorithm (y-axis) is illustrated with the colored bars

Despite these crucial findings, we acknowledge the limitations of our study, particularly regarding the lower discriminatory power of the QIAxcel system, which may have influenced allele calling and recrudescence classification. Higher-resolution techniques, such as deep amplicon sequencing or Applied Biosystems (ABI) capillary electrophoresis, could improve the accuracy of our results. Additionally, the absence of a gold-standard reference method for comparing our computational approaches may have impacted the interpretation of our findings.

In conclusion, our study suggests that PfPK2 and Poly-α are more effective at classifying recrudescence in high-transmission areas and could replace or complement *glurp* in these settings. The findings also highlight the limitations of the Bayesian algorithm in this context. Further validation of these markers in different malaria-endemic regions is necessary to optimize recrudescence classification methods and improve the reliability of TES. Our study underscores the need for flexible, adaptive approaches to PCR correction, taking into account regional transmission intensity and genetic diversity, to enhance recrudescence detection across diverse malaria transmission settings.

Methods

Study sites and sample collection

Dried blood spot (DBS) filter paper samples were collected from children aged 6 months to 10 years with recurrent uncomplicated *P. falciparum* malaria, confirmed by positive malaria microscopy. These children were enrolled in a TES assessing the efficacy of AL and DP. The study was conducted from September 2018 to February 2019 at three health centers in Uganda: Aduku Health Centre IV in Kwania district, Northern Uganda; Arua Regional Referral Hospital in Arua district, northwestern Uganda; and, Masafu District Hospital in Busia district, Eastern Uganda. All sites experience perennial malaria transmission with high transmission intensity. A 2018– 2019 malaria indicator survey showed 13% parasite prevalence among children aged 0–59 months in Kwania district, 22% in Arua district, and 21% in Busia district[16]. A total of 179 paired samples were genotyped, comprising 123 paired samples from the AL arm and 56 paired samples from the DP arm (Supplementary Table S1). Further details about the drug efficacy and safety study, are publicly available and described elsewhere[26].

Laboratory methods

DNA extraction

Genomic DNA was extracted from DBS filter paper samples using Chelex 100 Resin (Sigma-Aldrich, USA) as previously described[27]. Briefly, 6 mm discs were punched out from the DBS filter paper into 1.5 mL microcentrifuge tubes containing 1 mL of 1X phosphate-buffered saline (PBS) and incubated at 4 °C overnight. The discs were washed twice in 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 labeled 0.6 mL microcentrifuge tube with a 100 µL elution volume and stored at − 20 °C until further use.

P. falciparum msp-1, msp-2 and *glurp* genotyping

Parasite genotyping was conducted at the Infectious Diseases Research Collaboration (IDRC) molecular biology research laboratory in Uganda using nested PCR as previously described[28] adjusting the PCR reaction mixture to 25 µL. DNA from *P. falciparum* reference strains (3D7, and HB3) was used in each run as positive control[29]. Genotyping was done using nested PCR (Supplementary Table S4). A 3µL sample of the PCR product was then analysed on a 2% agarose gel to confirm the amplification.

The PCR products for the *msp-1* and *msp-2* allele families were analysed by capillary electrophoresis using a Qiaxcel DNA High-Resolution Kit (Qiagen) in the Qiaxcel Advanced system (Qiagen, Hilden, Germany). The analysis followed the AM420 protocol utilising the QX-DNA size marker 50–800 bp (Qiagen, Hilden, Germany) and the QX alignment marker 15 bp/1 kb (Qiagen, Hilden, Germany). The nested PCR products for *glurp* were analysed using the AM420 protocol with the QX DNA size marker 100 bp – 2.5 kb (Qiagen, Hilden, Germany) and the QX alignment marker 15 bp/3 kb (Qiagen, Hilden, Germany). The band sizes were determined with the Qiaxcel Screen Gel software (Version 1.5.0.16, Qiagen, Hilden, Germany) and the height cut off for minority clones was set at 10% for *msp-1* and *msp-2* and 20% for *glurp* of the dominant peak[13].

Microsatellite genotyping

Samples were genotyped for the microsatellites TA1, Poly-α, PfPK2, TA109, 2490, 313, 383 at the Centers for Disease Control and Prevention (CDC) Malaria Laboratory in Atlanta, USA. Fragment sizes were then measured using capillary electrophoresis on Applied Biosystems (ABI) 3033 and scored using GeneMarker® V2.6.3, as previously described[26]. For samples that produced more than one peak, the highest peak was defined as the dominant allele. Additional peaks were classified as minor alleles if their peak heights exceeded 200 relative fluorescence units (RFU) and were > 30% of the height of the dominant peak. This threshold was used to identify minor alleles, which may represent clones present at lower frequencies but still contribute to the genetic diversity of the infection. The identification of these additional minor alleles, including third and fourth alleles, was based on the relative peak heights at each microsatellite locus. Peaks that met these criteria were recorded as distinct alleles.

Genotyped samples were considered successful if they amplified on all markers (*msp-1*, *msp-2*, *glurp*, and microsatellites) on both day 0 and the day of failure. Samples that did not amplify on all markers were excluded from the final analysis.

Data analysis

To further refine the identified fragment sizes and determine the final alleles for each marker, we grouped the fragments based on predefined bin sizes. For the *msp-1* and *msp-2* allelic families, we used a bin size of 10; for *glurp*, a bin size of 50; for the double repeat microsatellites 313 and 383, a bin size of 1; and for the triple repeats microsatellites TA1, Poly-α, PfPK2, TA109, and 2490, a bin size of 2. Fragments with length differences within the specified bin sizes were grouped together. The grouping operation started with an offset identified to achieve the highest overlap between the final (theoretical) alleles and observed fragment sizes.

Genetic diversity. For each site, we determined the genetic diversity of the genotyped markers by calculating both the mean number of genotypes, allele richness (Ar), allele frequency and the number of effective alleles (N_e) at each locus using GENALEX 6.5 software[30]. Expected heterozygosity (H_e) which is the probability that two randomly selected individuals from a population will carry distinct alleles at each marker locus, was calculated using ARLEQUIN software version 3.11[31] with the following formula:

$$H_e = [n/(n-1)][1 - \sum p_i^2],$$

where 'n' and 'p_i' represent the number of isolates analysed and the frequency of the allele in a given population, respectively. H_e values range between 0 and 1 from no genetic diversity to high genetic diversity, respectively as previously described by Anderson et *al.*[18]. Parasite isolates with higher values of H_e and N_e are indicative of greater genetic diversity within the population[32,33].

P. falciparum MOI, defined as the number of distinct parasite clones simultaneously infecting an individual[34], was estimated for each sample at day 0 (day of the primary infection) under the different drug arms and across the three sites. For each site and marker, we then determined the mean MOI by identifying the highest number of alleles observed across any of the microsatellite markers used in the analysis. This maximum allele count was considered the MOI for that particular infection.

Recrudescence and reinfection classification. Three algorithms were applied to the identified alleles from all the samples, namely the 3/3 match counting algorithm, ≥ 2/3 match counting algorithm, and a Bayesian approach[10,15]. These algorithms were applied to several datasets, each combining different genotyped microsatellites as well as *glurp* with *msp-1* and *msp-2*. The 3/3 method classified an infection as recrudescence if one or more alleles occurred at all three genotyped markers on both day 0 and the day of the recurrent malaria infection. In contrast, a ≥ 2/3 match counting algorithm classified an infection as a recrudescence if one or more alleles were present in at least two of the three genotyped markers on both day 0 and the recurrence day. The Bayesian algorithm utilized allele information from all samples to estimate the probability of recrudescence for each case. A low probability indicated a greater likelihood of reinfection, whereas a probability closer to 1 suggested a high likelihood of recrudescence[15]. This approach highlights how different probability thresholds can influence the sensitivity (true positive rate) and specificity (true negative rate) of the algorithm. Specifically, a lower threshold may lead to the identification of more recrudescences, thereby enhancing sensitivity, while a higher threshold can improve specificity by reducing false positives. To classify recrudescence versus reinfection, probability thresholds of 0.7 and 0.8 were applied sequentially.

Statistical analysis

Genetic diversity and MOI across sites were compared using the Kruskal-Wallis test, while correlation between mean H_e and mean MOI was assessed using Spearman's rank correlation. The results of all three algorithms (3/3 match-counting, ≥ 2/3 match-counting, and Bayesian) were then compared using the Wilcoxon signed-rank test to assess their effectiveness in identifying recrudescences based on the hypothesis that there would be no difference in the number of recrudescences classified by the ≥ 2/3 and 3/3 algorithms for each treatment arm (both AL and DP). All the analyses were conducted using STATA version 17 (Stata Corporation, College Station, TX), with a statistical significance set at $P < 0.05$.

Data availability

The datasets generated and/or analysed in this study are available from the corresponding author upon reasonable request. All the analysis code used in the study and for generating all the figures is available at: https://github.com/SwissTPH/genotyping_comparison_Uganda/tree/main.

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References

1. Oladipo, H. J. et al. Increasing challenges of malaria control in sub-saharan Africa: Priorities for public health research and policymakers. *Ann. Med. Surg. (Lond).* **81**, 104366 (2022).
2. WHO. *World Malaria Report 2023* (Geneva, World Health Organisation, 2023).
3. Shibeshi, M. A., Kifle, Z. D. & Atnafie, S. A. Antimalarial drug resistance and novel targets for antimalarial drug discovery. *Infect. Drug Resist.* **13**, 4047–4060 (2020).
4. Zhong, A., Zhang, H. & Li, J. Insight into molecular diagnosis for antimalarial drug resistance of Plasmodium Falciparum parasites: A review. *Acta Trop.* **241**, 106870 (2023).
5. WHO. *Global Report on Antimalarial drug Efficacy and drug Resistance 2000–2010.Geneva* (World Health Organisation, 2010).
6. WHO. *Report on Antimalarial drug Efficacy, Resistance and Response: 10 Years of Surveillance (2010–2019)* (Geneva, World Health Organization, 2020).
7. Plucinski, M. M. et al. Variation in calculating and reporting antimalarial efficacy against Plasmodium Falciparum in Sub-saharan Africa: A systematic review of published reports. *Am. J. Trop. Med. Hyg.* **104** (5), 1820–1829 (2021).
8. Slater, M. Distinguishing recrudescences from new infections in antimalarial clinical trials: Major impact of interpretation of genotyping results on estimates of drug efficacy. *Am. J. Trop. Med. Hyg.* **73** (2), 256–262 (2005).
9. WHO. *Guidelines for the Treatment of Malaria 2015* (Geneva, World Health Organization,, 2015).
10. WHO, Informal consultation on methodology to distinguish reinfection from recrudescence in high malaria transmission areas: Report of a virtual meeting, 17–18 May 2021. Geneva, World Health Organisation, 2021.1. (2021).
11. Gruenberg, M., Lerch, A., Beck, H. P. & Felger, I. Amplicon deep sequencing improves *Plasmodium Falciparum* genotyping in clinical trials of antimalarial drugs. *Sci. Rep.* **9** (1), 17790 (2019).
12. WHO, Methods and techniques for clinical trials on antimalarial drug efficacy: Genotyping to identify parasite populations: informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29–31. Amsterdam, The Netherlands. 2008: World Health Organization.May (2007).
13. Messerli, C., Hofmann, N. E., Beck, H. P. & Felger, I. Critical evaluation of Molecular Monitoring in Malaria Drug Efficacy trials and pitfalls of length-polymorphic markers. *Antimicrob. Agents Chemother.* **61** (1), e01500–e01516 (2016).
14. Ishengoma, D. S. et al. Microsatellites reveal high polymorphism and high potential for use in anti-malarial efficacy studies in areas with different transmission intensities in mainland Tanzania. *Malar. J.* **23** (1), 79 (2024).
15. Plucinski, M. M., Morton, L., Bushman, M., Dimbu, P. R. & Udhayakumar, V. Robust algorithm for systematic classification of Malaria Late treatment failures as recrudescence or reinfection using microsatellite genotyping. *Antimicrob. Agents Chemother.* **59** (10), 6096–6100 (2015).
16. MoH, U. (2020). https://www.dhsprogram.com/pubs/pdf/MIS34/MIS34.pdf. 2020.
17. Ralinoro, F. et al. Genetic diversity of *Plasmodium falciparum* populations in three malaria transmission settings in Madagascar. *Malar. J.* **20** (1), 239 (2021).
18. Anderson, T. J. et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium Falciparum*. *Mol. Biol. Evol.* **17** (10), 1467–1482 (2000).
19. Amoah, L. E. et al. Population structure and diversity of *Plasmodium Falciparum* in children with asymptomatic malaria living in different ecological zones of Ghana. *BMC Infect. Dis.* **21** (1), 439 (2021).
20. Mwesigwa, A. et al. P. 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.* **23** (1), 97 (2024).
21. Nyachieo, A. *PlasmFalciparumiparum* genotyping by microsatellites as a method to distinguish between recrudescent and new infections. *Am. J. Trop. Med. Hyg.* **73** (1), 210–213 (2005).
22. Zhong, D. Molecular approaches to determine the multiplicity of Plasmodium infections. *Malar. J.* **17**, 1–9 (2018).
23. Mwingira, F. *PlasmFalciparumiparum msp1, msp2 and glurp a* llele frequency and diversisub-saharanaharan Africa. *Malar. J.* **10**, 1–10 (2011).
24. Schnoz, A. Genotyping methods to distinguish *Plasmodium Falciparum* recrudescence from new infection for the assessment of antimalarial drug efficacy: An observational, single-centre, comparison study. *Lancet Microbe.* **5** (11), 100914 (2024).
25. Jones, S. et al. Improving methods for analyzing antimalarial drug efficacy trials: Molecular correction based on length-polymorphic markers *msp-1, msp-2, and glurp*. *Antimicrob. Agents Chemother.* **63** (9). https://doi.org/10.1128/aac (2019). 00590 – 19.
26. Ebong, C. Efficacy and safety of artemether-lumefantrine and dihydroartemisinin-piperaquine for the treatment of uncomplicated *Plasmodium Falciparum* malaria and prevalence of molecular markers associated with artemisinin and partner drug resistance in Uganda. *Malar. J.* **20** (1), 1–12 (2021).
27. Hansson, H., Saidi, Q. & Alifrangis, M. *Preservation and Extraction of Malaria Parasite DNA from Dried Blood Spots, in Malaria Immunology: Targeting the Surface of Infected Erythrocytes* pp. 27–36 (Springer, 2022).
28. Congpuong, K. et al. Genetic diversity of the *msp-1, msp-2*, and *glurp* genes of *Plasmodium falciparum* isolates along the Thai-Myanmar borders. *Asian Pac. J. Trop. Biomed.* **4** (8), 598–602. (2014).
29. Baina, M. T. et al. Comparative study of *Plasmodium falciparum msp-1* and *msp-2* Genetic Diversity in Isolates from Rural and Urban Areas in the South of Brazzaville, Republic of Congo. Pathogens. **12** (5), 742. (2023).
30. Peakall, R. & Smouse, P. E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–an update. *Bioinformatics* **28** (19), 2537–2539 (2012).
31. Excoffier, L., Laval, G. & Schneider, S. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinform Online.* **1**, 47–50 (2007).

32. Roh, M. E. High genetic diversity of *Plasmodium Falciparum* in the low-transmission setting of the Kingdom of Eswatini. *J. Infect. Dis.* **220** (8), 1346–1354 (2019).
33. Nabet, C. et al. Genetic diversity of *Plasmodium Falciparum* in human malaria cases in Mali. *Malar. J.* **15**, 1–10 (2016).
34. Touray, A. O. Diversity and multiplicity of *P. falciparum* infections among asymptomatic school children in Mbita, Western Kenya. *Sci. Rep.* **10** (1), 5924 (2020).

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Author contributions
AM,SLN,JIN,JNK,CK, and PBK conceived the idea, planned, and designed the study. AM.BM performed laboratory assays. AM.MP and MG performed data analysis. AM wrote the first draft of the manuscript. MG. CN.SLC.SJ.EMB and PBK edited and provided continuous feedback on the manuscript. AM wrote the final draft of the manuscript.All authors reviewed the manuscript.

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Declarations

Ethics approval and consent to participate
The study was approved by the Makerere University School of Medicine Research and Ethics Committee (MakSOMREC #2021 – 152) and the Uganda National Council for Science and Technology (UNCST #HS2744ES). All procedures were conducted in accordance with the ethical guidelines and regulations of both MakSOMREC and UNCST. The study utilized dried blood spot (DBS) filter papers collected from children aged 6 months to 10 years. Informed assent was obtained from the children, and parental consent was granted for the reuse of their samples in future molecular parasite studies. Parents were fully informed about the study and provided written consent for their child's participation and the use of their samples.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-88892-7.

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APPENDIX VI. Data abstraction tool

Sample ID	Year	Site ID	Age	Sex	Parasite density	Temp	Hb	Parasite genetic diversity			Multiplicity of infection		Parasite population structure	
								Na	Ne	He	%ge polyclonal	Mean MOI	LD ($I^{A}_{S)}$)	F_{ST}
1														
2														
3														
4														
5														
6														
7														
8														
9														
10														
11														
12														
13														
14														
15														
16														
17														

APPENDIX VII. IDRC Administrative clearance

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IDRC Trainee/Student/Volunteer Project Application

Instructions: Please include the following information and limit the document to 1-2 pages (not inclusive of approval page).

Trainee/Student/Volunteer Name: MWESIGWA ALEX

University Affiliation: MAKERERE UNIVERSITY, COLLEGE OF HEALTH SCIENCES

PI or Mentor Name: PROF PAULINE BYAKIKA, DR JOANITER NANKABIRWA, DR SAMUEL L NSOBYA AND PROF CHARLES KARAMAGI.

Uganda Project Supervisor Name: , PROF PAULINE BYAKIKA, DR JOANITER NANKABIRWA, DR SAMUEL L NSOBYA AND PROF CHARLES KARAMAGI.

Local Peer Researcher (if relevant): NOT APPLICABLE

Name of Parent Project (if relevant): PRISM AND THERAPEUTIC EFFICACY STUDIES (TES) SAMPLES.

Title of trainee/student/volunteer project: *PLASMODIUM FALCIPARUM* GENETIC DIVERSITY, MULTIPLICITY OF INFECTION, GAMETOCYTEMIA AND RECRUDESCENCE IN AREAS OF VARIED MALARIA TRANSMISSION INTENSITY IN UGANDA.

Brief description of project activities: WE WILL EXTRACT P. FALCIPARUM GENOMIC DNA FROM DRIED BLOOD SPOT FILTER PAPERS

WE WILL AMPLIFICATION OF THE EXTRACTED P. FALCIPARUM GENOMIC DNA

THE AMPLIFIED P. FALCIPARUM GENOMIC DNA WILL THEN BE GENOTYPED USING MSP1, 2 AND GLURP AND MICROSATTELITE.

Describe Study population or Specimens to be studied: DRIED BLOOD SPOT FILTER PAPERS.

Proposed sites to carry out project activities (including laboratory space if applicable): IDRC MOLECULAR LABORATORY

Length of project: 2022-2023

Does project have IRB approval? (Describe IRB plan or reasons why not applicable):

YES THE PHD PROJECT PROPPSAL HAS BEEN APPROVED.

Is there a project budget or will the parent project cover expenses? (Attach the project budget):

NO THE PARENT STUDY WILL PROJECT WILL NOT COVER EXPENSES

Please indicate if funding already in place or if project will be submitted for funding (please include funder and submission date):

THE STUDENT HAS LIMITED SUPPORT FROM OPTIMAL STUDY LED BY PROF PAULINE BYAKIKA.

DocuSign Envelope ID: 4096229C-850D-4C8B-9BC5-DBBB917B2AA6

Will this project require a new subcontract to IDRC? If yes, who is the prime funding institution?

NO THE PROJECT WILL NOT REQUIRE A NEW SUBCONTARCT TO IDRC

What additional resources will be needed? Describe plan for each of the following: transportation, accommodation, procurement of suppliers, hiring personnel, any other resource needs:

STUDENT IS RESPONSIBLE FOR ALL HIS STUDY PROJECT ACTIVITIES.

Will project need data from existing study? Describe data needed (Note: Data sharing agreement may be required):

YES; WE WILL REQUIRE PARTICIPANTS DEMOGRAPHICS AND CLINICAL DATA FOR THE SELECTED SAMPLES

If new data to be collected, who will perform data collection and how will data be protected?:

NO NEW DATA WILL BE COLLECTED

Will this require IDRC Data Center support? Describe:

YES, PROVIDE PARTICIPANTS INFORMATION.

Please return the completed application form to Jacqueline Namyalo (jnamyalo@idrc-uganda.org) and Bridget (reachbridget@yahoo.com)

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IDRC Trainee/Student/Volunteer Project Application

Instructions: Please include the following information and limit the document to 1-2 pages (not inclusive of approval page).

Describe data ownership and authorship plans:

DATA WILL BE TREATED WITH HIGH LEVEL OF CONFIDENTIALITY, KEPT UNDER LOCK AND KEY, FINDINGS WILL USED FOR RESEARCH PURPOSES ONLY.

Signed by Mentor: DocuSigned by: Dr Jennifer Nankabirwa **Date:** 1/24/2022

INTERNAL USE ONLY:

DATE APPLICATION RECEIVED: _____

*APPROVALS (Review and Response within **2 weeks** of receiving application)*

Signed by Parent Project PI: DocuSigned by: Prof Moses Kamya Date: 1/24/2022

Uganda Supervisor: DocuSigned by: Pauline Byakika Date 1/28/2022

IDRC Administration: Date: 28/01/2022

IDRC Regulatory Office: Bridget Nyanzura Date: 1/30/2022

DocuSign Envelope ID: 4096229C-850D-4C88-90C5-D6[redacted]

Date: 1/30/2022

IDRC Lab Core (if applicable): _____

Date: 1/31/2022

Additional Comments:

Please return the completed application form to Jacqueline Namyalo (namyalo@idrc-uganda.org) and Bridget (reachbridget@yahoo.com)

APPENDIX VIII. SoMREC Approval

MAKERERE UNIVERSITY
P.O. Box 7072 Kampala, Uganda
E-mail: rresearch9@gmail.com
Phone: 256 414 533541
Fax: 256 414 541036/0414 532204
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICINE
RESEARCH ETHICS COMMITTEE

02/08/2021

To: Alex Mwesigwa

Makerere university
+256775135977

Type: Initial Review

Re: Mak-SOMREC-2021-152: PLASMODIUM FALCIPARUM GENETIC DIVERSITY, MULTIPLICITY OF INFECTION, GAMETOCYTEMIA AND RECRUDESCENCE IN AREAS OF VARIED MALARIA TRANSMISSION INTENSITY IN UGANDA., 2, 2021-07-14

I am pleased to inform you that at the **120** convened meeting on **28/06/2021**, the MAK School of Medicine REC (Mak-SOMREC), committee meeting, etc voted to approve the above referenced application.
Approval of the research is for the period of **02/08/2021** to **02/08/2022.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigators must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for re-review and approval **prior** to the activation of the changes.
3. Reports of unanticipated problems involving risks to participants or any new information which could change the risk benefit: ratio must be submitted to the REC.
4. Only approved consent forms are to be used in the enrollment of participants. All consent forms signed by participants and/or witnesses should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Continuing review application must be submitted to the REC **eight weeks** prior to the expiration date of **02/08/2022** in order to continue the study beyond the approved period. Failure to submit a continuing review application in a timely fashion may result in suspension or termination of the study.
6. The REC application number assigned to the research should be cited in any correspondence with the REC of record.
7. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of all documents approved in this application by MAK School of Medicine REC (Mak-SOMREC):

No.	Document Title	Language	Version Number	Version Date
1	Protocol	English	2	2021-07-14
2	Data collection tools	English	1	2021-06-16
3	Informed Consent forms	English	1	2021-06-16

Yours Sincerely



Ponsiano Ocama
For: MAK School of Medicine REC (Mak-SOMREC)

APPENDIX IX. UNCST Approval



Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS2744ES **12 April 2023**

Alex Mwesigwa
MAKERERE UNIVERSITY
Kampala

Re: Research Approval: **P. falciparum genetic diversity, multiplicity of infection, gametocytaemia and recrudescence in areas of varied malaria transmission intensity in Uganda.**

I am pleased to inform you that on **12/04/2023**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **12/04/2023** to **12/04/2024**.

Your research registration number with the UNCST is **HS2744ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project. As the Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. Keeping all co-investigators informed of the status of the research.
2. Submitting all changes, amendments, and addenda to the research protocol or the consent form (where applicable) to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority and a notification to the UNCST.
4. Unanticipated problems involving risks to research participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST notification after review by the REC.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

Please note that this approval includes all study related tools submitted as part of the application as shown below:

No.	Document Title	Language	Version Number	Version Date
	Project Proposal	English	1	
1	Approval Letter	English		
2	Administrative Clearance	English		
2	WAIVER OF CONSENT	ENGLISH	1	20 December 2021
3	DATA COLLECTION TOOLS	ENGLISH	1	23 March 2021
4	ASSENT FOR CHILDREN	ENGLISH	1	10 May 2013
5	Child future use of specimens	English	1	11 May 2017
6	Adult future use of specimen	English	1	03 October 2011
7	OPTIMAL CONSENT	English	1.2	26 April 2020
8	Community Engagement plan	English	1	05 April 2023

Yours sincerely,



Dr. Christopher Ddamulira
For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

LOCATION/CORRESPONDENCE

Plot 6 Kimera Road, Ntinda
P.O. Box 6884
KAMPALA, UGANDA

COMMUNICATION

TEL: (256) 414 705500
FAX: (256) 414-234579
EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

APPENDIX X. Study standard operating procedures

(a) *P. falciparum* DNA extraction

Materials:

- 1X PBS (Calcium and Magnesium Free pH7.4)
- 0.5% Tween-20 in 1X PBS: 2mL 10% Tween-20 in PBS + 38mL 1X PBS
- 2.5g/L Sodium azide in 1X PBS: 50mg sodium azide + 20mL 1X PBS
- 20% chelex (stored at room temperature)
- 1.5ml Eppendorf tubes
- O.5m DNA storage Eppendorf tubes

Procedure

1. Cut the DBS filter paper to 6mm using a scissors or hole puncher. The same blade or hole puncher can be used after rinsing with ddH_2O, punching clean filter paper 3X , and rinsing with ddH_2O again. (Appendix 1, blood volumes based on size of punch).

2. Combine the DBS filter paper punches with 1ml of 0.5% Tween-20 in PBS in a 1.5mL microfuge tube. This will elute your serum 1:120.

3. Arrange tubes in racks of 30 (depending on centrifuge). Shake rack overnight at room temperature, low speed.

4. Set up Matrix antibody storage tubes by adding 400uL of 2.5g/L sodium azide solution per Matrix tube.

5. Centrifuge DBS tubes for 5 seconds. Transfer 600uL of the now reddish PBS/Tween-20 to the antibody storage tubes, using a clean barrier tip for each tube. Store eluted serum (now at 1:200) at -80ºC.

6. Aspirate all remaining PBS/Tween-20 from the DBS tubes with a clean non-barrier tip attached to a Pasteur pipette at the end of a vacuum assembly. Change tips between tubes.

7. Add 1 ml of PBS/tube (no Tween-20), vortex briefly, and incubate at 4ºC for 15-30min. Turn on the heat block at this time to allow time for 95ºC to be reached.

8. Centrifuge and aspirate (as above) as much fluid as possible and afterwards use the tip to press the filter paper down into the lower third of the tube without packing it excessively. Change tips between tubes.

9. Vortex 20% chelex solution. Create a 2:1 solution of chelex to sterile water (1.75mL 20% chelex + 3.5mL PCR H_20 → enough for 35+ tubes). Transfer 150uL of 2:1 chelex, inverting the tube every 2-3 transfers. (NOTE: chelex quickly settles out of suspension, make sure you are not just transferring water).

10. Extract the parasite DNA by incubating the tubes for 10 min in a 95ºC heat-block, vigorously vortexing each sample every 2 minutes or so throughout the incubation. It is best to briefly uncap each tube before the first two vortexes to release pressure, or else the tubes will "pop".

11. After incubation, centrifuge the tubes for 5 minutes at high speed. Meanwhile, label 0.6mL tubes for transfer.

12. Transfer as much solution as possible from the spun tubes to labeled transfer tubes with a 200uL barrier-tip, not worrying if chelex is carried over as well.

13. Spin tubes for 10 minutes at high speed and then transfer the final, white-to-yellowish supernatant (avoiding the pelleted chelex) to the final Matrix tubes. Store at -20ºC.

(b) *P. falciparum* genotyping

(i) *msp-1*, *msp-2* and *glurp* genotyping

Primer sequences for *msp-1, msp-2* and *glurp*

Gene name	Primer ID	Primer sequence
MSP-1	M1OF	CTAGAAGCTTTAGAAGATGCAGTATT G

	M1OR	CTTAAATAGTATTCTAATTCAAGTGGA TCA
MSP-2	*MSP2-1* *MSP2-4*	ATGAAGGTAATTAAAACATTGTCTATT ATA ATATGGCAAAAGATAAAACAAGTGTT GCTG
GLURP	*GLURP E* *GLURP F*	ATGAATTTGAAGATGTTCACACTGAAC AAATATTACTATATCCTTTGCTATTCC

(ii) Master mix components and preparation

Component	**Stock concentration**	**Final concentration**	**Volume for one reaction (µL)**
PCR water	……..	……	16.8
Forward primer	1010 µM	0.2 µM	0.5
Reverse primer	10 µM	0.2 µM	0.5
Buffer	10X	1X	2.5
dNTPs (2 mM), 0.75 $MgCl_2$ (50mM),		200 µM	2.5
Taq Polymerase	5 U/µL	1.5 mM	0.2
Template DNA			2
Final Volume			25

(iii) PCR conditions

Msp-1

Stages	**Steps**	**Primary round**			**Nested round**		
		Temperature (^{0}C)	**Time**	**Cycles**	**Temperature (^{0}C)**	**Time**	**Cycles**
Initial denaturation		95	5 min	1	95	2 min	1
2. PCR	1. Denaturation	94	1 min	25	94		30
	2. Annealing	61	45 sec		61	30 sec	
	3. Extension	72	1.5 min		72	1 min	
3. Final elongation & Hold	1. Final elongation	72	5 min	1	72	5 min	
	2. Hold	4	∞		4	∞	

Msp-2

Stages	**Steps**	**Primary round**			**Nested round**		
		Temperature (^{0}C)	**Time**	**Cycles**	**Temperature (^{0}C)**	**Time**	**Cycles**

Initial denaturation		94	5 min	1	94	2 min	1
2. PCR	1. Denaturation	94	1.5 min	40	94	1.5	30
	2. Annealing	55	45 sec		55	45 sec	
	3. Extension	72	1.5 min		72	1.5 min	
3. Final elongation & Hold	Final elongation	72	10 min	1	72	10 min	
	2. Hold	4	∞		4	∞	

Glurp

Stages	**Steps**	**Primary round**			**Nested round**		
		Temperature (^{0}C)	**Time**	**Cycles**	**Temperature (^{0}C)**	**Time**	**Cycles**
Initial denaturation		95	5 min	1	95	2 min	1
2. PCR	1. Denaturation	94	1 min	25	94	1 min	30
	2. Annealing	58	2 min		58	2 min	
	3. Extension	72	2 min		72	2 min	
3. Final elongation	1. Final	72	10 min	1	72	10 min	

& Hold	elongation						
	2. Hold	4	∞		4	∞	

(c) Microsatellite genotyping

(i) Primer sequences used to amplify *Plasmodium falciparum* isolates

Primer Name	Sequence	Tag	Length of product in 3d7	Chromosome	Strand	Tandem repeats
Neu1_TA1-F_HEX	CCGTCATAAGTGCAGAGC	5HEX	186bp	6	Minus	3
Neu1_TAI_R	TTTTATCTTCATCCCCAC	None			Plus	
Neu1_TA1_F	CTACATGCCTAATGAGCA	None			Minus	
NEU3_POLYA_F	AAAATATAGACGAACAGA	None	151bp	4	Minus	3
NEU3_POLYA_R	ATCAGATAATTGTTGGTA	None			Plus	
NEU3_POLYA_R_FAM	GAAATTATAACTCTACCA	5FAM			Plus	
NEU7_PFPK2-F	CTTTCATCGATACTACGA	None			Minus	

NEU_PFPK2-R	CCTCAGACTGAAATGCAT	None	171bp	12	plus	3
NEU_PFPK2-R-HEX	AAAGAAGGAACAAGCAGA	5HEX			Plus	
NEU9_TA109_F	TAGGGAACATCATAAGGAT	None	174bp	6	Plus	3
NEU9_TA109_R	CCTATACCAAACATGCTAAA	None			Minus	
NEU9_TA109_F_FAM	GGTTAAATCAGGACAACAT	FAM			Plus	
NEU12_2490-F	TTCTAAATAGATCCAAAG	None	83bp	10	Plus	3
NEU12_2490-R	ATGATGTGCAGATGACGA	None			Minus	
NEU12_2490_R_FAM	AGAATTATTGAATGCAC	5FAM			Minus	
313_F_FAM	TCCCTTTTAAAATAGAAGAAA	5FAM	260bp	2	Plus	2
313_R	GATTATATGAAAGGATACATG	None			Minus	
383_F_HEX	AATAGGAACAAATCATATTG	5HEX	173bp	3	Plus	2

383_R	AGATATCCAGGTAATAAA AAG	NON E			minus	

(ii) Master mix components and preparation

Component	**Stock concentration**	**Final concentration**	**Volume for one reaction (µL)**
PCR water	……..	……	10.5
Forward primer	1010 µM	0.2 µM	0.3
Reverse primer	10 µM	0.2 µM	0.3
Buffer	10X	1X	1.5
dNTPs (2 mM), 0.75 $MgCl_2$ (50mM),		200 µM	0.3
Taq Polymerase	5 U/µL	1.5 mM	0.1
Template DNA			2
Final Volume			15 µL

(iii) PCR conditions

Microsatellites: Poly-α, TA1, TA109, PfPK2 and 2490

Stages	**Steps**	**Primary round**			**Nested round**		
		Temperature (^{0}C)	**Time**	**Cycles**	**Temperature (^{0}C)**	**Time**	**Cycles**
Initial denaturation		94	2 min	1	94	2 min	1
2. PCR	1. Denaturation	94	30 sec	25	94	20 sec	25
	2. Annealing	42 40	30 sec 30 sec		45	20 sec	
	3. Extension	65	40 sec		65	30 sec	
3. Final elongation & Hold	1. Final elongation	65	2 min	1	65	2 min	
	2. Hold	4	∞		4	∞	

Microsatellites: C2M34–313, and C3M69–383

Stages	**Steps**	**Temperature (^{0}C)**	**Time**	**Cycles**
Initial denaturation		94	2 min	1

1 step. PCR	1. Denaturation	94	30 sec	5
	2. Annealing	50	30 sec	
	3. Extension	60	30 sec	
2nd step PCR	Denaturation	94	30 sec	40
	Annealing	45	30 sec	
	Extension	60	30 sec	
3. Final elongation & Hold	1. Final elongation	60	2 min	1
	2. Hold	4	∞	

APPENDIX XI: Examiner's letter confirming compliance with corrections

MAKERERE UNIVERSITY
COLLEGE OF HEALTH SCIENCES
School of Biomedical Sciences
Department of Medical Microbiology
P.O. Box 7072 Kampala, Uganda
Tel: +256 414 541830
Fax: +256 414 533033

Your ref:

Our ref: Date: 25th June 2026

The Deputy Principal
College of Health Sciences

Thru: The Dean
School of Medicine

Dear Prof Idro,

Re: Review of Mwesigwa Alex's PhD Thesis, Reg. No. 2019/HD07/23845U

I was requested to oversee the corrections in Mwesigwa Alex's PhD thesis "**Assessment of *Plasmodium falciparum* genetic diversity, multiplicity of infection and genotyping algorithms for distinguishing recrudescence from reinfection in antimalarial drug efficacy studies in Uganda**"

I can confirm that Mr. Mwesigwa made the changes as was recommended by the examinations committee, and merits the award of a Doctor of Philosophy of Makerere University. His thesis flows well and I recommend that he can now proceed with the next steps in completing his PhD work.

I take this opportunity to congratulate Alex and wish him the best of luck in his next endeavours.

Please contact me at once, should you require any further details.

Yours sincerely

Hakim Sendagire MBChB, MSc, PhD
Senior Lecturer
+256 782 466 154
hakimsendagire@gmail.com