GENETIC DIVERSITY OF *ANOPHELES GAMBIAE'S* IMMUNE GENE AND VECTOR COMPETENCE TO *PLASMODIUM FALCIPARUM* IN WESTERN KENYA

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DECLARATION

Candidate

This thesis is my original work and has not been presented for a degree in any other University or for any other award.

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DEDICATION

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ACRONYMS AND ABBREVIATIONS

DBS	Dried blood spots
DNA	Deoxyribonucleic acid
ICEMR	International Center of Excellence for Malaria Rsearch
IRS	Indoor residual spraying
LLIN	Long-lasting insecticide-treated net
LRRs	Leucine rich repeat proteins
LRIM1	Leucine-rich repeat protein 1
LPSs	lipopolysaccharides
MEGA X	Molecular Evolutionary Genetics Analysis across computing platforms
NCBI	National Center for Biotechnology Information
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PGNs	Peptidoglycans
PSC	Pyrethrum spray catches
RT-PCR	Real-time polymerase chain reaction
TEP1	Thioecter containing protein 1
TED	Thioster domain

DEFINITION OF TERMS

Allele	A variant of a specific gene of a locus, on a chromosome
Codon	A sequence of three nucleotides that collectively make up a unit of genetic code in a DNA or RNA molecule.
Haplotypes	An inherited set of DNA variants in a single chromosome
Genetic diversity	Variety and range of genetic characteristics within a population or a species
Genotypes	The genetic makeup of an individual that determines phenotypes
Nonsynonymous mutations	Evolutionary mutation that alter the protein's amino acid sequence and are subjected to natural selection
Synonymous mutations	Neutral evolutionary mutations that do not change the protein's amino acid sequence.
Parsimony informative site	Sites with mutations in the DNA or protein sequence
Population structure	The organization of genetic variation
Vector competence	Ability of *Anopheles* vectors to transmit a *Plasmodium* parasites to a susceptible host, typically a human or mosquito

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ABSTRACT

Evolutionary pressures may introduce mosquito genotypes that adapt to new breeding habitats, favoring the selection of efficient malaria vectors increasing the risk of malaria transmission. Consequently, *Plasmodium falciparum* has evolved to evade the vector defenses mediated by Pfs47, impeding malaria control efforts. This study evaluated the genetic diversity of TEP1 and vector competence to *P. falciparum* in Homa Bay, Kakamega, Bungoma, and Kisumu counties. Cross-sectional entomological assessments and blood screening were conducted from 2017-2020. *Anopheles gambiae* adults and larvae were collected using pyrethrum spray catches (PSC) and plastic dippers, respectively. Adults were identified morphologically and further classified to siblings using polymerase chain reaction (PCR). The TEP1 alleles were determined using restriction fragment length polymorphisms-polymerase chain reaction (RFLP-PCR) and a representative sample sequenced for confirmation. Dried blood spots and slide smears were simultaneously collected from children between 5 and 15 years and tested by PCR and microscopy respectively. Chelex method was used to extract DNA from positive samples by microscopy and then PCR and sequencing were used to genotype Pfs47. Gametocyte carriers selected for membrane feeding experiments were identified using microscopy. Two TEP1 alleles (*S1 and *R2) and three genotypes (*S1/S1, *R2/S1, and *R2/R2) were identified in *Anopheles gambiae* and *Anopheles arabiensis*. Homozygous *S1/S1 and heterozygous *R2/S1 were widely distributed across all sites in both species. No significant difference was observed among populations and between species in TEP1 allele and genotype frequencies. Low population structure (F_{ST} = 0.019) across all sites corresponded to an effective migration index (Nm = 12.571) and low Nei's genetic distance values (<0.5) among subpopulation. Thirteen Pfs47 haplotypes were observed. Haplotype diversity ranged from 0.69 to 0.77 and nucleotide diversity; 0.10 to 0.12 across all sites. All sites displayed negative and significant Fu's Fs statistical values. The genetic differentiation index was not significant (F_{ST} = -0.00891, P>0.05) among *Plasmodium* populations The malaria infection prevalence in Chulaimbo was 19.7% (95% Cl: 0.003 – 0.007), 95% of the infections were *P. falciparum*. 16.9% had confirmed gametocytes. Six infectious haplotypes were identified out of 24 sequenced *P. falciparum* gametocyte-containing samples. Haplotypes with the L240I mutation were the most prevalent, with a frequency of 29.2%, then E27D with a frequency of 20.8%. The E27D and L240I mutation-carrying haplotypes had frequencies of 16.7%, while haplotypes with mutations S98T0 and E188D had frequencies of 4.2% and 8.3%, respectively. Haplotypes associated with positive midguts exhibited increased infectivity. These genes are potential targets for transmission blocking and should be considered when designing control interventions that focus on vectors expressing the resistant allele and prevalent infectious Pfs47 haplotypes

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Selection pressures such as environmental modifications are linked to an upsurge in the spread of malaria by providing suitable breeding environments for the vector's immature stages. The impact of these modifications on transmission varies with season and geographical locations depending on the existing local mosquito populations, disease prevalence and control interventions implemented. The successful transmission of *P. falciparum* by *Anopheles* mosquitoes depends on compatible vector-parasite combinations determined by complex interactions between the parasite and its vector (Lambrechts *et al.*, 2007) and vulnerability of these encounters with *Plasmodium* infections varies between species (Molina-Cruz *et al.*, 2015; Canepa *et al.*, 2016; Eldering *et al.*, 2016). *Plasmodium* parasites on the other hand adapt to diverse local *Anopheles* genotypes to successfully transmit malaria (Molina-Cruz and Barillas-Mury, 2014). Currently, at least 70 distinct species of *Anopheles* species are responsible for transmitting *Plasmodium* parasites around the world (Sinka, 2013).

Anopheles gambiae efficiently transmit malaria in sub-Saharan Africa. The complement-like thioester–containing protein 1 (TEP1) is an important mosquito immune gene effective in eliminating *Plasmodium* parasites and other pathogens by triggering responses to invaders thus determining vector competence (Blandin *et al.*, 2004; Blandin *et al.*, 2009; Volohonsky *et al.*, 2017; Levashina and Baxter, 2018) as well as playing a significant role in the mosquito male fertility by wearing off wornout sperm cells (Pompon and Levashina, 2015) in *An. gambiae.* The TEP1 gene is located on chromosome 3L and codes for a 1338 amino acid long protein. Allelic variations displayed in TEP1 within *Anopheles* species show differences in susceptibility to infections (Obbard *et al.*, 2008; Blandin *et al.*, 2009; Eldering *et al.*, 2016). The maintenance of TEP1 allele classes in specific *Anopheles* species vary with location and are driven by unknown environmental factors and hence the parasite adaptations. These factors may cause vectorial rearrangement exerting selection pressure that could change TEP1 allele frequencies and subsequently have implications on malaria transmission. Heterogeneity within vector populations can therefore shape parasite dynamics. However, ecological factors that naturally shape the genetic variability of TEP1 directly influencing vector competence is unclear (White *et al.*, 2011; Mancini *et al.*, 2015).

High densities of local vector populations could encourage the emergence of malaria vectors that are resistant or vulnerable to human *Plasmodium* infections. Genotyping the TEP1 protein in *An. gambiae* is therefore useful for surveillance especially in vectors that could influence the spread of malaria. *Plasmodium falciparum*, on the other hand, has adapted to counteract the mosquitoes' immune responses that are initiated by the Pfs47 gene. The *P. falciparum* parasite's Pfs47, located on chromosome 13, is responsible for its ability to elude the mosquito immune responses. It is a member of the 6-cystine protein family expressed on the female gametocytes and ookinetes surface (van Schaijk *et al.*, 2006). The Pfs47 exhibits significant polymorphisms, with various haplotypes worldwide implying a strong geographical genetic structure (Anthony *et al.*, 2007; Manske *et al.*, 2012). Parasites strains that express the *Pfs47* haplotypes resistant to TEP1 mediated-killing mechanisms increase infectivity (Eldering *et al.*, 2016). Consequently, interactions between diverse Pfs47 haplotypes and TEP1 allelic variations are important determinants of malaria infections in *Anopheles* vectors. Furthermore, interactions between *Anopheles* mosquitoes and *Plasmodium* parasites are a potential target for blocking transmission. As a result, genotyping local populations is critical for monitoring changes in mosquito densities that may explain variations in malaria prevalence across settings. This study established the distribution and genetic diversity of TEP1 in *An. gambiae* and vector competence to *Plasmodium* parasites in western Kenya.

1.2 Statement of the problem

Evolutionary forces including insecticide-based vector interventions, climate change and environmental modifications cause the selection of vector genotypes that are adapted in new breeding habitats, resulting in vectorial rearrangement, which exerts selection pressure on the TEP1 alleles. Hence, efficient vectors could thrive and continue to transmit malaria. Consequently, the migration of vectors between regions may introduce new Pfs47 variants into local vector populations, which causes geographical variation and the emergence of unique polymorphisms specific to each area. Despite the high mosquito densities, successful transmission is however dependent on infective *Plasmodium* parasites and competent vectors, which influence susceptibility to infections in local mosquito populations and, ultimately, malaria transmission dynamics.

1.3 Justification of the study

Allele variations of TEP1 play an important role in the elimination of *Plasmodium* parasites, influencing the vector's ability to transmit malaria. *Plasmodium falciparum*, on the other hand, is particularly adept at avoiding the mosquito's immune system, aided by Pfs47. The selection of certain mosquito genotypes by distinct *P. falciparum* haplotypes varies by geographic region resulting in significant variability in malaria transmission rates. However, the genetic processes governing *Plasmodium* infections and how mosquito genotypes influence the parasite's adaptations to distinct *Anopheles* species remain unknown. Understanding the associations between vectors and parasites are a potential target for vector control interventions. The findings will enhance a better understanding of the transmission dynamics and contribute to the development of targeted control and management strategies for malaria vectors.

1.4 Research questions

i. What is the distribution of TEP1 alleles in *Anopheles gambiae* in western, Kenya?

ii. What is the genetic diversity of *Pfs47* in *Plasmodium falciparum* in western, Kenya?

iii. What is the difference in infectivity of *Pfs47* haplotypes to *Anopheles gambiae* mosquitoes?

1.5 Null hypotheses

i. There is no difference in the distribution of TEP1 alleles in *Anopheles gambiae* in western Kenya.

ii. There is no genetic diversity of *Pfs47* in *Plasmodium falciparum* in western Kenya.

iii. There is no difference in infectivity of *Pfs47* haplotypes to *Anopheles gambiae.*

1.6 Objectives of the study

1.6.1 General objective

To evaluate the genetic diversity of *Anopheles gambiae* immunity gene
and vector competence to *Plasmodium falciparum* in western Kenya

1.6.2 Specific objectives

i. To determine the distribution of TEP1 alleles in *An. gambiae* in western Kenya
ii. To determine the genetic diversity of *Pfs47* in *P. falciparum* in western Kenya
iii. To determine the difference in infectivity of *Pfs47* haplotypes to *Anopheles gambiae*

1.7 Significance of the study

Understanding vector-parasite interactions, which have a direct effect on malaria transmission, requires knowledge of the polymorphisms in the TEP1 and *Pfs47* genes. Variations in the immunity gene TEP1 that affects vector competence have been linked to *Anopheles* mosquito resistance to *Plasmodium* parasites. The geographical distribution of TEP1 in *Anopheles gambiae* will inform researchers on interventions such as the use of transgenic mosquitoes expressing the TEP1*R allele that will prevent the growth of parasites in the vectors. *Plasmodium falciparum* on the other hand, facilitates effective transmission mediated by the *Pfs47* gene by disguising the parasite from mosquito immune receptors, hence important determinants of transmission. The development of ookinetes is inhibited by antibodies against *Pfs47* D2, which exhibit potent transmission-blocking activity independent of the human complement. Identifying the *Pfs47* haplotypes and their infectiousness to *An. gambiae* will act as a basis for potential transmission blocking interventions using gene drive systems. This study finding will therefore inform policy and practices for targeted vector control interventions that could complement existing techniques.

CHAPTER TWO

LITERATURE REVIEW

2.1 Recent Malaria trends

Globally, there was an estimated 241 million cases of malaria in 85 countries in 2020, up from 227 million cases in 2019, with most of the rise in occurrences originating in WHO African regions. The WHO African region, with an estimated 228 million cases in 2020, accounted for about 95% of cases and 627,000 malaria deaths with children below the age of 5 years accounting for 80% of the deaths. Malaria case incidence reduced from 59 in 2015 to 56 in 2019, before rising again to 59 in 2020 as a result of disruption to services during the COVID-19 pandemic (WHO, 2020). Sub-Saharan Africa accounted for 94% of reported cases in 2020; nonetheless, the WHO still considers the Eastern Mediterranean, South-East Asia, the Americas, and the Western Pacific to be at risk (WHO, 2020a). *Plasmodium falciparum* infections were the primary cause of the majority of reported cases in Africa (99.7%), the Eastern Mediterranean (71%), the Western Pacific (65%), and South-East Asia (50%) (WHO, 2020a). Malaria primarily affects underdeveloped areas in the world. Since more than 70% of Kenya's population is still at risk of infection, thus malaria continues to be a serious public health problem in Kenya (Division of National Malaria Programme Kenya (DNMP, 2021; USAID, 2022).

Five distinct species of the *Plasmodium* parasites have the ability to infect people including *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae.* A fifth species *Plasmodium knowlesi*, a fifth species, has been shown to have the ability to infect both monkeys and humans (Sabbatani *et al*., 2010). *Plasmodium falciparum* and *Plasmodium vivax* are widely recognized as the predominant etiological agents responsible for malaria in human populations. However, it is important to note that these two species exhibit distinct clinical manifestations and exhibit varying patterns of regional prevalence. The parasite *P. falciparum* is attributable for inducing severe illnesses within human populations. Malaria is most widespread in the western and coastal regions of the country, where the environment is warm and humid, providing suitable conditions for malaria vectors to breed. The prevalence along the lake regions is 27% majorly transmitted by *An. gambiae* s. l and *An. funestus* (Githeko *et al.,* 2012; Afrane *et al.,* 2016; Ogola *et al.,* 2019). The species occur throughout the year with peak populations coinciding with rising temperatures, rainfall patterns and human activities within the regions (Alonso *et al.*, 2011; Kapesa *et al.*, 2018; Krsulovic *et al.*, 2022). *Anopheles gambiae sensu lato* is a dominant and efficient malaria vector that constitutes of a complex morphologically indistinguishable species (Lanzaro and Lee, 2013). Infections in the western Kenyan highlands are characterized by high transmission, which varies significantly and is dependent on favorable vector breeding and malaria parasite transmission factors (Hay *et al.*, 2002; Hay *et al.*, 2005; Kapesa *et al.*, 2018). The primary option therefore is to control these vectors so as to reduce malaria incidences.

2.2 Plasmodium's life cycle in the mosquito and vertebral hosts

The *Anopheles* mosquito is held accountable for spreading malaria, caused by a eukaryotic Protist species, *Plasmodium.* It is most prevalent in tropical regions. The complex infection cycle begins with sporozoites being inoculated during feeding by an infected female *Anopheles*. The sporozoites penetrate in greater numbers (Mota *et al.*, 2001). Traversal movement in the liver occurs prior to the sporozoites' aggressive invasion (Vaughan and Kappe, 2017). Upon encountering a host, sporozoites establish a parasitophorous vacuole membrane (PVM) and undergo schizogony, a process in which they divide many times to produce offspring merozoites. These merozoites are then discharged into the bloodstream inside specialized compartments called merosomes (Venugopal *et al.*, 2020). Merozoites start a continual cycle of asexual reproduction in the bloodstream when they come into contact with erythrocytes. The merozoites that reproduce asymptotically will go through gametocytogenesis. Once they reach maturity, they move into peripheral circulation. The extracellular male and female gametes are formed in the midgut after the gametocytes have been ingested (Figure 2.1). A zygote is created when the macrogamete and microgamete fuse. The zygote matures into elongated, motile ookinetes, which travel via the midgut epithelium to produce oocysts, a tough, thick-walled stage of the parasite life cycle, below the basal lamina (Smith *et al*., 2014) Sporozoites in high numbers are released upon maturation and subsequent rupture of the oocysts, invading the hemolymph and subsequently inhabiting the salivary glands. Mosquitoes act as intermediate hosts, consuming the sporozoites before transmitting them to new hosts (Cirimotich *et al.*, 2010; Smith *et al.*, 2014)**.**

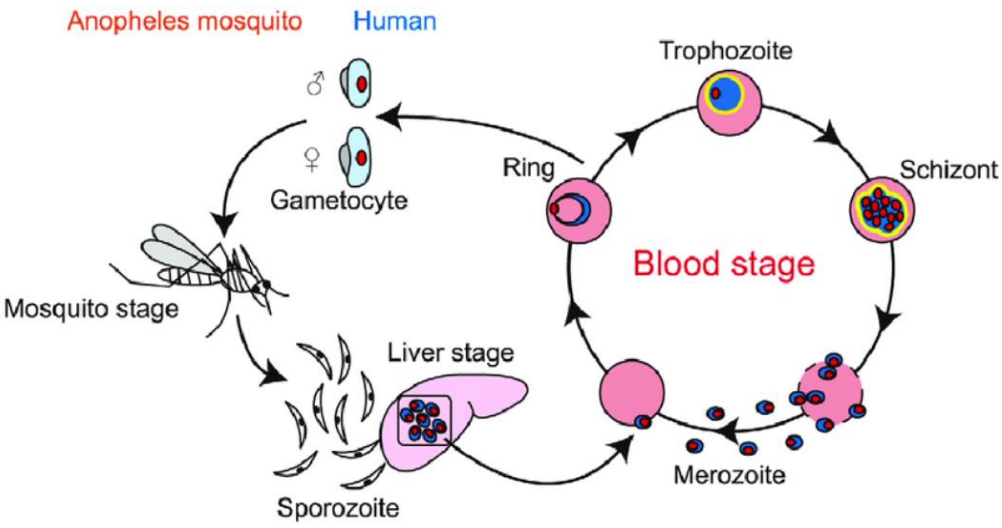

Figure 2.1: Life cycle of Plasmodium falciparum in the human definitive host and the mosquito intermediate host
(Niaré *et al*., 2002).

2.3 Plasmodium falciparum surface protein 47 (Pfs47)

Plasmodium falciparum is a predominant etiological agent accountable for malaria incidences throughout Africa (Gething *et al.*, 2012). Human migration from Africa to other continents may have contributed to the spread of this parasite, which has resulted in a worldwide occurrence of malaria (Liu *et al.*, 2010; Molina-Cruz *et al.*, 2016; Loy *et al.*, 2017). The parasite has genetically evolved to avoid many *Anopheles* mosquitoes mediated by Pfs47 surface protein compromising malaria control efforts. The Pfs47 gene facilitates *P. falciparum* to circumvent vector immune detection by a combination of genomic mapping, linkage group selection, and functional genetics (Molina-Cruz *et al.*, 2013). Furthermore, efficient transmission of *P. falciparum* parasites is contingent upon the compatibility between the Pfs47 haplotypes and TEP1 genotypes of local mosquito vector species within a particular continent and may be the cause of the strong geographical population structure worldwide (Molina-Cruz *et* *al.*, 2020).

Sequence similarities may vary between Pfs47 and other family of proteins with six-cysteine (6-Cys) including Pfs230 and Pfs48/45, influencing parasite fertility (Anthony *et al.*, 2007) and also serve as important targets for transmission blocking vaccines (Nikolaeva *et al.,* 2015; Arredondo and Kappe, 2017). The gamete surface protein *Pfs47* may be under selection for polymorphisms since *P. falciparum* demonstrates an enhanced ratio of non- synonymous to synonymous polymorphisms (Anthony *et al.*, 2007). The *Pfs47* genes in the GB4 and 7G8 *P. falciparum* parasite strains change only in four amino acids (T236I, S242L, V247A, and I248L), which determines the GB4 survival in the *An. gambiae* resistant strain (Anthony *et al.*, 2007). Majority of amino acid variations are found between two cysteines, separated by 30 amino acids (aa), in the subsequent domain (D2) of Pfs47. The Pfs47 surface protein directly interacts with the vector midgut proteins enabling evasion and subsequently the survival and spread of the disease(Degefa *et al*., 2017). Its potential in evading the mosquitos' immune responses makes it a target for innovative interventions for blocking malaria transmission. There are 42 different Pfs47 haplotypes reported in *P. falciparum* isolates globally; 32 of which are located in Africa, 1 is located in Papua New Guinea, 6 are located in Asia, and 3 in the Americas (Molina-Cruz *et al.*, 2015).

According to recent research, the same *Pfs47* haplotypes reported in western Kenya were also found in Brazil in South America, west and south Africa, and Papua New Guinea (Onyango *et al.,* 2021). There were four more *Pfs47* haplotypes found in Peru (the African control in this study) shared the same structural traits as Peru type 2, while the *Pfs47* Brazilian control shared traits with Peru type 1 (Villena *et al.*, 2021). These results corroborate previous research showing that Pfs47 has a high global population structure and is highly differentiated geographically, with fixation indices ranging from 0.60 to 0.88 (Anthony *et al.*, 2007; Molina-Cruz and Barillas-Mury, 2014; Molina-Cruz *et al.*, 2015; Molina-Cruz *et al.*, 2016). Consequently, it might be a possible candidate for blocking transmission within the vector.

2.4 Thioester-containing protein 1

Thioester-containing proteins (TEPs) are large (>100 KDa) glycoproteins discharged by hemocytes into the hemolymph (Blandin *et al.*, 2004). *Anopheles gambiae* has fifteen TEPs (TEP1 – TEP15), most come from species-specific expansions. It has been shown that TEPs are important for vector defenses (Christophides *et al.*, 2004). The highly reactive thioester motif is exposed by infection-induced proteolytic cleavage of TEP1, which targets the microbe and causes covalent attachment. *Anopheles gambiae* vectors contain a number of immunological TEPs, but TEP1 has received the most interest and has lately been investigated in additional *Anopheles* species vectors due to its ability to specifically kill *Plasmodium* parasites (Bartilol *et al.*, 2022b). The major regulator in *Anopheles* - *Plasmodium* interactions, which contributes to significant parasite losses in the vector, was found via functional experiments conducted in *An. gambiae*. *Plasmodium* ookinetes are specifically targeted for elimination by TEP1 in the basal lamina of the midgut epithelium (Crompton *et al.*, 2014).

The REL1/Toll, REL2/IMD, and JNK signaling pathways control TEP1, demonstrating its important function in immune defenses in the mosquito after which TEP1 attaches to the ookinetes surface forming a complex with leucine rich repeat proteins (LRRs) such as leucine-rich repeat protein 1 (LRIM1), and *Anopheles plasmodium*-responsive leucine-rich repeat protein 1 (APL1) (Schultz *et al.*, 2010) **(**Figure 2.2**)**. The peptide is released in an inactive form as a single chain and then undergoes activation by proteolytic cleavage (Clayton *et al.*, 2014). The leucine-rich repeat complex formed by the proteins LRIM1 and APL1C stabilizes the activated TEP1 protein after which TEP1 binds to bacteria in the hemolymph and ookinetes in the midgut, neutralizing the parasite (Fraiture *et al.*, 2009; Povelones *et al.*, 2009).

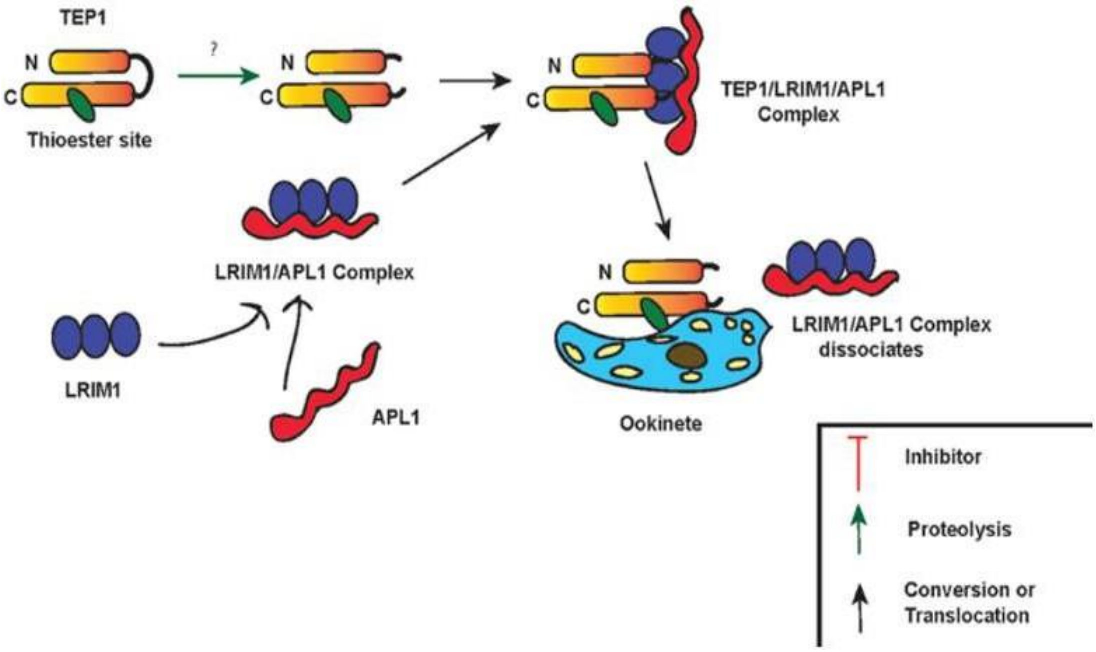

Figure 2.2: Thioester-containing protein 1 activity in the mosquito.

Thioester- containing protein 1 is triggered in the hemolymph and cut by unknown proteases to form two fragments; TEP1-N and TEP1-C which remain connected after proteolytic cleavage. The two LRR proteins (LRIM1 and APL1) maintain TEP1 maturity forming a complex. The TEP1 then recognizes the *Plasmodium* parasite and separates from the LRR proteins by an unidentified process before binding to and killing the parasite (Shokal and Eleftherianos, 2017).

Thioester-containing protein 1 is structurally and functionally similar to complement factor C3 (TED) and consists of eight MG domains, a linker (LNK)/protease-sensitive loop, a CUB domain, and a thioester (TED). The absence of the complement component anaphylatoxin domain in TEP1 suggests that TEP1 activation is distinct from that of vertebrate complement factors (Baxter *et al.*, 2007). The TEP1 gene has two distinct allele classes; TEP1*S and TEP1*R, which are susceptible and resistant to *Plasmodium* infections respectively, causing significant variation among vector populations (Blandin *et al.*, 2004; Blandin *et al.*, 2009; White *et al.*, 2011; Eldering *et al.*, 2016). Additionally, vectorial capacity of TEP1 is linked to allelic divergence (Rono, 2017; Gildenhard *et al.*, 2019). For instance, TEP1*R1 is linked to increased refractoriness to infection as compared to the susceptible alleles (TEP1*S1-S3) (Blandin *et al.*, 2009). These variations may result from selective pressures acting on the TEP1 gene which ultimately influence vector competence.

Knockdown studies using double-stranded RNA further support the distinct functions of TEP1 in parasite killing. Thus, a five-fold decrease in oocyst densities was seen after silencing TEP1, which added the *P. berghei* oocysts proportion of developing in the midgut in vulnerable mosquitoes but showed no effect on refractory insects (Blandin *et al.*, 2004). The distribution of TEP1 alleles in the *An. gambiae* complex across Africa has been characterized (Gildenhard *et al.*, 2019; Bartilol *et al.*, 2022b; Onyango *et al.*, 2022), yet, not much is known about how these allelic variants functionality influence vector competence and, ultimately, malaria transmission. Henceforth, understanding the molecular pathways driving TEP1-mediated immune defenses is crucial, particularly those that affect vector competence and may lead to targeted vector-based malaria prevention.

2.5 Vector-parasite interactions in *Anopheles* species

Anopheles gambiae complex is primarily accountable for transmitting *Plasmodium falciparum* in most parts of Africa. The nature and intensity of mutual vector-parasite interactions during different phases impact the success of transmission. However, the abundance and diversity of malaria parasites and vectors make it difficult to comprehend these complex interactions between *Plasmodium* and *Anopheles*. Furthermore, factors that influence the success of infection in vectors has recently attracted a lot of interest (Sinden *et al.,* 2004; Cirimotich *et al.*, 2010; Le *et al.*, 2012; Molina- Cruz *et al.,* 2012; Redmond *et al.,* 2015; Bennink *et al.*, 2016; Lefevre *et al.*, 2018;), but it is still unclear what causes variation in other important aspects of transmission.

Vector competence is a combined assessment of a vectors' susceptibility to infection and parasite infectivity to its host. It involves both parasite adaptations against the vector's defenses and mosquito infection- fighting mechanisms. For instance, different *Anopheles gambiae* strains exhibit a variety of susceptibility for specific parasite genotypes, and diverse *Plasmodium* isolates similarly differ in their infectivity to certain mosquito strains (Blandin *et al.*, 2009; Harris *et al.*, 2012; Molina-Cruz *et al.*, 2012; Molina-Cruz *et al.*, 2015; Molina-Cruz *et al.*, 2020;), however, not all interactions are incompatible, some represent mosquito immune evasion in its vector (Molina-Cruz *et al.*, 2020). Also, despite numerous research showing vectors and parasite genetic associations, knowledge of this interactions and transmission dynamics in the field is lacking. Crompton *et al* (2014) demonstrated that *Anopheles* vectors and *P. falciparum* parasites are immunological targets with different and stage-specific responses. Several barriers must be overcome before the *Plasmodium* parasite establishes an infection in its vector (Lombardo and Christophides, 2016). Moreover, mosquitoes are continually exposed to infection mainly during a blood meal and within minutes of exposure, they develop an innate humoral and cellular immune response that eliminate pathogens through lysis, melanization and hemolytic mediated phagocytosis to fight infections (Hillyer *et al.*, 2007). Aside from phagocytosis, other immune effectors produced by hemocytes and fat bodies into the hemolymph, Nitric oxide (NO)-mediated killing mechanisms and oxidative defenses are also implicated in nodule formation, agglutination, and encapsulation according to Osta *et al* (2004), which are triggered through various immune signaling pathways (De Gregorio *et al.*, 2002; Garver *et al.*, 2012; Garver *et al.*, 2013; Clayton *et al.*, 2014; Ramphul *et al.*, 2015).

2.5.1 Signaling pathways for Anti-Plasmodial Immunity

Mosquitoes are protected by signaling pathways, which also regulate dangerous microbes. Antimicrobial peptides (AMPs) are proteins that may be synthesized in response to pathogen invasion and may help neutralize the invaders. Once a mosquito is infected by a pathogen, an immune response starts with the host's specific proteins known as pattern recognition receptors (PRRs) that detect the infectious pathogen (Janeway and Medzhitov, 2002) by binding to conserved molecular structures called PAMPs. The PRRs then transfer the immunological signal to other components of the immune responses. Insect pathogens respond to a wide variety of PAMPs, such as peptidoglycans (PGNs), lipopolysaccharides (LPSs), and b-1, 3 glucans, which are shared among both pathogenic and non-pathogenic microbes. Consequently, signaling molecules are triggered after pathogen detection prompting them for appropriate immune responses. Additionally, their corresponding PAMPs have not been clearly established yet the fact that mosquito innate immune responses to parasitic infections are triggered is evidence that they actually exist (Buchon *et al.*, 2009). Several signaling pathways that involve vector-pathogen interactions are then activated during the elimination processes. The primary signaling mechanisms in defenses against *Plasmodium* parasites are the Toll, Immunodeficiency (IMD), and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways (De Gregorio *et al.*, 2002).

2.5.1.1 Toll and Immunodeficiency (IMD) immune signaling pathways

Anti-*plasmodium* immune defenses are primarily controlled by the Toll and IMD pathways that target the ookinetes stage of *Plasmodium* (Frolet *et al.*, 2006). Besides *Plasmodium* parasites, they are also elicited by bacteria, fungi, and viruses (Ramirez and Dimopoulos, 2010). The transcriptional regulation of the splicing factors Caspar and IRSF1 by the Toll and IMD pathways in *Anopheles* vectors determines the synthesis of pathogen-specific connector variant sequences of the hyper-variable pattern recognition receptor *AgDscam*, hence mediating species-specific defenses against *Plasmodium* and bacteria (Figure 2.3) (Dong *et al.*, 2012). Pathogen-associated molecular patterns (PAMPs) and their encounters with pattern recognition receptors (PRRs) activate transcription factors Rel1 and Rel2 in the Toll and IMD pathways, respectively. As a result, NF-*k*B regulates immune effector genes transcription that are then introduced into the nucleus (Cirimotich *et al.*, 2010; Valanne *et al.*, 2011). The thioester-containing protein 1 (TEP1), leucine-rich repeat protein members (LRRD7/APL2) and fibrinogen immunolectin 9 (FBN9) are important immune effector genes in the killing of malaria parasites in *An. gambiae* Keelen strain (Garver *et al.*, 2009; Dong *et al.*, 2012). Limitations of understanding signaling pathways is that most of research is conducted using rodent parasites though the mechanisms may not be the same in eliminating *P. falciparum*. The most effective approach for getting rid of *P. falciparum* parasites is through the IMD signaling pathway unlike Toll and other pathways that were explored to identify mechanisms in which pathogens are dealt with within the vectors (Cirimotich *et al.*, 2010).

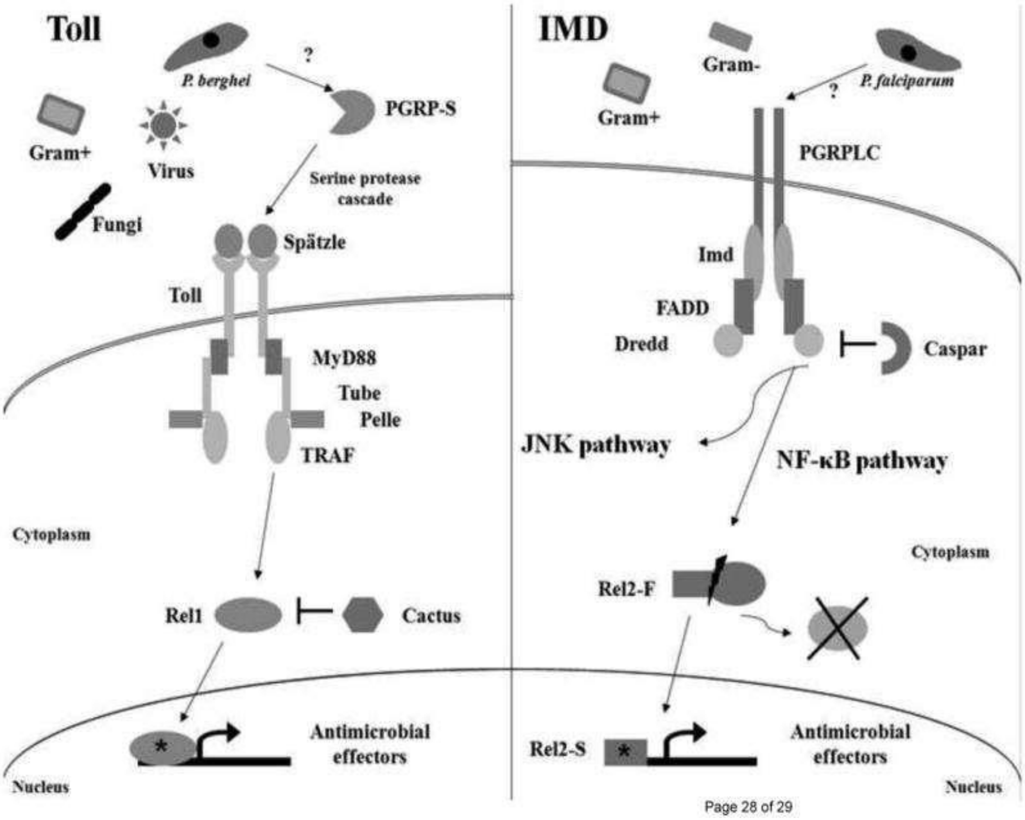

Figure 2.3: The Toll and IMD signaling pathways involved in the mosquitos *Plasmodium* defenses.

The Toll pathway is activated when the ligand Spätzle binds to the Toll transmembrane receptor after being recognized by soluble (Peptidoglycan recognition proteins) PGRP molecules as a microbe or an unidentified *Plasmodium* ligand. This sets off a chain of biochemical processes that leads to the activation (*) and nuclear translocation of Rel1, upregulating the transcription of immune genes that are in charge of destroying invading pathogens. The IMD pathway is activated when the transmembrane PGRPLC receptor binds to a peptidoglycan or an unidentified *Plasmodium* ligand, causing Rel2-F to be cleaved and active Rel2-S (*) transferred into the nucleus. When the IMD pathway is activated, a separate group of *anti-Plasmodium* genes are up-regulated responsible for eliminating *Plasmodium* parasite (Cirimotich *et al*., 2010).

2.5.1.2 Janus Kinase signaling pathway (JAK-STAT)

The Janus Kinase (Jak) enzymes and the transcription factors (STAT) are responsible for controlling the initiation of the Janus Kinase pathway. Immune responses to viruses in *Drosophila melanogaster* and *Aedes aegypti* are also known to be strongly influenced by the Jak/Stat system according to Buchon *et al.* (2009) and Souza-Neto *et al* (2009). The interactions between *Anopheles* and *Plasmodium* have, however, received little study. Moreover, the *An. gambiae* genome has two Stat transcription factors, STAT-A and STAT-B. The transcription factor STAT-A, whose activity is controlled by STAT-B, is a key part of the mosquito's defenses against *Plasmodium* parasites (Christophides *et al.*, 2002). In the mosquito's defense against *Plasmodium*, the transcription factor STAT-A, whose activity is regulated by STAT-B, is key.

The JAK-STAT pathway is triggered by the binding of cytokine ligand UPD to the transmembrane receptor DOME and the eventual nuclear translocation of STAT-A into the nucleus. As a result, a transcriptional activation of anti-P*lasmodium* effector is expressed. The mechanism mediates *P. falciparum* and *P. berghei* parasites killing at a later stage of infection, following midgut invasion in *An. gambiae* mosquitoes (Gupta *et al.*, 2009). Although STAT-A depletion enhanced *P. berghei* oocyst intensity, depletion of the negative regulator SOCS activated the pathway and reduced infection levels (Gupta *et al.*, 2009). More research is needed on the involvement of the Jak/Stat pathway in triggering *Anopheles* immunity against the malaria parasite. This immune mechanism can be considered as a potential target for the invention of malaria control strategies, together with the Imd/REL2 and Toll/REL1 pathways.

Additional processes have also been connected to mosquitos' resistance to *Plasmodium* infections (Surachetpong *et al.*, 2009). The precise mechanisms by which the pathways distinguish between multiple activation tactics, how those alternate techniques affect downstream gene transcription, and how they affect microbial elimination are unknown. Furthermore, there's dearth knowledge of the time and location at which these pathways in the mosquito's body are engaged, as well as which stage of the parasite the reaction is aimed at. As a result, immunological signaling pathways are prospective targets for genetic alteration to produce mosquitoes with powerful immune systems that can eliminate parasites.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

This study was executed in Bungoma, Kakamega, Kisumu, and Homa Bay counties with various endemicities and ecosystems (Figure 3. 1). These counties' continuous research on vector ecology and malaria epidemiology resulted to the selection of five sentinel sites namely Kimaeti located at 00.54058°N, 034.56411°E, with an altitude ranging from 1386 to 1,545 meters above sea level in Bungoma county, Iguhu located at 34°45′E, 0°10′N; and an elevation of 1430–1580 meters above sea level in Kakamega county, Kombewa (34°30′E, 0°07′N; 1150–1300 meters above sea level) and Chulaimbo (0.03572°S, 34.621°E) in Kisumu county, and Kendu Bay located at 34.64190°E-0.38000°S; with an altitude of 1134–1330 meters above sea level in Homa Bay county. Incidences of *Plasmodium falciparum* is particularly high in the studied regions, which is mostly transmitted by *Anopheles gambiae, Anopheles funestus,* and *Anopheles arabiensis* (Bayoh *et al.*, 2011; ; Ochomo *et al.*, 2013; Zhong *et al.*, 2020; Debrah *et al.*, 2021).

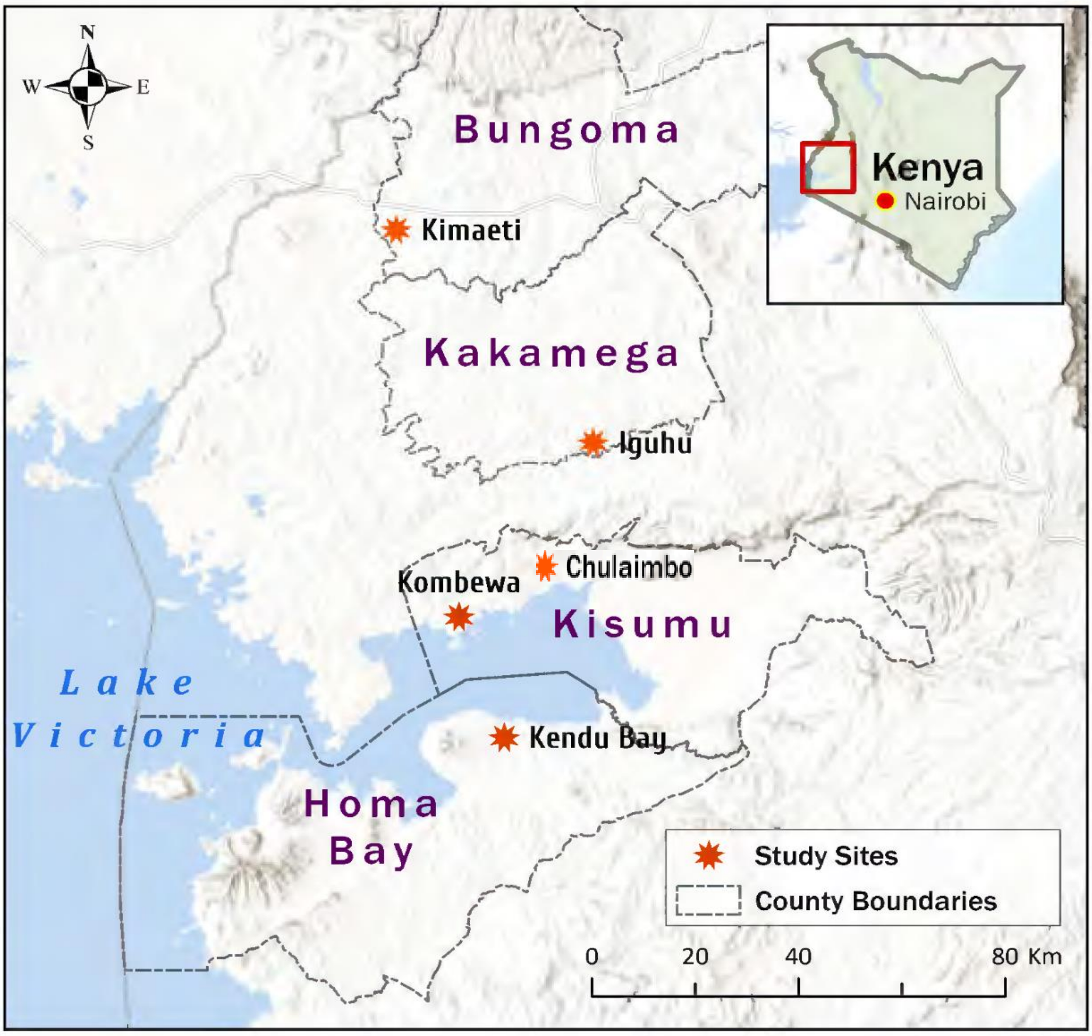

Figure 3. 1: A map showing the locations of the research sites in western Kenya

3.1.2 Kimaeti study site

Kimaeti is a malaria epidemic prone highland region. Malakasi and Myanga markets in Bungoma County are to the north and south of Kimaeti, respectively. It is a well-liked region recognized for its big animal market and religious activities. Kimaeti exhibits a bimodal precipitation pattern, characterized by a prolonged period of rainfall spanning from April to July. These months also happen to be when malaria is most prevalent. Additionally, Kimaeti experiences a brief wet season occurring between October and

November. In January through March, during the hot and dry season, transmission is at its lowest. Daily temperatures range from 25.2^0C to 15.6^0C, and an average of 1573mm of rainfall every year. The most dominant malaria vector species are *Anopheles gambiae* and *Anopheles funestus*. Kimaeti engages in considerable tobacco growing, as evidenced by the vast tobacco fields and numerous drying kilns in the region.

3.1.3 Iguhu study site

Iguhu is a malaria epidemic prone highland in Kakamega County, located at latitude 010'.8" N, longitude $34^0$45'.3" E, and an elevation of 1,430-1,580 meters above sea level (Munyekenye *et al.,* 2005). The Yala River cuts through Iguhu, which is characterized by low, flat valleys that become inundated during the wet season. This site receives an average of 2,000 millimeters of precipitation per year, and experiences extended rains between March to May, and sporadic rains in October to December. This region's average annual temperature is 20.8 degrees Celsius (Githeko *et al.,* 2006). Most wetlands have been converted into farmlands (Ndenga *et al.,* 2006). The river banks have remnant forest from a bigger forest destroyed for habitation whereas the major malaria transmitters in this site are *An. gambiae* s. l and *An. funestus*.Ondeto *et al*. (2017)

3.1.4 Kombewa study site

The semi-arid region of Kombewa in Central Seme, Kisumu County, is prone to malaria because of its poor drainage and sometimes flowing swamps. Located at an altitude of 1,150–1,300 m, its coordinates are 0°07'N, 34°30'E. The average yearly rainfall is 1,400 mm, between April and May and October and December. Temperature ranges between 18.5^0C and 29.7^0C. Predominant vectors in this site are

An. arabiensis, *An. gambiae s. s* and *An. funestus*. Most of the residents are either fisherman or small-time merchants.

3.1.5 Chulaimbo study site

Chulaimbo is situated in the lake region 35 kilometers west of Kisumu City (0.03572°S, 34.621°E). It is an area where *Plasmodium falciparum* transmission is stable and endemic. The transmission is moderate in this area. The elevation ranges from 1,328 to 1,381 meters above sea level, and an average rainfall of 1200mm to 1300mm per year. Temperatures range from 20^0C to 35^0C, and humidity is between 50 and 68%. The residents are small- scale subsistence farmers who cultivate crops like maize, beans, and vegetables as well as keep livestock. *Anopheles arabiensis* and *An. gambiae s. s* are the major malaria vectors in this site.

3.1.6 Kendu bay study site

Kendu Bay, located between 1,134 - 1,330 meters above sea level in the Winam Gulf of Lake Victoria, is a lowland region with a semi-arid climate where malaria is endemic. This area relies on the Kimira-Oluch irrigation system, which is located in Rachuonyo North and Rangwe in Homa Bay County. The Kimira-Oluch irrigation system is located in the Rachuonyo constituency, which has a population of 307,126 people but a catchment population of 21,083 (KNSB, 2009). According to the Kenya Information Guide (2015), the area receives 1100mm of rainfall each year with a temperature range of 26^0C to 34^0C and an annual temperature of 22.5^0C. March through May has the heaviest rainfall, while September through November experiences fewer but more intense downpours. Bala hot springs and semi-arid vegetation like euphorbia and cacti are the area's defining features with *An. arabiensis* and *An. gambiae s. s* being the most predominant vectors in the region.

3.2 Study design

Pyrethrum spray captures (PSC) were employed in a cross-sectional investigation to catch *Anopheles* adult mosquitos from thirty selected houses randomly while dippers were used for larval collection from 45 breeding sites per study region. The availability or lack of larvae in each setting was documented after five to twenty dips. Random sampling from a large number of breeding sites was used to increase the likelihood of including varied breeding sources and decrease the likelihood of selecting related larvae. Sampling took place concurrently in February and March during the dry season and again in May, one month following the beginning of the extended rainy season.

A cross-sectional community-based survey was conducted to obtain *P. falciparum* positive participants between the ages of 5and 15 years during the wet and dry seasons. Samples were collected once per season per participant in schools and households. Thick and thin blood smears were collected and screened using microscopy and PCR respectively. Participants who were found positive for *P. falciparum* gametocytes were then enrolled for membrane feeding assays. Their dried blood spots were then used to confirm *Plasmodium* species and to genotype Pfs47.

3.3 Inclusion criteria

All participants who were children aged 5 to 15 whose parents or legal guardians gave consent in writing for them to take part in the current research.

3.4 Exclusion criteria

Children that refused to be enrolled in this research and those whose parents' or guardians' consent was not obtained were omitted.

3.5 Sample size

Fisher's exact formulae (Fisher, 2006) was used to calculate the sample size based on the prevalence of 50% estimation as the proportion.

$$n = \frac{z^2 \times p\,(q)}{d^2}$$

Where:

n = minimum sample size required

z = Z score for normal standard deviation for a 95% confidence interval (1.96)

p = Chance of success due to prevalence variation (0.5%)

q = (1-p)

d = margin of error of the confidence interval at +/- 5%

$$= \frac{(1.96)^2 x 0.5(1-0.5)}{(0.05)^2} = 384 \text{ participants}$$

3.6 Ethical considerations

The research entailed the utilization of human participants for experimental purposes. This work received ethical approval from the Maseno University's Ethical Review Committee (Appendix 1) after which a permit was issued by the National Council of Science and Technology (NACOSTI) (Appendix II). The Kenyatta University Graduate School Board gave their approval to the study proposal (Appendix III). Following an explanation of the study's goals and methodology, the Ministry of Health and local authorities (chiefs and village elders) written approval was also obtained before the study commenced (Appendix IV). Community leaders and study participants received an extensive overview of the objectives, study methods, risks, and benefits prior to the start of data collection. Minor children's parents or legal guardians gave their consent on their behalf. Patients' identities were not recorded to prevent violations of the ethical code. Household heads had the option to drop out of the study at any time.

CHAPTER FOUR

ALLELE DISTRIBUTION OF THIOESTER-CONTAINING PROTEIN 1 IN *ANOPHELES GAMBIAE* IN WESTERN KENYA

4.1 Introduction

Anopheles gambiae mosquitoes are carriers for malaria parasites in sub-Saharan Africa (Sinka *et al.,* 2010; Lanzaro and Lee, 2013). Vector control measures (Gimnig *et al.*, 2003; Zhou *et al.*, 2013), climate change (Lindsay and Birley, 1996; Minakawa *et al.*, 2002; Tanser *et al.*, 2003; Tonnang *et al.*, 2010; Dao *et al.*, 2014), and environmental alterations may lead to the emergence of vector genotypes or species that thrive in new breeding habitats. These variables could exert selection pressure on the TEP1 gene leading to a change in alleles frequency and distribution. As a result, effective vectors might proliferate and keep on transmitting the disease. Although there are increased vector densities, transmission is dependent on availability of infectious parasites. The ability of the vector to disseminate malaria depends on its susceptibility or resistance to infection by the *Plasmodium* parasite, both of which are regulated by TEP1.

The transmission of malaria by *Anopheles gambiae* is subject to the effect of genetic alterations in the TEP1 gene (Sinden *et al.*, 2004; Le *et al.*, 2012). The variations could be as a result of selective forces operating on the TEP1 gene. Two frequent factors that have been identified in relation to the transmission of the *Plasmodium* parasite are climate change and vector control efforts (Le *et al.*, 2012). The TEP1 gene was reported to target the *Plasmodium* parasite in the early stages of infection in the mosquito host mostly the ookinetes (Blandin *et al.*, 2008; Blandin *et al.*, 2009) either by melanization or lysis (Blandin *et al.*, 2004; Volohonsky *et al.*, 2017) effectively reducing oocysts and sporozoite numbers in the vector. The impact of these allelic polymorphisms in vector competence on malaria transmission, however, is poorly understood (Lefevre *et al.*, 2018; Levashina and Baxter, 2018). Furthermore, it is unknown how the TEP1 allele is distributed in parts of western Kenya where malaria transmission intensity varies. In order to monitor infections in vectors that directly affect malaria transmission, it is important to understand the molecular mechanisms governing mosquito genotypes and Plasmodium adaptations to various Anopheles species.

The complement-like thioester-containing protein 1 (TEP1) plays a key role in the immunity against pathogens (Richman *et al.*, 1997; Vizioli *et al.*, 2000; Povelones *et al.*, 2013; Levashina and Baxter, 2018). The TEP1 is a highly polymorphic protein that codes for a 1338 amino acid long protein and is found in the thioester domain (TED) on chromosome 3L. It contributes to phenotypic divergence and exhibits genetic variations linked to different genotypes in its resistance to *Plasmodium* parasites (Obbard *et al.*, 2008; White *et al.*, 2011; Fabrigar *et al.*, 2016;). Recently, six allelic classes have been identified in the African *Anopheles gambiae* complex: TEP1*S1, TEP1*S2, TEP1*S3, TEP1*R1, TEP1*R2, and TEP1*R3 (Blandin *et al.*, 2004; Blandin *et al.*, 2009; Gildenhard *et al.*, 2019). The most prevalent TEP1 alleles discovered in Africa are TEP1*S1 and TEP1*R2. However, the TEP1*S1 lacks a clearly defined geographic structure (Gildenhard *et al.*, 2019). Impaired sperm cells in male mosquitoes are eliminated by the TEP1*S2 allele found in the 4Arr strain, which is unique to *Anopheles coluzzii* (Pompon and Levashina, 2015) bringing forth varying vector population abundance. The G3 strain is linked to infection susceptibility to *P. berghei* via the fixed TEP1*S3 allele, which is closely related to TEP1*S1 (Blandin *et al.*, 2009). According to research conducted in *An. coluzzii* in West Africa, TEP1*R1 found in the L3-5 strain exhibits the highest level of *Plasmodium* resistance and is associated with melanization.(Collins *et al.*, 1986; Blandin *et al.*, 2009; Pompon and Levashina, 2015; Gildenhard *et al.*, 2019). A recently discovered allele, TEP1*R3, is exclusive to the Kenyan coast found *An. merus* saline water mosquito. It is unclear whether selective factors are responsible for these variations in the genetic makeup of native *An. gambiae* populations in various ecological settings. Additionally, if selective pressures directly impact variations in their susceptibility to *Plasmodium* parasites. Genotyping TEP1 in local vector populations is therefore important for monitoring changes in abundance that could explain potential malaria prevalence in varying endemicities and is a potential tool for developing vector control interventions. Furthermore, an improved understanding of the dynamics of malaria transmission would be achieved by determining how changes in the environment, vector control, and underlying molecular mechanisms affect vector competence. The objective of the current research was to contribute to the designing of targeted vector control techniques by mapping the distribution of the TEP1 immunity gene in *An. gambiae* s.l. vectors in malaria-endemic areas of western Kenya.

4.2 Materials and methods

4.2.1 Study sites

This study was conducted in four counties in western Kenya namely, Bungoma, Kakamega, Kisumu, and Homa Bay (Figure 3.1). Two malaria epidemic-prone highland sites including Kimaeti and Iguhu and two lowland sites located around Lake

Victoria; Kombewa and Kendu Bay. The climate in western Kenya consists of long and short rainy seasons that malaria transmission peaks between March to May and October to November respectively. Temperature ranges from a minimum of 14-18^{0}C to a maximum of 30-36^{0}C and average rainfall ranges between 1740mm and 1940mm annually. *Plasmodium falciparum* is the most common cause of malaria and is transmitted by *An. arabiensis, An. gambiae* and *An. funestus* (Githeko *et al.*, 2006; Zhou *et al.*, 2011). The key vector control interventions are long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) (Gimnig *et al.*, 2016). Indoor residual spray was conducted in the entire Homa Bay County once a year in 2017 and 2018 making the Kendu Bay site unique, unlike the other sampling sites.

4.2.2 Study design

Anopheles adults and larvae were collected in a cross-sectional study design from 30 randomly selected houses and 45 breeding habitats per site respectively. Sampling of the adults and larvae were concurrently conducted during the middle of the dry season in February-March and four weeks after the start of the long rainy season in May–July between 2017 and 2020. Collections were conducted between 0630h and 1000h in the morning and transported to the insectary and laboratory at the International Center of Excellence for Malaria Research (ICEMR), Homa bay, Kenya.

4.2.3 Larval sampling and DNA extraction

Larval sampling was conducted using 350 ml standard dippers and hand pipettes (WHO, 1992). A maximum of 10 dips was taken at each habitat and the presence or absence of larvae was recorded. To avoid collecting siblings from the same pool, larvae were randomly sampled from different breeding habitats. Collected larvae were labeled by habitat type and identified morphologically using the referenced keys (Coetzee, 2020), briefly, taxonomic keys were used to identify distinct features including the morphology of the head capsule, the shape and arrangement of the antennae, the structure of the siphon and angle upon which the larvae rests on the water to identify species. Polymerase chain reactions was then conducted using the Chelex resin (chelex® -100); (a styrene divinylbenzene copolymers containing paired iminodiacetate ions) method following a protocol by Musapa *et al* (2013). Briefly, deionized water was added into single mosquito sample tubes and ground into a uniform suspension. Phosphate buffer saline 1X and 10% Saponin was then added to sample homogenates, mixed gently, and incubated at room temperature for 20 minutes. The suspension was then centrifuged and the supernatant discarded. The pellets were then resuspended in PBS 1X and centrifuged, supernatant discarded, and gently vortexed. The pellets were then suspended in sterile deionized water and 20% Chelex-resin suspension in deionized water. The samples were incubated at 85°C for 10 minutes, centrifuged at 20,000 x g for a minute, and DNA transferred into prelabelled storage vials.

4.2.3.1 Adult mosquito rearing techniques

The field collected immature stages (larvae and pupae) were reared to adults according to the standardized rearing procedures (Das *et al.*, 2007). Briefly, larvae were transferred into colored small basins, and a pinch of pulverized fish meal added to the rearing trays. A pipette was used to collect the pupae from the larvae, which were then placed inside adult mosquito cages and allowed to emerge. Emerged adults were then anesthetized using chloroform and identified using the morphological keys and further confirmed to sibling species by PCR. The insectary was maintained at 28°C and 80%

relative humidity with a 12-hour cycle between day and night.

4.2.4 Adult sampling

Anopheles mosquitoes were collected using Pyrethrum Spray catch (PSC) from 30 randomly selected houses per site between 2017 and 2020 during the dry and rainy seasons. Collections were conducted between 0630 and 1000hrs in the morning and transported to the International Center of Excellence for Malaria Research (ICEMR), Homa Bay, Kenya. Samples were stored at -20^{o}C in 1.5 ml Eppendorf tubes containing silica gel and assigned a unique code for further molecular processing. *Anopheles gambiae* mosquitoes were identified to sibling species using PCR as described in section 3.2.3.

4.2.5 Identification of mosquito sibling species

Deoxyribonucleic acid collected from 627 *Anopheles gambiae* female mosquitoes were selected at random from larvae and adult field collections and used to identify mosquitoes to sibling species as described by (Scott *et al.*, 1993). In brief, DNA fragments of species analytical length from sample DNA and Universal, *Anopheles gambiae* and *Anopheles arabiensis* primers were amplified using polymerase chain reaction (PCR) based on ribosomal DNA (rDNA) sequences targeting the 18S rDNA coding region. Polymerase chain reactions were performed using the universal-UN(5´-GTGTGCCCCTTCCTCGATGT-3´), *An. gambiae-AG*(5´-AAGTGTCCTTCTCCATCCTA-3´), and *An. Arabiensis-AR* (5´-AAGTGTCCTTCTCCATCCTA-3´) primers. A single reaction consisted of 6.5 µl of Taqman Master Mix 2X (Promega Corp., Madison, WI, USA), 0.5 µl of each primer (10 µM), 2 µl of sample DNA (40 ng/µl), and 4 µl of nuclease-free water for a total reaction volume of 14 µl. Polymerase chain reaction amplifications were performed under the following conditions: 95 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension cycle of 72 °C for 6 min. All the amplicons were assessed by gel electrophoresis in 1.5% w/v agarose gel.

4.2.6 Molecular characterization of TEP1 alleles in *Anopheles gambiae*

Genomic DNA (gDNA) was extracted from whole female *An. gambiae* mosquitoes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described by Gildenhard *et al* (2019). Briefly, the first PCR was carried out with Nest 1 primers VB003-(GATGTGGTGAGCAGAATATGG) and VB004-(ACATCAATTTGCTCCGAGTT) targeting 892 base pairs, and the second PCR was carried out on 5µl of the resulting Nest 1 product with Nest 2 primers VB001- (ATCTAATCGACAAAGCTACGAATTT) and VB002-(CTTCAGTTGAACGGTGTAGTCGTT) producing a final fragment length of 758 base pairs targeting the TEP1-TED domain. Both PCR reaction conditions were set as denaturation at 95˚C for 3 minutes, 35 cycles of 94˚C for 30 seconds, annealing at 55˚C for 30 seconds, extension at 72˚C for 30 seconds, and a final step at 72˚C for 6 min using DreamTaq Green Master Mix (Thermo Fisher Scientific). PCR products were then digested by restriction enzymes *Bam* HI, *Hind* III, or *Bse* NI (New England BioLabs Inc) according to the manufacturer's instructions. The PCR amplicons were resolved in 2.5% agarose gel electrophoresis. The TEP 1 allelic classes were then determined by fragment size of restriction enzyme digestions. A subset of samples with identified TEP1 alleles was further confirmed through sequencing of respective Nested II amplicons. Sequencing was done using 3700/3730 BigDye® Terminator v3.1 Sequencing Standard kit (ABI PRISM® 3700 DNA Analyzer).

4.2.7 Thioester-containing protein1 (TEP1) alleles characterization analysis

Descriptive statistical analyses were performed using GraphPad Prism v.8.0.1 Software and SPSS version 25 for Windows. Statistical significance was set at P ≤ 0.05. TEP1 allele frequencies observed heterozygosity (Ho), and expected heterozygosity (He), the inbreeding coefficients (F*is*), departure from Hardy-Weinberg expectations were analyzed using GeneAlex version 6.053 software (Peakall, 2006) DNA sequences of TEP1 haplotypes were compared with published sequences. Basic Local Alignment Search Tool (BLASTN) was used to retrieve sequences from the National Center for Biotechnology Information (NCBI) database with a high similarity index to each of the haplotype sequences. The retrieved sequences with accession numbers AF291654.1, FN431783.1, FN431782.1, FN431785.1, FN431784.1, and MF098591.1 together with the identified haplotype sequences in this study were aligned. MView web-based tools (Brown *et al.,* 1998) were used to conduct the alignment of the sequences and to calculate pairwise sequence identity and similarity. Phylogenetic analysis of the representative sequenced and GenBank retrieved TEP1 sequences was performed using MEGA 7.0 software (Kumar *et al.,* 2016) in this study were aligned. MView web-based tools (Brown *et al.*, 1998) were used to conduct the alignment of the sequences and to calculate pairwise sequence identity and similarity. Phylogenetic analysis of the representative sequenced and Gene Bank retrieved TEP1 sequences was performed using MEGA 7.0 software (Kumar *et al.,* 2016)

4.3 Results

4.3.1 Species composition of *An. gambiae* s. l across western Kenya

A total of 627 *An. gambiae s.l.* adults randomly selected from the field collected pool by using an online random number generator. The samples were molecularly identified to sibling species based on species-specific conventional PCR. The results show *An. arabiensis* are identified at 315bp and *An. gambiae* s. s as 390bp (Figure 4.1). The mosquito species identified were *An. gambiae* and *An. arabiensis* constituting 49.28% (309/627) and 50.72% (318/627) of the total samples genotyped respectively. A higher number of *An. gambiae* mosquitoes were observed from Bungoma (62.5%), Kakamega (65.9%) and Kisumu (74.2%) unlike Homa Bay that had higher numbers of *An. arabiensis* (82.7%) (Figure 4.2). A significant difference in species abundance (*An. gambiae* versus *An. arabiensis*) was observed in all study sites (χ^2 =592.012 *df* =1, P<0.0001).

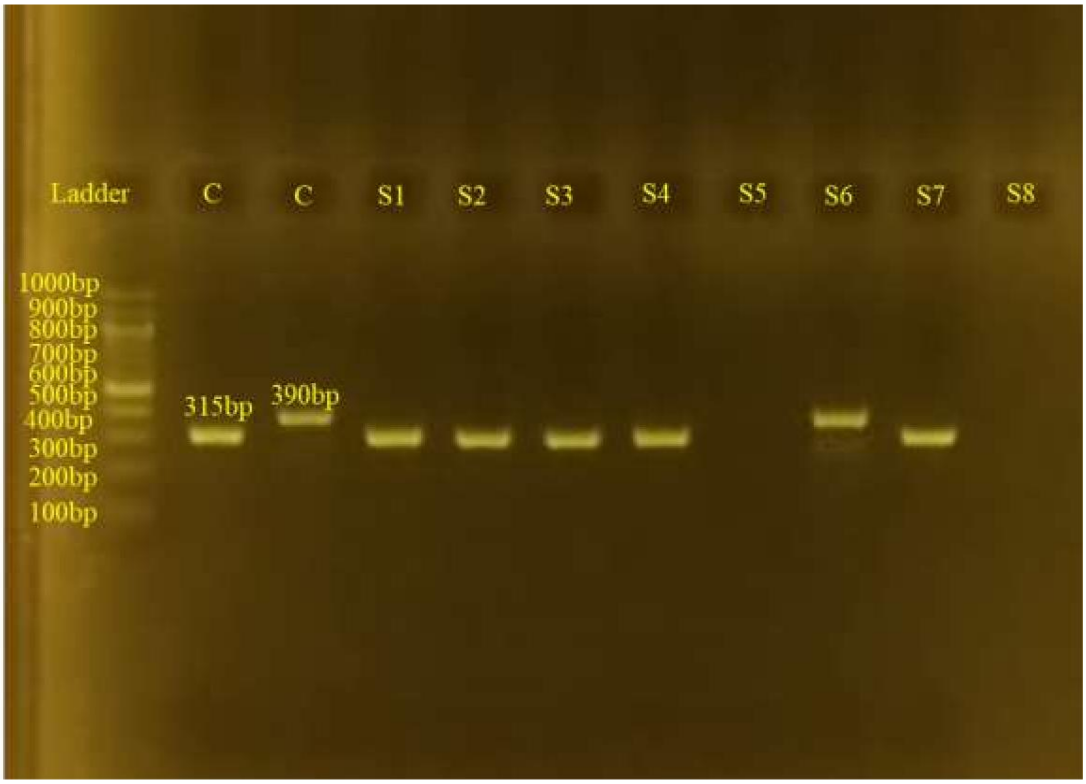

Figure 4.1: *Anopheles gambiae s.* l sibling species PCR gel. Lane 1- *An. arabiensis* control (C1), Lane 2- *An. gambiae s.s* control (C2), Lanes 3-10 represents the *An. gambiae s. l* samples (S1-S8). Samples on Lanes S5 and S8 did not amplify.

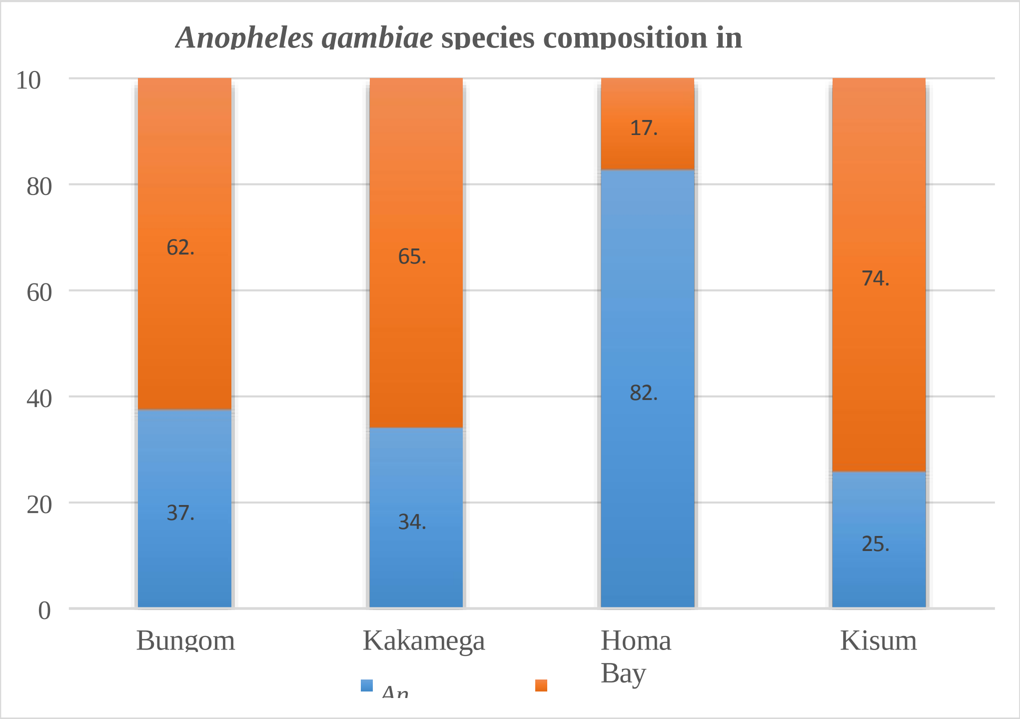

Figure 4.2: Molecular determined *Anopheles gambiae* species composition in western Kenya.

4.3.2 TEP1 allele distribution in western Kenya

Two TEP1 alleles (TEP1*S1, and TEP1*R2) were identified with average frequencies of 84.9% and 15.1%, respectively. *Anopheles arabiensis* populations from Homa Bay had the highest TEP1*S1 allele frequency (89%, 95%CI: 85.8% - 92.2%) which significantly differed from observed proportions in Kisumu (86.4%, 95%CI: 79.8% - 92.9%), Kakamega (84.5%, 95%CI: 74.9% - 94.1%) and Bungoma (74.4%, 95%CI: 64.5% - 84.3%) (Two-tailed $p<0.0001$). Among *An. gambiae s.s*, populations from Bungoma displayed the highest TEP1*S1 allele frequency (93.1%, 95%CI: 88.7% - 97.5%) followed by Homa Bay (84.6%, 95%CI: 76.4% - 92.8%), Kakamega (83.9%, 95%CI: 77% - 90.8%), and Kisumu (83.5%, 95%CI: 79.4%% - 87.7%) respectively (Figure 4.3). The observed TEP1*S1 allele frequency in Bungoma significantly differed from Kakamega (two-tailed P=0.047) and Kisumu (two-tailed p<0.0001). The highest TEP1*R2 allele frequency among *An. arabiensis* was observed in vector populations from Bungoma (26%, 95%CI: 15.7% - 35.5%) followed by Kakamega (15.5%, 95%CI: 5.91% - 25.1%), Kisumu (13.6%, 95%CI: 7.12% - 20.2%), and Homa Bay (11%, 95%CI: 8.06% - 14.5%). In *An. gambiae* the TEP1*R2 allele frequency was highest in populations from Kisumu and Kakamega displaying allele frequencies of 16.5%, 95%CI: 12.3% - 20.5% and 16.1%, 95%CI: 9.16% - 23% respectively, followed by Homa Bay (15.4%, 95%CI: 7.20% - 23.6%) and Bungoma (7%, 95%CI: 7.20% - 23.6%) respectively. No significant differences in allele frequency were observed between species (P=0.799) and between site variation (P>0.05).

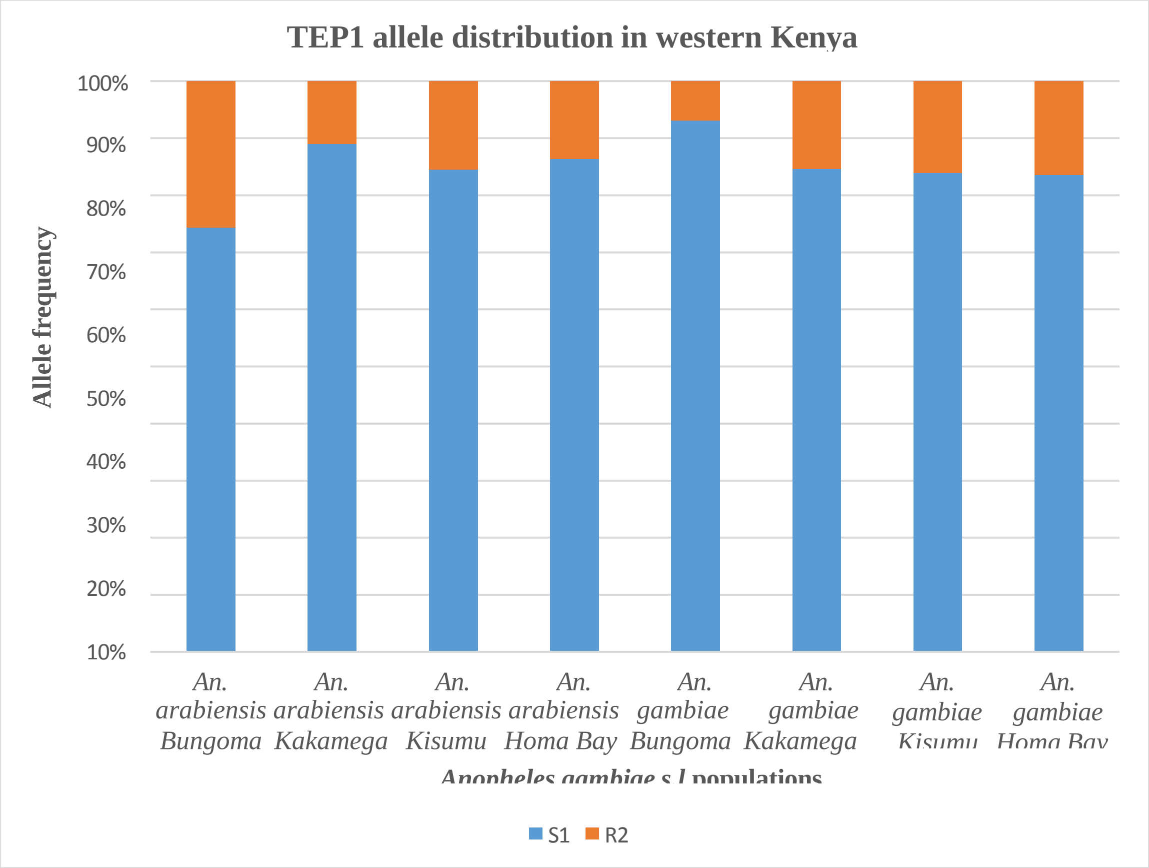

Figure 4.3: Distribution of TEP1 alleles identified in *An. gambiae s.s* and *An. arabiensis* in western Kenya

4.3.3 TEP1 genotype distribution in western Kenya

A total of three genotypes were identified in populations of *An. gambiae* s. l in western Kenya. Out of the three genotypes, two were homozygous TEP1*S1/S1 and TEP1*R2/R2 and one heterozygous TEP1*R2/S1. Homozygote TEP1*S1/S1 and heterozygote TEP1*R2/S1 genotypes had distinct frequencies (Figure 4.4). The TEP1*S1/S1 and TEP1*R2/S1 genotypes were commonly present among species in all sites at an average frequency of 71.75% and 26.61% respectively. TEP1*R2/R2 although rare, was only present in *An. arabiensis* from Bungoma (2.6%), Kakamega (3.4%) and Homa Bay (1.6%) and *An. gambiae s.s* from Kakamega (3.6%) and Kisumu (1.9%) but in the lowest average frequency of 1.64% (Figure 4.4). The TEP1*S1/S1 genotype was predominant followed by TEP1*R2/S1 but in low varied frequencies among species across all sampling sites. The TEP1*S1/S1 genotype frequency was highest in *An. gambiae* as compared to *An. arabiensis* from all sites except Kakamega populations that displayed higher TEP1*S1/S1 frequencies in *An. arabiensis* (75.9%) than in *An. gambiae* (53.6%) The distribution of TEP1*R2/S1 genotypes was highest in *An. arabiensis* than *An. gambiae* in all sites except populations from Kakamega where higher genotype frequencies (42.9%) were observed in *An. gambiae* than in *An. arabiensis* (20.7%) (Figure 4.4).

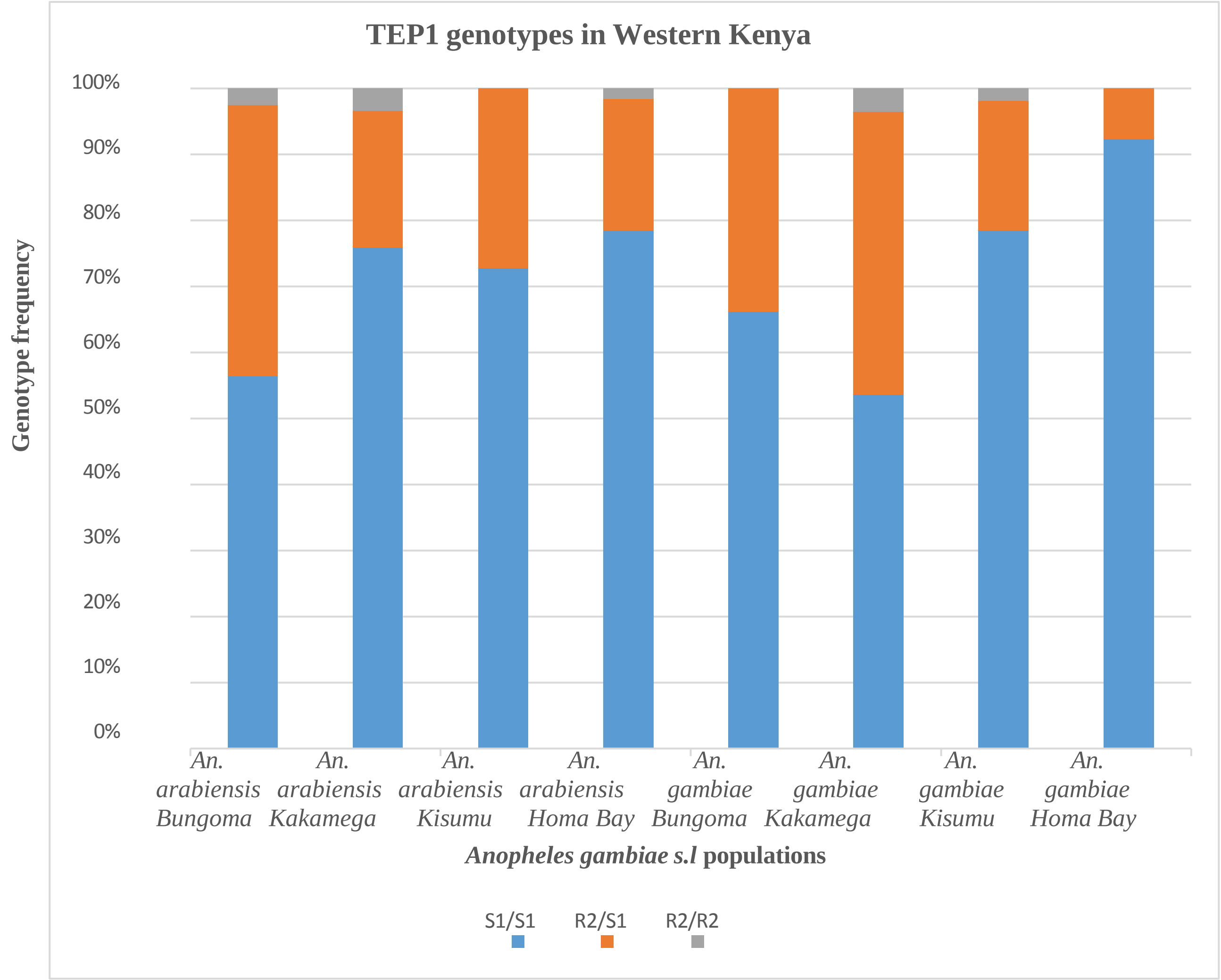

Figure 4.4: Thioester-containing protein 1 (TEP1) genotypes identified in *An. gambiae* and *An. arabiensis* in western Kenya

The observed RFLP results (Figure 4.5) for each TEP1 allele were confirmed by respective sequences upon alignment with reference sequences from the NCBI database. The TEP1*S1 and TEP1*R2 sequences had 100% identity matrix to AF291654.1 and FN431784.1 respectively. A significant difference in genotype frequency was observed among sites in *An. gambiae* populations (Fisher's exact test two-sided P-value < 0.001, n=309) whereas no significant difference was observed among sites in *An. arabiensis* population (Fisher's exact test two-sided P=0.07, n=318).

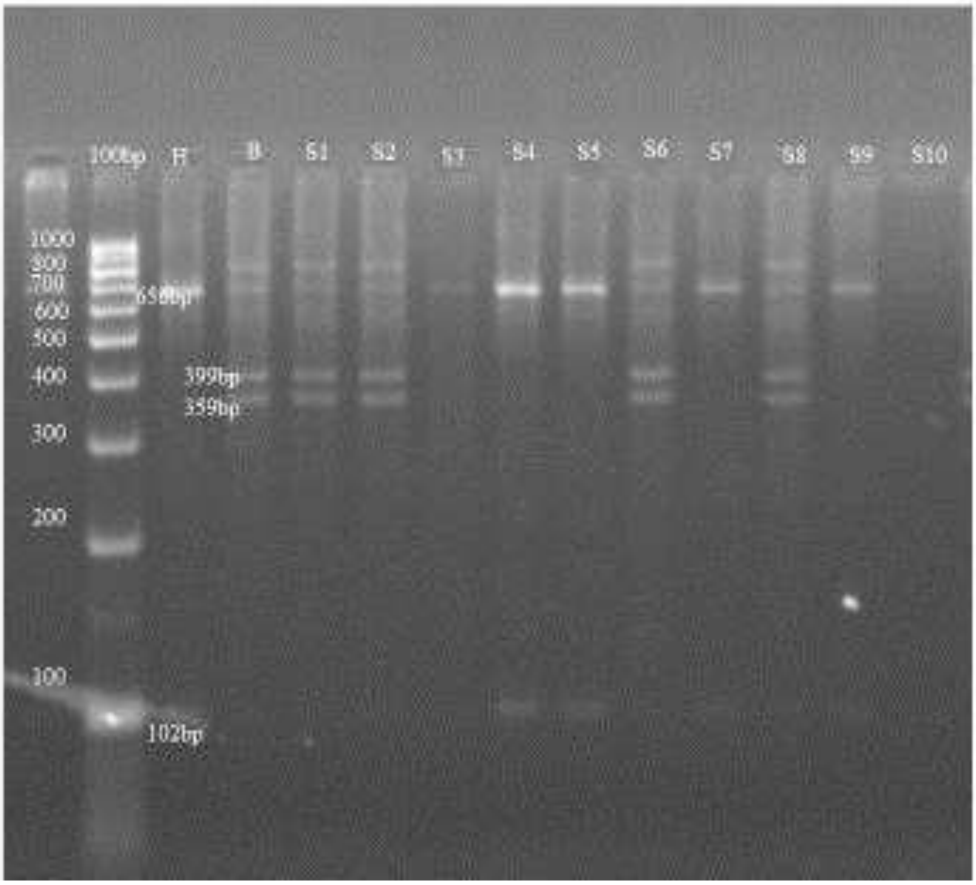

Figure 4.5: The thioester-containing protein1 genotyping RFLP-PCR results. Lanes 1 and 2 represent the ladder whereas lanes # and Lane 4 represent the TEP1 susceptible (656;102) and Tep1 resistant (399;359) alleles respectively after enzyme digestion

4.3.4 Evolutionary relationship based on TEP1 gene

The phylogenetic analysis of TEP1 sequences showed that alleles were clustered into susceptible and resistant groups (TEP1 is either susceptible or resistant to *Plasmodium* parasites) with high bootstrap values ranging from 72% to 100%. The TEP1*S1 allele identified in *An. gambiae s. s* (AG) and *An. arabiensis* (AR) from western Kenya (green dots) study sites shared a common lineage with TEP1*S1 (AF291654) from Suakoko, Liberia (Green square) and TEP1*S3 (FN431782) (purple square) whereas

TEP1*R2 (red circle) and TEP1*R1 (yellow square) independently evolved from TEP1*R3 (MF035809) (brown square) which shared common ancestral lineage with the Susceptible (S) alleles. Both the susceptible and resistant alleles evolved from a susceptible ancestry (Figure 4.6).

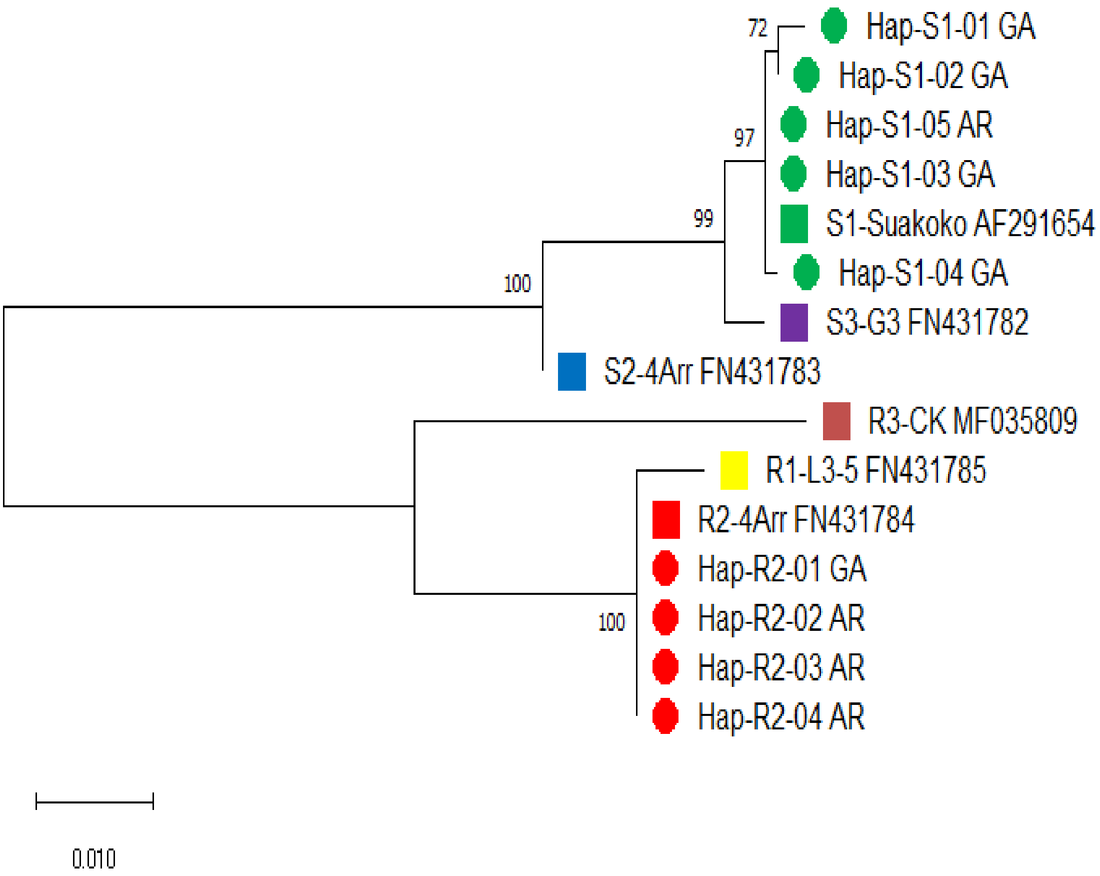

Figure 4.6: Phylogenetic relationship between TEP1 allele classes

4.3.5 Heterozygosity and departure from Hardy Weinberg Equilibrium (HWE)

The mean observed heterozygosity (H_O) of the TEP domain in *An. gambiae* and *An. arabiensis* across all sites was 0.270±0.035 with a mean (He) expected heterozygosity of 0.251±0.025. There were minor differences in the *An. arabiensis* observed heterozygosity ranges of 0.188–0.462 and the *An. gambiae* ranges of 0.138–0.321. Populations of *An. arabiensis* from Bungoma and Kisumu, as well as *An. gambiae* from

Bungoma, Kakamega, and Homa Bay, also displayed comparable tendencies of higher observed heterozygosity than the expected heterozygosity with negative *FIS* values (Table 4.1). A deviation was observed among *An. gambiae* from Kisumu and *An. arabiensis* from Kakamega and Homa Bay which displayed slightly higher expected heterozygosity than observed heterozygosity signifying the presence of inbreeding among these populations.

Table 4.1: Genetic diversity of *An. gambiae* s. s (GA) and *An. arabiensis* (AR) in western Kenya

Population	**N**	**Na**	***Ho***	***He***	**F**
AR-Bungoma	39	2.000	0.462	0.381	-0.210
AR-Homa Bay	186	2.000	0.188	0.196	0.041
AR-Kakamega	29	2.000	0.241	0.262	0.079
AR-Kisumu	55	2.000	0.236	0.236	-0.004
GA-Bungoma	65	2.000	0.138	0.129	-0.074
GA-Homa Bay	39	2.000	0.308	0.260	-0.182
GA-Kakamega	56	2.000	0.321	0.270	-0.191
GA-Kisumu	158	2.000	0.266	0.275	0.033

N represents the total number of mosquitoes sampled per study site, Na- Number of alleles per site, Ho- Observed heterozygosity, He- Expected heterozygosity, F- Fixation index The *FIS* showed a negative and non-significant value in *An. arabiensis* population from Bungoma (-0.210) and Kisumu (-0.004) and *An. gambiae* from Bungoma (-0.074), Kakamega (-0.191), and Homa Bay (-0.182). None of the analyzed population was at HWE as all the computed values were nonsignificant (P>0.05). The computed HWE values for *An. arabiensis* across the four localities ranged from 0.001

to 0.307 whereas for *An. gambiae* ranged 0.174 to 2.053 which was >1.

4.3.6 Population structure

The pairwise Wright's fixation index (*F*ST) values revealed a low genetic differentiation among *An. arabiensis* and *An. gambiae.* Zero value represented complete Panmixis between species in the subpopulations. The *F*ST values ranged from no subdivision to moderate differentiation (0.000 - 0.036) among *An. arabiensis* from the four study sites (Table 4.2). A moderate differentiation in *An. arabiensis* was observed between Bungoma and Homa Bay subpopulations (*F*ST = 0.036). The *F*ST values ranged from 0.000 to 0.022 among the *An. gambiae* subpopulations across the four regions. No population differentiation was observed between Kakamega and Homa Bay, Kisumu and Homa Bay, and Kakamega and Kisumu subpopulations (*F*ST =0). All pairwise *F*ST values for *An. gambiae and An. arabiensis* from all regions across western Kenya demonstrated low population differentiation (0 ≤ 0.05) except *An. arabiensis and An. gambiae* from Bungoma that showed moderate differentiation (0.05 ≥ 0.15). The overall low levels in population structure (*F*ST = 0.019) across all sites were supported by the high level of gene flow (Nm= 12.571) and low Nei's genetic distance values (<0.5) among the subpopulation.

Table 4.2: Pairwise comparison of FST among An. gambiae and An. Arabiensis populations in western Kenya

Population	**AR-HB**	**AR-BGM**	**AR-KK**	**AR-KSM**	**GA-BGM**	**GA-HB**	**GA-KK**	**GA-KSM**
AR-BGM		0						
AR-HB	0	0.036						
AR-KK	0.004	0.016	0					
AR-KSM	0.002	0.023	0.001	0				
GA-BGM	0.005	0.064	0.019	0.012	0			
GA-HB	0.004	0.016	0	0.001	0.018	0		
GA-KK	0.005	0.014	0	0.001	0.021	0	0	
GA-KSM	0.006	0.013	0	0.002	0.022	0	0	0

AR- *An. arabiensis,* GA- *An. gambiae,* BGM-Bungoma, HB- Homa Bay, KK-Kakamega, KSM- Kisumu

The analysis of molecular variance (AMOVA) results revealed that 99 percent of the observed variations in allele frequency were within each of the mosquitoes sampled (n=627) within respective populations, and a 1% variation was observed among the eight populations, but no variations were observed among each mosquito (Table 4.3)

Table 4.3: Analysis of molecular variance of the TEP1 gene in *An. g ambiae* populations circulating in western Kenya

Source	*df*	SS	MS	Est. Var.	%
Among Pops	7	2.306	0.329	0.001	1%
Among Individuals	619	71.992	0.116	0.000	0%
Within Individuals	627	77.000	0.123	0.123	99%
Total	1253	151.298		0.124	100%

df, degrees of freedom; SS, sum of squares; MS, mean squares.

4.4 Discussion

This study demonstrated variable frequencies of two common alleles TEP1*S1 and TEP1*R2 identified in *An. gambiae s. s* and *An. arabiensis.* Homozygous TEP1*S1/S1 and heterozygous TEP1*R2/S1 genotypes were also frequently identified across species and sites in western Kenya, as compared to the heterozygous genotype TEP1*R2/R2, which was rare and detected at very low frequencies. The majority, TEP1*S1, followed by TEP1*R2 alleles lacked a clearly defined distribution in each site analyzed. A high similarity index was observed among sequenced alleles that were initially identified by RFLP-PCR and sequences retrieved from NCBI. Consistent with previous reports, TEP1*R2 and TEP1*S1 were the most common identified alleles (Rono, 2017; Gildenhard *et al.,* 2019) which may have been because they are conserved and indicate ancestral alleles that have been passed down through generations with time. Furthermore, these alleles may have been maintained in the local populations due to selection pressure that confer some advantage to choosing these alleles or adaptations to their environments, however, their roles and significance in vector competence is still not clear (Obbard *et al.,* 2008; Blandin *et al*., 2009; White *et al.,* 2011; Mancini *et al.,* 2015).Low TEP1*R allele frequencies observed in these malaria-endemic areas in our study sites may be a product of selective pressures in the TEP1 gene resulting in functional variations that select for susceptible mosquitoes to *Plasmodium* infection (Le *et al*., 2012; Eldering *et al.,* 2016; Rono, 2017) as well as encourage evolutionary processes in the TEP1 loci (Obbard *et al*., 2008). Additionally, western Kenya still has relatively high malaria cases according to a recent study by Ochwedo *et al* (2021).

Implemented vector control interventions such as insecticide treated nets, indoor residual spraying, and environmental factors may determine the population structure. ITNs and IRS are two commonly used vector control interventions in Africa, and they have a direct impact on vector densities and species composition (Russell *et al*., 2011; WHO, 2020b). For example, IRS was deployed in Homa Bay to supplement the existing malaria interventions. The pre-spray period constituted 83% *An. funestus* and 16% *An. gambiae* s.l. However, consistent with this study, there was a drift in the local species composition with 99% of vectors in the post-spray period being *An. arabiensis* in the same sites (Russell *et al.,* 2011; Orondo *et al.,* 2021) Indoor interventions targeting *An. gambiae* complex remain the preferred method of lowering malaria transmission risk in endemic areas. *An. gambiae* s. s. is an anthropophilic indoor malaria vector (Gillies and De Meillon, 1968) and is susceptible to *P. falciparum,* which may explain higher TEP1*S1/S1 frequencies unlike *An. arabiensis* that is zoophilic and an outdoor dweller (Ochwedo *et al.,* 2021) which harbored TEP1*R2/S1 genotypes. The susceptibility rate between the three TEP1 genotypes identified among

An. arabiensis and *An. gambiae s. s.*, would have been confirmed by examining receptivity and sporozoite prevalence, which were, however, outside the scope of this study. Understanding the underlying molecular mechanisms that determine vector competence remains important and will thereafter contribute towards developing new vector control interventions and also complement existing control methods.

Selective forces acting on the TEP1 gene may be the reason for the low resistant allele (TEP1*R) frequencies observed in the study sites of the current research in comparison to susceptible allele frequencies observed. Functional variations would therefore favor mosquitoes that are more susceptible to *Plasmodium* infections, exerting evolutionary processes in the TEP1 locus (Le *et al.*, 2012; Eldering *et al.*, 2016; Rono, 2017; Bartilol *et al.*, 2022a). The TEP1*R2 and TEP1*S1 alleles are maintained in *An. gambiae* and *An. arabiensis* by TEP1*R2/R2, TEP1*S1/S1 and TEP1*R2/S1. This might be evidence that the TEP1*R2 and TEP1*S1 alleles offer certain benefits. The TEP1*R2 and TEP1*S1 alleles, may play conserved functional roles that have not been identified. Additionally, higher preference of *An. gambiae* for human blood may be the cause of its higher TEP1*S1/S1 frequencies. On the other hand, *An. arabiensis*, a zoophilic and opportunistic feeder, harbors the TEP1*R2/S1 and TEP1*R2/R2 genotypes, indicating that *An. gambiae* may be a more efficient malaria vector in western Kenya.

Only two species from the *Anopheles gambiae* complex identified in the current study were *An. gambiae* and *An. arabiensis*. The two species predominate in western Kenya, according to previous surveys done in this areas (Ochomo *et al.*, 2013; Machani *et al.*, 2020; Orondo *et al.*, 2021; Owuor *et al.*, 2021). Eighty three percent of the samples from Homa Bay had *Anopheles arabiensis,* whereas sites in Bungoma, Kakamega, and Kisumu were *An. gambiae.* The significant proportions of *An. arabiensis* observed in Homa Bay could be attributed to the mass indoor residual spray (IRS) that was conducted during the study period. The pre-spray period constituted 83% *An. funestus* and 16% *An. gambiae s.l.* However, consistent with this study, there was a drift in the local species composition with 99% of vectors in the post-spray period being *An. arabiensis* in the same sites (Russell *et al.*, 2011; Orondo *et al.*, 2021).

Populations of *Anopheles gambiae* and *Anopheles arabiensis* showed very minimal genetic variation and population structure between species and sites. There may not be any physical barriers separating the sites, as shown by the total FST values for the pairwise comparison for all populations show that the allele frequencies within each population is most likely similar hence the low genetic heterogeneity between species and sites represented in sites of the current study. This observation, however, does not support the idea that ecological changes and continuing interventions have an impact on the frequency of TEP1 alleles. The high levels of gene flow across the sampling sites are demonstrated by the effective migration index (Nm=12.571), which is consistent with the low levels of genetic differentiation.

Expected heterozygosity values for *An. arabiensis* from Homa Bay and Kakamega and *An. gambiae* from Kisumu were higher than the observed heterozygosity, indicating the presence of null alleles. The FIS results for *An. arabiensis* from Homa Bay (0.041) and Kakamega (0.079) and *An. gambiae* from Kisumu (0.033) infer possible inbreeding and non-random mating of individuals within those populations. The insignificant deviations from Hardy Weinberg Equilibrium imply that the TEP1 loci is under strong selection and confirm other forces such as natural mutations (Horton *et al.*, 2010; Harris *et al.*, 2012) and gene flow that may directly be shaping TEP1 alleles in *An. gambiae s. l* mosquitoes in western Kenya. Furthermore, ecological niches contributing towards selection forces acting on genetic variations shape the population structure of the local species populations in time and hence the adaptations of these malaria vectors to available breeding habitats (Smith *et al.*, 2015). The majority of the genetic variations were found within individual mosquito populations, indicating a high level of genetic diversity within each group. The 1% identified among the eight populations may reflect modest levels of genetic divergence as a result of variations between mosquito populations, indicating some level of genetic differentiation among populations. Furthermore, individual differences among mosquito populations did not have a significant influence on genetic diversity, hence no variations were identified among individuals within each population.

4.5 Conclusion

Anopeheles gambiae s. l mosquitoes from western Kenyan sites had two common alleles, TEP1*S1 and TEP1*R2, and their associated genotypes, homozygous TEP1*S1/S1 and TEP1*R2/R2, heterozygous TEP1*R2/S1. The findings of the current study indicate minimal genetic variation and a low population structure of TEP1 in *Anopheles gambiae* s.s and *An. arabiensis* in the study sites.

CHAPTER FIVE

GENETIC DIVERSITY AND POPULATION STRUCTURE OF PFS47 IN ISOLATES FROM WESTERN KENYA

5.1 Introduction

In the African region, *Plasmodium falciparum* is responsible for the vast majority of malaria infections and fatalities, accounting for 99.7% of anticipated malaria cases in 2018 (WHO, 2019). Malaria is mostly a concern in the lowlands of Kenya's endemic Lake regions (risk category equal to or greater than 20%), where transmission occurs throughout the year. (Githeko *et al.*, 1996; WHO, 2017; Division of National Malaria Programme Kenya (DNMP), 2021;). The combination of control interventions used in these regions includes long-lasting insecticidal nets, indoor residual spray and Artemisinin-based combination treatments (Bhatt *et al.*, 2015). Yet, there is still continuous transmission of the *P. falciparum* among the vulnerable populations.

The spread of malaria is a result of the parasites' adaptation to indigenous vectors in different geographical regions (Molina-Cruz and Barillas-Mury, 2014). Hence, over 70 *Anopheles* species transmit *P. falciparum* malaria (Sinka, 2013). The mosquito immune system can significantly deter successful malaria transmission and is critical for controlling the vector capacity (Crompton *et al.*, 2014). For a complete transmission circuit, *Plasmodium* parasites have to overcome immune responses mounted by diverse *Anopheles* vectors (Smith *et al.*, 2014; Ramphul *et al.*, 2015; Belachew, 2018). The Pfs47 gene is a surface protein in *P. falciparum* expressed on the surface of female gametocytes, gametes, zygotes and ookinetes (Molina-Cruz *et al.*, 2017) that interacts with the mosquito midgut making the parasite invisible to the vector’s immune system thus providing the parasite with an immune evasion mechanism (Molina-Cruz *et al.*, 2020) limiting the efforts to effectively control and eliminate malaria. Hence, Pfs47 is a potential molecular target of importance in designing appropriate interventions for malaria (Molina-Cruz *et al.*, 2012; Molina-Cruz *et al.*, 2015; Molina-Cruz *et al.*, 2020).

The Pfs47 gene is exceptionally polymorphic with a strong geographical genetic structure and diversity (Anthony *et al.*, 2007; Manske *et al.*, 2012). It exhibits haplotypes that are naturally selected by the anopheline vectors in varying geographical regions causing significant variations in malaria transmission (Anthony *et al.*, 2007; Molina-Cruz *et al.*, 2015). *Plasmodium falciparum* isolates from African strains have consistently displayed high levels of genetic diversity (Collins *et al.*, 1986; Molina-Cruz *et al.*, 2012; Yalcindag *et al.*, 2012) and a strong geographic structure in the Pfs47 gene from laboratory and field isolates (Anthony *et al.*, 2005; Anthony *et al.*, 2007) as well as haplotypes found circulating within the major malaria vectors; *An. gambiae* and *An. funestus* populations. These results indicate that compatible Pfs47 haplotypes are naturally selected within vector populations in Africa triggered by the mosquito’s immune pressures (Anthony *et al.*, 2007). Previous findings identified 42 Pfs47 haplotypes that exhibit high dN/dS worldwide (Molina-Cruz *et al.*, 2015) whereby the evolutionary relationships between these revealed 32 haplotypes exclusively from Africa, Papua New Guinea, the Americas, and Asia Guinea, the Americas, and Asia (Liu *et al.*, 2010). These polymorphisms may therefore have a significant impact on the trends in malaria transmission dynamics and the parasite history.

It is important to consider the genetic diversity and population structure of *Plasmodium falciparum* when developing successful malaria control measures. The degree of transmission and levels of inbreeding in different endemicities (Anderson *et al.*, 2000; Susomboon *et al.*, 2008), movement of people and vectors (Schultz *et al.*, 2010), geographical features that restrict or promote gene flow (Anderson *et al.,* 2000), and locally implemented control efforts are some of the factors that have an impact on genetic diversity and population structure (Anthony *et al.*, 2005; Zhong *et al.*, 2007). The underlying mechanisms by which *Plasmodium* parasites adapt to predominant malaria vectors from different regions and endemicities are not well understood. Therefore, it is important to carry out local *Plasmodium* parasite population genetics research which will greatly enhance our knowledge of transmission dynamics and contribute towards developing more effective malaria management and control strategies. The genetic diversity, haplotype distribution, and population structure of Pfs47 were evaluated along with their effects on malaria infections in endemic areas in Western Kenya.

5.2 Materials and methods

5.2.1 Study area and sampling

Cross-sectional mass blood screening was conducted in children between the ages of five to fifteen from January to August 2018 and January to March 2019 in malaria-endemic lowlands in Western Kenya. The regions include Kombewa (34°30′E, 00°07′N; altitude ranges 1,170–1,300 m above sea level), Chulaimbo, a rural site 19 km west of Kisumu City (0.03572°S, 34.621°E, altitude ranges 1328-1381 m above sea level) and Kendu Bay (0.3800 S, 34, 6419 E, altitude 1300 m) (Figure 5.1). Malaria transmission is perennial in the lowlands and the major *Plasmodium* transmitters in these regions include *An. gambiae* and *An. arabiensis* (Ochomo *et al.*, 2013). Malaria incidence in the lowlands is consistently high and is characterized by flat land with vast malaria breeding habitats especially during the rainy season (Mutuku *et al.*, 2009).

Kombewa is semi-arid with poor drainage and semi-permanent swampy streams and an average monthly temperature range of 18.4^0C - 29.1^0C (Ndenga *et al.*, 2006). Malaria is holoendemic in this region and transmission occurs throughout the year. The economic activities in Kombewa involve subsistence farming, animal husbandry, and fishing (Sifuna *et al.*, 2014). Chulaimbo has a sporadic water supply system, and limited sewer and waste disposal, and a mean annual temperature range of 12 ^{0}C–35 ^{0}C. The region experiences an average annual rainfall of 1352 mm and an average relative humidity range of 66–83%. Most residents are small-scale subsistence farmers. Kendu Bay has extensive environmental modifications and human migration and experiences semi-arid climatic conditions and depends on the Kimira-Oluch irrigation scheme for the production of crops.

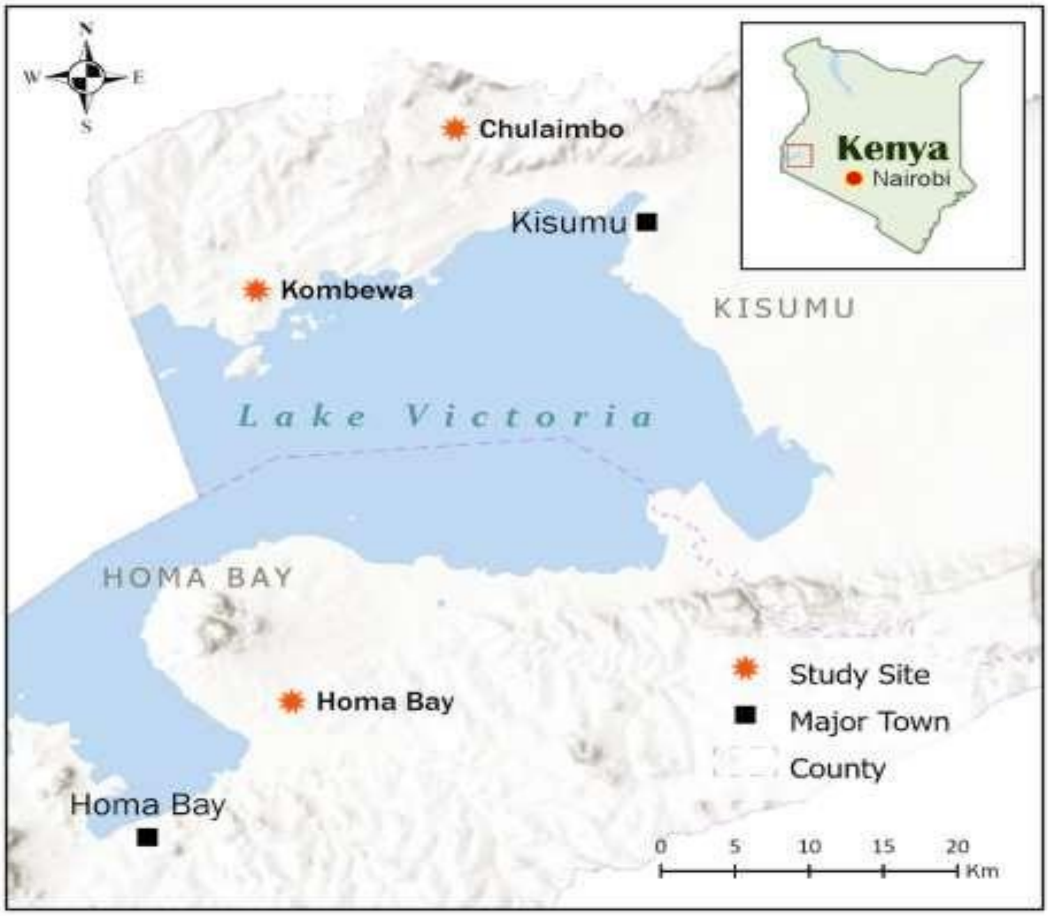

Figure 5. 1: A Map of Western Kenya showing the sampling locations.

The map was generated using ArcGIS Pro 2.6 software. Map source: ESRI, CGIAR, and USGS (available at: www.esri.com).

5.2.2 Parasitological evaluations

Blood samples were collected from school going children 5 to 15 years who had consented to participate in the study. Dried blood spots (DBS) were collected by finger prick on well-labeled Whatman® 903 Protein Saver Cards (GE Healthcare WB100014) containing the participants' details. Approximately, 50 µl of blood was drawn and placed onto the Whatman® 903 Protein Saver Cards and allowed to air dry before they were individually stored and preserved at -20 ° C for molecular analyses.

Thick and thin smears were simultaneously prepared for *Plasmodium* species identification and parasite counts. Blood films were stained using 10% Giemsa and examined in a compound microscope to determine the presence of *Plasmodium* parasites. Only the 125 DBS from *P. falciparum* positive participants were used to genotype Pfs47 and later sequenced to determine haplotypes distribution.

5.2.3 DNA extraction and *Plasmodium* species identification

Plasmodium falciparum parasite DNA was extracted from the DBS using Chelex method (Bereczky *et al.*, 2005). Briefly, 3 mm discs cut from each of the DBS were soaked in 10% Saponin and Phosphate buffer saline (PBS), and incubated overnight at 4^0C. The preparation was washed twice in 1xPBS and boiled in 20% (wt/vol) chelex suspension (styrene-divinylbenzene co-polymer containing iminodiacetic acid groups). The mixture was vortexed and centrifuged and used to identify *Plasmodium* species as described by Veron *et al* (2009). In brief, multiplex real-time PCR (RT-PCR) was run in a final volume of 12 µl containing 2 µl of sample DNA, 6 µl of PerfeCTa® qPCR ToughMix™, Low ROX™ Master mix (2X), 0.5 µl of each species-specific probe (*P. falciparum, P. ovale* and *P. malariae*), 0.4 µl of each species-specific forward primers (10 µM), 0.4 µl of each species-specific reverse primers (10 µM) and 0.1 µl of double-distilled water (Table 5.1). The thermal profile used was 50 °C for 2 min, (95 °C for 2 min, 95 °C for 3 sec and 58 °C for 30 sec) for 45 cycles. After species confirmation by RT-PCR, 125 samples that were infected with *P. falciparum* parasites only were randomly selected for genotyping the Pfs47 gene.

Table 5.1: Primers and probes sequences used for *Plasmodium* species identification PCR

Reagents	**Sequences**	**Specifications**
Pf-probes	5'FAM-CATAACAGACGGGTAGTCAT-MGB3'	(FAM-MGB)
Pm-probes	5' HEX-ATGAGTGTTTC'I'I'ITAGATAGC-MGB3'	(HEX-MGB)
Po-probes	5'NED-CGAAAGGAA'I'I'ITCTTATT-MGB3'	(NED-MGB)
Pf forward primer	5' ATTGCTI'ITGAGAGGTI'ITGTTACTTT3'	
Pf reverse primer	3'GCTGTAGTATTCAAACACAATGAACTCAA5'	
Pm forward primer	5'AGTTAAGGGAGTGAAGACGATCAGA3'	
Pm reverse primer	3'CAACCCAAAGACTITGATTTCTCATAA5'	
Po forward primer	5'AACCCAAAGACTTTGATTTCTCATAA3'	
Po reverse primer	3'CCGACTAGGTI'ITGGATGAAAGATI'I'IT5'	

5.2.4 Genotyping *Plasmodium falciparum* surface protein 47 (Pfs47)

Genotyping Pfs47 was performed following the method as described by Anthony *et al* (2007) with modifications. Briefly, forward 5’ATGTGTATGGGAAGAATGATCAG3’ and reverse 5’ACAAGTTCATTCATATGCTAACATA3’ primers were used to amplify the entire coding region with 1320 base pairs from the DNA of the 125 *P. falciparum* positive samples (Table 5. 1). A final reaction volume of 12 µl was constituted by adding 6 µl of Dream Taq Green PCR Master Mix (2X), 0.5 µl of each of the forward and reverse primer, 3 µl of double distilled PCR grade water, and 2 µl of sample DNA. The PCR conditions for genotyping Pfs47 were set as follows; 95 ^{o}C for 3min, 35X (94 ^{o}C for 30 sec, 50 ^{o}C for 30 sec, 68 ^{o}C for 90 sec), and 72 ^{o}C for 6 minutes prior to sequence, amplicons quality and size were determined by visualization of PCR products in 1.5% w/v gel under UV trans illuminator. The amplicons were cleaned and sequenced directly using Big Dye terminator chemistry v3.1, PCR primers, and PRISM® 3730xl genetic analyzer (Applied Biosystems, CA, USA). Paired reads from the sequencer were edited and assembled using BioEdit software (version 7.2.5) before further analysis.

5.2.5 Data analysis

The ClustalW method (integrated into the Mega X Programme) was used to align 125 established sequences with the reference sequence Pf3D7_1346800. Genetic diversity parameters such as nucleotide diversity, mean pairwise differences, polymorphic sites, haplotype diversity, and linkage disequilibrium were calculated using the DnaSP software version 6.12.03. The haplotypes network displaying the distribution per site was created using Population Analysis with Reticulate Trees (Popart) version 1.7 software. GeneAlex version 6.5 software was used to infer allele frequency, genotypic frequency, and population genetics (fixation index, gene flow, and Analysis of molecular variance). The analysis of molecular variance (AMOVA) was divided into populations within populations and individuals within groups in the Kombewa, Chulaimbo and Homa Bay *P. falciparum* populations

5.3 Results

A total of 1518 children were tested from the three study sites for malaria parasites. *Plasmodium falciparum* parasites were detected in 20.5 percent (309/1518) of the participants who underwent screening. Chulaimbo, Homa Bay, and Kombewa had respective prevalence of 25.5%, 8.9%, and 56.8% for *P. falciparum*. A total of 125 samples (67 female and 58 male) were chosen at random for *Pfs47* sequencing and analysis.

5.3.1 Genetic diversity indices of *Pfs47* across Western Kenya

Western Kenyan *Plasmodium falciparum* parasites (n=125) were successfully sequenced from Homa Bay (n=62), Chulaimbo (n=30), and Kombewa (n=33). In comparison to Pf3D7_1346800, nucleotide sequence analysis of the *Pfs47* revealed eight segregating sites, six of which were parsimony informative sites, and two singletons or SNPs. Single nucleotide polymorphisms (SNPs) were observed at mutation loci 581 and 814 whereas 81, 564, 718, 742, 815, and 910 were parsimony informative.

The Homa Bay parasites (11) had the greatest number of haplotypes and segregating sites out of the 13 haplotypes identified in western Kenya, followed by Kombewa (10) and Chulaimbo (7) parasites. The haplotype diversity (Hd) and nucleotide diversity (π) in western Kenya were 0.74±0.03 and 0.11±0.01, respectively, whereas population-wise, Hd values varied from 0.69 to 0.77, and nucleotide diversity was mainly low (0.10±0.02 to 0.12±0.01) across all sites (Table 5.2). *Plasmodium falciparum Pfs47* parasite populations from Homa Bay (0.77), Kombewa (0.76), and Chulaimbo (0.69), showed a comparatively higher level of genetic diversity. (Table 5.2). The Tajima's D values for the *Pfs47* were all negative and not statistically significant, deviating from the expected neutral model. The findings of all neutrality tests—Fu’s Fs (-5.90*), Fu and Li's D (-0.49*), and Fu and Li's F (-0.53*) were equally negative but significant ($P<0.05$).

Table 5.2: Genetic diversity indices and neutrality tests based on Pfs47 sequences

Population	n	*S*	H	Hd	π (10^{-2})	*k*	Fu's *Fs*	Tajima's *D*	Fu and Li's *D*	Fu and Li's *F*
Homa Bay	62	7	11	0.77±0.04	0.12±0.01	1.22	-4.95*	-0.46	-0.39*	-0.49*
Kombewa	33	7	10	0.76±0.07	0.11±0.02	1.21	-5.47*	-0.87	-0.16*	-0.44*
Chulaimbo	30	5	7	0.69±0.08	0.10±0.02	1.05	-2.58*	-0.47	-0.61*	-0.66*
Western Kenya	125	44	13	0.74±0.03	0.11±0.01	1.17	-5.90*	-0.49	-0.42*	-0.53*

n, number of samples sequenced; *S*, number of polymorphic (segregating) sites; *H*, number of Haplotypes; *H*d, Haplotype diversity; π, nucleotide diversity; *k*, mean number of pairwise differences. *; significance level $P<0.05$

Most of the *P. falciparum Pfs47* sequences from the Homa Bay, Chulaimbo, and Kombewa dimorphic loci were found at locus 81 Domain 1 (D1) with >0.5 allele frequency (Table 5.3). The Homa Bay parasite population exhibited mutations at locus 81 with 61.29%, loci 564, 581, 718, 742, 815, and loci 910 with 6.45%, 1.61%, 20.97%, 6.45%, 1.61%, and 4.84%, respectively. None of the sequences from Homa Bay and Chulaimbo had mutations at locus 814. There were no mutations at loci 581(D2), 814(D3), and 910(D3) in the Chulaimbo parasite sequences, however there were mutations at loci 81, 564, 718, 742, and 815, with respective percentages of 70%, 13.33%, 13.33%, 3.33%, and 3.33%. The *P. falciparum* populations sequenced from Kombewa had no mutations at locus 581, but exhibited mutations at loci 81, 564, 718, 742, 814, 815, and 910, respectively, with percentages of 66.67%, 9.09%, 9.09%, 3.03%, 6.06%, and 3.03%.

Table 5.3: Pfs47 allele frequencies per mutation loci

Mutated loci	**Pfs47 Domains**	**Genotypic frequencies % (n)**		
		Homa Bay (n=62)	**Chulaimbo (n=30)**	**Kombewa (n=33)**
81	D1	61.29 (38)	70 (21)	66.67 (22)
564	D2	6.45 (4)	13.33 (4)	9.09 (3)
581	D2	1.61 (1)	0	0
718	D2	20.97 (13)	13.33 (4)	9.09 (3)
742	D2	6.45 (4)	3.33 (1)	9.09 (3)
814	D3	0	0	3.03 (1)
815	D3	1.61 (1)	3.33 (1)	6.06 (2)
910	D3	4.84 (3)	0	3.03 (1)

n is the total number of sequences analyzed The *Pfs47* amino acid chain exhibited 8 nonsynonymous alterations as a result of all the detected base substitutions at the 8 loci: E27D, E188D, P194H, L240I, I248L, N272I, N272Y, and I304L (Table 5.4). The codon variations in the corresponding haplotypes showed that *P. falciparum* parasite populations from all sites shared Hap_1, 2, 3, 5, and 6 as prevalent haplotypes. In all populations, the haplotype (Hap_3) was the most prevalent. Only the Homa Bay populations were exclusively associated to the haplotypes Hap_7, 9, and 10, while Kombewa parasites were the only population that had Hap_13.

Table 5.4: Haplotype mutations and frequency per site

Haplotype	**Codon changes**	**Frequency per site**			**Total frequency**
		Homa Bay	**Chulaimbo**	**Kombewa**	
Hap_1	E27D and P194H	27	16	15	58
Hap_2	P194H and L240I	8	2	2	12
Hap_3	P194H	10	5	7	22
Hap_4	P194H and I304L	3	0	1	4
Hap_5	E188D and P194H	1	2	1	4
Hap_6	E27D, P194H and L240I	5	2	1	8
Hap_7	P194H and N272I	1	0	0	1
Hap_8	E27D, P194H and I248L	3	0	1	4
Hap_9	E27D, E188D, P194H and I248L	1	0	0	1
Hap_10	Same as 3D7* (No mutations)	1	0	0	1
Hap_11	E27D, E188D and P194H	2	2	2	6
Hap_12	E27D, P194H, I248L and N272I	0	1	2	3
Hap_13	E27D, P194H and N272Y	0	0	1	1

*NCBI Reference Sequence of 6-cysteine protein (*Plasmodium falciparum* 3D7): XP_001350182.1s

The transmission clustering system (TCS) network illustrates the distribution of *Pfs47* haplotypes in *P. falciparum* throughout the three regions, which include Homa Bay, Kombewa, and Chulaimbo. All other haplotypes identified in the study sites at varying frequencies. The 13 different haplotypes in the TCS network profile were all related by a single mutation step whereas the size of the circles represents the frequency (Figure 5.2).

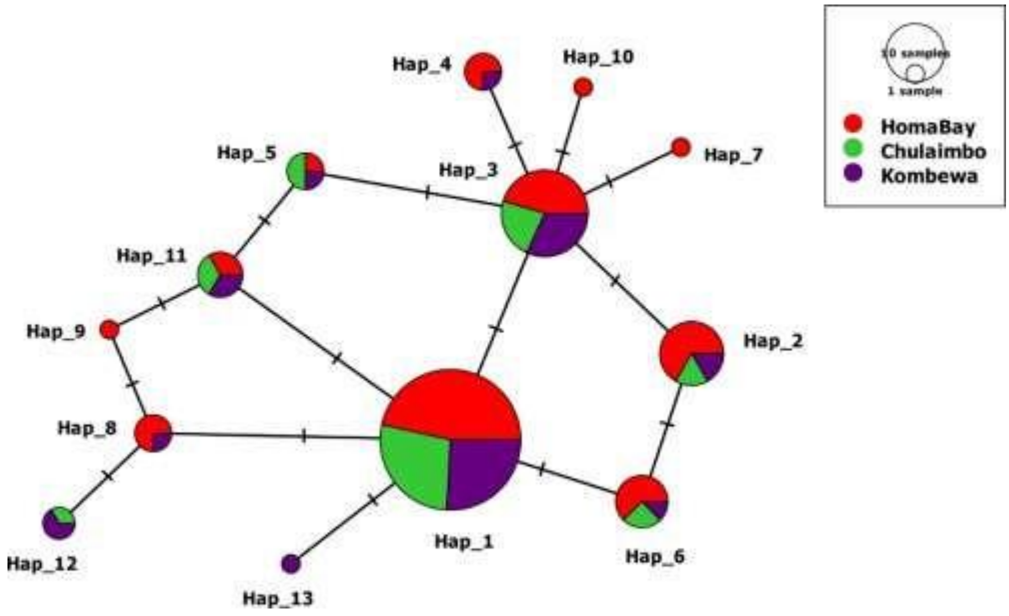

Figure 5.2: TCS-network analysis of the relationship between Pfs47 haplotypes (Hap_1-Hap_13) in D1, D2 and D3 from parasites in malaria-endemic areas of western Kenya.

Based on observed sequence differences in *P. falciparum Pfs47,* the TCS-network shows the distribution of haplotypes. The network displays the distribution of *Pfs47* haplotypes in western Kenya from Homa Bay (red), Kombewa (purple), and Chulaimbo (green) regions. The hatch mark indicates the number of mutations that led to each haplotype, and the size of the circle indicates the frequency of each haplotype.

5.3.2 Phylogenetic relationship of *Pfs47* haplotypes

All haplotypes arose from common ancestry. Out of the sequences retrieved from the gene bank, Pfs47 haplotypes from western Kenya have a common ancestral lineage with haplotypes from Brazil/Cambodia and Asia accession numbers (KT892016 Brazil/Cambodia, and EF137239-K1 Asia) (Figure 5.3). The haplotypes Hap_2, 6, and 11 and haplotypes from Papua New Guinea accession number (KT892040-PNG) are drifting away from the ancestor indicative of recent haplotypes. The rest of the haplotypes identified in this study from Western Kenya are close to ancestry. The other African countries retrieved from the gene bank accession numbers (KT892026-Gambia, KT892027- Ghana, KT892028- Ghana, KT892029-Guinea Bissau, KT892032-Liberia, KT892033-South Africa, and KT892034-Sudan) evolved independently from the haplotypes identified in Western Kenya. (Figure 5.3).

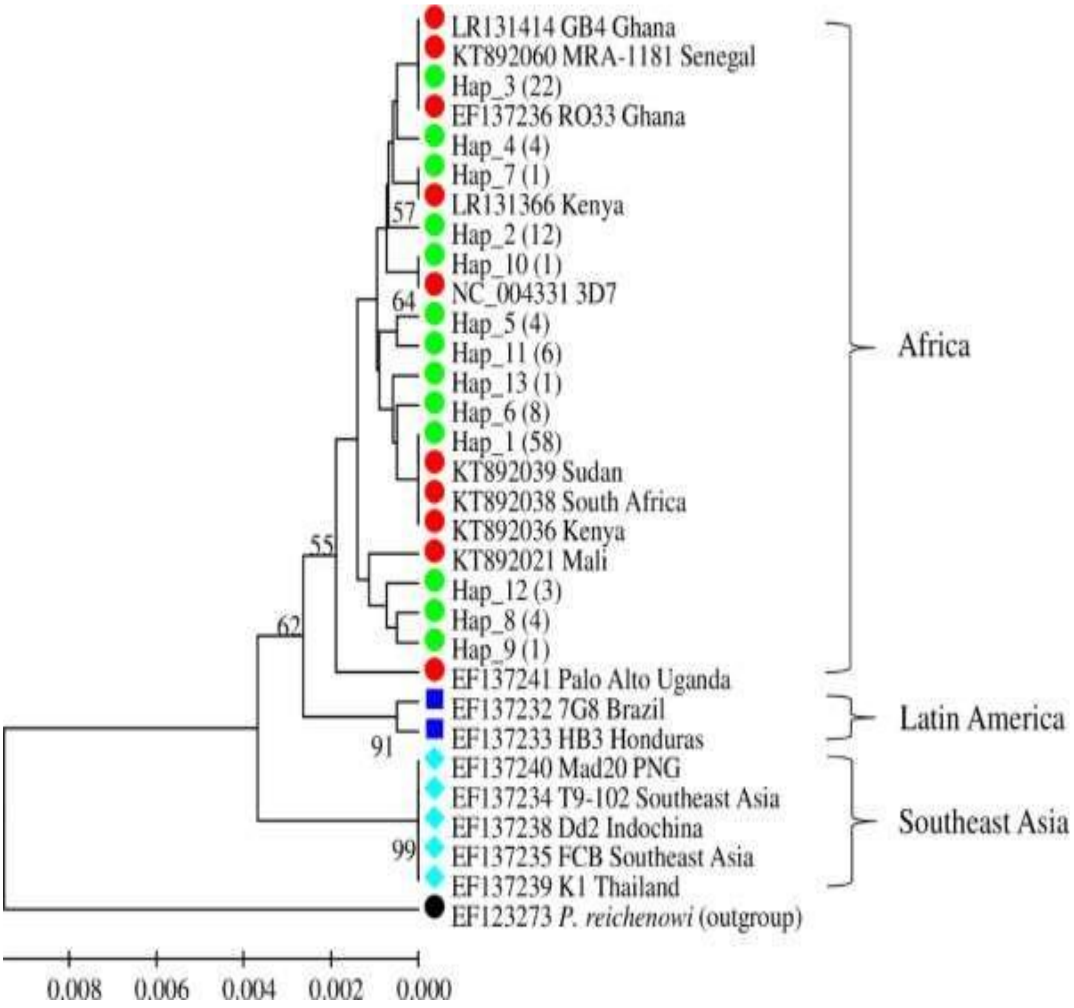

Figure 5.3: Phylogenetic inference using UPGMA method.

The branches coincide with the proportion of duplicate trees (50% bootstrap cut-off value) where the associated taxa clustered during the bootstrap test (1000 repetitions). The evolutionary distances, which are measured in base substitutions per site, were calculated using the Kimura 2-parameter technique. Green dots represent the haplotypes identified in Western Kenya, red dots represent haplotypes from other African countries, blue represents haplotypes from other continents and light blue represents haplotypes shared between regions. Twenty-one sequences were retrieved from Gene Bank: (KT892060-Senegal; LQ878280-Thailand/Senegal; KT892036-Mali; KT892040-PNG; KT892021-Brazil; KT892020-Brazil; KT892019-Brazil PaloAlto; EF137241-Brazil PaloAlto; KT892053- PNG; KT892015-Brazil; EF137233-HB3 Honduras; EF137232-7G8 Brazil; KT892026-Gambia; KT892027-Ghana; KT892028-Ghana; KT892029-Guinea Bissau; KT892032- Liberia; KT892033-South Africa; KT892034-Sudan; KT892016-Brazil/Cambodia; EF137239-K1 Asia) were used.

5.3.3 Differentiation and population structure in *Plasmodium falciparum* strains determined by Pfs47 sequences

Populations of *P. falciparum* from Homa Bay, Kombewa, and Chulaimbo did not differ significantly. Chi-square test results for comparisons across populations were all not significant ($P>0.05$): Kombewa in comparison Chulaimbo ($x^2=4.232$, df =1, P=0.895); Kombewa versus Homa Bay ($x^2=10.91$, df =1, P=0.618); Homa Bay versus Chulaimbo ($x^2=10.39$, df =0.582). All Nm values in western Kenya were more than two (Table 5.5). When the populations of Kombewa and Chulaimbo were compared to Homa Bay separately, they exhibited GammaSt Nm values comparable to Homa Bay. No genetic variation across the populations since all pairwise F_{ST} values were zero.

Table 5.5: Population structure among *Plasmodium falciparum* across Western Kenya

Populations		χ^2	P-value	*df*	*F*ST	GammaSt Nm
Kombewa	**Chulaimbo**	4.232	0.895	9	-0.023	51.000
Kombewa	**Kendu Bay**	10.911	0.618	13	-0.004	34.200
Homa Bay	**Chulaimbo**	10.394	0.5815	12	-0.003	34.620

χ^2 Chi-square; *df*, degrees of freedom; *F*ST, fixation index; Nm, gene flow estimate

The analysis of molecular variance (AMOVA) results shows that all observed variations in allele frequency were from among the individuals in the *P. falciparum* parasite populations per site and no variation (0%) was observed among each population and within individuals in a population (Table 5.6). Wright's F-statistic revealed a low population structure across parasite populations from all the sites while the total genetic differentiation index (*FST*) was -0.00891 (P>0.05) in the study sites.

Table 5.6: Analysis of molecular variance of Pfs47 gene in Plasmodium falciparum population circulating in Homa Bay, Chulaimbo and Kombewa

Global AMOVA results

Source of variation	*df*	**Sum of squares**	**Mean Square**	**Established Variation**	**Percentage of variation**
Among populations	2	1.68	0.84	0.00	0
Among Individual	122	143.44	1.18	0.59	100
Within Individuals	125	0.00	0.00	0.00	0
Total	249	145.12		0.59	100

df, degrees of freedom

5.4 Discussion

The current study conducted in western Kenya *Plasmodium falciparum* parasites provides important insights into the genetic diversity of Pfs47. This study identified a total of 13 haplotypes. The *Pfs47* gene showed substantial haplotype diversity (H*d*) ranging from 0.69 to 0.77 but low nucleotide diversity (π) ranging from 0.10±0.02 to 0.12±0.01 across sites within the human population. The comparatively higher levels of genetic diversity correspond to the observed mean pairwise differences and haplotype diversity per study site. The variation in genetic diversity indices of Pfs47 per site corroborates previous results that linked various parasite genetics to levels of malaria transmission intensities (Babiker *et al.*, 1997; Peyerl-Hoffmann *et al.*, 2001).

Plasmodium falciparum is diverse and has varying patterns of population genetic characteristics that correlate with local endemicities and transmission intensity (Anderson *et al*., 2000). Reports have shown that the population genetic diversity of *P. falciparum* tends to be low in hypo to meso-malaria endemic regions and high in hyperendemic regions (Anderson *et al*., 2000; OraLee *et al*., 2011). These results also demonstrate similar trends where parasites from Chulaimbo a meso-endemic region had the lowest genetic diversity compared to the Homa Bay and Kombewa *P. falciparum* populations. In this study, the Pfs47 gene has displayed relatively diverse haplotypes with low nucleotide diversity being observed across sites within Western Kenya. Homa Bay (hyperendemic) had the highest nucleotide diversity followed by Kombewa (holoendemic) then Chulaimbo (mesoendemic).

The high diversity corresponds to the observed mean pairwise differences and haplotype diversity per study site. The variation in genetic diversity indices of Pfs47 per site corroborates previous results that linked various parasite genetics to levels of malaria transmission intensities (Babiker *et al*., 1997; Peyerl-Hoffmann *et al*., 2001). Most (50%) of the observed mutations occurred within immunogenic domain two (D2) of Pfs47 antigen which is in agreement with findings from previous studies (Molina-Cruz *et al.,* 2015). The variations within D2 of Pfs47 antigen has been hypothesized to be vital and aid parasite in escaping nitration or TEP1 mediated killing (Canepa *et al.,* 2016). Domain 3 had only three mutations while domain 1 had one which was much pronounced or had high allele frequencies across the three study regions. All the sequences from each site except one from Homa Bay had at least one of the loci mutations. The sequence lacking mutation corresponds to NF54 wild strains that were reported to have over 90% chance of survival in the *An. gambiae* R strain mosquitoes (Molina-Cruz *et al*., 2015; Canepa *et al*., 2016).

Out of the 13 observed Pfs47 haplotypes, 6 haplotypes were shared in the three regions representing the different transmission intensities. However, haplotypes harboring mutation codon E27D were predominant in each site and seem to be highly selected or most infective within the Western Kenya P. falciparum populations. This finding reaffirms parasites having mutation codon E27D to be the most predominant Pfs47 haplotype only found in *P. falciparum* parasites circulating in Africa (Molina-Cruz *et al*.,2015). Consistent with findings from another study (Molina-Cruz *et al.,* 2015) other common haplotypes most of which had mutations in D2 and were found in parasites from the three study regions were E188D, L240I, I248L, and N272Y. Among the four mutation codons, I248L is more conservative and results in a change of methyl group position within the side chain also identified by Canepa *et al* (2016) and Eldering *et al* (2016) in *P. falciparum* African strains. The mutations are shown to slightly increase infection rates to 4% non-silenced and further 75% in *An. albimanus* with silenced LRIM1 (Canepa *et al.*, 2016). Apart from mutation codon I304L described here for the first time, the other seven have been described in parasites circulating in Africa, Asia, America, and Papua New Guinea (Molina-Cruz *et al.*, 2015). Haplotypes with mutation codon I304L were unique to the Kombewa and Homa Bay parasite populations whereas those with mutation codon P194H and N272I were private to Homa Bay and Kombewa populations respectively. Compared to the Pfs47 orthologue Pvs47 (PVX_083240), both share a 38.5%-38.7% amino acid identity, and the haplotype distribution exhibit a geographical population structure indicative of alleles favored by natural selection in a given region. Not all the observed mutations in Pfs47 were present in Pvs47, however, in amino acid sequences Pfs47 from the study sites had two mutation sites (27 and 240) at same loci position as the one described in Pvs47 (Tachibana *et al.,* 2015).

All the inter-population comparisons displayed non-significant differences across the three *P. falciparum* populations thus confirming a weak population structure. The weak population structure or lack of significant difference in nucleotide diversity indices may be as a result of considerable gene flow, lack of geographical barriers, and inbreeding characterizing parasites at various sites in Western Kenya. Furthermore, these sites have a vast network of roads that facilitate movement and trade across these study regions. Human movement may also affect the parasite population structure by introducing an admixture of *P. falciparum* strains (Shanks *et al.,* 2005; Bonizzoni *et al*., 2009) as a result of a weak structure as illustrated in this study. *Plasmodium falciparum* populations from Homa Bay and Kombewa showed the strongest evidence of endemic structure. This is consistent with other studies conducted in the African continent where *P. falciparum* is diverse and has varying patterns of population genetic characteristics that correlate with local endemicities and transmission intensity (Anderson *et al.*, 2000). These results also demonstrate similar trends where parasites from Chulaimbo a mesoendemic region had the lowest genetic diversity compared to the Homa Bay and Kombewa *P. falciparum* strains.

5.5 Conclusion

There was no genetic differentiation among the *Plasmodium falciparum* parasite populations from Homa Bay, Kombewa and Chulaimbo. The *Pfs47* polymorphic region (1061 base pairs) exhibited thirteen haplotypes and eight SNPs, resulting in seven amino acid changes; E27D, E188D, P194H, L240I, I248L, N272I, and I304L. There was evidence of an abundance of rare haplotypes, a recent population expansion or a selective sweep caused by genetic hitchhiking. The absence of a synonymous substitution is also evidence of natural selection in the amino acid polymorphisms within the Pfs47 gene.

CHAPTER SIX

COMPATIBILITY OF PFS47 INFECTIOUS HAPLOTYPES TO *ANOPHELES GAMBIAE* IN WESTERN KENYA

6.1 Introduction

Insecticide resistance (Ondeto *et al.*, 2017; Huijben and Paaijmans, 2018; Sternberg and Thomas, 2018 ; Orondo *et al.*, 2021) and outdoor transmission (Okello *et al.*, 2006; Meyers *et al.*, 2016; Sherrard-Smith *et al.*, 2019; Keïta *et al.*, 2021) have compromised the efficacy of primary malaria control interventions necessitating the development of new or improved targeted strategies that could complement the control of malaria such as transmission-blocking approaches. Molecular mechanisms underlying *Plasmodium* infections and mosquito genotypes influencing parasite adaptations to diverse *Anopheles* species are vital in understanding *Anopheles*-*Plasmodium* compatibility interactions.

Malaria transmission majorly depends on competent vectors and compatible infectious parasites to influence susceptibility in local *Anopheles* populations (Molina-Cruz *et al.*, 2015). The mosquito immune factors including recognition receptors, cellular and humoral components influence the infectiousness of gametocytes to vectors (Cirimotich *et al.*, 2010). The likelihood of infection after ingesting gametocytes from an infected person is determined by a combination of factors such as the mosquitos' immune responses (Richman *et al.*, 1997; Michel and Kafatos, 2005; Cirimotich *et al.*, 2010; Nilsson *et al.*, 2015). The thioester-containing protein 1(TEP1) is a key immunological gene that exhibits allelic variations (Obbard *et al.*, 2008) and also prevents pathogens including *Plasmodium* infections in mosquitoes (Clayton *et al.*, 2014), hence altering vector competence and malaria infectivity (Blandin *et al.*, 2004;

Blandin *et al.*, 2009).

The Pfs47 gene is a mechanism used by the malaria parasite *P. falciparum* to avoid the immune responses of the vectors (Molina-Cruz *et al.*, 2013; Molina-Cruz *et al.*, 2020). The gene exhibits haplotypes that innately favor particular mosquito receptors, resulting in significant transmission variability (Molina-Cruz *et al.*, 2015). Previous studies have shown that certain Pfs47 haplotypes have evolved to interact with specific midgut vector receptors in mosquitoes, enabling the malaria parasite to evade the mosquito's immune system. This evasion mechanism enhances the chances of the parasite surviving and development within the mosquito, ultimately increasing the risk of transmitting malaria to humans when an infected mosquito subsequently takes a blood meal (Molina-Cruz *et al.*, 2020). The immune system of the vector, however, recognizes and eliminates incompatible haplotypes. Furthermore, *Anopheles* populations dominating in a particular area may have exerted selection pressures that alter the genetic diversity of Pfs47 haplotypes, resulting in parasite adaptations to local vector species. According to Sinka *et al*, (2010) over 70 *Anopheles* species now transmit *P. falciparum* malaria around the world.

From a molecular standpoint, the associations between Pfs47 in *P. falciparum* and the TEP1 immunity gene in *Anopheles* mosquitoes may play a significant role in defining malaria infections since specific Pfs47 haplotypes can avoid the mosquito's immune system, elevating the risk of infection. Understanding the complex and poorly understood *Anopheles-Plasmodium* interactions form a basis for identifying host factor knowledge gaps related to vector competence and serve as potential targets for malaria transmission- blocking therapies.

6.2 Materials and methods

6.2.1 Study site and population

Chulaimbo is a rural site 19 km west of Kisumu City (0.03572°S, 34.621°E, altitude ranges 1328-1381 m above sea level). The mean annual temperature is between 12^0C - 35°C, average annual rainfall of 1352 mm and an average relative humidity range of 66–83%. Malaria transmission in this area is endemic with *Plasmodium falciparum* as the dominant species in the area (Idris *et al.*, 2016). Most residents are small-scale subsistence farmers. The region has a sporadic water supply system, limited sewer and waste disposal.

A cross-sectional survey was conducted during the dry season (January to March, 2020) and the wet season (October to December, 2019 and October to December 2020). Children between the ages of 5 and 15 years with parental consent were screened from villages in Chulaimbo, Kisumu County (Plate 6.1).

6.2.2 Mosquitoes used for the study

Membrane feeding experiments were carried out on female *Anopheles gambiae* mosquitoes (Kisumu strain) that were reared in the Centre of excellence for malaria research (ICEMR) insectary between three and five days after emergence (Plate 6.2). This colony was maintained in ICEMR, Homa Bay in western Kenya. The mosquitoes were reared at temperatures of 27-29^0C, 69-80% relative humidity (RH), and a 12 hours light and 12 hours dark cycle. The colony was then constantly maintained on 10% sucrose (Das *et al.*, 2007).

Plate 6.1: A; Blood is drawn from a *Plasmodium falciparum* gametocyte carrier, B; Infected blood transfer into the hemotek feeders prior to feeding, C; Anopheles gambiae mosquitoes ingesting blood under a dark cloth and the hemotek feeding system

6.2.3 Identifying gametocyte carriers

Parasitological assessments to detect *P. falciparum* gametocyte carriers were conducted in school children between the ages of 5 and 15 who agreed and had their guardians' permission. Blood samples were obtained from the children using finger pricks on well- labeled Whatman® 903 Protein Saver Cards (GE Healthcare WB100014) with the participants' information including the code, age, sex and date. A total of 50 µl of blood was collected and placed onto the Whatman® 903 Protein Saver Cards, which were then air dried before being stored at -20ºC in zip lock bags for molecular analyses. Simultaneously, thick and thin smears were prepared. Blood films were stained with 10% GIEMSA and *Plasmodium* parasites identified using a compound microscope. Malaria parasites counts were read against 500 white blood cells. Gametocyte densities were determined for all *P. falciparum* positive participants by counting the number of gametocytes per 500 leukocytes by microscopy and expressed as parasites per μl assuming a standard white blood cell (WBC) concentration of 8000/μl (McKenzie *et al.*, 2005). Two skilled Microscopists took two readings per slide smear. A senior external Microscopists then selected 20% of the slides for quality control verification. Membrane feeding was limited to gametocyte positive subjects only. Individuals who tested positive for malaria and had symptoms were then referred to the nearest local health center and treated.

6.2.4 Blood draws for mosquito feeding assays

Gametocyte carriers from screened volunteers positive for *P. falciparum* gametocytes donated blood for the whole blood and serum replaced experiments. Blood was drawn intravenously by a professional phlebotomist using butterfly needles. Approximately 3 ml of blood was collected by venipuncture in heparinized tubes for each volunteer. An aliquot of 1.5ml of blood was immediately aliquoted into pre-warmed hemotek feeders (1ml capacity) at 37^0C while another 1.5ml was immediately transferred into 2ml Eppendorf tubes and centrifuged at 2000 rounds per minute for 2 minutes. The supernatant of serum was discarded and replaced with a naïve human serum type AB (Bio Whittaker, Cambrex Bio Science Walkersville, MD, USA). Replaced blood was quickly transferred to the feeders to allow the starved mosquitoes to feed.

6.2.5 Mosquito infections using membrane feeding assays

Only participants blood positive for gametocytes was used to feed insectary reared *An. gambiae* KSM strain mosquitoes using membrane feeding assays (serum replacement and whole blood experiments) (Bousema *et al.*, 2012). Aggressive 3-5 ay old female *Anopheles gambiae* (Kisumu strain) mosquitoes were starved for 6-8hrs prior to feeding on infected blood. Whole blood and serum replacement experiments were conducted for each participant. Each cup contained 100 mosquitoes. The mosquitoes were allowed to feed from different feeders of the same infected blood for 15-30 minutes through a parafilm membrane. All membrane feeding procedures were conducted at 37^0C using the hemotek system. Only fully engorged blood-fed mosquitoes were sorted and unfed mosquitoes discarded by freezing them for 15 minutes at -20^0C. Infected mosquitoes were transferred to cages and maintained at 27-29^0C temperatures and 69-80% relative humidity and a 12 hours light and 12 hours dark cycle. They were given 6% sucrose and maintained to days 8 and 9 post- feeding. Only mosquitoes that survived were dissected for oocysts enumeration. The study participants were treated with artemether-lumefantrine (Coartem®) according to the Ministry of Health guideline after the membrane feeding exercise.

6.2.5.1 Oocysts enumeration

All infected mosquitoes that survived were dissected on the 8th or 9th day post-feeding under a dissecting microscope as described by Afrane *et al* (2008). Briefly, each mosquito gut was carefully pulled out from the abdomen in 0.5% mercurochrome allowed to stain for 10 minutes. The midguts were then examined for oocysts under a light microscope. The number of oocysts observed were counted and recorded per mosquito gut. The oocysts load was expressed as the number of oocysts per infected mosquito. Mosquito carcasses were labeled and preserved for further molecular assays.

6.2.6 Data analysis

Data from the participants was tabulated in Microsoft Excel V16. Computing descriptive statistics (sum, mean, standard deviation, standard error, and 95 % confidence interval) and comparing means were done using GraphPad Prism v.8.0.1 and SPSS version 25 for Windows software. The Shapiro–Wilk normality test was used to check data normality before performing multiple mean comparisons and chi-square tests. BioEdit version 7.2.6.1 was used to construct sequenced paired raw reads, which were then aligned using the ClustalW algorithm in MEGA X software. Data were considered statistically significant at $P< 0.05$.

6.3 Results

6.3.1 Parasitological surveys used to identify gametocyte carriers

Out of 4481 children that were tested for malaria, 885 tested positive, representing a 19.7% infection prevalence (95% CI: 0.003-0.007). The majority of the positive cases were attributed to *P. falciparum* infections, accounting for 95% (841) of the total infections. Other malaria parasite species identified in the study included P. *malariae* (1.6%), *P. ovale* (0.3%), and mixed infections involving *P. falciparum* and *P. malariae* (Pf/Pm) or *P. ovale* (Pf/Po), each accounting for 2.7% of the infections. Out of the 841 confirmed *P. falciparum* positive infections, 142 participants had *P. falciparum* gametocytes that were confirmed by microscopy. The overall gametocyte density was 37.3 gametocytes/µl of blood. The gametocyte prevalence was 6.9% and density ranged from 16-176 gametocytes/µl of blood treating each infection as an individual entity.

The odds of finding microscopic gametocyte infections were significantly high during the dry season (OR 1.37, 95% CI, P=0.001) compared to the wet season (Table 6.1). Males were 1.23 times more likely than females to harbor microscopic gametocyte infections. No significant variations in gametocyte prevalence by age was observed P=0.649. Children between the ages of 8 and 15 years had the highest gametocyte densities.

Table 6.1: Gametocyte prevalence by season and gender

Parameter		N	Gametocyte Density/µl	Gametocyte Prevalence n (%)	OR (95% CI)	P-Value
Season	Wet	3690	30.88 (26.67 - 35.74)	682 (18.5)	1.37 (1.14-1.64)	0.001
	Dry	791*	44.11(28.43 - 68.42)	187 (23.6)		
Gender	Female	2262	32.03 (26.15 -39.23)	403 (17.8)	1.23 (1.06-1.42)	0.007
	Male	2219*	31.97 (26.30 - 38.84)	466 (21)		

*Reference categories

6.3.2 Mosquito infections rates by assay

Membrane feeding assays were performed on 109 of 142 children who tested positive for *P. falciparum* gametocytes. On day 9 after feeding, only 34% of individuals with gametocytes infected mosquitos. A total of 3894 mosquitoes were dissected, 1960 in serum replacement and 1934 in whole blood to evaluate infection rates. Thirty-seven paired membrane feeding experiments from the same donor had infection rates of 0.8% (15/1960) and 0.5% (9/1934), with oocyst densities of 1 and 1.8 in serum replacement and whole blood, respectively. (Table 6.2). The difference in both experiments was however not significant (P=0.738).

Table 6.2: Infection prevalence for paired experiments (n=37)

Experiment types	Number exposed	Feeding rate (%)	Dissected (%)	Number Infected	Prevalence of infection (%)	Total oocysts count	Oocyst density/midgut
Serum replacement	3760	65.20%	1960 (82.2)	15	0.8	15	1
Whole blood	3760	56.60%	1934 (80.2)	9	0.5	16	1.8

6.3.3 Prevalence of infectious Pfs47 haplotypes

Six genetic haplotypes from 24 *P. falciparum* gametocyte sequences, both with and without specific codon variants, have been identified as potentially infectious. Haplotypes with the dimorphic codons E27D and L240I, haplotypes containing S98T, haplotypes containing E27D, haplotypes with the codon L240I, haplotypes with the codon E188D, and haplotypes without dimorphic codons (Figure 6.2). Genotyped parasite DNA from blood infected with Hap_4 (with dimorphic codon L2401) was the most frequent at 7 (29.2%) with positive oocyst results followed by Hap_3 (E27D) and Hap_6 (with no polymorphic sites) each with 5 (20.8%). Infectious haplotypes with E27D and L240I mutations (Hap_1) were observed at a frequency of 4 (16.7%) whereas Hap_2 (S98T0) and Hap_5 (E188D) was each presented at a frequency of 1 (4.2%) and 2 (8.3%) respectively.

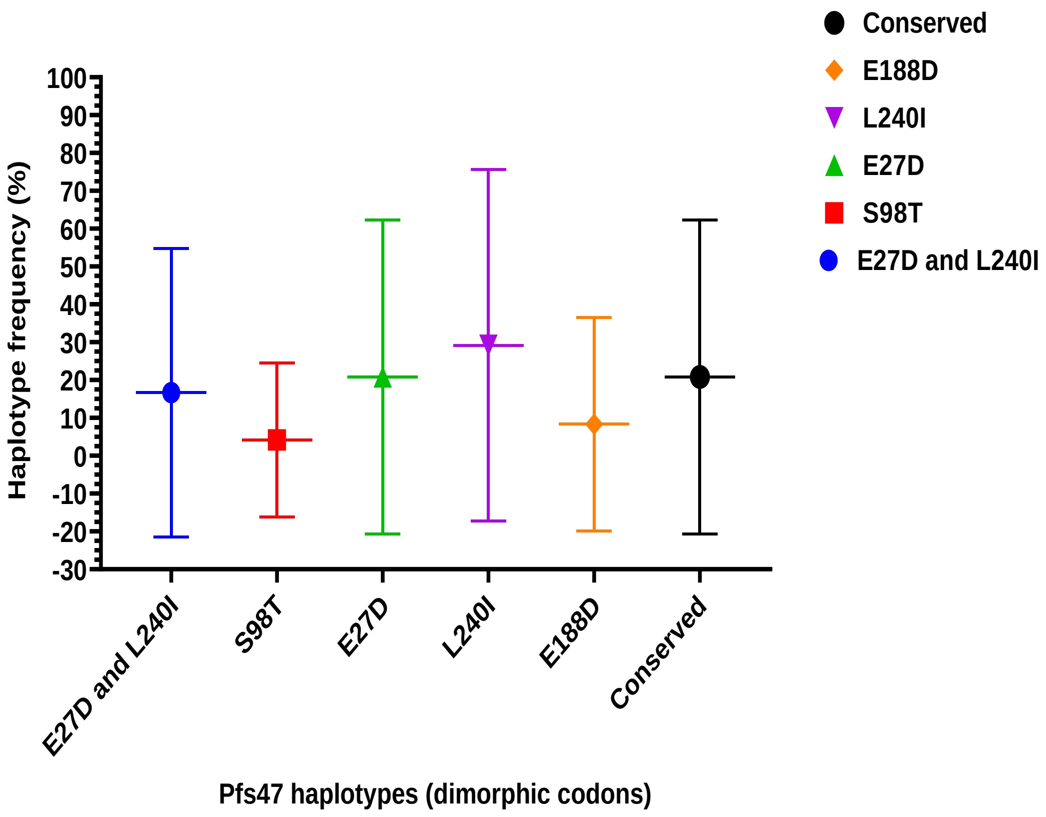

Figure 6.1: Frequency of haplotypes with positive oocyst results.

6.4 Discussion

This study established the compatibility of probable infectious *Pfs47* haplotypes to *An. gambiae* Kisumu strain mosquitoes reared in the insectary. Children aged 5 to 15 years from the same cohort and study site had the highest frequency of positive oocyst results, although being detected at a low frequency (6.7%) in the blood infected with haplotypes containing dimorphic codon L240I. Despite the combination of the two mutation codons E27D and L240I, this haplotype did not appear to increase infectivity based on the frequency observed. On the other hand, haplotypes containing codon E27D and those without polymorphic sites infected the same proportion of mosquitoes, showing limited compatibility to the *An. gambiae* s. s mosquitoes used.

The fact that six Pfs47 haplotypes with mutation codons observed in this study infected mosquitoes at low frequencies shows that they could be compatible with other vector species when transmitting malaria. Apart from vectors and gametocyte compatibility human antibodies against gametocytes play a critical role in transmission blocking or reduction capabilities in addition to their compatibility (Miura *et al.*, 2013; Fabra-García *et al.*, 2023; Simons *et al.*, 2023).

A previous study identified thirteen Pfs47 haplotypes; haplotypes bearing E27D mutations were the majority (53.3%) followed by those without mutation sites (16.7%) whereas other haplotypes occurred at a frequency of 6.7% or lower (Onyango *et al.*, 2021). All *An. gambiae s.s* used were reared in the insectary and harbored the homozygous TEP1* S1/S1 genotype in higher frequencies than the heterozygous TEP1*R2/S1 genotypes. The high prevalence of TEP1* S1/S1 observed indicates that the mosquito population used were mostly susceptible to *Plasmodium* gametocytes (Rono, 2017: Bartilol *et al.*, 2022a) and may have been highly compatible with gametocytes containing the L240I dimorphism following the “lock-and-key” theory as described by Molina-cruz *et al* (2015). Interactions between *P. falciparum* strains from different continents and mosquitos from Africa, Asia, and the Americas resulted in parasites infecting vectors from the same locality considerably more successfully than vectors from another continent which may be due to the selection of compatible parasites by indigenous vectors.

A weak association between gametocyte density and infection rates was observed despite exposing numerous mosquitoes with infected blood. The low infection rates in the mosquito may have been an indication that the serum replacement and whole blood experiments did not have a significant difference on infection rates. The infectiousness of gametocytes can be validated by exposing *Anopheles* vectors to parasites using membrane feeding assays. However, MFAs have clearly demonstrated variances in gametocyte prevalence and their infectiousness, which may be caused by inherent vector factors reported by Koella and Sorense (2002). Other factors may include physiological and environmental variables, antibody levels (Smith *et al.*, 2014) and surface antigens among them Pfs47, which is expressed by *P. falciparum* gametocytes (Crompton *et al.*, 2014), as well as other human host factors (Drakeley *et al.*, 1999; Blandin *et al.*, 2008; Timinao *et al.*, 2021). These factors might contribute to the low infection rates as well as the weak associations between gametocyte density and infection rates reported in the current study. This finding corroborated previous investigations by Bousema and Drakeley (2011) that also documented low infection rates reported in the current study. and weak association between gametocyte densities and mosquito infection rates that also varied with low gametocyte densities.

To better understand malaria transmission dynamics, molecular interactions underlying mosquito immune responses to *P. falciparum* infections and the immune evasion tactics is of importance. According to Molina-cruz *et al* (2017; 2015; 2016), the *Pfs47* gene has undergone natural selection as a result of adaptations to diverse anopheline species found in different continents hence a strong population structure. Parasites with compatible Pfs47 haplotypes can evade complement activation and survive within invaded midgut cells (Ramphul *et al.*, 2015). According to previous investigations*, Plasmodium falciparum* strains from different continents interacted with mosquitoes from Africa, Asia, and the Americas to indicate that the parasites infected the vectors from the same site far more potently than those from another continent (Molina-Cruz *et al.*, 2015). Subsequently, different recombinant *P. falciparum* parasites, each containing a Pfs47 haplotype from a different region worldwide with the same genetic makeup, were synthesized in order to test the hypothesis that Pfs47 haplotypes directly influence the parasite survival in indigenous vectors to a region. The findings implied that the Pfs47 haplotype replacement was sufficient to alter compatibility with several vectors(Canepa *et al.*, 2016). However, further investigations should be conducted on indigenous *Anopheles* vectors combinations with infectious Pfs47 haplotypes as a potential target that may focus on disrupting malaria transmission in the mosquito host.

6.5 Conclusions

The Pfs47 haplotypes that contained mutation codons E27D, L240I, S98T, E188D and those conserved (haplotypes with no mutations like the 3D7 *P. falciparum* strain) were found to be potentially infectious to *Anopheles gambiae s. l.* Despite some haplotypes having a combination of the mutations in the Pfs47 gene the parasites' ability to infect and be transmitted by *Anopheles* mosquitoes was not enhanced in western Kenya.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion

The aim of this study was to determine the genetic diversity of TEP1 and *Pfs47* in *An. gambiae* s. l and *P. falciparum* parasites respectively in western Kenya, in addition to understanding the parasite and vector interactions with regards to vector competence. The TEP1 distribution in *An. gambiae* s. s and *An. Arabiensis* from western Kenya identified TEP1*S1/S1, TEP1*R2/R2, and TEP1*R2/S1 genotypes and TEP1*S1 and TEP1*R2 alleles, with TEP*S1 being most prevalent than the resistant allele TEP*R2. Apart from *Anophelese merus*, which was identified along the Kenyan coast and exhibited the TEP1*R3 allele, and *An. coluzzii*, which was identified in Gambia, Mali, Burkina Faso, and Cameroon and exhibited the TEP1*R1 allele, the distribution of TEP1 in other members of the *An. gambiae* complex identified was consistent with the findings of this study (Rono, 2017; Gildenhard *et al.*, 2019; Bartilol *et al.*, 2022a; Hamid-Adiamoh *et al.*, 2023;).

The high TEP1*S1 allele frequency in *An. gambiae s.s* and *An. arabiensis*, demonstrated that both species have varying frequencies of genetic variations associated with *Plasmodium* parasite susceptibility hence a varied genetic diversity within populations. These genetic variations could influence the malaria transmission dynamics in different regions where these mosquitoes are present (Le *et al.*, 2012; Eldering *et al.*, 2016). The preservation and widespread distribution of TEP1*S1 and TEP1*R2 alleles may have substantial implications for malaria transmission dynamics. Furthermore, the genotypes widely distributed may provide mosquitoes with certain advantages such as more effective immune responses against *Plasmodium* parasites, which may influence their ability to spread malaria.

The overall F*st* values of TEP1 in *Anopheles gambiae s. l* indicate minimal genetic variation between study sites. This finding reveals the absence of significant barriers hindering genetic exchange, hence facilitating a continuous gene flow leading to minimal genetic differentiation between species. The effective migration indexes further reinforce these findings, indicating a high level of gene flow among populations (Horton *et al.,* 2010; Harris *et al.*, 2012). The small departures from Hardy-Weinberg Equilibrium (HWE) at the TEP1 loci indicate that particular TEP1 alleles are beneficial and are preferred by selection pressures, resulting in deviations from expected equilibrium frequencies (Crawford *et al.*, 2012). Furthermore, the findings suggest that other processes, such as natural mutations and interbreeding, may be involved in shaping TEP1 allele structure. This implies that genetic alterations caused by mutations, and the genetic material exchanged through interbreeding may be contributing to the observed genetic diversity at the TEP1 loci.

Overall, this study illuminated the genetic diversity of TEP1 in *An. gambiae s. l* in western Kenya. Understanding the variations in TEP1 and the distribution in different mosquito populations is vital in devising effective malaria control strategies, such as genetically manipulating mosquitoes to reduce their ability to transmit the disease. Additionally, these findings enhance our understanding of mosquito immunity and provide valuable insights into the complex interactions between mosquitoes and malaria parasites. The current research characterized thirteen haplotypes in *Pfs47* within the polymorphic region (1061bp). The high level of haplotype diversity observed within the Pfs47 gene suggests that the *P. falciparum* parasite is continuously developing and adapting to varied settings and host populations. These findings support previous investigations on the genetic diversity of malaria parasites, demonstrating the genetic diversity of the Pfs47 protein (Molina-Cruz and Barillas-Mury, 2014; Molina-Cruz *et al.*, 2016; Onyango *et al.*, 2021). This polymorphism is most likely caused by selection pressures imposed by the human immune system and the biology of the mosquito vector.

Eight single nucleotide polymorphisms (SNPs) resulted in 7 amino acid changes: E27D, E188D, P194H, L240I, I248L, N272I, and I304L within the Pfs47 sequence. Prevalent haplotypes E188D, L240I, I248L, and N272Y, the majority of which contained mutations in Domain 2 (D2) were identified in *P. falciparum* isolates from western Kenya. The other seven mutations were identified in parasites present throughout Africa, Asia, America, and Papua New Guinea (Molina-Cruz *et al.*, 2015), with an exception of the haplotype with mutation codon I304L documented in this study for the first time. Most of the observed mutations occurred within immunogenic domain two (D2) of *Pfs47*. This region is known to be highly polymorphic, containing the polymorphisms that determine mosquito compatibility at the Pfs47 protein's C-terminus (Molina-Cruz and Barillas-Mury, 2022). Moreover, antibodies targeting the D2 domain of the Pfs47 protein can effectively block transmission, consequently, a potential target for future malaria transmission-blocking interventions (Canepa *et al.*, 2018; Molina-Cruz and Barillas-Mury, 2022). Antibodies prevent the parasite from completing the life cycle within the mosquito by blocking the development of ookinetes, thus decreasing the number of malaria parasites transmitted to the human host. The ability of these antibodies to inhibit transmission is not dependent on the human complement system and cannot be attributed simply to complement's role in the immune response, but that other mechanisms are involved in mediating the transmission- blocking process (Canepa *et al.*, 2018).

The research findings demonstrate an excess of rare alleles at high frequencies. Similar trends observed in another study indicating that this could be as a result of a recent positive selection or a population expansion within populations (Amegashie *et al.*, 2020). Furthermore, the population's richness of rare haplotypes is indicated by low Fu's F values which is consistent with recent population growth since it indicates an excess of mutations that have not yet reached high frequencies in the populations. According to Pfs47 sequence analysis, there was a considerable gene flow between the local populations of *P. falciparum* with a pairwise index of differentiation (F*st*) less than 0.05. Previous studies have proposed that gene movement among *P. falciparum* parasites is probably linked to the mixing of human populations as a result of migration (Duffy *et al.*, 2017). Despite variations in the ecology and epidemiology among the sites, substantial human migration between sites (Ndeda, 2019) could be responsible for the observed Pfs47 gene flow in the area.

The presence of rare alleles in the lowlands comprises the parasite population. Despite the significant level of genetic variations seen due to nonsynonymous nucleotide and amino acid alterations, the *P. falciparum* isolates retained a shared gene pool, resulting in a relatively homogeneous parasite population. Nonsynonymous substitutions observed indicated that the amino acid polymorphisms in the *Pfs47* antigen are being selected for by vectors. Due to adaptations to various anopheline species present in different continents, the *Pfs47* gene has undergone natural selection, resulting in a significant population structure (Molina-Cruz and Barillas-Mury, 2014; Molina-Cruz *et al.*, 2015; Molina-Cruz *et al.*, 2016). The Pfs47-invading midgut cells enable parasite persistence by inhibiting complement activation (Ramphul *et al.*, 2015). The selection pressure exerted by *Anopheles* vectors may have an impact on the distribution of Pfs47 haplotypes. The polymorphisms may be vital in evading TEP1-mediated immune responses to the vectors.

The ability of potentially infectious Pfs47 haplotypes to interact effectively with *An. gambiae* s.s mosquitoes was also established in the current study. The importance of this research stems from distinct Pfs47 haplotypes, characterized by mutation codons, exhibiting limited infection rates within mosquitoes. Within the same cohort and study site, children aged 5 to 15 exhibited the highest occurrence of positive oocyst outcomes (Khagayi *et al.*, 2019). These outcomes, characterized by a modest frequency of 6.7%, were linked to infected blood containing the Pfs47 haplotype containing the L240I dimorphic codon. According to the observed frequency, the Pfs47 haplotype containing both E27D and L240I mutation codons did not enhance infectivity. In contrast, haplotypes having the mutation codon E27D and haplotypes lacking polymorphic sites had equal mosquito infection rates, indicating limited compatibility with the *An. gambiae s. s*.

The TEP1*S1/S1 genotype's high frequency shows that the mosquito population used was predominately vulnerable to *Plasmodium* infections (Rono, 2017). Additionally, it might have been especially compatible with parasites that contained L240I dimorphisms. A previous study on the prevalence of asexual parasites in the western

Kenyan, haplotypes with the E27D mutation were found to be the most widespread (53.3%), followed by those without mutation sites (16.7%), and the remaining haplotypes were found to be at a frequency of 6.7% or lower (Onyango *et al.*, 2021), this haplotype, however, had no infectious activity. The high frequency of the TEP1*S1/S1 genotype suggests that the mosquito population employed was mostly susceptible to *Plasmodium* infections (Rono, 2017). There is evidence, nevertheless that *P. falciparum* parasite populations in Africa differ in their ability to circumvent the TEP1-mediated immune response, with the most significant modifications reported in resistant *Anopheles gambiae* mosquitoes (Eldering *et al.*, 2016). It may also have been compatible with parasites carrying L240I dimorphisms hence the high infection frequencies observed. On the contrary, Pfs47 haplotype with mutation I248L also identified in this region were completely melanized in *Anopheles gambiae* R strain after infection unlike in *An. stephensi* that showed infectious activity and *An. albimanus* though exhibited low infection rates of 4% (Canepa *et al.*, 2016).

7.2 Conclusions

i. The distribution of TEP1 alleles depicts two common TEP1 alleles (TEP1*S1 and TEP1*R2) and three corresponding genotypes (TEP1*S1/S1, TEP1*R2/S1, and TEP1*R2/R2) in *An. gambiae* mosquitoes in western Kenya.

ii. There is a high genetic diversity and population structure of Pfs47in *P. falciparum* populations found in western Kenya.

iii. The infectious Pfs47 haplotypes in *Plasmodium falciparum* in western Kenya vary in their infectivity to *An. gambiae* mosquitoes.

7.3 Recommendations

i. Use acquired TEP1 distribution data to inform the development of policies and targeted interventions depending on the genotypes and transmission setting.

ii. Establish long-term genetic surveillance programs to monitor changes in the genetic diversity and population structure of Pfs47 over time.

iii. Strengthen vector control strategies especially in regions exhibiting high prevalence of infectious Pfs47 haplotypes by monitoring targeted interventions

7.4 Suggestions for further research

i. Characterize the distribution of TEP1 immune related genes and their impact on vector competence

ii. Conduct longitudinal studies to track changes in the Pfs47 genetic diversity and population structure over time

iii. Conduct field studies to determine the association between specific Pfs47 haplotypes prevalence of in wild populations and their transmission rates.

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APPENDICES

Appendix I: Ethical Approval letter

MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 11th September, 2019

TO: Dr. Harrysone Atieli
Department of Public Health
School of Public Health and Community Development
Maseno University
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00456/17

RE: Environmental Modification in Sub-Saharan Africa: Changing Epidemiology, Transmission and Pathogenesis of *Plasmodium falciparum* and *Plasmodium vivax* Malaria. Proposal Reference Number: MSU/DRPI/MUERC/ 00456/17

This is to inform you that the Maseno University Ethics Review Committee (MUERC) considered your valued application for extension of ethics approval of your study. The Committee commended the progress made and granted an **approval for continuation** of the study effective this 11th day of September, 2019 for a period of one (1) year

Please note that authorization to conduct this study will automatically expire on 10th September, 2020. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 15th August, 2020.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 15th August, 2020.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to the MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

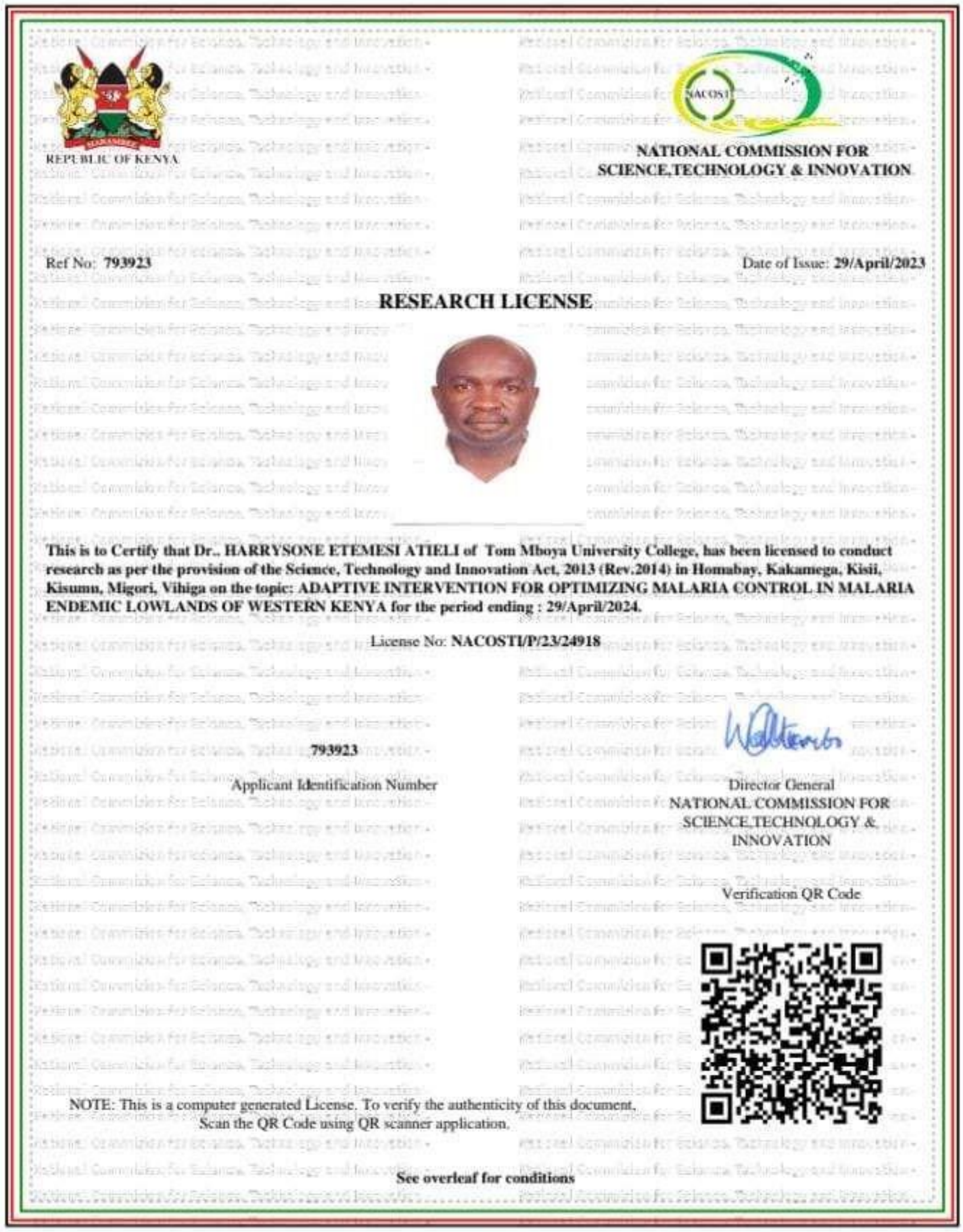
SECRETARY
11 SEP 2019

Dr. Bernard Guyah
Ag. Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Appendix II: Permit from the National Commission for Science, Technology and Innovation



REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **793923**

Date of Issue: **29/April/2023**

RESEARCH LICENSE

This is to Certify that Dr.. HARRYSONE ETEMESI ATIELI of Tom Mboya University College, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Homabay, Kakamega, Kisii, Kisumu, Migori, Vihiga on the topic: ADAPTIVE INTERVENTION FOR OPTIMIZING MALARIA CONTROL IN MALARIA ENDEMIC LOWLANDS OF WESTERN KENYA for the period ending : 29/April/2024.

License No: **NACOSTI/P/23/24918**

793923

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY &
INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document,
Scan the QR Code using QR scanner application.

See overleaf for conditions

Appendix III: Kenyatta university Research approval letter

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 8710901 Ext. 57530

OUR REF: I84/39140/2017

Date: 11th March, 2021

The Director General,
National Commission for Science, Technology & Innovation
P.O. Box 30623-00100,
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR ONYANGO S. AKINYI REG. NO. I84/39140/2017

I write to introduce Onyango who is a Postgraduate Student of this University. The student is registered for Degree programme in the **Department of Zoological Sciences**

Onyango intends to conduct research for project entitled, **"Genetic Diversity of *Anopheles gambiae* Immunity Gene and Vector Competence to *Plasmodium falciparum* in Homa Bay and Kisumu Counties, Kenya"**

Any assistance given will be highly appreciated.

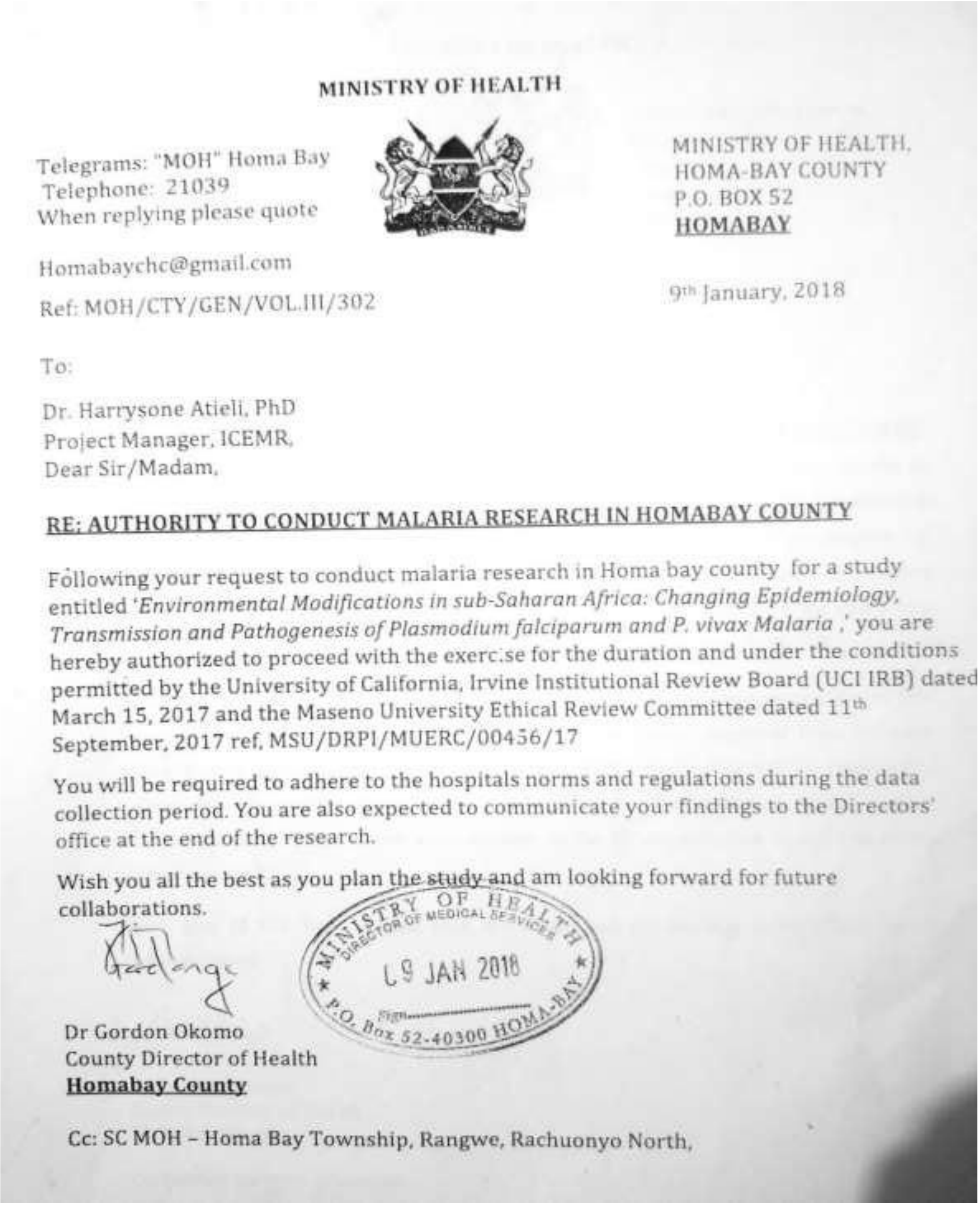
Yours faithfully,

PROF. ELISHIBA KIMANI
DEAN, GRADUATE SCHOOL

RM/cao

Committed to Creativity, Excellence & Self-Reliance

Appendix IV: Ethical clearance from the Ministry of Health



MINISTRY OF HEALTH

Telegrams: "MOH" Homa Bay
Telephone: 21039
When replying please quote

Homabaychc@gmail.com

MINISTRY OF HEALTH,
HOMA-BAY COUNTY
P.O. BOX 52
HOMABAY

Ref: MOH/CTY/GEN/VOL.III/302

9th January, 2018

To:

Dr. Harrysone Atieli, PhD
Project Manager, ICEMR,
Dear Sir/Madam,

RE: AUTHORITY TO CONDUCT MALARIA RESEARCH IN HOMABAY COUNTY

Following your request to conduct malaria research in Homa bay county for a study entitled '*Environmental Modifications in sub-Saharan Africa: Changing Epidemiology, Transmission and Pathogenesis of Plasmodium falciparum and P. vivax Malaria* ,' you are hereby authorized to proceed with the exercise for the duration and under the conditions permitted by the University of California, Irvine Institutional Review Board (UCI IRB) dated March 15, 2017 and the Maseno University Ethical Review Committee dated 11th September, 2017 ref. MSU/DRPI/MUERC/00436/17

You will be required to adhere to the hospitals norms and regulations during the data collection period. You are also expected to communicate your findings to the Directors' office at the end of the research.

Wish you all the best as you plan the study and am looking forward for future collaborations.

DIRECTOR OF MEDICAL SERVICES
09 JAN 2018

Dr Gordon Okomo
County Director of Health
Homabay County

Cc: SC MOH – Homa Bay Township, Rangwe, Rachuonyo North,

Appendix V: Assent Form

Genetic Diversity of *Anopheles Gambiae* Immunity Gene and Vector Competence to *Plasmodium falciparum* in Western Kenya

This consent form will be explained and signed by each study participant

Name of Volunteer:__________________________________

Age of Volunteer:__________________________________

Principal Investigator: Shirley Akinyi Onyango. Supervisors: Prof. Andrew K. Githeko and Prof. Elizabethe kokwaro. P.O. Box 30197-00100. Tel: +254722290696

The purpose of study

The purpose of this study is to determine polymorphisms in Thioester containing protein1(TEP1) and *Plasmodium faicliparum* surface protein 47 (Pfs47) and their interactions from different rural areas of western Kenya with varying transmission intensities.

Procedures involved

Approximately 4ml of blood will be drawn from the vein in heparin microtainers. 100ul of the blood drawn from participants will be used to prepare dried blood spots and 200ul will be used to prepare thick and thin smears. 3ml will be used for serum replacement and whole blood indirect mosquito membrane feeding assays to asses *Plasmodium* parasites infectivity to mosquitoes.. All participants will have a unique identifier that links them to their laboratory results, demography and location. Samples will be analyzed by microscopy and qPCR at the international center of excellence for malaria research (ICEMR). unauthorized tests for the study will not be carried out on the blood samples.

Discomforts and risks

The finger prick procedure is slightly uncomfortable. For each individual, sterile needle and a syringe (followed by sterile ethanol) will be utilized. The operations will represent very minimal risk of infection by other infections.

Benefit to participants

Because enrolment is voluntary, there is no financial benefit for your participation.

Confidentiality

To the degree permitted by law, information about you will be kept strictly confidential. Your identity will be coded but not linked to any published results. Your code number and identity will be retained in the Principal Investigator's locked file.

Freedom to withdraw

Your participation in this study is voluntary and you may discontinue your participation at any time without prejudice and without affecting future health care.

Who to contact

If you have questions about the study or your participation in this study, you may contact the investigators on the contact given above. For any questions pertaining to rights as a research participant, contact person is: **The Secretary, Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722290696, 0721543976, 0733230878;**

Email address: muerc-secretariate@maseno.ac.ke; muerc- secretariate@gmail.com

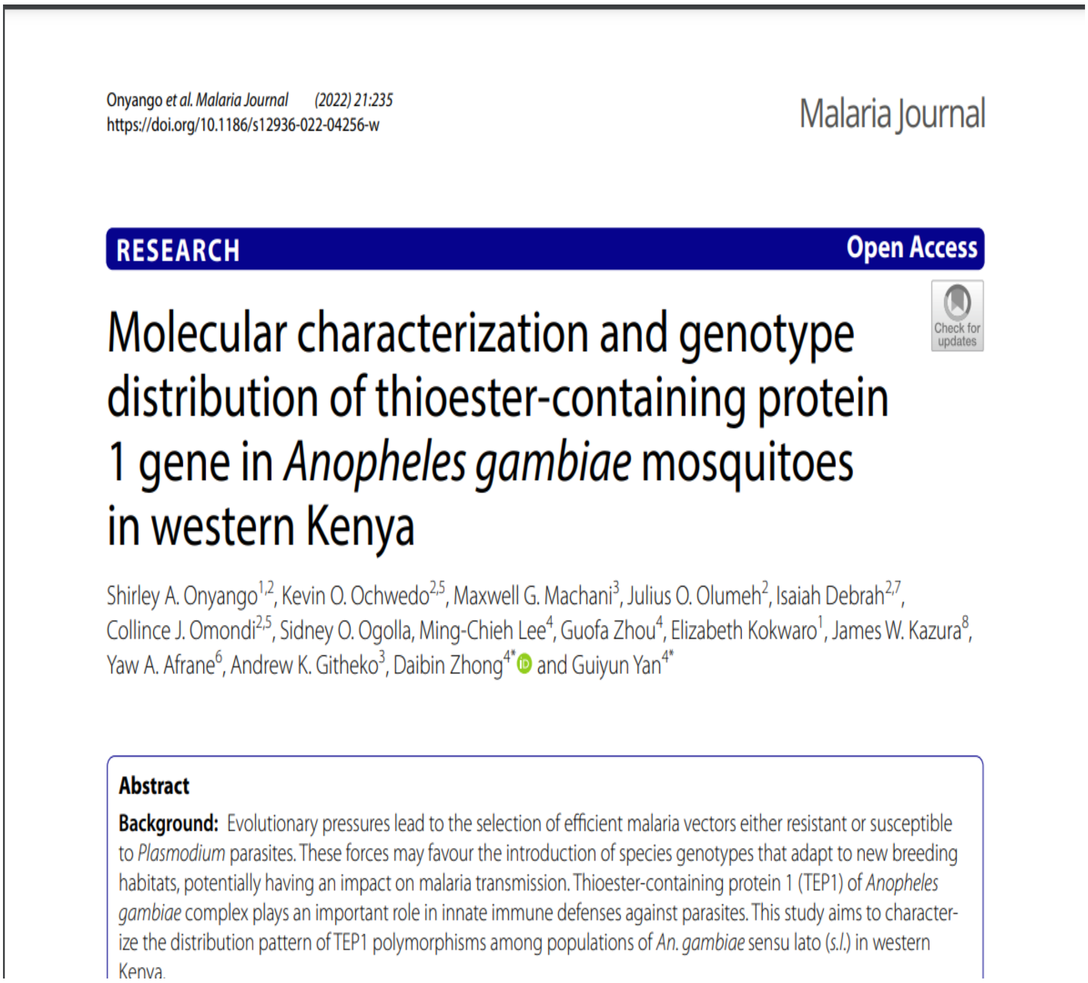
I have read and understand this consent form, and I am willing to participate in the study.

Participant’s Name Participant’s Signature (consent) Date

Parent/Guardian’s Name Parent/Guardian’s Signature (Assent) Date

Investigator’s Name (type or print) Investigator’s Signature Date

Appendix VI: Publication 1



Onyango *et al. Malaria Journal* (2022) 21:235
https://doi.org/10.1186/s12936-022-04256-w

Malaria Journal

RESEARCH Open Access

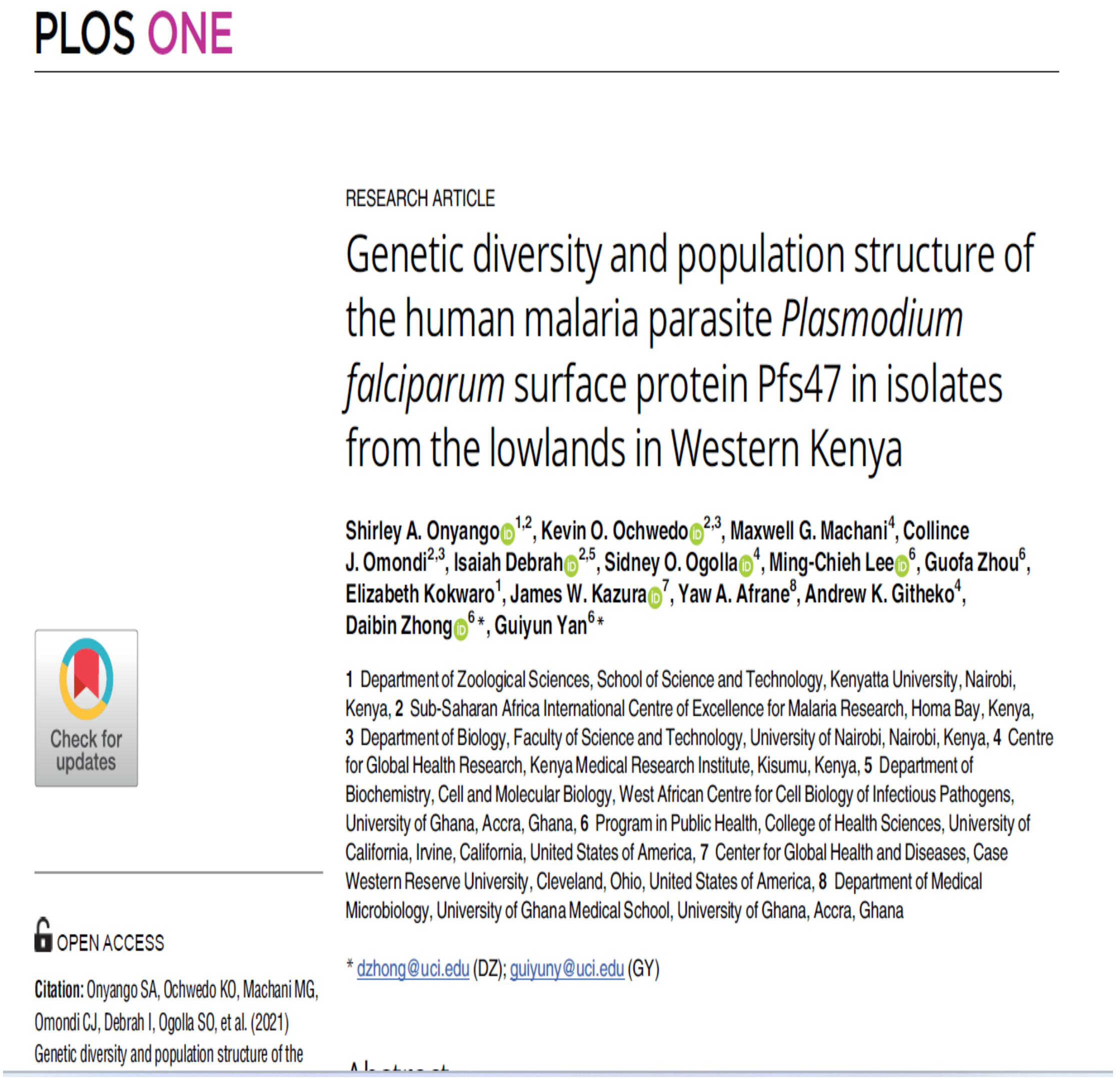
Molecular characterization and genotype distribution of thioester-containing protein 1 gene in *Anopheles gambiae* mosquitoes in western Kenya

Shirley A. Onyango[1,2], Kevin O. Ochwedo[2,5], Maxwell G. Machani[3], Julius O. Olumeh[2], Isaiah Debrah[2,7], Collince J. Omondi[2,5], Sidney O. Ogolla, Ming-Chieh Lee[4], Guofa Zhou[4], Elizabeth Kokwaro[1], James W. Kazura[8], Yaw A. Afrane[6], Andrew K. Githeko[3], Daibin Zhong[4*] and Guiyun Yan[4*]

Abstract

Background: Evolutionary pressures lead to the selection of efficient malaria vectors either resistant or susceptible to *Plasmodium* parasites. These forces may favour the introduction of species genotypes that adapt to new breeding habitats, potentially having an impact on malaria transmission. Thioester-containing protein 1 (TEP1) of *Anopheles gambiae* complex plays an important role in innate immune defenses against parasites. This study aims to characterize the distribution pattern of TEP1 polymorphisms among populations of *An. gambiae* sensu lato (*s.l.*) in western Kenya.

Appendix VII: Publication 2



PLOS ONE

RESEARCH ARTICLE

Genetic diversity and population structure of the human malaria parasite *Plasmodium falciparum* surface protein Pfs47 in isolates from the lowlands in Western Kenya

Shirley A. Onyango[1,2], **Kevin O. Ochwedo**[2,3], **Maxwell G. Machani**[4], **Collince J. Omondi**[2,3], **Isaiah Debrah**[2,5], **Sidney O. Ogolla**[4], **Ming-Chieh Lee**[6], **Guofa Zhou**[6], **Elizabeth Kokwaro**[1], **James W. Kazura**[7], **Yaw A. Afrane**[8], **Andrew K. Githeko**[4], **Daibin Zhong**[6]*, **Guiyun Yan**[6]*

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* dzhong@uci.edu (DZ); guiyuny@uci.edu (GY)

Check for updates

Citation: Onyango SA, Ochwedo KO, Machani MG, Omondi CJ, Debrah I, Ogolla SO, et al. (2021) Genetic diversity and population structure of the