THE UNIVERSITY OF LIVERPOOL

School of Tropical Medicine

Landscape genetics of *Anopheles gambiae* in southern Ghana.

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Dedication

I dedicate this study to my mum, Mary Araba Quantson and my Dad, Joseph Kofi Essandoh, and to my awesome princesses; Charity, Suzzy, Modesta and Khadijah.

Abstract

Insecticide application via indoor residual spraying (IRS) and long-lasting insecticide-treated bednets (LLIN) has been a key component in combating malaria in sub-Saharan Africa, with pyrethroids being the traditionally recommended insecticides. However, the resistance of *Anopheles gambiae s.s.* and *An. coluzzii* to pyrethroids is high and widespread, especially in West Africa, underpinned in part by high frequencies of the L1014F target-site mutation in the voltage-gated sodium channel (*Vgsc*). Carbamate (CB) and organophosphate (OP) resistance is generally lower but appears to be spreading via increasing frequencies of acetylcholinesterase (*Ace*-1) target site mutations involving the G119S polymorphism. Whilst *Vgsc*-1014F often nears fixation, *Ace*-1-119S is far more heterogeneous with complex copy number variant (CNV) dynamics. This study seeks to identify the determinants of *Ace-1*-119S spatial and temporal distribution and use population genetics to quantify the fine-scale population structure of *An. gambiae s.s and An. coluzzii* to understand how genetic barriers and gene flow may determine the current and future geographical spread.

The study across southern Ghana demonstrated that ecological zones and land-use activities are important determinants of *Ace-1*-119S and *Vgsc*-1014F distributions. The *Vgsc*-1014F is associated with rural, mining and cash crop plantations, while *Ace-1*-119S is associated with urban agricultural activities. These human-influenced environmental factors are likely to influence selection for, and the spread of the insecticide resistance mutations. Interesting, whilst almost all past studies have shown that insecticidal mortality increased with mosquito age, this was not the case for pirimiphos methyl. Indeed, mortality was well predicted by *Ace-1* mutant type but not age, suggesting that age-dependent costs of resistance may be low, and more broadly that age-specific resistance is not a universal phenomenon and may depend on insecticide class and resistance mechanisms.

The study also showed that informative molecular markers such as *Ace-1* can be used for sensitive temporal surveillance to track the changes in resistance in populations missed by standard diagnostic dose assays. In the focal area of Accra from which the time series of samples were collected, *Ace*-1-119S frequencies within *An. gambiae s.s.* and *An. coluzzii* appeared destined for fixation but deviated from this path in recent years. This resulted from the co-existence of homogeneous and heterogeneous *Ace-1* duplications, with balancing of mutant polymorphisms likely a result of different costs and benefits of each. There was also evidence of shifting *Ace*-1 duplication type dynamics that are operating in the opposite direction in each species – presumably bringing each towards a common equilibrium.

Finally, *Anopheles gambiae s.s.* and *An. coluzzii* sampled from 34 sites spanning the four ecological zones, and land-use types were genotyped using around 10,000 markers generated by low-coverage genome sequencing. Results showed a striking lack of close relatives among mosquitoes, despite larval sampling, suggesting that the strategy of collecting from multiple sites within (on average) a 3.0-km radius successfully yielded unrelated mosquitoes. There was low genetic differentiation and no population structure within both *An. coluzzii* and *An. gambiae s.s.* across southern Ghana. However, *An. coluzzii* showed isolation by distance at a local sampling scale, suggesting there is distance-limited dispersal, and that patterns reflect contemporary gene flow. In *An. gambiae s.s,* no isolation by distance was detectable, even at a smaller geographical distance of separation suggesting the implication of historical signals for the observed no population structure. The results also showed that distance is not the primary cause of *Ace-1* differentiation, but rather habitat differentiation which may correlate with distance. The findings of this study have given insight into phenotypic resistance of primary *Anopheles* malaria vectors to CB and particularly OP and how underlying target-site resistance mechanisms may be selected by environmental factors to modulate resistance and its spread. This information provides guidelines for vector control and resistance management.

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List of abbreviations

AMOVA	Analysis of molecular variance
BNARI	Biotechnology and Nuclear Agricultural Research Institute
CYP450	Cytochrome P450 enzymes
CI	Confidence interval
CB	Carbamate
DDT	Dichlorodiphenyltrichloroethane
df	Degree of freedom
DNA	Deoxyribose nucleic acid
GABA	Gamma aminobutyric acid
GLM	Generalized linear model
GST	Glutathione S- transferase
HWE	Hardy-Weinberg Equilibrium
IRM	Insecticide resistance management
IRS	Indoor residual spraying
ITN	Insecticide-treated bed nets
Kdr	Knockdown resistance
LLIN	Long-lasting insecticide-treated net
LSTM	Liverpool School of Tropical Medicine
MAF	Minor allele frequency
N	Number
MCMC	Markov Chain Monte Carlo
NMCP	National Malaria Control Programme
OP	Organophosphate
OR	Odds ratio
p	95% Probability value
PCoA	principal coordinate analysis
PCR	Polymerase chain reaction
PM	Pirimiphos methyl
PMI	President's Malaria Initiative
PO	Posterior odds
LD	Linkage disequilibrium
RBM	Roll Back Malaria
RIDL	Release of Insects with a Dominant Lethal
SIT	Sterile Insect Technique
s.l	*sensu lato*
s.s	*sensu stricto*
UPGMA	Unweighted Pair-Group method
UV	Ultraviolet
VCM	Vector control method
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
X^2	Chi-squared

Chapter One: Introduction and Literature Review

1.0 Introduction

1.1 Malaria impact

Malaria is an infection caused by a parasitic protozoon of the genus *Plasmodium* and transmitted through the bite of female *Anopheles* mosquitoes. It remains the most important vector-borne disease as it counts for 212 million cases with an estimated 429000 deaths in 2015, particularly among children between two and ten years (WHO, 2017). The repeated clinical consequences of infection during early life place a significant burden on individual households, the health system and, ultimately, the economic development of communities and nations (Sachs and Malaney, 2002). Although there has been a significant decline of malaria cases and deaths globally since 2010 as more countries are moving towards elimination, the gains have stalled since 2016 and even some regions have recorded elevated indigenous malaria cases between 2014 and 2016 (WHO, 2017). The stagnation of the reduction is maybe attributed partly to inadequate funding and drug and insecticide resistance (Alonso and Noor, 2017; WHO, 2017). Currently, malaria is endemic in 91 countries and territories (Figure 1.1) and the direct cost of malaria control, prevention and elimination globally in 2015 alone amounted to 2.1 billion dollars. It is therefore acknowledged that the persistence of malaria in the tropical and sub-tropical regions greatly contributes to a perpetual state of depressed economic growth (Sachs et al., 2001).

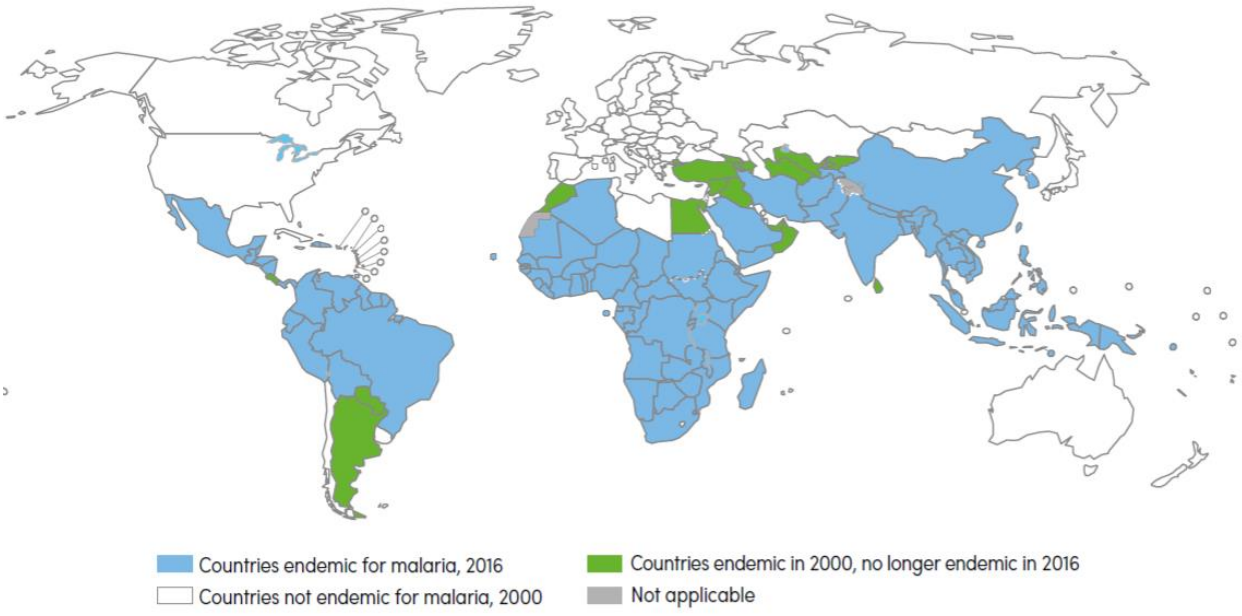


Figure 1.1: Countries endemic for malaria in 2000 and 2016 (WHO, 2017).

1.2 Malaria parasites and their distribution

Plasmodium species are obligate parasites that require an invertebrate host and a vertebrate host (intermediate host) to complete their life cycle. Currently, over 100 *Plasmodium* species have been described but only five accounts for all malarial infections in humans, these are *P. falciparum*, *P. ovale, P. vivax, P. malariae* and *P. knowlesi*. *Plasmodium falciparum* is predominantly in Sub-Saharan Africa where it is responsible for most malarial infections and is associated with most morbidity and mortality (Greenwood et al., 2005). *Plasmodium vivax* and *P. ovale* can cause relapses after months of infection and they are associated with substantial morbidity, rather than mortality. *Plasmodium vivax* is responsible for most cases in Central and Southern America and Asia (Greenwood et al, 2005) but it is less prevalent in Africa, restricted mainly to Ethiopia, Eritrea and Sudan (Meara *et al.*, 2010), while *P. ovale* is predominantly in Africa. *Plasmodium malariae* causes little morbidity but has a wide global distribution. *Plasmodium knowlesi* is confined to Southeast Asia where it naturally infects macaque monkeys and can cause zoonotic malaria in humans (White, 2008)

1.3 Major Malaria vectors and their distribution

About 70 Anopheline species have been identified as being capable of transmitting *Plasmodium* parasites under natural conditions. However, only a subset of them are of a major public health concern as they are physiologically competent, bite humans and live long enough to support the completion of sporogonic development of the parasite (Sinka *et al.*, 2012). Some of the most epidemiologically important vectors of malaria in the various tropical and semi-tropical regions are *Anopheles punctulatus* and *An. koliensis* in the Oceanic (Killeen et al, 2013), *An. stephensi*, *An. culicifacies* and *An. punctulatus* in Asia (Basseri et al, 2012)*, An. gambiae s.s., An. coluzzii, An. arabiensis* and *An. funestus* from Africa (Huho et al, 2013), and *An. darlingi, An. punctimacula, An. nunetzovari* and *An. albimanus* in Latin America (reviewed in Killeen, 2014) (Figure 1.2). The most important vectors often exhibit highly anthropophilic and endophilic behaviour.

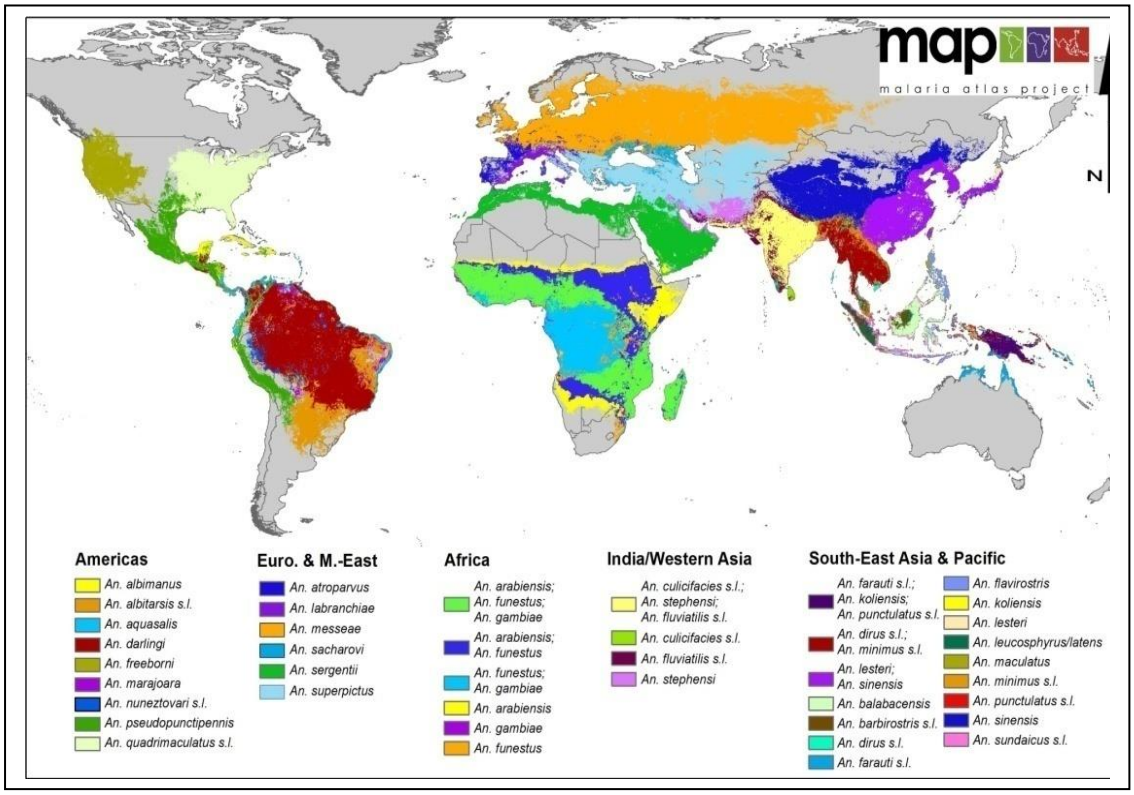


Figure 1.2: Global distribution dominant malaria vectors (Sinka, 2013)

1.4 *Anopheles gambiae s.s.* and *Anopheles coluzzii*

The *Anopheles gambiae* complex consists of at least eight sibling species, these are *An. arabiensis, An. melas, An. merus, An. bwambae, An. quadrimaculatus, An. amharicus, An. gambiae sensu stricto*, and *An. coluzzii* (Coetzee, 2004; Coetzee *et al.*, 2013). These anopheline species are morphologically identical but differ markedly in their genetic makeup and eco-ethological malaria transmission capabilities (Della Torre *et al.*, 2002; Neafsey *et al.*, 2015). The diversity of the vectorial system will influence malaria transmission dynamics as they differ in their vectorial capacities, and also interventions strategies such as insecticide application are less likely to have a homogeneous effect (Coluzzi, 1984).

In West Africa, two species, *An. coluzzii* and *An. gambiae s.s.* (which were previously known as M-form and S-form respectively based on the molecular analysis of sequence polymorphism in ribosomal DNA loci) are the most important malaria vectors due to their widespread distribution, highly anthropophilic and endophilic behaviour. Geographically, both species co-exist in West and Central Africa, whereas only *An. gambiae s.s.* is found in Eastern Africa (della Torre et al, 2005; Nwane *et al.*, 2013), and it is hypothesised that the distribution pattern is associated with the occupation of habitats of different permanence (Lehmann and Diabate, 2008a). The most remarkable phenotypic difference documented between the two

species is larval habitat segregation (Costantini *et al.*, 2009; Gimonneau *et al.*, 2012). Ecological studies have shown that *An. coluzzii* preferentially breeds in permanent freshwater collections mainly resulting from anthropogenic activities such as irrigations and it is reproductively active throughout the year, whereas *An. gambiae s.s.* thrives in temporary breeding sites such as rain-filled puddles, quarries etc. and are present only during the rainy season (Costantini et al. 2009). The adaptation to different larval habitats has been suggested to play a key role in the speciation process (Coluzzi *et al.*, 2002)

Hybrids of *An. coluzzii* and *An. gambiae s.s.* are readily obtained in the laboratory, although they are generally rare in the wild (Simard et al., 2009) but they exist in varying frequencies spatially and temporally. A study along the coastal regions of West Africa has shown that hybrid frequencies ranging between 5% and 97% exist within populations where *An. gambiae s.s.* and *An. coluzzii* coexist (Lee *et al.*, 2013). Another study in Guinea-Bissau and Gambia reported a hybrid frequency as high as 20% (Caputo *et al.*, 2011). Earlier genome-wide comparisons of *An. gambiae s.s.* and *An. coluzzii* reported a low level of divergence (independent genetic changes through time resulting in reproductive isolation) and identified three regions of divergence that are located in chromosome X, 2L and 2R (Turner et al, 2005). However, subsequent studies found no evidence supporting the designation of the 2L genomic island of divergence as a speciation island (Clarkson *et al.*, 2014), while the chromosome-X island of divergence is associated with assortative mating (a non-random mating in which mate choices are established based on phenotype) between the species pair, thereby, confirming this genomic region as a primary candidate location for genes involved in reproductive isolation (Lee *et al.*, 2013; Aboagye-Antwi *et al.*, 2015; Caputo *et al.*, 2015).

It has also been found that assortative mating is unstable and undergoes periodic disintegration resulting in massive hybridization (crossbreeding between individuals of different species), however, the patterns of hybridization and introgression (the transfer of genes from one species to another) vary spatially (Lee *et al.*, 2013), and it likely that the differences in the local environmental conditions that influence mate selection and hybrid survival may account for the variations (Aldridge, 2005; Teeter *et al.*, 2010). Although there is no marked disparity between the species pair in terms of their host preference and vectorial competence (Wondji *et al.*, 2005; Gnémé *et al.*, 2013), adaptive habitat divergence may alter some aspects of their physiological and behavioural processes which may affect vectorial capacity and consequently have an impact on malaria epidemiology and control (Coluzzi, 1984; White *et al.*, 2011). The divergence implies that the genetic tools for controlling vector populations or spreading refractory genes are less likely to be effective as the species pair share little current gene flow.

1.5 Ecological factors that affect the life history of *An. gambiae s.s.* and *An. coluzzii*

1.5.1 Climatic factors

The ecology of anopheline vectors influences the incidence, seasonal transmission and geographical range of malaria (Mordecai *et al.*, 2013). The development, survival and reproduction of malaria vectors are contingent on environmental conditions such as ambient temperature, rainfall and humidity. Temperature, for instance, is a key driver of mosquito population dynamics and malaria transmission (Beck-Johnson *et al.*, 2013) since each life stage as well as the gonotrophic cycle is dependent on temperature. Temperature affects numerous important mosquito and parasite history traits that work together to influence transmission intensity, including biting rate and the survival of the parasite within the vector (Blanford *et al.*, 2013). Studies have also shown that mosquitoes are affected not only by average temperature but also by the degree of the daily temperature variation (Blanford *et al.*, 2013). Another environmental factor that influences mosquito biology and impacts malaria transmission is rainfall. Rainfall is essential in creating suitable habitat for mosquitoes to breed, as it provides the water required for mosquitoes to lay eggs and for their larva to mature thereby increasing the vector density. It also creates a relatively high humidity setting which consequently increases the mosquito lifespan and their activities. Relatively elevated temperatures and rainfall are the reasons for the endemicity of malaria in warmer and wetter parts of the tropics and semi-tropics (Hay *et al.*, 2013).

1.5.2 Non-climatic factors

1.5.2.1 Urbanization

Urbanization is characterised by physical landscape modification and transformation of the environment through demand for resources and improved communications (Alirol *et al.*, 2011). Generally, the incidence of malaria in urban settings is lower than that of rural settings. An extensive review by Hay *et al.*, (2013) and Tatem *et al.*, (2013) concluded that urbanization affects anopheline species in the environment in terms of their diversity, abundance, survival rates, infection rates with *P. falciparum* and man-biting rates, as such a relatively low number of people acquire the infection, become ill or die of its consequence in urban settings (Figure 1.3). For instance, a study in Burkina Faso reported an entomological inoculation rate (EIR)

(a metric that estimates the number of bites by infectious mosquitoes per person per unit time) of *An. gambiae s.l* ranging from 0 to 7.7 in the urban areas, while the surrounding villages (rural areas) recorded 82.0 to 442.0 (Rossi *et al.*, 1986). The most plausible explanation is lower vector densities due to scarcity of clean freshwater breeding sites in an urban environment as more spaces are inundated by houses, and increasing pollution of few remnant breeding sites (Trape et al, 1987; Tatem *et al.*, 2013), hence, restricting the dispersion opportunities of adult mosquitoes. Other explanations include better access to health care services, better-quality "mosquito-proof" housing and an increased ratio of humans to mosquitoes that decreases the man-biting rate (Klinkenberg *et al.*, 2008).

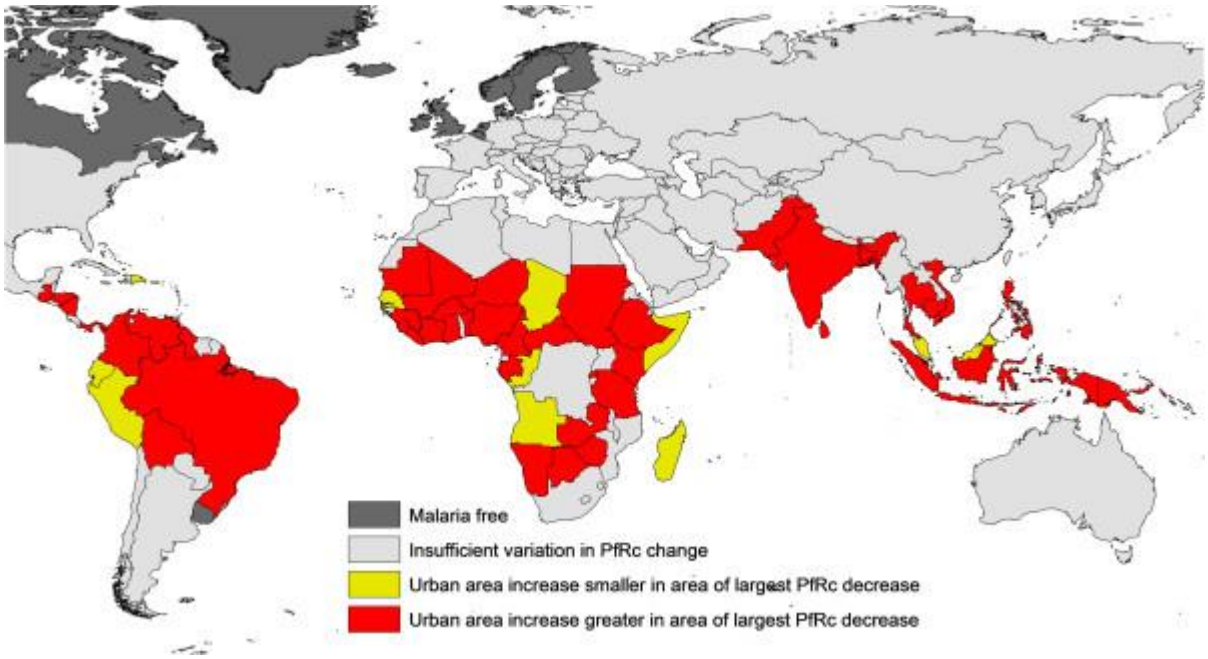


Figure 1.3: *Plasmodium falciparum* basic reproductive number (PfRC) changes and urbanization (Tatem *et al*., 2013). This figure shows that changes in urbanization in malaria-endemic countries influence the rate of infection.

However, unplanned and usually rapid urbanisation of areas within or on the outskirts of cities may provide suitable conditions for the survival of mosquitoes and the spread of malaria. This may explain why malaria transmission persists in African cities and, in some cases, at even higher levels than in rural areas (Matthys *et al.*, 2006).

Another worrying issue is that *An. gambiae s.s.* and *An. coluzzii* are adapting to a wide range of water pollution in urban settings, and this colonization of mosquitoes in otherwise unsuitable habitats is enhancing niche expansion (Tene Fossog *et al.*, 2013). In Cameroon, *An. gambiae* species pair exhibit a clinal pattern of habitat segregation along urbanization gradient, thus, *An. coluzzii* has adapted to polluted urban settings whilst *An. gambiae s.s.* thrives in the surrounding rural areas, and the pair co-exist in sympatry in the peri-urban areas

(Kamdem *et al.*, 2012). Pollutants (organic waste, petroleum products and heavy metals) in urban water bodies are likely to influence insecticide resistance in mosquitoes. It has been found that mosquitoes emerging from polluted environment possess elevated levels of metabolic enzymes and therefore they can detoxify a wide range of xenobiotics including insecticides (Djouaka *et al.*, 2007; Poupardin *et al.*, 2012; Fossog Tene *et al.*, 2013). For instance, studies have found that mosquito populations in urban areas are genetically differentiated from rural populations and show strong signatures of recent selection at loci harbouring xenobiotic resistance genes (Kamdem *et al.*, 2017).

1.5.2.2 Agricultural practice: Irrigation

Irrigation is a pivotal agricultural practice to increase crop production for ensuring food security and alleviating poverty in Africa. However, irrigation schemes are highly associated with malaria as they provide ideal breeding sites for mosquito vectors (Ijumba and Lindsay, 2001). Irrigated rice fields may also extend the breeding season of mosquitoes, and hence increase the malaria transmission duration (Ijumba and Lindsay, 2001). In arid areas, irrigation elevates relative humidity that enhances the survival of mosquitoes. A study in Ivory Coast reported that an interruption of irrigated rice farming resulted in a significant reduction in the number of infective bites per person per annum (Koudou *et al.*, 2010).

Conversely, it is also argued that the relationship between irrigation and malaria is not straightforward as increased vector densities may not necessarily mean increased malaria cases. Thus, transmission intensity in irrigated areas may be higher, similar or less than in neighbouring villages outside the irrigation scheme (Ijumba and Lindsay, 2001). For instance, it has been found that higher mosquito densities in irrigated areas may lead to increased inter-species competition and reduced longevity (Dolo *et al.*, 2004). In some cases, *An. arabiensis,* which is highly zoophilic, has a lower vectorial capacity and thrives better in irrigated areas, replaces highly anthropophilic malaria vector *An. funestus* (Ijumba and Lindsay, 2001). An extensive review on the effect of irrigation and dams on the burden of malaria concluded that malaria transmission depends on the contextual determinants of malaria, including epidemiologic setting, socioeconomic factors, vector management and health-seeking behaviour (Keiser *et al.*, 2005). Irrigation may result in increased malaria cases in unstable transmission areas such as highlands and deserts fringes of Africa, but in most sub-Saharan Africa where malaria transmission is stable, irrigation schemes may have negligible impact on malaria transmission (Ijumba and Lindsay, 2001). However, improved socioeconomic conditions offset the biological impact of irrigation on malaria increase.

1.6 Malaria control by IRS and ITN

Insecticide application via indoor residual spraying (IRS) and treatment of bednets (ITNs) and more recently long-lasting insecticidal nets (LLINs) has been a key component in combating malaria in Sub-Saharan Africa due to its proven efficacy, relatively cost-effectiveness, and high community benefit (Bhatt *et al.*, 2015). Furthermore, LLINs are, in particular, widely accepted by householders as it protects them not only against mosquitoes but also other nuisance arthropods. However, a drawback is LLIN use depends on community participation and user education which require effective and regular usage campaign (WHO, 2009). The IRS and LLIN operate by reducing human-vector contact and increasing the adult vector mortality as they target the adult mosquito which is the most epidemiologically important vector stage. They impact the vector population by reducing the longevity thereby effectively altering the age structure of the vector population such that fewer mosquitoes live long enough to become infective with the *Plasmodium* parasite (Cook and Sinkins, 2010). The IRS and LLIN are executed singly or in combination depending on the epidemiology of the disease in the local setting, acceptability of residents, timely access to communities and minimum coverage and the rapidity of the impact required (WHOPES, 2002). The substantial reduction in malaria incidence in Sub-Saharan Africa is attributed to the high coverage of LLIN (and IRS) programs (Bhatt *et al.*, 2015; World Health Organization, 2020) as shown in Figure 1.4.

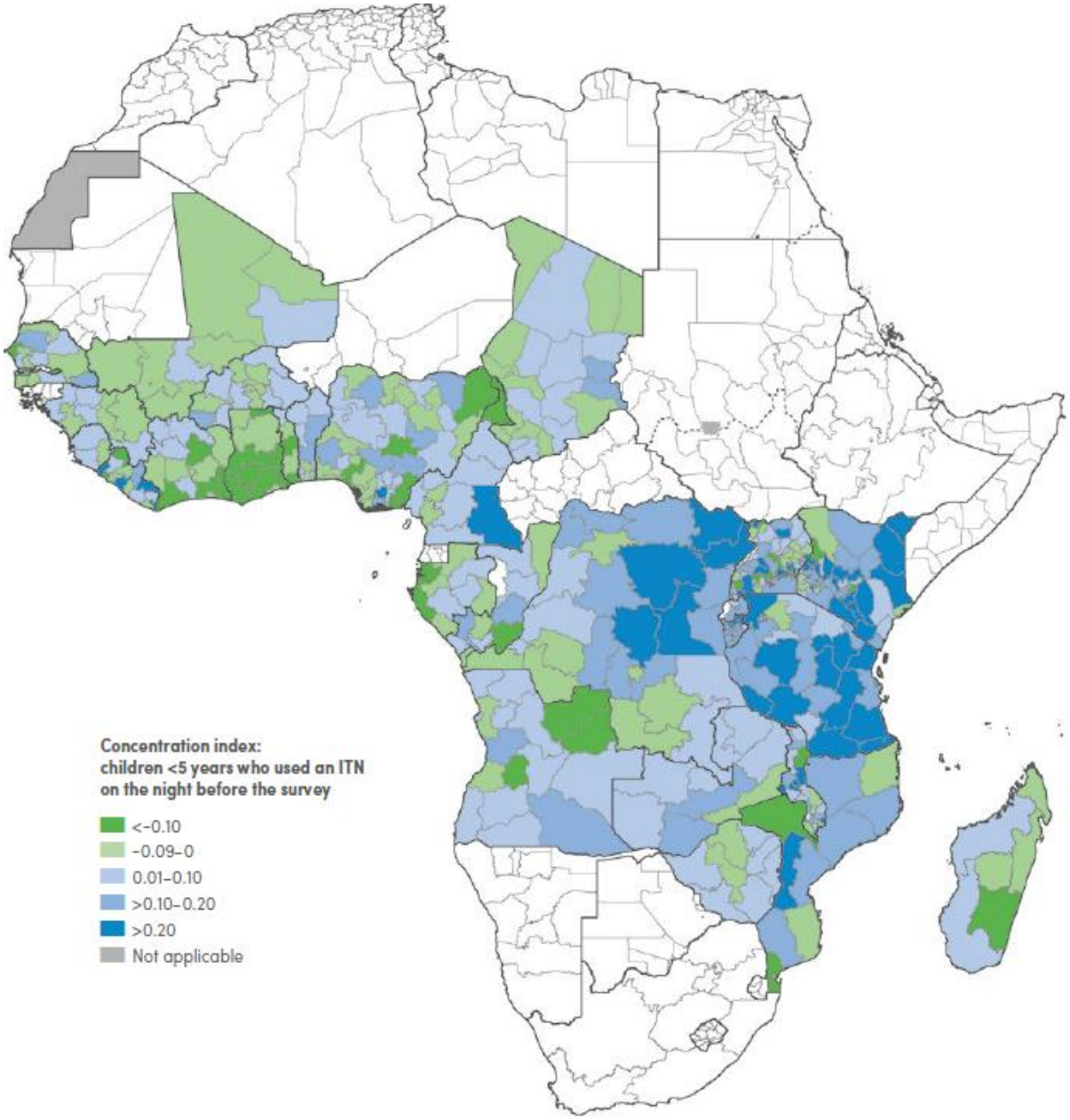


Figure 1.4: Concentration index of ITN use by children aged under five years in sub-Saharan Africa between 2015 and 2019 (World Health Organization, 2020).

1.7 Public health insecticides and their mode of action

The four classes of insecticides traditionally deployed in public health for IRS are organochlorines (e.g DDT), pyrethroids, organophosphates and carbamates. However, only pyrethroids are recommended for ITN due to their low mammalian toxicity and high excito-repellent properties (Zaim *et al*, 2000). Pyrethroids and DDT act by prolonging the opening of

the voltage-gated sodium channel (*Vgsc*) resulting in hyper-excitation, paralysis and eventual death. Organophosphates and carbamates, on the other hand, operate by inhibiting the release of the acetylcholinesterase (AChE) at cholinergic synapses, thereby preventing the termination of nerve impulses, resulting in insect death (McCaffery and Nauen, 2006).

There are also new generation insecticides that represent new classes of chemistry for public health and these include clothianidin and chlorfenapyr. Clothianidin is a neonicotinoid insecticide that is widely used in crop protection and veterinary medicine (Ngufor *et al.*, 2017). It acts as an agonist on nicotinic acetylcholine receptors causing a range of symptoms from hyper-excitation to lethargy and paralysis (Tomizawa and Casida, 2005). Neonicotinoids also have a very low affinity for vertebrate nicotinic receptors relative to insect nicotinic receptors, and therefore exhibit a very low mammalian toxicity profile (Tomizawa and Casida, 2005). Chlorfenapyr, a halogenated pyrrole, targets the oxidative pathways in the insect's mitochondria by short-circuiting the mitochondrial proton gradient thereby disrupting ATP production, loss of energy leading to cell dysfunction and subsequent death of the organism (Black *et al.*, 1994; Ngufor *et al.*, 2016).

1.8 Insecticide resistance

Insecticide Resistance Action Committee (IRAC) defines resistance as "the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended" (McCaffery and Nauen, 2006). Resistant strains emerge via the survival and reproduction of individuals carrying a genome altered by mechanisms that allow survival after exposure to an insecticide (Brattsten et al.,1986). The insecticide exerts selective pressure that increases the frequency of the allele expressed as resistance within the exposed population (Brattsten et al.,1986). Consequently, continued insecticide application elevates the proportion of resistant individuals such that they become increasingly difficult to control (Nauen, 2007) as shown in Figure 1.5.

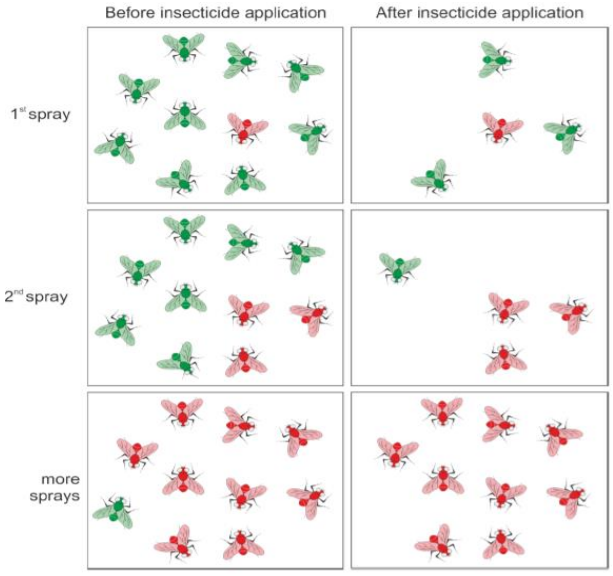


Figure 1.5: Illustrating the increase of insecticide resistance levels in a pest population (Panini *et al.*, 2016).

Insecticide resistance is an inevitable adverse side effect of mosquito control programs since the introduction of insecticides for wide-scale deployment in the 1940s for malaria control (Brown *et al.*, 2013). It has been reported that more than 100 mosquito species including 56 species of anopheline and 39 species of culicine mosquitoes are known to have developed resistance to one or more insecticides globally (WHO, 2015). According to WHO (2016) report on insecticide resistance in malaria vectors, between 2010 and 2016, of 76 malaria-endemic countries, 61 countries had detected resistance in at least one major malaria vector to at least one class of insecticide used for adult malaria vector control (Figure 1.6).

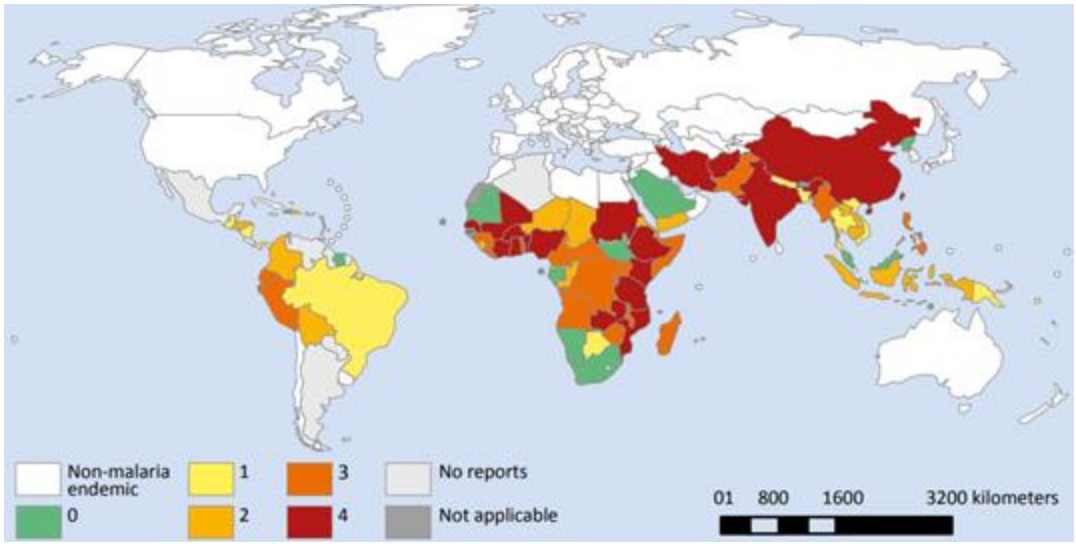


Figure 1.6: Map showing the number of insecticide classes to which resistance in malaria vectors was reported, by country, for the period 2010–2016 (WHO, 2016)

Insecticide resistance does not necessarily imply a reduction in the effectiveness of insecticide in operational terms. For instance, a study conducted in areas with pyrethroid-resistant mosquitoes in Benin, Cameroon, India, Kenya, and Sudan reported no significant decrease in the efficacy of pyrethroid-bednets (Kleinschmidt *et al.*, 2018), indicating that field impact of insecticide resistance may be a complex phenomenon which may depend on an array of ambient, biological and technical factors. However, resistance is a major cause for concern not only in public health but also in agriculture due to the overwhelming socioeconomic impact posed by arthropod disease vectors and pests, and insecticide application being one of the most rapid and effective vector control tools available.

1.9 Insecticide resistance management

The practice of using a particular insecticide until resistance emerges has eroded the number of suitable/available insecticides for vector control (Corbel and N'Guessan, 2013). The failure of the Global Malaria Eradication Campaign in the 1950s is attributed to the sole reliance on DDT for vector control. The emergence and spread of resistance coupled with the adverse environmental impact of DDT derailed the campaign (Enayati and Hemingway, 2010). Insecticide resistance management is a strategy to adopt in reducing the vector population to an acceptable level while maintaining the long-term efficacy of the insecticides deployed. However, effective management of insecticides could slow the emergence and spread of resistance.

According to IRAC, effective resistance management requires a sound understanding of the vectors' biology and the monitoring of vector populations, and the adoption of strategies to reduce selection pressure exerted by a particular mode of action on vector populations (McCaffery and Nauen, 2006). Selection pressure can be reduced through multiple-attack strategies, thus, achieving control using several insecticides with different modes of action in rotation, mosaic or mixture. The underlying principle is that if the resistance to each of the compounds is independent, then cases of multiple resistance (vector population developing resistance to different insecticides classes at the same time) to several will be rare (Georghiou, 2005; McCaffery and Nauen, 2006). It implies that it will be difficult for mosquitoes to develop resistance to insecticide classes with different target sites at the same. A multiple-attack strategy has been proposed as a resistance management tool in anopheline species (Enayati and Hemingway, 2010). However, its impact depends on the proper choice of chemicals based on their mode of action, the potential mechanisms of resistance, and the prior exposure of the

target population to insecticidal selection pressure and the presence of a significant fitness differentiation between resistant and susceptible individuals (Georghiou, 2005).

Another strategy proposed by IRAC is the use of synergists as they are essential in prolonging the lifespan of insecticides where metabolic mediation is implicated in resistance development (McCaffery and Nauen, 2006). The synergists operate by inhibiting the enzymes (cytochrome P450s and esterases) that metabolise insecticides within the insect, thereby enhancing the effect of the insecticide deployed. Examples of synergists are piperonyl butoxide (PBO), S,S,S-tributyl phosphoro-trithioate (DEF), and N-Octyl bicycloheptene dicarboximide (MGK-264) (McCaffery and Nauen, 2006). Recent randomised control trials in Tanzania have revealed that pyrethroid-PBO nets appear to provide improved protection against malaria in areas with high insecticide resistance (Protopopoff *et al.*, 2018). Experimental hut studies in Ivory Coast (Oumbouke *et al.*, 2019) and Togo (Ketoh *et al.*, 2018) have also shown that LLINs with PBO provide additional protection against pyrethroid-resistance *An. gambiae s.l* with regards to blood-feeding inhibition and increase in mosquito mortality compared to a pyrethroid-only net. However, a recent study in Cameroon has reported that PBO-based net is not effective in controlling the glutathione-S-transferase-mediated pyrethroid-resistant *An. funestus* (Menze *et al.*, 2020). As such, WHO has recommended that a pyrethroid-PBO net should be used in places where the main malaria vectors had confirmed pyrethroid resistance of moderate-intensity conferred, at least in part, by monooxygenase-mediated resistance mechanism (WHO, 2017). Resistance monitoring is an integral component of resistance management as it allows early detection of resistance or prediction of future resistance in a population, identifying selective environments and providing information critical for strategic insecticide deployment (McCaffery and Nauen, 2006) so that rational insecticide choices can be made.

1.10 Insecticide resistance mechanisms in *Anopheles gambiae*

The mechanism of insecticide resistance exhibited by an organism depends on the prevailing pressure, the mode of action of the insecticide in use and the genetic variation in the population (McCaffery and Nauen, 2006; Panini *et al.*, 2016). The two main insecticide resistance mechanisms in *An. gambiae s.l.* are the metabolic resistance and target-site mutation, while other subsidiary mechanisms include lower chemical penetration (cuticular resistance) and insecticide avoidance (behavioural resistance).

1.10.1 Metabolic Resistance

It is the reduction in the sensitivity to insecticides due to the elevation of the activities of detoxifying enzymes or sequestration of insecticides before reaching their targets to trigger the desired effect or effluxion of the insecticides. These enzyme systems evolved to allow insects to feed off plants by detoxifying xenobiotics and plant toxins (Panini *et al.*, 2016). This may explain the quick emergence of metabolic resistance against a very broad spectrum of insecticides, particularly, those derived directly and indirectly from plants (Isman and Grieneisen, 2014; Hawkins *et al.*, 2019). Resistant insects metabolise the insecticide faster as they possess forms of the enzyme with a higher catalytic rate, or higher quantities of the enzymes as a result of elevated transcription or gene amplification (Panini *et al.*, 2016). Three main enzyme groups are involved in the detoxification process: esterases (COE), monooxygenase (P450) and glutathione S-transferases (GST).

1.10.1.1 Esterases

Esterases detoxify a broad range of xenobiotics via enzymatic cleavage or sequestration rather than metabolizing the xenobiotics/insecticides (Hemingway and Ranson, 2000). They can also provide a narrow range of insecticide resistance through the metabolism of a few insecticides with an ester bond (Hemingway and Karunaratne, 1998). This involves the hydrolysis of ester insecticides into their corresponding acid and alcohol compounds thereby increasing the polarity of the insecticidal metabolites so that they are excreted more readily from the insect body (Panini *et al.*, 2016). Esterases have been implicated in pyrethroid, organophosphate and carbamate resistance (Hemingway and Ranson, 2000), and potential involvement in neonicotinoid resistance in many insect species (Zhu and Luttrell, 2015). Esterase-mediated detoxification has been reported in pyrethroid-resistant populations of *An. gambiae s.s., An. coluzzii* and *An. arabiensis* (Vulule *et al.*, 1999; Casimiro, 2006).

1.10.1.2 Monooxygenases

Monoooxygenases are microsomal oxidases that include mixed-function oxidases (MFOs) such as Cytochrome P450s. The MFOs are involved in the detoxification of xenobiotics as well as the metabolism of endogenous substances such as hormones, pheromones or fatty acids (Panini *et al.*, 2016), as such, they occur in the fat body, Malpighian tubes, and the midgut of insects. Cytochrome P450s are a large family of enzymes with broad

substrate specificity. They can catalyse different reactions such as epoxidation, hydroxylation, N-dealkylation, O-dealkylation or desulphurisation; and therefore, they play an essential role in the metabolism of many insecticide classes (Panini *et al.*, 2016). Three families of cytochrome P450s (*CYP4*, *CYP6* and *CYP9*) have been implicated in insecticide-resistant mosquitoes as a result of elevated transcriptional levels (Feyereisen, 2015), with the *CYP6* family accounting for the majority of the resistance (reviewed in Donnelly *et al.*, 2016). In *An. funestus* the over-expressions of *CYP6P9*, *CYP6P4* and *CYP6M7* are implicated in pyrethroid resistance (Hargreaves *et al.*, 2000; Casimiro, 2006; Mulamba *et al.*, 2014), whereas *CYP6Z1* confers cross-resistance to both pyrethroids and carbamates (Ibrahim *et al.*, 2016). In *An. gambiae s.s.*, *CYP6P3* and *CYP6M2* are associated with pyrethroid resistance (Djouaka *et al.*, 2008; Stevenson *et al.*, 2011), whereas *CYP6M2* confers resistance to DDT (Mitchell *et al.*, 2014), and cross-resistance to carbamate (Edi *et al.*, 2014). It has been found that the extreme carbamate resistance in Tiassale *An. gambiae* population results from the coupling of over-expressed target site allelic variants with elevated *CYP6* P450 expression (Edi *et al.*, 2014). Some common P450s implicated in metabolic resistance in *An. gambiae s.*s and *An. coluzzii* in Africa are shown in Table 1.1.

Table 1.1: Some common P450s involved in insecticide metabolism and their substrates in *An. gambiae s.*s and *An. coluzzii* in Africa

Region	**Countries**	**P450s**	**Substrates**	**Reference**
Central	Cameroon	*CYP6Z3, CYP6Z1, CYP12F2, CYP6P4, CYP6GA1*	Bendiocarb	(Antonio-Nkondjio *et al.*, 2016)
	Cameroon	*CYP6M2, CYP6P3, CYP6Z3*	DDT, Pyrethroids	(Toé *et al.*, 2015)
East	Ethiopia	*CYP6P4*	DDT, Pyrethroid	(Simma *et al.*, 2019)
	Tanzania	*CYP6P3, CYP6P4, CYP6AA1* and *CYP9K1*	Pyrethroid	(Matowo *et al.*, 2021)
	Kenya	*CYP325A3, CYP6Z1*	Permethrin	(David *et al.*, 2005)
West	Nigeria	*CYP325A3, CYP6Z3*	DDT, Pyrethroids	(Muhammad *et al.*, 2021)
	Nigeria	*CYP6M2, CYP9K1, CYP6P4* and *CYP6Z1*	DDT, Pyrethroids, Bendiocarb	(Haruna *et al.*, 2020)
	Ghana	*CYP6AG1, CYP6Z3 CYP6P4*	DDT, Pyrethroid	(Mitchell *et al.*, 2014)
	Côte d'Ivoire	*CYP6M2, CYP6P3, CYP6Z3*	Bendiocarb, DDT, Pyrethroid	(Mitchell *et al.*, 2014)
	Southern Senegal	*CYP6Z1* and *CYP6Z2*	DDT, Pyrethroids	(Gueye *et al.*, 2020)

1.10.1.3 Glutathione S-transferases (GSTs)

Glutathione S-transferases (GSTs) are a diverse family of enzymes that play an essential role in the detoxification of both endogenous and xenobiotic compounds (Hemingway and Ranson, 2000). The GSTs have a broad range of substrates and have been associated with resistance to different classes of insecticides, including organophosphates, pyrethroids and DDT(Panini *et al.*, 2016). For instance, elevated GST levels have been implicated in DDT and pyrethroid resistance in *An. gambiae, An. arabiensis, An. funestus* and houseflies (Hargreaves *et al.*, 2003; Enayati, Ranson and Hemingway, 2005; Djouaka *et al.*, 2011) The GST-mediated resistance is usually due to elevated levels of enzymes as a result of gene amplification or overexpression (Vontas *et al.*, 2002; Panini *et al.*, 2016)

1.10.2 Target-site resistance

This mechanism causes a reduction in the rate at which the insecticide binds to its target site as a result of non-synonymous point mutations altering protein structure (Hemingway and Ranson, 2000). The mutation is selected such that the resultant amino acid alteration does not cause a loss of the primary function of the target site. Target sites include sodium channel, acetylcholinesterase and gamma-aminobutyric acid (GABA) receptor, which are traditionally the most important ones for vector control insecticides. However, other minor target sites are tabulated below (Table 1.2).

Table 1.2: Minor target sites and their mode of action classified by IRAC (Resistance and Committee, 2018)

Main Group and Primary Site of Action	**Chemical Sub-group**	**Active Ingredients**
Nicotinic acetylcholine receptor (nAChR) competitive modulators *Nerve action*	Neonicotinoids	Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid, Thiamethoxam,
Nicotinic acetylcholine receptor (nAChR) allosteric modulators *Nerve action*	Spinosyns	Spinetoram, Spinosad
Glutamate-gated chloride channel (GluCl) allosteric modulators *Nerve and muscle action*	Avermectins, Milbemycins	Abamectin, Emamectin benzoate, Lepimectin, Milbemectin
Juvenile hormone mimics *Growth regulation*	Juvenile hormone Analogues	Hydroprene, Kinoprene, Methoprene
Chordotonal organ TRPV channel modulators *Nerve action*	Pyridine azomethine Derivatives	Pymetrozine, Pyrifluquinazon

1.10.2.1 Voltage-gated sodium channel (*Vgsc*)

The target-site resistance resulting from the point mutation in the *Vgsc* gene conferring resistance and cross-resistance to pyrethroid and DDT is called knockdown resistance (*kdr*). Two alternative *kdr* mutations have been reported at the same codon of *An. gambiae s.s.* and *An coluzzii*, these are *Vgsc-L1014F* and (Martinez-Torres *et al.*, 1998) and *Vgsc-L1014S* (Ranson *et al.*, 2000). The L1014F is most widely distributed in West African *An. gambiae s.l.* populations (Awolola *et al.*, 2007; Namountougou *et al.*, 2012; Hien *et al.*, 2017; Camara *et al.*, 2018); the mutation was initially observed in *An. gambiae s.s.* and later emerged in *An. coluzzii*, as a result of introgression (Clarkson *et al.*, 2014). In contrast, the emergence of the *Vgsc*-L1014F mutation within *An. arabiensis* resulted from a *de novo* mutation event (Diabate *et al.*, 2004). The *Vgsc*-L1014S mutation, which was initially predominant in East Africa (Ranson *et al.*, 2000; Verhaeghen *et al.*, 2006) has been reported in West Africa and Central Africa within *An. arabiensis*, *An. coluzzii* and *An. gambiae s.s.* populations (Djègbè *et al.*, 2011; Badolo *et al.*, 2012; Namountougou *et al.*, 2012). A third substitution, N1575Y, has been documented in West and Central Africa, and it operates in synergy with *L1014F* to enhance pyrethroid resistance (Jones *et al.*, 2012). Recent studies, using Illumina whole-genome sequence data from the *Anopheles gambiae* 1000 Genomes Project (Ag1000G), have provided a comprehensive account of genetic variation in the *Vgsc* gene in mosquito populations from eight African countries, whereby 20 non-synonymous nucleotide substitutions have been described in *Anopheles* mosquitoes (Clarkson *et al.*, 2018) as shown in Table 1.3.

Table 1.3: Non-synonymous nucleotide variation in the voltage-gated sodium channel gene.

	Variant		Population allele frequency (%)								
Position[1]	*Ag*[2]	Domain[4]	AO*Ac*	BF*Ac*	GN*Ag*	BF*Ag*	CM*Ag*	GA*Ag*	UG*Ag*	KE	GW
2,390,177 G>A	R254K	IL45	0	0	0	0	32	21	0	0	0
2,391,228 G>C	V402L	IS6	0	7	0	0	0	0	0	0	0
2,391,228 G>T	V402L	IS6	0	7	0	0	0	0	0	0	0
2,399,997 G>C	D466H	LI/II	0	0	0	0	7	0	0	0	0
2,400,071 G>A	M490I	LI/II	0	0	0	0	0	0	0	18	0
2,400,071 G>T	M490I	LI/II	0	0	0	0	0	0	0	0	0
2,416,980 C>T	T791M	IIS1	0	1	13	14	0	0	0	0	0
2,424,384 C>T	A1125V	LII/III	9	0	0	0	0	0	0	0	0
2,425,077 G>A	V1254I	LII/III	0	0	0	0	0	0	0	0	5
2,429,617 T>C	I1527T	IIIS6	0	14	0	0	0	0	0	0	0
2,429,897 A>G	E1597G	LIII/IV	0	0	6	4	0	0	0	0	0
2,429,915 A>C	K1603T	IVS1	0	5	0	0	0	0	0	0	0
2,430,424 G>T	A1746S	IVS5	0	0	11	13	0	0	0	0	0
2,430,817 G>A	V1853I	COOH	0	0	8	5	0	0	0	0	0
2,430,863 T>C	I1868T	COOH	0	0	18	25	0	0	0	0	0
2,430,880 C>T	P1874S	COOH	0	21	0	0	0	0	0	0	0
2,430,881 C>T	P1874L	COOH	0	7	45	26	0	0	0	0	0
2,431,019 T>C	F1920S	COOH	0	0	0	0	1	4	0	0	0
2,431,061 C>T	A1934V	COOH	0	12	0	0	0	0	0	0	0
2,431,079 T>C	I1940T	COOH	0	4	0	0	7	0	0	0	0

*AO=Angola; BF=Burkina Faso; GN=Guinea; CM=Cameroon; GA=Gabon; UG=Uganda; KE=Kenya; GW=Guinea-Bissau; Ac= *An. coluzzii*; Ag= *An. gambiae.* *Domain shows the location of the variant within the protein structure i.e the transmembrane segments are named according to domain number (in Roman numerals) (Clarkson *et al.*, 2018).

1.10.2.2 Gamma-aminobutyric acid (GABA) receptor

Mutations in the *Rdl* locus coding for a gamma-aminobutyric acid (GABA) receptor are implicated in dieldrin resistance (Hemingway and Ranson, 2000). Two amino acid substitutions (A302S and A302G) at the M2 transmembrane domain of a GABA gene have been associated with dieldrin resistance in *Drosophila melanogaster* (ffrench-Constant *et al.*, 2000). In mosquitoes, the alanine-glycine substitution (A296G) has been detected in *An. gambiae*, while the alanine-serine substitution developed independently in the same codon in a dieldrin-resistant strain of *An. arabiensis*, and *An. stephensi* (Du *et al.*, 2005). Although cyclodiene insecticides have been stopped for agricultural and public health use, the resistance allele is still found in insect field populations (Aronstein, Ode and Ffrench-Constant, 1995). This observation may be due to the lack of any clear fitness cost associated with the resistance phenotype, and it is also likely that *Rdl* alleles are being maintained at high frequencies by balancing selection (ffrench-Constant, 1994).

1.10.2.3 Acetylcholinesterase

Acetylcholinesterase (coded by the *Ace-1* gene) is an essential enzyme of the central nervous system that is targeted by both carbamate and organophosphate insecticides. Resistance and cross-resistance to both classes of insecticides have been reported in several mosquito species due to insensitive acetylcholinesterase as a result of single point mutations in the *Ace-1* gene (Weill et al 2003). To date, four *Ace-1* gene substitutions implicated in resistance have been identified: G119S, F290V, F331W (Alout *et al.*, 2009) and N485I (Ibrahim *et al.*, 2016). These few positions in the *Ace-1* gene suggest that mutational options within this highly evolutionarily conserved gene are likely to be limited (Oakeshott *et al.*, 2005; Weetman *et al.*, 2015). The phenylalanine-to-tryptophan (F331W) substitution has been observed only in resistant *Cx. tritaeniorhynchus* in East Asia (Alout, Berthomieu, Cui, *et al.*, 2007). The phenylalanine-to-valine (F290V) substitution has been reported in a *Cx. pipiens* population in Cyprus (Alout *et al.*, 2007). The asparagine-to-isoleucine (N485I) substitution which confers resistance to carbamates has been observed only in *An. funestus* populations in southern Africa (Ibrahim *et al.*, 2016). The glycine-to-serine (G119S) substitution has been reported in numerous anopheline and culicine species including *An. gambiae*, *An. albimanus*,

Cx. vishnui and *Cx. pipiens* (Alout *et al.*, 2007; Djogbénou *et al.*, 2008; Essandoh *et al.*, 2013). The *Ace-1-119S* mutation in *An. gambiae s.s.* and *An. coluzzii* is restricted to West Africa, and it is widespread (http://anopheles.irmapper.com). More worryingly is the onset of *Ace-1* duplication, perhaps, to suppress the fitness cost associated with *Ace-1-119S* or enhance resistance (Djogbénou *et al.,* 2008).

1.10.3 Cuticular resistance

The rate of insecticide penetration depends on the physicochemical characteristics of the insect cuticle, which vary markedly between species and life stages (Brattsten et al., 1986) and so penetration resistance arises when the insects modify the structure of their cuticle to slow down the absorption or reduce the quantity of insecticide into their body (Panini *et al.*, 2016) (Figure 1.7). Although penetration resistance protects insects from a wide range of xenobiotics, it confers low levels of resistance on its own (Panini *et al.*, 2016). As such, it usually functions in combination with other forms of resistance to enhancing their effects. Two main mechanisms have been implicated in penetration resistance, these are (1) cuticle thickening and (2) alteration of cuticle composition (Balabanidou *et al.*, 2018). Cuticular thickening due to high deposition of hydrocarbons in the epicuticle *of An. gambiae* has been shown to provide appreciable levels of resistance to multiple insecticide classes (Balabanidou *et al.*, 2018). Studies on cuticles of South African *An*. *funestus* population have shown that pyrethroid-resistant females possess thicker cuticle than their susceptible counterparts, and this observation is likely to have developed as an auxiliary to the primary mode of pyrethroid resistance which is based on enzyme-mediated detoxification (Wood *et al.*, 2010). *Anopheles gambiae* populations from West Africa that are resistant to pyrethroids and DDT are exhibiting thickening in all their chitin layers (Yahouédo *et al.*, 2017).

Alteration of cuticular composition usually results in elevated insecticide resistance due to inhibition of insecticide penetration capacity (Balabanidou *et al.*, 2018). Although numerous factors have been implicated in this mechanism, the two main ones are (i) over-expression of laccase 2 leading to cuticular hardening (i.e sclerotization and pigmentation) and (ii) over-expression ABC transporters leading to enhance translocation of cuticular materials into the epidermis (Pignatelli *et al.*, 2017). For instance, in *Cx. pipiens*, a laccase-2 gene was found to be up-regulated significantly in resistant mosquitoes than in susceptible ones. The laccase-2 gene induces extra sclerotization of the cuticle of larvae and pupae of resistant mosquitoes (Balabanidou *et al.*, 2018).

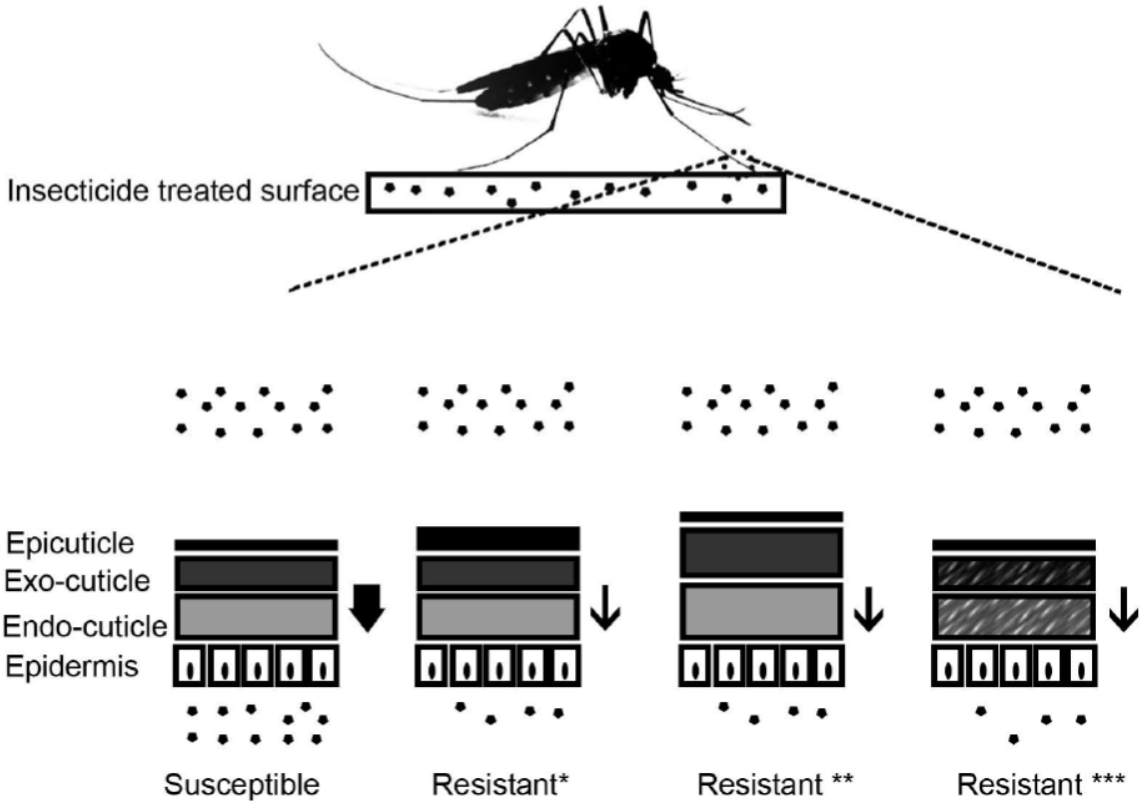


Figure 1.7: Diagrammatic illustration of penetration (cuticular) resistance showing the various components of the cuticle and how they act as barriers to insecticide intake (Balabanidou *et al.*, 2018)**.**

1.10.4 Behavioural resistance

Behavioural resistance occurs when insects alter their behaviour (thus, shifts in biting time, location and host preference) to avoid coming into contact with insecticides. Indoor application of insecticides has been found to alter the biting patterns and the endophagic frequency in some mosquitoes (Gatton *et al.*, 2013). For instance, a study has shown that *An. funestus* are shifting from indoor to outdoor biting preferences in Tanzania due to wide coverage of pyrethroid-treated nets (Russell *et al.*, 2011). In Benin, there has been a significant change in the host-seeking behaviour of the *An. funestus* as scaling up of LLINs at community level caused a shift from night biting to early-morning biting (Moiroux *et al.*, 2012). Similarly, in Bioko Island, *An. gambiae s.l.* are shifting to earlier outdoor feeding and it is postulated to be associated with high coverage of LLINs inducing a change from endophily to exophily (Reddy *et al.*, 2011). Behavioural resistance poses a great threat to malaria control in Africa as the current strategy is mainly based on the indoor application of insecticides, which is effective only when mosquito vectors are endophagic, endophilic and bite late in the evening (Corbel and N'Guessan, 2013).

1.11 The current distribution of pyrethroid and DDT resistance and Kdr resistance in *An. gambiae S.l* in West Africa

Resistance to pyrethroids and DDT has been reported in *An. gambiae s.l.* in West Africa and it is widespread (Figure 1.8) with mechanisms involving metabolic enzyme mediation and *kdr* resistance (mainly L1014F). In Senegal, high levels of resistance to DDT (mortality range: 5.8 – 52.9) and pyrethroids (permethrin 19.1- 43.3%, deltamethrin 37.7 – 60%, lambda-cyhalothrin 18.9 - 52.9% and alphacypermethrin 84.3 - 85.1%) with an implication of L1014F, L1014S, and N1575Y mutations, and elevation of expression levels of CYP6Z1 and CYP6Z2 genes (Gueye *et al.*, 2020). In southern and central Mali, high levels of pyrethroid resistance in *An. gambiae s.l* have been reported, with the mean mortality rates for permethrin, deltamethrin and alpha-cypermethrin being 24.8%, 33.6% and 49.2% respectively, and some mosquitoes even surviving at the 10X the diagnostic dose (Sovi *et al.*, 2020). In Guinea, the average mortalities of *An. gambiae s.l*. to permethrin and deltamethrin were 4.0% and 86.0%, with implicated mechanisms including fixation of L1014F mutation and overexpression of *CYP6M2*, *CYP6P3* and *GSTD3* genes. In south-east Nigeria, mortality rates of *An. gambiae s.l.* to DDT and deltamethrin were 13.0% and 57.0 % respectively, with implicated L1014F mutation frequency ranging from 60.0% to 90.0% (mortality of 13.0%) (Chukwuekezie *et al.*, 2020). In Ivory Coast, a nationwide study reported a varying range of pyrethroid and DDT resistance in *An. gambiae s.l.* (permethrin 0 – 96.9%, deltamethrin 0.9 - 97.0%, lambdacyhalothrin 3.7 - 67.7% and alphacypermethrin 2.0 - 82.9%, and DDT 0.3 - 58.5%) (Camara *et al.*, 2018). In western Burkina Faso, mortality ranges of *An. gambiae s.l.* to deltamethrin and DDT exposure were 10.0 – 38.0% and 2.7 – 59.6% respectively, with L1014F mutation and elevated levels of cytochrome P450 and GSTs as implicated mechanisms (Namountougou *et al.*, 2019). In Benin, *An. gambiae s.l.* resistance to pyrethroids has been reported, with a mortality range of 25 – 83% for deltamethrin and 6–55% for permethrin, and the L1014F mutation frequency range of 67 – 88% and elevated levels of oxidase and esterase activity (Salako *et al.*, 2018). In Ghana, a three-year monitoring of resistance in an urban vegetable-grown suburb recorded average mortalities of 9.2%, 1.7% and 2.7% for deltamethrin, permethrin and DDT respectively, and the average frequency of *Vgsc*-1014F was 99% (Pwalia *et al.*, 2019).

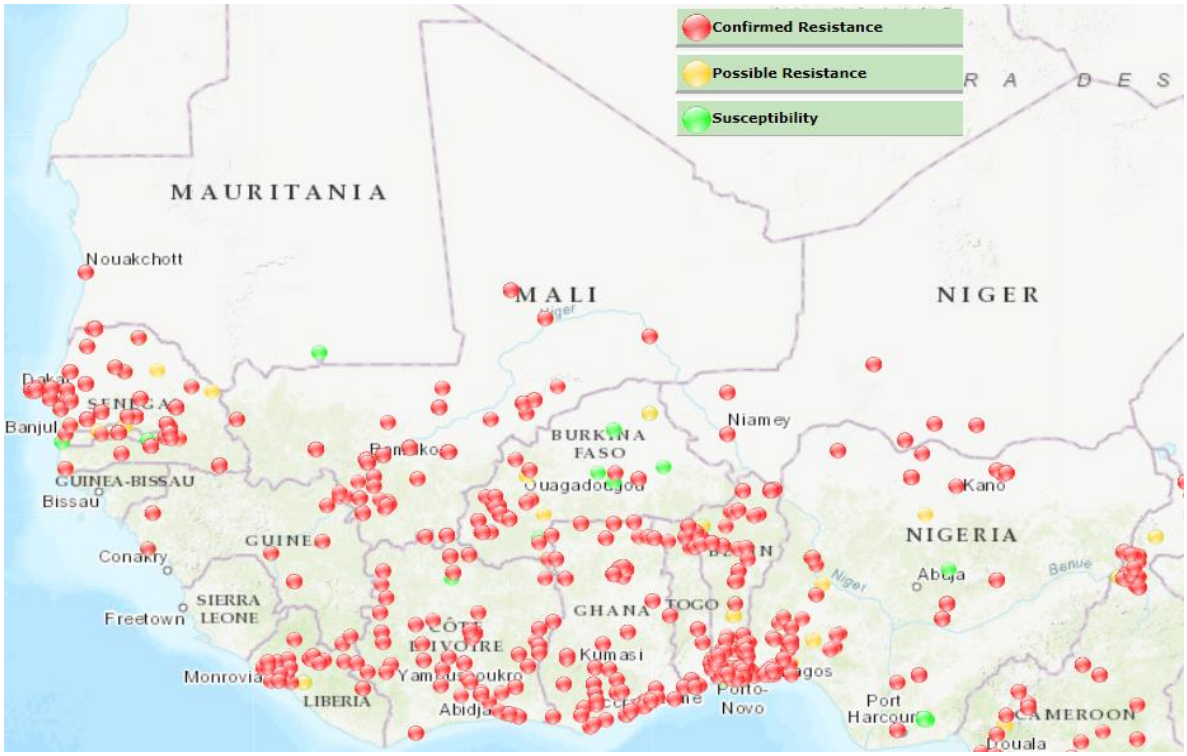


Figure 1.8: Map of West Africa showing phenotypic resistance distribution of *An. gambiae s.l.* to pyrethroids and DDT (http://anopheles.irmapper.com)

1.12 The current distribution of carbamate and organophosphate resistance and *Ace-1-119S* in *An. gambiae S.l.* in West Africa

Resistance to carbamate and organophosphate in *Anopheles* mosquitoes has been reported in most places in West Africa, but it is of lower levels compared to pyrethroid and DDT. In southern, eastern and central Africa, *Ace-1-119S* resistance alleles have not been detected in Anopheles populations (Choi *et al.*, 2014; Matowo *et al.*, 2014; Abeku *et al.*, 2017; Alemayehu *et al.*, 2017; Nardini *et al.*, 2017; Olé Sangba *et al.*, 2017). In West Africa, CB and OP resistance (Figure 1.9) and the *Ace-1*-119S mutation have been observed in *An. gambiae s.l.* but of varying frequencies which may be due to differences in selection pressures prevailing in the various regions and/or potential involvement of metabolic resistance and/or an alternative *Ace-1* mutation such as F290V (Alout et al., 2007). In Nigeria, for instance, carbamate and organophosphate resistance has been observed in *An. gambiae s.l.* but *Ace-1*-119S mutation has not yet been detected (Oduola *et al.*, 2012; Abdu, Manu and Deeni, 2017).

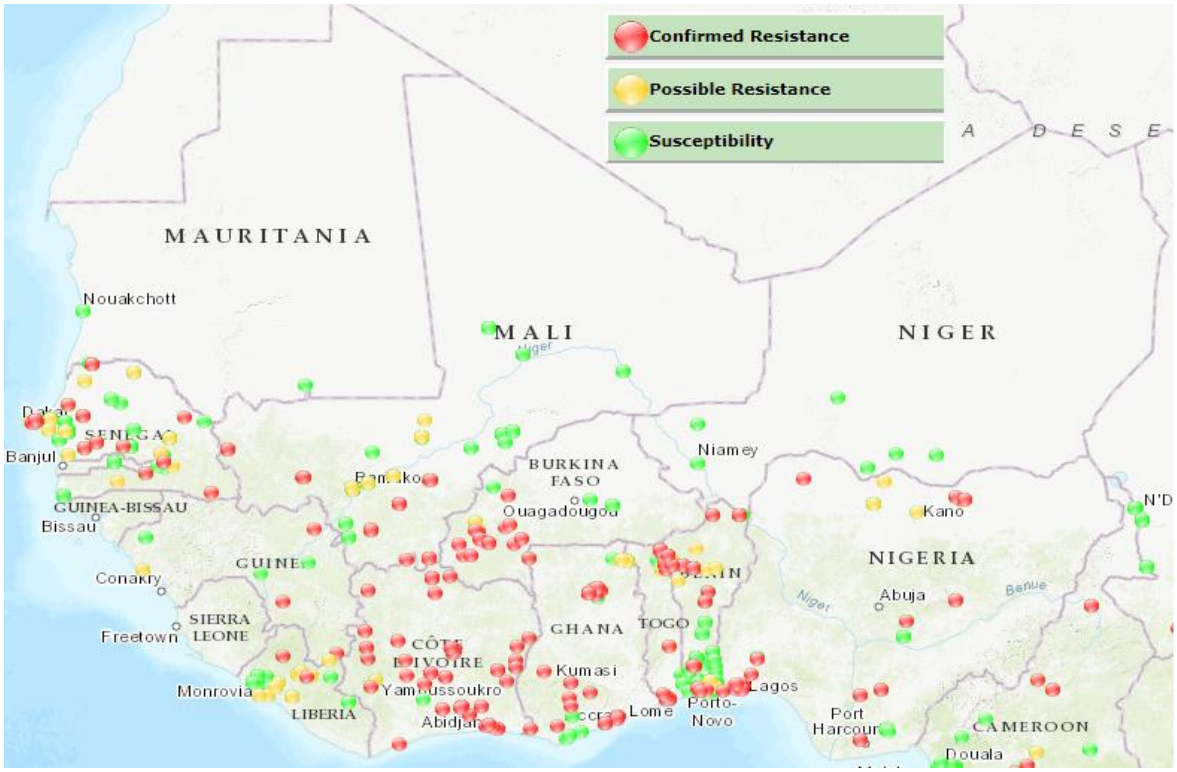


Figure 1.9: Map of West Africa showing phenotypic resistance distribution of *An. gambiae s.l.* to Carbamate and Organophosphate insecticides (http://anopheles.irmapper.com**)**

1.13 The fitness of cost of *Ace-1* (G119S) mutation

The possession of *Ace-1-119S* is advantageous in CB and OP treated areas as it hastens metamorphosis and consequently adult emergence in *An. gambiae s.s.* and *An. coluzzii* (Djogbénou *et al.*, 2010). However, the *Ace-1-119S* mutation is associated with high fitness costs that affect various physiological functions and life history and developmental traits. In *Cx. pipiens*, for instance, the catalytic activity of acetylcholinesterase in *Ace-1-119S* resistant individuals was found to be 60% less than that of the susceptible forms (Labbé, Berthomieu, *et al.*, 2007), and is associated with lower male reproductive success and female survival during overwintering (Weill *et al.*, 2004). In *Cx. quinquefasciatus*, higher levels of pre-adult mortality (Berticat *et al.*, 2008) and compromised blood-feeding success (Djègbè *et al.*, 2011) in mosquitoes possessing the *Ace-1-119S* resistance mutation. Similarly, in *An. gambiae s.s.* and *An. coluzzii*, higher pupal mortality and smaller body size in *Ace-1-119S* resistant mosquitoes than in susceptible strains (Djogbénou *et al.*, 2010). The high fitness cost may explain why fewer homozygotes than heterozygotes are found in most populations where *Ace-1-119S* is detected (Alou *et al.*, 2010).

1.14 *Ace-1* Duplication

Gene duplication is generally regarded as a necessary source of material for the origin of evolutionary novelties (new gene functions and expression patterns) (Lynch and Conery, 2001; Zhang, 2003). It plays a pivotal role in the plasticity of genome or species in changing environments particularly in diploid individuals where no more than two variations (alleles) exist within any locus (Magadum *et al.*, 2013). Gene duplications have been reported in several mosquitoes and they are likely to be non-neutral events when they arise since their immediate effect is to increase the duplicated gene expression (Labbé *et al.*, 2014) which may lead to significant changes in essential cellular balance and processes if the gene dosage is not down-regulated (Kondrashov *et al.*, 2002; Veitia, 2005).

Gene duplications have been detected in two of the major insecticide target sites in mosquitoes, *Vgsc* and *Ace*-1. The *Vgsc* duplication, involving the pairing of distinct alleles, has been detected in the *Aedes aegypti* in Brazil (Martins *et al.*, 2013) and the *Culex quinquefasciatus* in Tanzania (Martins *et al.*, 2017). The *Vgsc* duplication, in both cases, has a restricted distribution and a low frequency, and presently, its phenotypic impact remains unknown (Weetman *et al.*, 2018). The G119S is the most important of the three mutant codons (the others: F290V and F331W) associated with *Ace*-1 resistance in mosquitoes. The *Ace*-1-G119S is associated with high fitness costs, however, in *Cx. pipiens* and *Cx. quinquefasciatus*, it appears that the *Ace-1* gene duplication has been selected to act as a compensatory mechanism to restore, at least, part of the normal catalytic activity of acetylcholinesterase in the presence of the *Ace*-1-119S mutation (Labbé *et al.*, 2007).

There are two types of gene duplications: homogeneous and heterogeneous duplication. Homogeneous duplications (amplification) are usually associated with an increase in the quantity of protein produced (Milesi *et al.*, 2017) as seen in human adaptation to a starch-rich diet via greater amylase production (Axelsson *et al.*, 2013), and resistance to xenobiotics via elevated levels of detoxification (Guillemaud *et al.*, 1998). Most homogeneous gene duplications are associated with deleterious pleiotropic effects on gene function and biochemical disruptions (Kondrashov *et al.*, 2002; Kondrashov and Kondrashov, 2006). For instance, *Ace-1* gene amplification produces higher levels of AChE1, resulting in excessively rapid degradation of the neurotransmitter acetylcholine (Lee and Kwon, 2004). As such, homogeneous duplications are usually subjected to purifying selection (Kondrashov and Kondrashov, 2006; Schrider *et al.*, 2013). Heterogeneous duplication, on the other hand, involves the combination of two functionally divergent alleles of a single locus, and it is favoured in cases of overdominance (a phenomenon whereby the heterozygous individuals are the fittest) (Haldane, 1954) and it allows the fixation of both alleles. Few cases of adaptive

processes mediated by heterogeneous duplications have been reported, and to date, all are associated with genes that are target-sites of insecticides, for example, rdl in *Drosophila melanogaster* (Remnant *et al.*, 2013), and *Ace-1* in *Cx. pipiens* (Labbé *et al.*, 2007) and *An. gambiae* (Djogbénou, Dabiré, *et al.*, 2008).

Ace-1 gene duplication was first reported in *An. gambiae* in West Africa by Djogbénou et al., (2008). Using heterozygote excess at the *Ace*-1 locus, it was estimated that the *Ace-1* gene duplication frequency reaches up to 0.65 in *An. gambiae* populations of Burkina Faso and Ivory Coast (Djogbénou *et al.*, 2009). Although this estimation method provided a useful indicator but not definitive due to the possibility of selection for heterozygotes in the field (Weetman *et al.*, 2018). Recent studies have shown that both homogeneous and heterogeneous duplications of *Ace-1* exist in both *An. gambiae s.s.* and *An. coluzzii* (Assogba *et al.*, 2016; Assogba *et al.*, 2018). Genomic studies have shown that additional copies of *Ace-1* alleles in both *An. gambiae s.s.* and *An. coluzzii* lack sequence variations indicating that the duplication is a very recent event (Djogbénou *et al.*, 2008; Assogba et al 2016) and also possible introgression of *Ace-1* duplication between the species pair and subsequent spread via a hard selective sweep (Assogba *et al.*, 2018).

Heterogeneous *Ace-1* duplication is widespread in West Africa (Djogbénou, Chandre, *et al.*, 2008a; Djogbénou *et al.*, 2009, 2015), and its phenotypic consequence is similar to a standard *Ace-1*-119S heterozygote, providing an intermediate level of resistance and a lower fitness cost (Assogba *et al.*, 2015). For instance, in Tiassale, Ivory Coast, it has been found that *An. coluzzii* individuals possessing heterogeneous *Ace*-1 duplication had a significant survival advantage and this explains the near-complete heterozygosity (Edi *et al.*, 2014). It is therefore postulated that *An. gambiae* individuals that possess heterogeneous *Ace-1* duplication are likely to be selected in mosaics of treated and untreated areas, as they may represent the best resistance/cost trade-off (Assogba *et al.*, 2016). Homogeneous *Ace*-1 duplication is postulated to be selected in high-insecticide-dosage environments and it influences CB/OP resistance via higher copy numbers of *Ace-1*-119S despite the fitness cost (Assogba *et al.*, 2016). Recent genomic studies have shown that a large chromosomal segment encompassing *Ace-1* locus has duplicated, however, in some mosquitoes possessing the *Ace*-1-119S allele, there are internal deletions of ten flanking (surrounding) genes of *Ace*-1 within the chromosomal segment (Assogba *et al.*, 2018). It is hypothesised that these internal deletions might have occurred to reduce a wasteful gene expression which may result in a biochemical cellular imbalance (Assogba *et al.*, 2016).

1.15 Impact of insecticide resistance on malaria transmission

The emergence of insecticide resistance is regarded as a threat to malaria control because it increases the number of vectors that survive the insecticide treatment (Rivero *et al.*, 2010). The epidemiological assessment of the impact of insecticide resistance is very complex due to variations in the methodology deployed to measure and report resistance (Ranson *et al.*, 2000), the multivariate nature of transmission and the fact that randomised controlled trials cannot be conducted for insecticide resistance. As such, entomological indices are used as a proxy measure of the epidemiological impact of insecticide resistance (Strode *et al.*, 2014).

To date, there are few studies showing evidence of resistance impacting operational malaria control programmes. In KwaZulu-Natal, South Africa, a switch from DDT to deltamethrin for IRS resulted in a four-fold increase in malaria cases, and this was due to *An. funestus* resistance to pyrethroids (Maharaj *et al.*, 2005). However, the reintroduction of DDT led to a sharp decline in malaria cases which was maintained in subsequent years. Similarly, in Ghana (Coleman *et al.*, 2017) and Uganda (Kigozi *et al.*, 2012), a switch from DDT and pyrethroids to OPs for IRS resulted in a significant reduction in malaria cases in Uganda and Ghana. In Benin, pyrethroid resistance in *An. gambiae* showed a marked reduction in the efficacy of LLIN (N'Guessan *et al.*, 2007; Asidi *et al.*, 2012). Another worrying issue is that the possession of target-site mutations (*Vgsc- 1014F* and *Ace-1-119S*) appears to make *An. coluzzii* and *An. gambiae s.s.* more susceptible to *P. falciparum* (Alout *et al.*, 2013) and this may increase the parasite transmission in resistant *An. gambiae*. For instance, a study conducted in Tanzania found that *P. falciparum* infection rate was three times higher in pyrethroid-resistant populations than in the susceptible counterparts (Kabula *et al.*, 2016), suggesting that insecticide resistance may enhance vector competence for malaria parasites.

On the contrary, the selection of insecticide resistance in mosquito vectors is thought to interfere with the development and transmission of parasites due to pleiotropic effects on vector longevity, vector competence, and vector feeding behaviour (Rivero *et al.*, 2010) and consequently reduce the capacity of the vectors to transmit the malaria parasite. In Uganda, the exposure of resistant *An. gambiae* populations to a sub-lethal dose of deltamethrin inhibited the growth of *P. falciparum* compared to populations exposed to untreated nets (Kristan *et al.*, 2016). Another study from Bioko Island reported that *Anopheles* mosquitoes that were homozygous for *Vgsc-1014F* appeared less likely to be able to transmit malaria than susceptible individuals (Hemingway *et al.*, 2013).

Insecticide resistance may also have a smaller effect than expected on malaria control (Ranson *et al.*, 2011). In Malawi, for instance, LLINs significantly reduced malaria episodes among children in areas where the predominant malaria vector species, *An. funestus* and *An. arabiensis* exhibited moderate to high levels of pyrethroid resistance (Lindblade *et al.*, 2015). Similarly, in Burundi (Protopopoff *et al.*, 2008) and Ivory Coast (Henry *et al.*, 2005), the distribution of LLINs reduced malaria cases despite a high *Vgsc-1014S* and *Vgsc-1014F* frequency respectively. Data from recent studies found no association between malaria incidence and *kdr* frequency, and between malaria incidence and bioassay survivorship (Ochomo *et al.*, 2017; Kafy *et al.*, 2017). This is because LLIN in good condition protects from mosquito bites, therefore, reducing malaria transmission irrespective of insecticide presence (Eisele and Steketee, 2011). Furthermore, the effectiveness of insecticides on resistant mosquito populations is critically dependent on age (Alout *et al.*, 2014). As such, insecticides that are ineffective on younger *kdr* homozygous mosquitoes may still be partially efficient in controlling malaria transmission since infected mosquitoes possessing resistant alleles have less chance to survive after insecticide exposure (Alout *et al.*, 2014). Hence, the selection of insecticide resistance in vector population by operational control might not be associated with an increase in malaria cases. This may explain why to date no studies have directly correlated insecticide resistance and vectorial capacity.

1.16 Insecticide resistance and agricultural use of pesticides

The rapid human population growth has led to an intensification of crop production with concomitant increases in pesticide application (Overgaard, 2006). It is estimated that approximately 90% of global insecticide production is used for agriculture (WHO, 1989). In Sub-Saharan Africa, studies have shown a possible link between *An. gambiae s.l.* resistance and the use of agricultural pesticides (Diabate *et al.*, 2002; Hien *et al.*, 2017). For instance, it has been shown that mosquito larvae exposed to sub-lethal pesticides frequently appear more tolerant to insecticides through the induction of their detoxifying system (Akogbéto *et al.*, 2006). Insecticidal products such as pyrethroids are used extensively to control agricultural pests and this practice has possibly elevated the selection pressure resulting in pyrethroid resistance in *An. gambiae s.l.* as the same insecticide class is deployed in public health against malaria vectors (Chouaïbou *et al.*, 2016a; Hien *et al.*, 2017). In Burkina Faso, the extensive use of pyrethroids in cotton-growing areas is associated with the emergence of resistance in *An. gambiae s.l.* (Dabiré *et al.*, 2014; Hien *et al.*, 2017; Namountougou *et al.*, 2019). In Tanzania, a study comparing urban agricultural and low pollution areas reported high levels of resistance in *An. gambiae s.s.* in areas with intensive agriculture (Nkya *et al.*, 2014).

1.17 Population genetics involved in insecticide resistance

Alleles conferring resistance to insecticides show marked differences in frequency between *An. gambiae s.s.* and *An. coluzzii* even in places where both species exist in sympatry. This disparity has been ascribed to assortative mating (the tendency for organisms to choose mates that are more similar or dissimilar to themselves in phenotype characteristics than would be expected by chance) which is also fuelling their speciation (Lee *et al.*, 2013; Lee *et al.*, 2013; Main *et al.*, 2015; Norris *et al.*, 2015). For instance, the *Vgsc*-1014F allele is more frequent in *An. gambiae s.s.* than in *An. coluzzii* in sympatry from Côte d'Ivoire, Nigeria, Burkina Faso and Ghana (Martinez-Torres *et al.*, 1998; Yawson *et al.*, 2007; Ibrahim *et al.*, 2014; Hien *et al.*, 2017; Zoh *et al.*, 2018). However, the *Vgsc*-1014F allele is present in both species as a result of introgression of the 2L divergence island through interspecific hybridization, as it was previously present only in *An. gambiae s.s.* (Weill *et al.*, 2000; Lee *et al.*, 2013; Norris *et al.*, 2015; Hanemaaijer *et al.*, 2019). The reproductive isolation maintained by assortative mating occasionally breaks down, leading to the emergence of F1 hybrids. This appreciable degree of hybridization is likely to allow several adaptively and epidemiologically important genetic exchanges to occur between the species pair (Lee *et al.*, 2013; Hanemaaijer *et al.*, 2019). In Mali, for instance, widespread usage of ITNs in 2005 presumably altered the hybrid fitness bottleneck and enhanced adaptive introgression of *Vgsc*-1014F from *An. gambiae s.s.* into *An. coluzzii* (Tripet *et al.*, 2006; Norris *et al.*, 2015).

The target-site resistance mechanism associated with CB/OP in between *An. coluzzii* and *An. gambiae s.s.* is the *Ace-1*-119S allele, and more recently via an *Ace*-1 duplication event (Djogbénou *et al.*, 2009; Assogba *et al.*, 2015; Assogba *et al.*, 2018). The *Ace*-1-119S has been detected in both *An. coluzzii* and *An. gambiae s.s.* but at different frequencies, thus, it is significantly higher in the *An. gambiae s.s.*, perhaps reflecting the discrepancies in the insecticide selection pressures the species pair experience in their habitats (Djogbénou et al., 2008). It is postulated that the distribution of *Ace*-1-119S alleles and *Ace*-1 duplication within the species pair may be due to genetic introgression, as observed in *kdr*. Therefore, interspecies hybridization plays a pivotal role in the evolution and spread of OP/CB resistance.

1.18 Effects of selection and genetic drift on cline and adaptation of resistance alleles

A cline is a predictable gradient of a phenotypic or genetic character within species, usually across a range of geographic distances. It is influenced by adaptive factors such as

natural selection (local or global), and non-adaptive factors such as genetic drift, gene flow, and demography history (Adrion *et al.*, 2015). The effects of adaptive and non-adaptive factors in the formation of cline are not mutually exclusive and can combine to influence adaptations and cline formation (Labbe *et al.*, 2005).

Natural Selection plays a strong role in maintaining cline partly due to spatial heterogeneity in environmental/climatic factors. For instance, despite unhindered gene flow among populations, the persistence of latitudinal and altitudinal clines in chromosomal inversions in *Drosophila melanogaster* for decades is associated with climatic variation (Adrion *et al.,* 2015). In the Australian east coast, clinal variation (creating locally-adapted phenotypes) in the thoracic pigmentation of *D. melanogaster* is accounted primarily by differential expression of in *ebony* locus within inversion I*n3R(P)*, and latitude (Telonis-Scott *et al.*, 2011). In *An. gambiae s.s* populations in West Africa, a cline in chromosome 2 inversions is associated with spatial variation in aridity and thermal stress (Cheng *et al.*, 2012, 2018). A longitudinal study on OP resistance alleles (*Est-2* and *Est-3*, coding for detoxifying esterase) along a transect in the Montpellier area of southern France showed that *Est-2* was more dominant than *Est-3* in moderately treated areas (Labbe *et al.*, 2005). In southern Ghana, a cline was observed in phenotypic and genotypic resistance to OP/CB in *An. gambiae s.s.* and *An. coluzzii*. Thus, high levels of *Ace-1*-119S frequencies found at the central belt correspond to areas with intense agricultural and industrial activities, and then the frequencies gradually decline towards the eastern and western belts (Essandoh *et al.,* 2013). These findings lay emphasis on selection acting at the local level.

Genetic drift is often regarded to have negligible effect in the establishment of cline as it reduces local diversity making selection ineffective, hence interfering with local adaptations (Polechová and Barton, 2011). However, a local genetic drift in conjunction with spatially restricted gene flow can create clines in allele frequencies at a single locus (Vasemagi et al., 2006). Genetic drift also shifts clines in space (from side to side), changing their width, and distorting their shape (Polechová and Barton, 2011). Furthermore, in non-additive traits resulting from epistasis, clines may occur often in a particular direction because random changes in allele frequencies at one locus can have a disproportionate influence on the distribution of phenotypes within a population (Santangelo et al., 2018). For instance, using a spatial simulation to examine the establishment of phenotypic clines of cyanogenesis (results from an epistatic interaction between two Mendelian-inherited loci) polymorphism along urbanization gradients in white clover, *Trifolium repens*, showed that the natural populations are prone to decrease in cyanogenesis frequencies through drift (Santangelo *et al.*, 2018).

Adaptive and non-adaptive processes in combination with ecological conditions (landscape features and environmental factors) are essential for the evolution of resistant alleles. The heterogeneity in the landscape may create a degradation by gene flow of adaptation to the local settings or genetic background (Polechová and Barton, 2011).

1.19 Landscape genetics and insecticide resistance management

Insecticide resistance in *An. gambiae s.s.* and *An. coluzzii* can be heterogeneous even across relatively small distances (Ranson *et al.,* 2009), which may be due to variation in selection pressure exerted or mosquito population differentiation across habitat types (Coluzzii, 1984). Studies have identified subdivisions within *An. gambiae* population that are attributed to distances, major geographical features (Lehmann *et al.,* 2003) and habitat (Lanzaro and Lee, 2013), and these divisions are likely to influence insecticide resistance. Studies correlating genetic and environmental variables will help resolve how patterns of gene flow are influenced by landscape features, and how resistance mutations may spread. These will provide information for the design of control programmes that reduce the impact of insecticide resistance.

To develop effective tools to make vector intervention strategies more targeted, there is a need to understand the population structure of *An. gambiae* and how the possible strata are connected in heterogeneous landscapes to facilitate the tracking and prediction of the spread of genes conferring insecticide resistance (Lehmann *et al.,* 2003). These require studies that blend genetic variations, spatial patterns and ecological processes (such as migration), which the field of landscape genetics has emerged to address. Landscape genetics involves the detection of genetic discontinuities and correlating them with landscape and environmental features (Manel *et al.,* 2003).

Chapter Two: Identification of environmental drivers of target-site resistance in southern Ghana.

Abstract

Resistance of *Anopheles gambiae s.s.* and *An. coluzzii* to pyrethroids and DDT is near-ubiquitous in West Africa with often high frequencies of the L1014F target-site mutation in the voltage-gated sodium channel (*Vgsc*) playing a substantial role. Though less common, carbamate (CB) and organophosphate (OP) resistance is also spreading with an implication of increasing frequencies of the G119S target-site mutation (in acetylcholinesterase (*Ace*-1)). Whilst *Vgsc* 1014F often nears fixation, *Ace*-1-119S is far more heterogeneous, offering opportunities to understand determinants of its spatial distribution and inform control decisions.

Anopheles gambiae s.l. larvae were sampled from 51 sites across southern Ghana between May and November 2016, and environmental parameters such as settlement types, ecological zones, larval source and land use were recorded. Bioassays were performed on adult female mosquitoes from a subset of the sites to assess their phenotypic resistance status to bendiocarb, fenitrothion, DDT, permethrin and deltamethrin. Species diagnostics and *Ace-1*-119S and *Vgsc*-1014F genotype characterizations using PCRs and TaqMan qPCR assays respectively. *Anopheles gambiae s.l.* were found to be more resistant to DDT and the pyrethroids than to bendiocarb or fenitrothion. *An. gambiae s.s.* and *An. coluzzii* were found to be the two main species but with varying frequencies, whilst only 12 specimens of *An. melas* were recorded in the total samples from five sites within the mangrove swamp. The *Vgsc*-1014F mutation was at much higher frequencies than *Ace*-1-119S. Both *Vgsc*-1014F and *Ace*-1-119S were found at significantly higher frequencies in *An. gambiae s.s.* than *An. coluzzii*. Ecological zones and land-use activities were found to be the principal determinants of *Ace-1*-119S and *Vgsc*-1014F distributions. The *Vgsc*-1014F is associated with rural, mining and cash crop plantations, while *Ace-1*-119S associated with urban and agricultural activities. This study shows that environmental factors are likely to influence the selection of insecticide resistance mutations, and also suggests why *Ace-1 119S* has spread in West Africa during a period of limited use of AChE-targeting insecticides for mosquito control.

2.1 Introduction

Malaria is hyperendemic and perennial in Ghana, with marked seasonal variations (PMI, 2019). It is estimated that 12.0 million clinical malaria episodes and about 12000 deaths occurred in Ghana in 2018 (WHO, 2019). Malaria accounts for the majority of outpatient department visits and about 11% of mortality in children under the age of five in Ghana (WHO, 2019). It is acknowledged to be the principal cause of poverty and low productivity in the nation. To alleviate the malaria burden, the National Malaria Control Programme in collaboration with PMI and Global Fund has established operational strategies, such as diagnosis with rapid diagnostic test kits, provision of sulfadoxine-pyrimethamine for the intermittent preventive treatment of pregnant women, administration of artemisinin-based combination therapies, distribution of LLINs and scale-up of IRS (PMI, 2019). Unfortunately, the effectiveness of IRS and LLIN/ITN programmes in Ghana and other sub-Saharan African countries is being threatened due to the emergence and spread of insecticide resistance in malaria vectors (WHO, 2019).

For close to a decade, IRS has relied mainly on pyrethroids and DDT, although there is currently an ongoing US President's Malaria Initiative (PMI) effort to reduce the sole reliance on OPs for IRS through the use of Sumishield (clothianidin), with susceptibility tests being performed across West Africa (Oxborough *et al.*, 2019). However, the incessant reliance on pyrethroids to control public health and agricultural insects has caused *An. gambiae s.s.* and *An. coluzzii* to develop resistance and cross-resistance to DDT (Camara *et al.*, 2018; Dia *et al.*, 2018). In West Africa, resistance to pyrethroids and DDT is widespread in *An. gambiae s.s.* and *An. coluzzii* and the primary mechanisms include the target-site mutations (L1014F and L1014S) at the *Vgsc* gene, and elevation of metabolic enzymes such as cytochrome P450s (Samb *et al.*, 2016; Chouaïbou *et al.*, 2017; Zoh *et al.*, 2018; Koukpo *et al.*, 2019). The IRS with pyrethroids often shows substandard efficacy against *An. gambiae s.l.*(Katureebe *et al.*, 2016; Abeku *et al.*, 2017; Sherrard-Smith *et al.*, 2018) and killing by standard long-lasting insecticide-treated nets (LLINs) (treated with only pyrethroids) also appears compromised (Asidi *et al.*, 2012; Ochomo *et al.*, 2013; Protopopoff *et al.*, 2018), though personal protection from a physical barrier may persist (Kleinschmidt *et al.*, 2018). As such the long-term efficacy of pyrethroids and sustainability of IRS/LLIN programmes will depend on a blended usage of other insecticide classes with a different mode of action via mixture, rotation or mosaic, with the underlying principle being that if the resistance to each of the compounds is independent, then cases of multiple resistance will be extremely rare. This principle has encouraged the use of CB and OP insecticides for IRS.

Unfortunately, *An. gambiae s.s.* and *An. coluzzii* have developed resistance to both CB and OP insecticides in West Africa, with the best-known mechanisms being glycine-to-serine mutation (G119S) in *Ace*-1 gene (Essandoh *et al.*, 2013; Accrobessy *et al.*, 2017; Hien *et al.*, 2017; Zoh *et al.*, 2018) and amplification of the mutated *Ace*-1 allele (Djogbénou *et al.*, 2008b; Assogba *et al.*, 2015, 2016). The *Ace*-1-119S mutation is a strong predictor for both CB/OP resistance in the species pair (Essandoh *et al.*, 2013). For CB, upregulation of metabolic enzymes in the species pair has also been implicated in their resistance (Edi *et al.*, 2014). Presently, CB and OP resistance is less prevalent compared to pyrethroids and DDT, but it is on the increase (Aizoun *et al.*, 2014; Accrobessy *et al.*, 2017; Camara *et al.*, 2018; Poda *et al.*, 2018; Zoh *et al.*, 2018; Ahadji-dabla *et al.*, 2019). *An. gambiae s.s.* and *An. coluzzii* resistance to CBs/OPs poses a great threat to malaria control as it reduces the number of insecticide options available for vector intervention, emphasising the need for use of newly available adulticides such as the neonicotinoid clothianidin (Oxborough *et al.*, 2019).

In Ghana, high levels of pyrethroid and DDT resistance with a concomitant *Vgsc* L1014F mutant allele near fixation have been reported in several *An. gambiae s.s.* and *An. coluzzii* populations (Adasi and Hemingway, 2008; Baffour-Awuah *et al.*, 2016; Chabi *et al.*, 2018; Egyir *et al.*, 2019; Pwalia *et al.*, 2019), this is perhaps not surprising as pyrethroids are extensively used in both agriculture and public health. The CB and OP insecticides, on the other hand, have been seldom used in public health, however, pockets of phenotypic resistance in *An. gambiae s.s.* and *An. coluzzii* have been reported nationwide (Figure 2.1). For instance, a study in Madina, a suburb of Accra, southern Ghana, reported *Ace*-1-119S frequency in sympatric populations of *An. coluzzii* and *An. gambiae s.s.* to be 10% and 35% respectively (Essandoh *et al.*, 2013). An extensive study across southern Ghana reported average *Ace*-1-119S frequencies in *An. coluzzii* and *An. gambiae s.s.* as 4.3% and 23.5% respectively, with higher frequencies restricted to the central-eastern belt of southern Ghana, which includes the Greater Accra Region (Essandoh *et al.*, 2013). A recent study in Opeibea, a suburb of Accra characterised by vegetable cultivation, also reported an average frequency of *Ace*-1-119S to be 0.76 for three years (Pwalia *et al.*, 2019). These results indicate that CB/OP resistance and *Ace*-1-119S are heterogeneous across Ghana, with hotspots (areas with relatively high levels of resistance) of high mutant frequency and phenotypic resistance.

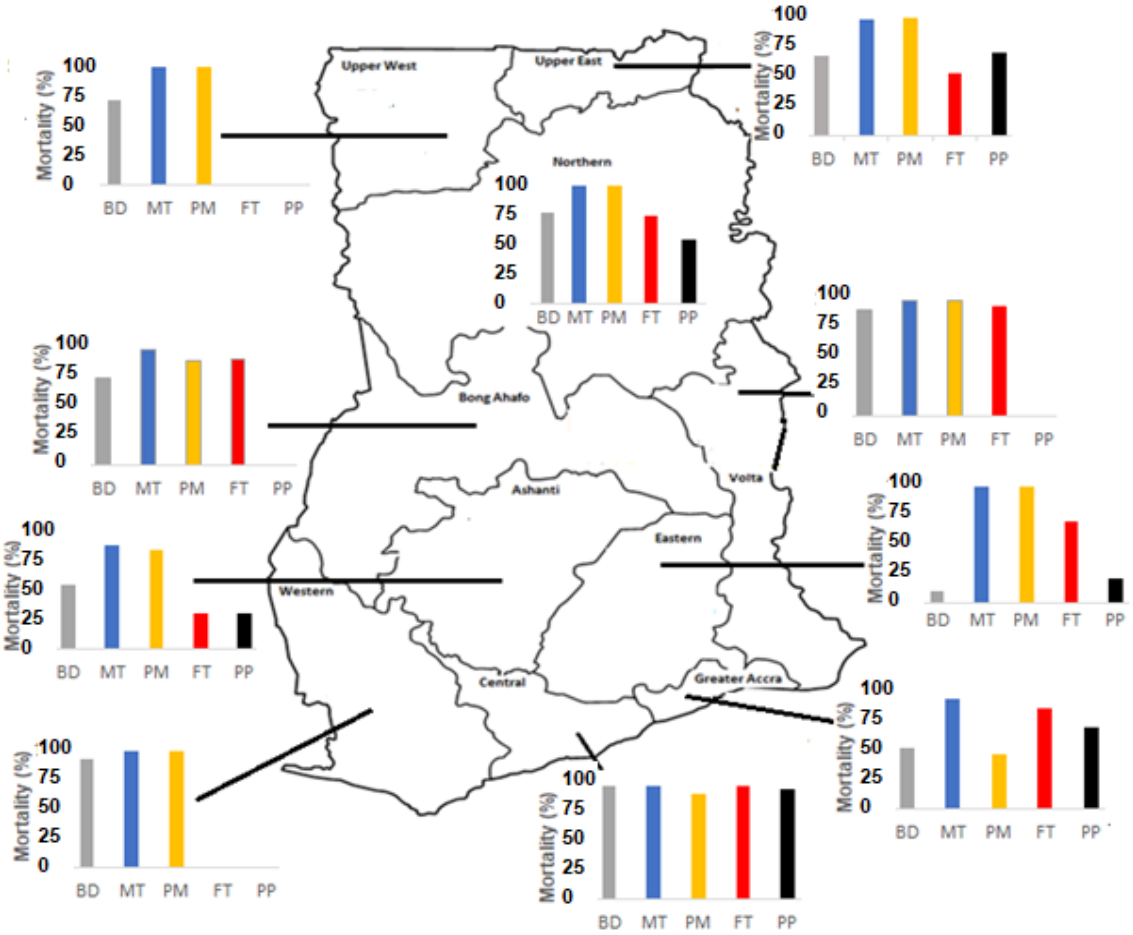


Figure 2.1: Map showing nationwide phenotypic resistance distribution of *An. gambiae s.l.* to carbamates and organophosphates. The bioassay data were recorded in the PMI regional sentinel sites in 2017 (PMI, 2018) according to the WHO resistance level at 80%. BD= Bendiocarb, MT= Malathion, PM= Pirimiphos-methyl, FT = Fenitrothion and PP = Propoxur. The blanks in the graphs indicate no bioassay results.

Insecticide resistance threatens the use of chemical formulations for malaria vector control, as in the insecticide-treated areas, resistant mosquitoes are more likely to survive longer to impact vectorial capacity and influence malaria transmission dynamics than their susceptible counterparts (Cook *et al.*, 2008; Cook and Sinkins, 2010). The emergence of resistance mutations in anopheline mosquitoes is direct via selection pressure exerted by insecticides, yet several ecological factors are thought to contribute to how mosquitoes respond to insecticides (Afrane *et al.*, 2008; Mordecai *et al.*, 2013; Owusu *et al.*, 2017). It is therefore necessary to understand the spatial distribution of resistance mutations and identify the areas that may serve as hotspots to facilitate their spread.

Southern Ghana is characterized by four major ecological zones, numerous habitat types (Yawson *et al.*, 2007), human settlements and primary land-use activities. Human settlement (rural/peri-urban/urban) and some agricultural practices such as irrigation and

fertilizer/pesticide applications have been shown to have a direct impact on malaria vectors, in terms of their densities (Ijumba and Lindsay, 2001) and responses to insecticide treatments (Overgaard, 2006; De Silva and Marshall, 2012; Chouaïbou *et al.*, 2016b; Hien *et al.*, 2017), however, they may vary considerably among the various ecological zones and habitat types.

In Ghana, pyrethroid-IRS operation implemented in the Northern Region led to a significant reduction in the sporozoite and entomological inoculation rates (EIRs), however, this gain was threatened by insecticide resistance thereby necessitating a switch to pirimiphos-methyl (marketed as Actellic) (Coleman *et al.*, 2017; Gogue *et al.*, 2020). As the Ghana National Malaria Control Programme (NMCP) is planning to scale up IRS with pirimiphos-methyl and increase the distribution of pyrethroid-treated LLIN, it will require up-to-date information on malaria vector distributions, resistance patterns and mechanisms for tracking changes temporally and spatially. This study seeks to provide the fine-scale spatial pattern of the *Vgsc* and *Ace*-1 resistance mutation and to investigate their co-occurrence as a partial proxy for pyrethroid and CB/OP resistance respectively, and also to examine the impact of anthropogenic and natural environmental heterogeneity on the distribution of insecticide resistance mutations in *An. gambiae s.s.* and *An. coluzzii* in Ghana.This study will provide essential information to guide the local implementation of vector interventions.

Research questions:

1. What are the current distributions of *An. coluzzii and An. gambiae s.s.* across southern Ghana?

2. What are the distribution patterns of insecticide resistance loci, *Vgsc* (L1014F, L1014S and N1575Y) and *Ace-1* genes (*G119S*) in *An. coluzzii and An. gambiae s.s* across southern Ghana?

3. What are the environmental factors and human activities that are influencing insecticide resistance distributions across southern Ghana?

4. Which areas are serving as the possible resistance hotspots?

2.2 Methods

2.2.1 Study area

Field sampling was performed in southern Ghana and comprised 51 study sites (Figure 2.2a) spanning all the four major ecological zones (coastal savannah (13 sites), mangrove swamp (5 sites), deciduous forest (12 sites) and rainforest (21 sites) (Figure 2.2b), five administrative regions (Western, Central, Greater Accra, Eastern and Volta Region), and major human land-uses, to ensure broad coverage encompassing environmental variation. Southern Ghana is characterised by a bimodal rainfall distribution pattern with mean annual rainfall ranging from 750mm (in the coastal savannah) to 2200mm (in the tropical rainforest) (FAO and MOFA, 2018). The major rainy season occurs from March to mid-July with a peak in June; the minor rainy season occurs between early September and early November. Mean temperature varies from 28.7 to 35 ^{0}C; mean relative humidity is 84% during the rainy season and 40% during the dry season (FAO and MOFA, 2018).

2.2.2 Larval collection and maintenance

An average of 50 *Anopheles gambiae* larvae was collected from each sampling site (Figure 2.2) between May and November 2016. The larval stage was targeted to facilitate collecting few individuals from many breeding sites within an approximate radius of three kilometres (3.0 km) in each study area. The GPS readings, nature of the larval niche and other environmental variables such as settlement types, and land-use that are likely to lead to the creation of mosquito breeding sites and associated with the use of pesticides/chemical pollutants such as plantations, vegetable farming (subsequently 'vegefarm'), domestic gardening and mining were recorded. Larvae were collected from a variety of habitats such as roadside puddles, gutters, refuse damp, irrigation sites and domestic water tanks (Figure 2.3) using dippers and transported to the laboratory in partly filled plastic containers. Larvae were sorted using Pasteur pipettes and kept in plastic trays covered with nets and fed with ground fish food (Tetramin flake) and those that metamorphosed into pupae were collected in a beaker and placed into collection-specific mosquito cages for them to emerge into adults. Cotton wool pads soaked with 10% sugar solution were used to feed the adult mosquitoes.

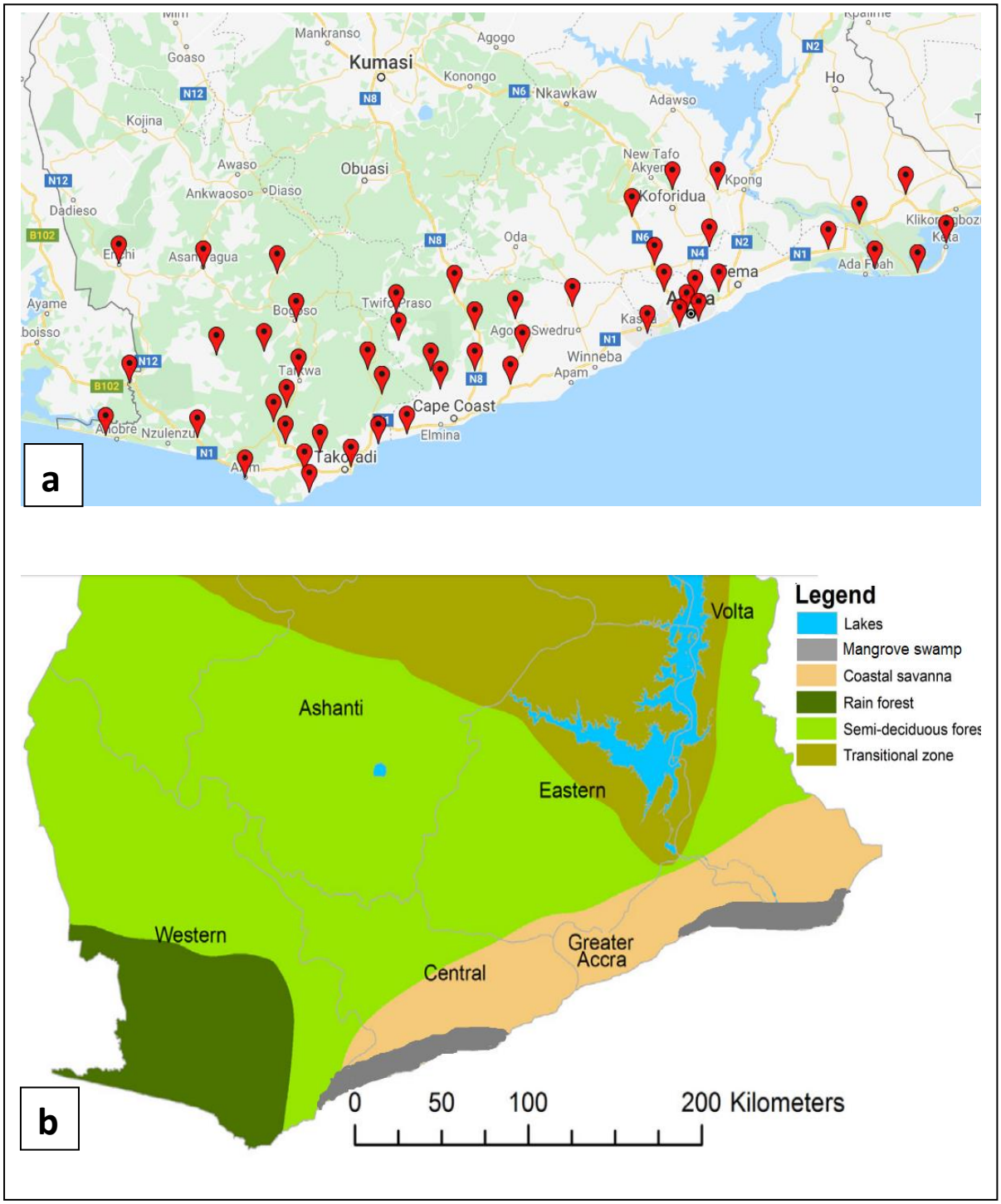


Figure 2.2: The map of southern Ghana showing (a) the mosquito sampling sites and (b) the major ecological zones (Rhebergen *et al.*, 2016).

Figure 2.3 : Variety of mosquito breeding sites where larvae samples were collected: (a) a gutter close to a refuse damp, (b) a farm road puddle and (c) a roadside pool.

2.2.3 Phenotypic resistance

Bioassays were performed to assess phenotypic resistance on mosquitoes sampled from 15 study sites (Figure 2.4) where collection numbers and time permitted. Approximately twenty-five 3-5-day-old adult female mosquitoes were aspirated into WHO bioassay holding tubes and then exposed to the recommended dosage of insecticide-treated papers of permethrin, deltamethrin, fenitrothion, bendiocarb or DDT for one hour (WHO, 2018). Four replicates were used along with a control tube containing paper lacking insecticide. A thermo-hygrometer was used to record the temperature and humidity. The mosquitoes were kept under observation for 24 hours, supplied with 10% sugar solution, and the mortality was recorded after the recovery period. A mortality rate less than 90% indicates that the tested population is resistant, whereas mortality higher than 98% indicates that the population is susceptible (WHO, 2018).

Study site keys

1 = Half-Assini	6 = Dixcove	11 = Agona Dunkwa
2 = Nkwanta	7 = Takoradi	12 = Tuba
3 = Axim	8 = Shama	13 = Dzorwulu
4 = Tarkwa	9 = Twifo Praso	14 = Ashaiman
5 = Mpohor	10 = Assin Foso	15 = Keta

Figure 2.4: Map of southern Ghana showing where bioassays were performed on samples.

2.2.4 Species diagnostics

An average of 20 mosquitoes from each study site was selected randomly and DNA was extracted from each using Nexttec kits following the manufacturer's (nexttec™) protocol. Species characterizations into *An. gambiae* complexes were performed using PCR protocol described by Scott et al., (1993) (Appendix 1) and further characterization into *An. coluzzii* and *An. gambiae s.s.* using protocols described by Santolamazza et al., (2008) (Appendix 2). The PCR products were visualised under ultraviolet (UV) light after electrophoresis using 2% agarose gel stained with Peqgreen dye manufactured by Peqlab Biotechnologie. In populations with an even mixture of *An. coluzzii* and *An. gambiae s.s.*, the mosquito sample was increased to improve confidence in the estimation.

2.2.5 Genotyping of the *Ace-1* mutation and *Vgsc* gene mutations

Mosquitoes of known species were genotyped individually at single nucleotide substitutions using the fluorescence-based TaqMan assay described by Bass *et al.*, (2010) for *Vgsc-L1014F, Vgsc-L1014S* and *Ace-1-G119S*, and the protocol described by Jones *et al.*, (2012) for *Vgsc-N1575Y*, on an Agilent MxPro 3005 thermal cycler (Appendix 4). The Fam probe detects the mutant (resistant allele (serine)), while the Hex probe detects the wild type (susceptible allele (glycine)). The genotypes were scored from bi-directional scattered plots produced by the MxPro software at the endpoint cycle of amplification.

2.2.6 Data analysis

Bioassay results for phenotypic tests were reported as proportions of dead mosquitoes with 95% confidence intervals. The differences in the susceptibility of mosquitoes to fenitrothion and permethrin were compared using Mann-Whitney tests. Chi-square tests (or Fisher's exact tests when a variate is < 5 in the contingency table) were used to analyse the association between anopheline species, environmental parameters and resistance genotypes or allele frequencies using the XLstat add-in for Microsoft Excel. A Wilcoxon test was used to compare the proportions of *Ace-1*-119S and *Vgsc*-119S.

A categorical principal component analysis (PCA) was performed in SPSS version 25 to investigate relationships among species, environmental variables and resistance marker genotypes. The analysis was restricted to construct two novel independent composite

variables (PC1, 2) that explained maximal variance in the data set, with which correlations of the original variables could be visualised. The reliability of the model was assessed using Cronbach's alpha value. The Cronbach's alpha value ranges between 0 and 1, with $\alpha \geq 0.7$ is regarded as acceptable reliability, whilst $\alpha \leq 0.5$ indicates poor reliability (Tavakol and Dennick, 2011)

To test the hypothesis of isolation by distance, i.e. there is a clinal decrease in genetic similarity with geographic distance, the latitude and longitude coordinates of the 51 study sites were used to generate a pairwise distance matrix using Geographic Distance Matrix Generator software (Ersts, https://www.biodiversityinformatics.amnh.org/open_source/gdmg/) . A simple Mantel test (a test used to correlate two matrices) was performed (with 100,000 permutations) to test the correlation between matrices of geographical distance and pair-wise differences in the proportion of *An. gambiae s.s.*, *Ace-1*-119S alleles or *Vgsc*-1014F alleles using ZT software (ZT is a command-line user interface software used to perform simple and partial Mantel tests) (Bonnet and Van De Peer, 2002). Partial Mantel tests (they are used to test the correlation between matrices *A* and *B* while controlling the effect of a third matrix *C*) were also performed to assess how well species and *Vgsc*-1014F correlate with distance while holding the *Ace-1*-119S frequency constant.

Multinomial logistic regression was used to generate a model for the categorical outcomes of the *Ace*-1-119S genotypes and *Vgsc*-1014F genotypes, using settlement types (rural, peri-urban, urban), ecological zones (mangrove swamp, coastal savannah, deciduous forest and rainforest) and land-use activities (domestic gardening, plantation, vegefarm and mining) as predictors and only significant predictors were kept in the models. The effects of the predictors were reported as odds ratios with 95% confidence intervals and associated p-values. The goodness-of-fit measure of the models was assessed using Nagelkerkes pseudo-R^2, which is the proportion of the variance explained by the model (Bo *et al.,* 2006).

2.3 Results

2.3.1 Species distributions

A total of 3540 females were collected, of which 1037 *An. gambiae s.l.* mosquitoes were characterised to species level using PCR. A total of 1025 identified as *An. gambiae* species pair (comprising *An. coluzzii* and *An. gambiae s.s.)*, while 12 were *An. melas* (found only in five sites within mangrove swamp in the Western and Volta region). Further characterization of 1025 *An. gambiae* specimens found that 57.0% (CI: 54.0 - 60.0%) were *An. coluzzii*, while 42.8% (CI: 39.8 - 46.0%) were *An. gambiae s.s.*, with only two hybrids recorded (0.2%; CI: 0.02%-0.7%), both from the same site (Agona Dunkwa). Five non-mixed sites in forest areas harboured only *An. gambiae s.s.*, while 13 sites harboured only *An. coluzzii*, with co-existence in 33 of the 51 sampling sites, but with varying frequencies (Table 2.1 and Figure 2.5). There were also non-random distributions of species across the major primary land-use activities ($\chi^2 = 38.4$, df = 2, $p < 0.001$), with *An. coluzzii* being predominant in areas with commercial mining, plantation areas (coconut, oil palm, cocoa and rubber), and vegefarms, whilst *An. gambiae s.s.* predominated areas with gardening (Figure 2.6a). The settlement type also had a significant impact on the distribution of the species pair ($\chi^2 = 37.4$, df = 2, $p < 0.001$), with *An. coluzzii* being more common in urban and peri-urban areas (Figure 2.6b). The species pair showed significant variability in distribution across ecological zones ($\chi^2 = 225.0$, df = 3, $p < 0.001$), with *An. coluzzii* being predominant in the mangrove swamp and tropical rainforest, and *An. gambiae* s.s. in deciduous forest, with more balanced frequencies in coastal savannah (Figure 2.6c).

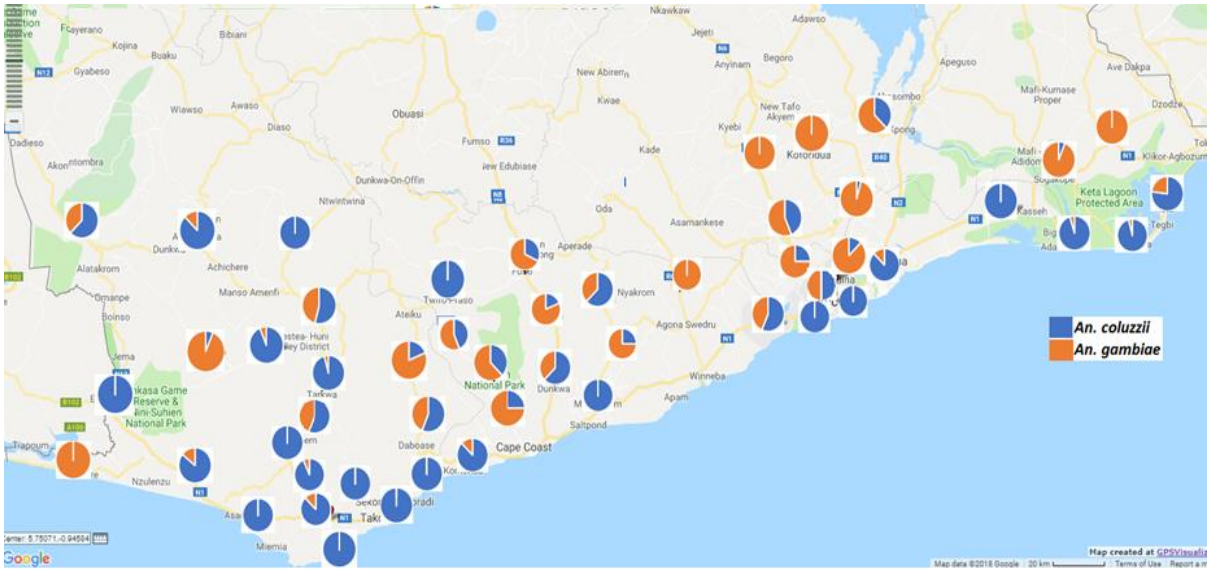


Figure 2.5: Spatial distribution of *An. gambiae s.s.* and *An. coluzzii* across southern Ghana.

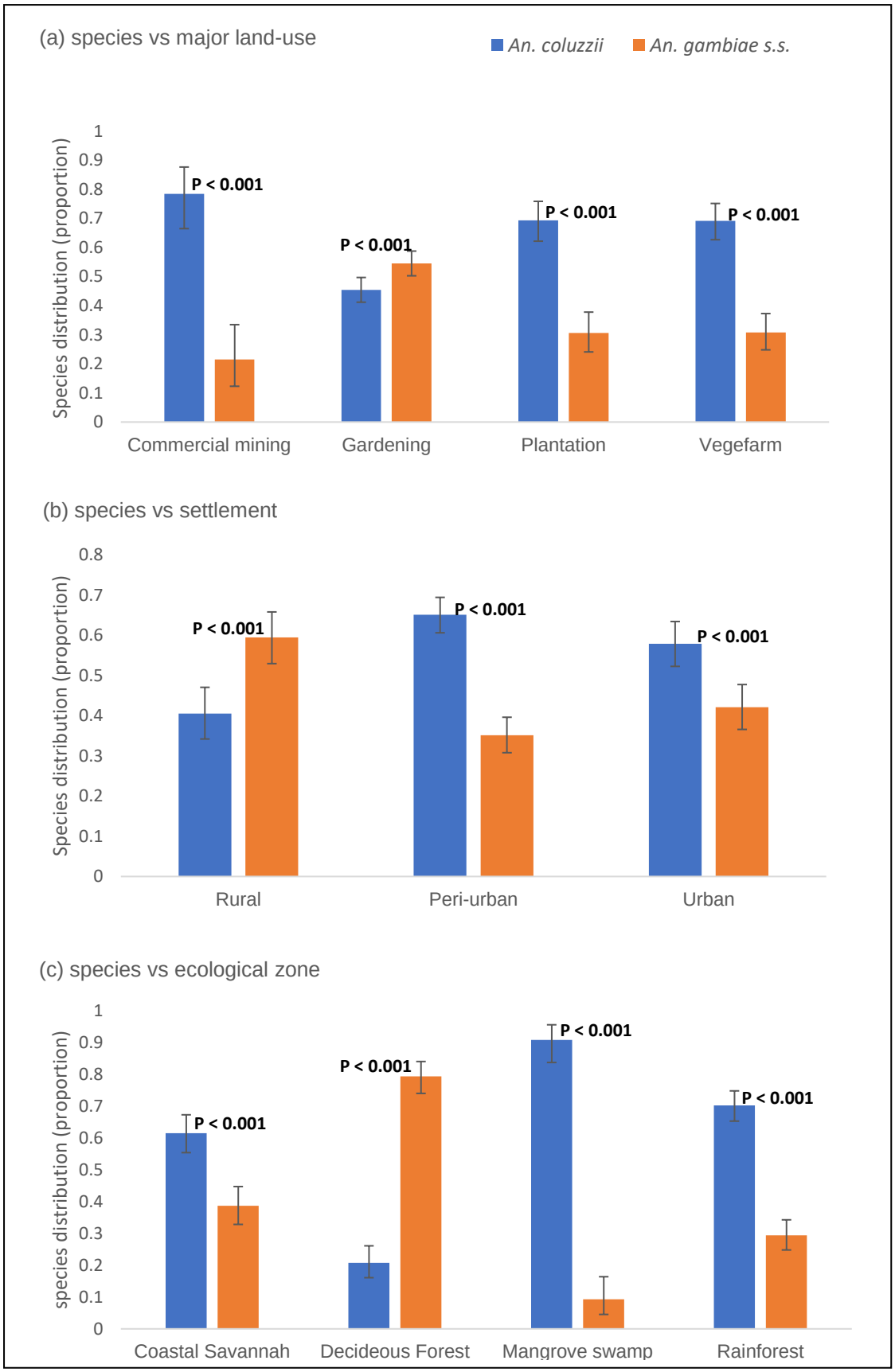


Figure 2.6: Bar charts showing the distribution of species in terms of (a) major primary land-use activities (b) settlement types and (c) ecological zones of the sampling sites across southern Ghana. The error bars indicate a 95% confidence interval and the p-value is the significant difference between the species based on chi-square.

Table 2.1: Details and species compositions of the collection sites across southern Ghana

Site	Ecological zone	Settlement type	Major land-use	Latitude	longitude	*An. coluzzii* (N / %)	*An. gambiae s.s.* (N / %)
Abura Dunkwa	Deciduous Forest	Rural	Gardening	5.3430N	1.1787W	10 (62.5)	6 (37.5)
Ada Foah	Mangrove swamp	Peri-urban	Vegefarm	5.8035N	0.6641E	20 (95.2)	1 (4.8)
Agona Dunkwa	Deciduous Forest	Rural	Gardening	5.6338N	0.7281W	0 (0)	40 (100)
Agona Nkwanta	Rainforest	Peri-urban	Gardening	4.8880N	1.9660W	14 (87.5)	2 (12.5)
Ajumako	Deciduous Forest	Peri-urban	Gardening	5.4256N	0.9595W	10 (62.5)	6 (37.5)
Akatsi	Coastal Savannah	Peri-urban	Gardening	6.1361N	0.8051E	0 (0)	23 (100)
Amasaman	Deciduous Forest	Urban	Gardening	5.6979N	0.3087W	4 (25.0)	12 (75.0)
Anloga	Mangrove swamp	Peri-urban	Vegefarm	5.7863N	0.8651E	23 (95.8)	1 (4.2)
Anyinase	Rainforest	Peri-urban	Plantation (rubber)	5.0394N	2.4603W	12 (85.7)	2 (14.3)
Asankrangwa	Rainforest	Peri-urban	Gardening	5.8027N	2.4334W	14 (87.5)	2 (12.5)
Ashaiman	Coastal Savannah	Peri-urban	Vegefarm	5.6953N	0.0524W	35 (87.5)	5 (12.5)
Asikumah	Deciduous Forest	Peri-urban	Gardening	5.5802N	0.9961W	4 (25.0)	12 (75.0)
Assin Foso	Deciduous Forest	Peri-urban	Gardening	5.6936N	1.2733W	9 (32.1)	19 (67.9)
Assin Kyina	Deciduous Forest	Rural	Gardening	5.5297N	1.1804W	3 (18.8)	13 (81.3)
Axim	Rainforest	Urban	Plantation (rubber)	4.8638N	2.2392W	16 (100)	0 (0)
Bogoso	Rainforest	Urban	Commercial mining	5.5685N	2.0026W	13 (52.0)	12 (48.0)
Bonsaso	Rainforest	Rural	Plantation (rubber)	5.1804N	2.0482W	9 (56.3)	7 (43.8)
Dixcove	Coastal Savannah	Peri-urban	Gardening	4.7948N	1.9467W	24 (100)	0 (0)
Dodowa	Deciduous Forest	Peri-urban	Vegefarm	5.8982N	0.1012W	1 (4.2)	23 (95.8)
Dzorwulu	Coastal Savannah	Urban	Vegefarm	5.6050N	0.2059W	10 (55.6)	8 (44.4)
Elubo	Rainforest	Peri-urban	Gardening	5.2894N	2.7750W	12 (100)	0 (0)
Enchi	Rainforest	Peri-urban	Plantation (cocoa)	5.8232N	2.8230W	15 (68.2)	7 (31.8)
Half-Assini	Coastal Savannah	Urban	Plantation(Coconut)	5.0513N	2.8833W	0 (0)	16 (100)
Jukwa	Rainforest	Rural	Gardening	5.2609N	1.3390W	4 (25.0)	12 (75.0)
Kakum	Rainforest	Rural	Gardening	5.3421N	1.3850W	6 (37.3)	10 (62.7)
Keta	Mangrove swamp	Urban	Vegefarm	5.9175N	0.9916E	20 (76.9)	6 (23.1)
Koforidua	Deciduous Forest	Urban	Gardening	6.1547N	0.2703W	0 (0)	40 (100)

Komenda	Mangrove swamp	Peri-urban	Gardening	5.0557N	1.4944W	14 (87.5)	2 (12.5)
Kpong	Deciduous Forest	Peri-urban	Gardening	6.1547N	0.0628W	6 (37.5)	10 (62.5)
Labadi	Coastal Savannah	Urban	Gardening	5.5672N	0.1507W	16 (100)	0 (0)
Madina	Coastal Savannah	Urban	Gardening	5.6697N	0.1672W	4 (12.5)	28 (87.5)
Mamprobi	Coastal Savannah	Urban	Vegefarm	5.5410N	0.2377W	16 (100)	0 (0)
Mankessim	Coastal Savannah	Peri-urban	Gardening	5.2807N	1.0176W	16 (100)	0 (0)
Matsepe	Coastal Savannah	Urban	Vegefarm	5.8877N	0.4500E	16 (100)	0 (0)
Mpohor	Rainforest	Rural	Plantation (oilpalm)	4.9743N	1.8950W	24 (100)	0 (0)
Nkwanta	Rainforest	Rural	Plantation (cocoa)	5.4156N	2.3711W	1 (6.2)	15 (93.8)
Nsawan	Deciduous Forest	Peri-urban	Gardening	5.8167N	0.3539W	9 (50.0)	9 (50.0)
Nsuaem	Rainforest	Peri-urban	Plantation (rubber)	5.0117N	2.0553W	14 (83.3)	1 (6.7)
Prestea	Rainforest	Peri-urban	Commercial mining	5.4324N	2.1520W	15 (93.7)	1 (6.3)
Sekyere Krobo	Rainforest	Rural	Gardening	5.2404N	1.6072W	9 (56.2)	7 (43.8)
Shama	Mangrove swamp	Peri-urban	Gardening	5.0118N	1.6285W	21 (100)	0 (0)
Simpa	Rainforest	Rural	Plantation (rubber)	5.11067N	2.1095W	15 (100)	0 (0)
Sogakope	Coastal Savannah	Peri-urban	Vegefarm	6.0056N	0.5914E	1 (6.2)	15 (93.8)
Suhum	Deciduous Forest	Peri-urban	Gardening	6.0350N	0.4576W	0 (100)	24 (100)
Takoradi	Coastal Savannah	Urban	Gardening	4.9108N	1.7544W	16 (100)	0 (0)
Tarkwa	Rainforest	Urban	Commercial mining	5.3138N	1.9923W	23 (93.8)	1 (4.2)
Tuba	Coastal Savannah	Urban	Vegefarm	5.5131N	0.3867W	13 (56.5)	10 (43.5)
Twifo Hemang	Rainforest	Rural	Plantation (cocoa)	5.4802N	1.5307W	7 (43.7)	9 (56.3)
Twifo Praso	Rainforest	Peri-urban	Gardening	5.6076N	1.5458 W	16 (100)	0 (0)
Wassa Akropong	Rainforest	Urban	Plantation (cocoa)	5.7805N	2.0902W	16 (100)	0 (0)
Wassa Essaman	Rainforest	Rural	Gardening	5.3501N	1.6780W	8 (26.7)	22 (73.3)

2.3.2 Phenotypic resistance

A total of 4350 mosquito samples from 15 study sites were tested for their phenotypic susceptibility to the four insecticide classes. Limitations in collection sizes meant it was seldom possible to test all classes and therefore the primary focus was on insecticides with contrasting target sites (VGSC vs. AChE). Susceptibility varies significantly among the study sites (permethrin, (χ^2 = 503.7, df = 14, p < 0. 001) and fenitrothion (χ^2 = 440.6, df = 11, p < 0. 001). In sites where all the susceptibility tests were performed for all the four main insecticide classes, *An. gambiae* s.l. were generally more resistant to pyrethroids (permethrin) and DDT than to CB (bendiocarb) and OP (fenitrothion) (Appendix 5). *An. gambiae s.l.* from Dzorwulu, a suburb of Accra, exhibited the least susceptibility to both permethrin and fenitrothion, while most susceptible to permethrin and fenitrothion were mosquitoes from Nkwanta and Axim respectively (Figure 2.7). There was a significant difference between the mosquito susceptibility to permethrin and fenitrothion (Wilcoxon, p = 0.002), however, there was no significant correlation between the susceptibility of the two insecticides per study site (Spearman's Rho, r_s = 0.55, p = 0.061).

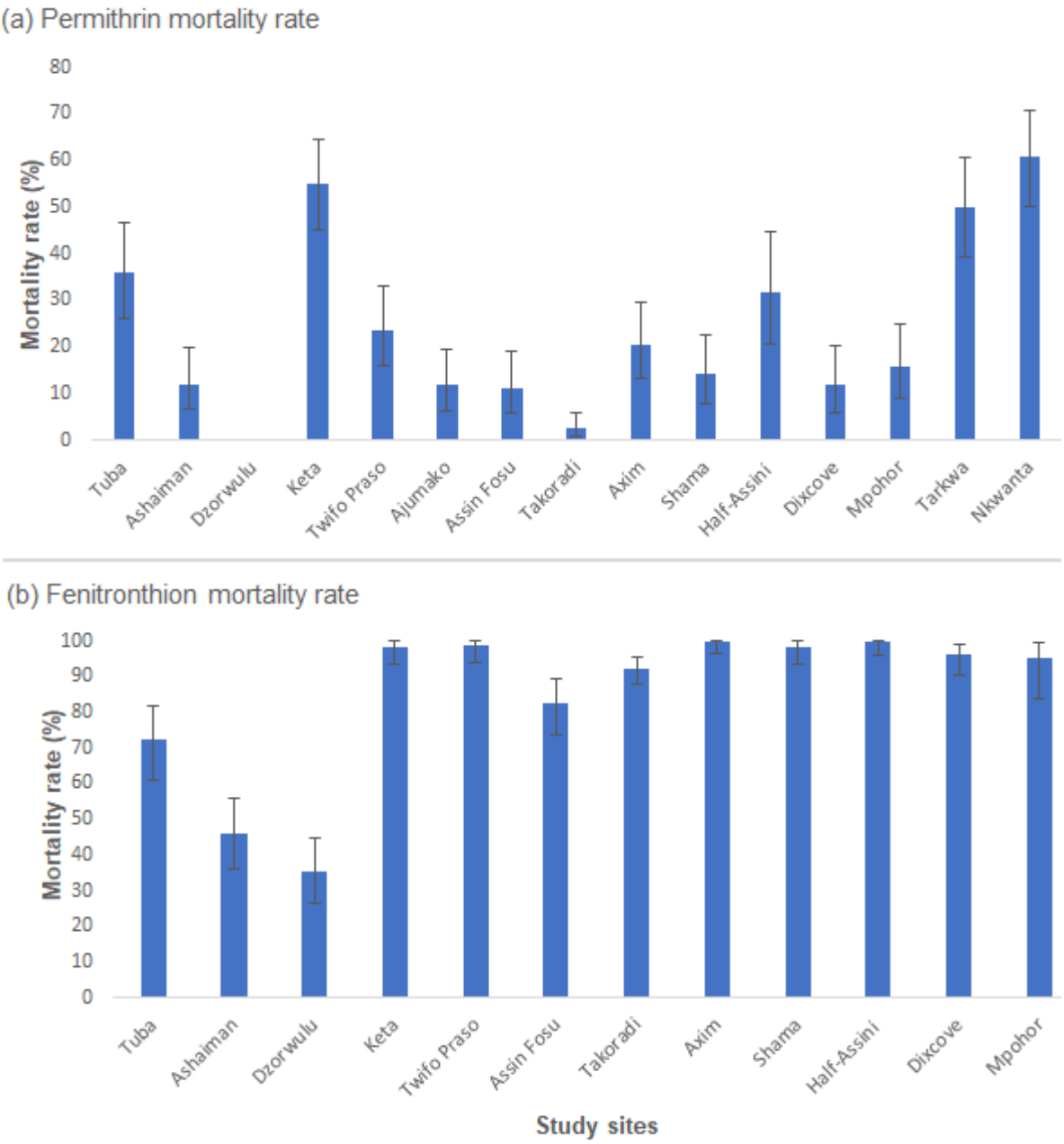


Figure 2.7: The phenotype resistance (mortality rate) with 95% confidence interval (error bars) in *Anopheles gambiae s.l.* to (a) permethrin and (b) fenitrothion**.** Dzorwulus recorded zero mortality rate for permethrin

2.3.3 *Vsgc* and *Ace*-1 genotype and allele distributions

The *Vsgc-1014F* allele was detected in high proportions in almost every study site and fixed in the populations of *An. coluzzii* and *An. gambiae s.s.* in two and 26 study sites respectively Figure 2.8 and Appendix 6. The *Vsgc-1014F* allele was significantly higher in *An. gambiae s.s.* (89.5%, 95% CI: 87.6 - 91.6%) than in *An. coluzzii* (83.6%, 95%CI: 79.2 - 83.6%), (χ^2 = 26.8, df = 1, p < 0.001). The *Vsgc-1014F* distribution according to settlement showed

significant difference in *An. gambiae s.s.* (χ^2 = 8.0, df = 2, p = 0.018) but not in *An. coluzzii* (χ^2 = 3.4, df = 2, p = 0.184), with rural areas recording the highest frequency in both *An. coluzzii* (84.9%, 95% CI: 79.0 – 89.6%) and *An. gambiae s.s.* (93.6, 95%CI: 90.1 – 96.2). For land-use, plantation and commercial mining areas recorded the highest *Vsgc-1014F* allelic frequencies in *An. coluzzii* (89.5%, 95% CI: 85.1 – 93.0%) and *An. gambiae s.s.* (92.9, 95%CI: 76.5 – 99.1) respectively (Table 2.2).

The *Vsgc-1014F* genotypic frequencies (*FF*, *LF* and *LL*) varied between species pair even in sites where they coexist. In both species, the genotype with the highest proportion was found to be *FF*, however, it was markedly higher in *An. gambiae s.s.* (84.2%, 95%CI: 76.6 – 84.2%) than in *An. coluzzii* (65.8%, 95%CI: 61.8 - 69.6%). The *LF* was found to be more common in *An. coluzzii* (31.5%, 95%CI: 27.9 - 35.5%) than in *An. gambiae s.s.* (19.1%, 95%CI: 15.6 – 23.1%). The *LL* frequency was also higher in *An. coluzzii* (2.7%, 95%CI: 1.6 – 4.4%) than in *An. gambiae s.s.* (0.7%, 95%CI: 0.1 – 2.0%). The observed genotypic difference between the species pair was statistically significant (χ^2 = 25.7, df = 2, p < 0.001). The *Vsgc-1014F* genotypes show significant differences in terms of settlements and ecological zones and land use in *An. coluzzii* (Table 2.2). The spatial distribution of the *Vsgc-1014F* allele shows it is widespread across southern Ghana (Figure 2.9). Using pooled samples across study sites, *Vsgc-1014F* genotypic frequencies within each of the species pair showed no deviations from Hardy-Weinberg equilibrium (*An. coluzzii*, χ^2 = 1.19, df = 2, p = 0.275, and *An. gambiae s.s.*, χ^2 = 1.19, df = 0.70, p = 0.403) (Table 2.3), which may suggest that the populations of *An. coluzzii* and *An. gambiae s.s* across southern Ghana are in panmixia.

No *Vsgc-1014S* allele was detected in the populations of both species. For the *Vgsc-1575Y* allele, it was detected at low frequencies in both species but significantly higher in *An. gambiae s.s.* (10.3%, 95%CI: 8.4 – 12.4%) than in *An. coluzzii* (0.17%, 95%CI: 0.05 – 0.62%), (p < 0.001) (Table 2.3). In *An. coluzzii*, only two heterozygotes of *Vgsc-1575Y* were recorded. *In An. gambiae s.s.*, of 86 individuals found to be possessing *Vgsc-1575Y*, four were homozygotes.

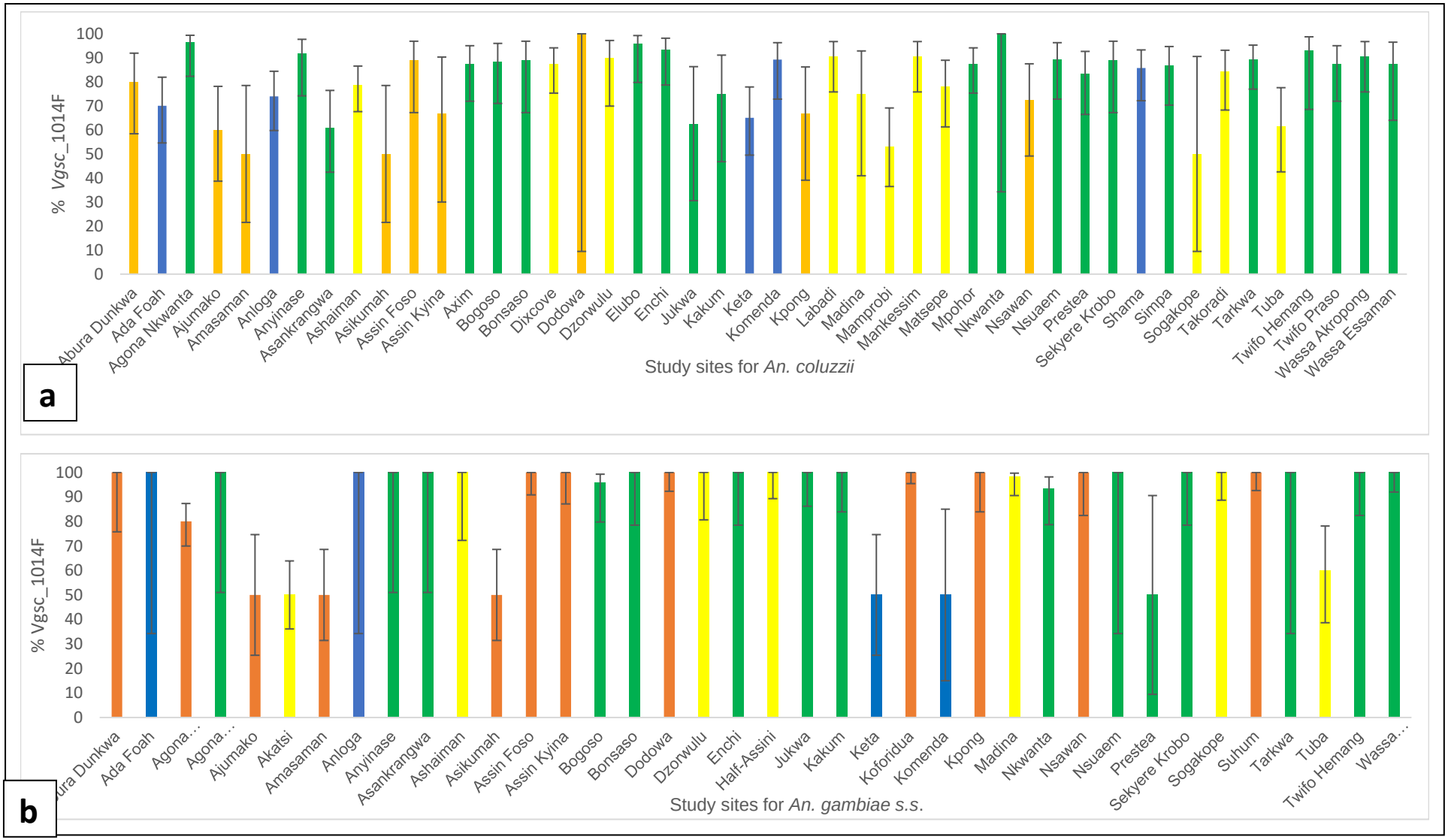


Figure 2.8: Distribution of *Vgsc-L1014F* allelic frequency with 95% confidence interval in (a) *An. coluzzii* and (b) *An. gambiae s.s.* The colour coding of the bar chats indicates the ecological zones; blue = mangrove strand, green = rainforest, yellow = coastal savannah and orange = deciduous forest

Figure 2.9: The distribution of *Vgsc*-1014 L allelic frequency in *An. gambiae s.l.* among the study sites across southern Ghana

Table 2.2: The distribution of *Vgsc*-1014F genotypes in terms of settlements, ecological zones and land-use types and their corresponding Fisher's exact test

(a) Species	Genotype		Land-use				Exact p-value
			Mining (N / %)	Gardening (N / %)	Plantation (N / %)	Vegefarm (N / %)	
An. coluzzii	*Vgsc_1014F*	LL	0 (0)	6 (3.5)	0 (0)	10 (6.5)	< 0.001
		LF	13 (25.5)	76 (30.5)	27 (20.9)	68 (43.9)	
		FF	38 (74.5)	167(67.0)	102 (79.1)	77(49.6)	
An. gambiae s.s.	*Vgsc_1014F*	LL	0 (0)	1(0.3)	1(1.8)	1 (1.4)	< 0.001
		LF	2 (14.3)	70 (23.4)	0 (0)	12 (17.4)	
		FF	12 (85.7)	228 (76.3)	56 (98.2)	56 (81.2)	

(b) Species	Genotype		Ecological zone				Exact p-value
			Mangrove Swamp (N / %)	Coastal Savannah (N / %)	Deciduous forest (N / %)	Rain forest (N / %)	
An. coluzzii	*Vgsc*_1014F	LL	6 (6.1)	5 (3.0)	1(1.8)	4 (1.5)	< 0.001
		LF	35 (35.7)	59 (35.3)	31(55.4)	59 (22.4)	
		FF	57(58.2)	103 (61.7)	24(42.8)	200 (76.1)	
An. gambiae s.s.	*Vgsc*_1014F	LL	2 (20.0)	0 (0)	0 (0)	1 (0.9)	< 0.001
		LF	4 (40.0)	32 (30.5)	46 (21.5)	2 (1.8)	
		FF	4 (40.0)	73 (69.5)	168 (78.5)	107(97.3)	

(C) Species	Genotype		Settlement			Exact p-value
			Rural (N / %)	Per-urban (N / %)	Urban (N / %)	
An. coluzzii	Vgsc_1014F	LL	1 (1.1)	12 (3.9)	3 (1.6)	0.059
		LF	27 (28.1)	86 (28.2)	71 (38.8)	
		FF	68 (70.8)	207 (67.9)	109 (59.6)	
An. gambiae s.s.	Vgsc_1014F	LL	1 (0.7)	1 (0.7)	1 (0.8)	0.014
		LF	16 (11.4)	42 (25.4)	26 (19.5)	
		FF	124 (87.9)	122 (73.9)	106 (79.7)	

(d) Hardy-Weinberg Equilibrium

Species	Genotype		Observed	Expected	p-value
An. coluzzii	Vgsc_1014F	LL	16	20.0	0.275
		LF	184	176.1	
		FF	384	388.0	
An. gambiae s.s.	Vgsc_1014F	LL	3	4.6	0.403
		LF	84	80.8	
		FF	352	353.7	

Table 2.3: The distribution of *Vgsc*-1575Y genotypes in *An*. *gambiae s.s*. and *An. coluzzii*

Vgsc_NY genotype	Species		Total	Exact p-value
	An. coluzzii **(N / %)**	***An. gambiae s.s.*** **(N / %)**		
NN	582 (99.7)	353 (80.4)	935 (91.4)	< 0.001
NY	2 (0.3)	82 (18.7)	84 (8.2)	
YY	0 (0)	4 (0.9)	4 (0.4)	
Total	584	439	1023	

The Ace-1-119S mutation frequency was significantly higher in *An. gambiae s.s.* (33.8%, 95%CI: 30.7-37.1%) than in *An. coluzzii* (17.6%, 95%CI: 15.4-19.9%), (χ^2 = 68.6, df = 1, p < 0.001). The highest *Ace*-1-119S allelic frequencies in *An. gambiae s.s.* and *An. coluzzii* were found in Ashaiman (study site 44 in Figure 2.11) and Ada Foah (study site 48 in Figure 2.11) respectively (Figure 2.10). However, *Ace-1-119S* was not detected in populations from six study sites Figure 2.10 and Appendix 7. The *Ace-1-119S* allelic frequency varied significantly among settlement types in both species (χ^2 = 27.5, df = 2, p < 0.001), with the highest proportions recorded in urban areas (Table 2.4). The *Ace-1-119S* allelic proportions differ significantly among land-use in both species (χ^2 = 168.80, df = 3, p < 0.001), with vegefarm areas recording the highest *Ace-1-119S* allelic proportions in both species, whilst areas characterised by large-scale mining recorded the lowest *Ace-1-119S* proportions. The *Ace-1-119S* allelic proportions also differ significantly among ecological zones (χ^2 = 165.41, df = 3, p < 0.001) with highest proportions in *An. gambiae s.s.* and *An. coluzzii* found in deciduous forest and coastal savannah respectively. The *Ace-1-119S* genotypic frequencies (*GG*, *GS* and *SS*) varied significantly between the species pair (χ^2 = 72.7, df = 2, p < 0.001), even in sites where they coexist (Wilcoxon, p = 0.005). However, the possession of *Ace-1-119S* correlated moderately between the species pair in sites where both species coexisted (Spearman's Rho, r_s = 0.48, p = 0.006). The *Ace-1SS* individuals were found in 1.9% (95%CI: 0.9 - 3.3%) of *An. coluzzii* population, and were reported in only three sites, while 11.8% (95%CI: 9.0 -15.2%) were in *An. gambiae s.s.* population and they were reported in 14 sites.

Using pooled samples across study sites, *Ace-1-119S* genotypic frequencies within *An. coluzzii* populations showed marginal deviations from Hardy-Weinberg equilibrium (χ^2 = 3.99, df = 2, p = 0.046), which was attributable to an excess of heterozygotes (Table 2.4d). However, this result is interpreted with caution as pooling sites that are genetically distinct can artificially create excess heterozygotes. The *An. gambiae s.s.* populations, on the other hand, showed no deviation from HWE (χ^2 =15.21, df = 2, p = 0.706).

The *Ace-1-119S* genotypes exhibited significant differences in terms of settlements, ecological zones and land-use types in both species (Table 2.4). A Wilcoxon test for pairwise comparison

of the proportions showed that *Vgsc-1014F* is significantly higher than *Ace-1-119S* ($p < 0.001$). The proportions of the two resistant alleles correlated weakly and negatively, but this was not significant ($R^2 = -0.21$, $p = 0.073$).

Table 2.4: The distribution of *Ace*-1-119S genotypes in terms of settlements, ecological zones and land-use types

(a)		**Land-use**				
Species	***Ace*-1-119S Genotype**	**Mining (N / %)**	**Gardening (N / %)**	**Plantation (N / %)**	**Vegefarm (N / %)**	**Exact p-value**
An. coluzzii	*GG*	46 (90.2)	203 (81.5)	116 (89.9)	25 (16.1)	< 0.001
	GS	5 (7.8)	45 (18.1)	13 (10.1)	120 (77.4)	
	SS	0 (0)	1 (0.4)	0 (0)	10 (6.5)	
An. gambiae s.s.	*GG*	6 (42.9)	114 (38.1)	49 (85.9)	25 (36.3)	< 0.001
	GS	6 (42.9)	147 (49.2)	7 (12.3)	33 (47.8)	
	SS	2 (14.2)	38 (12.7)	1 (1.8)	11(15.9)	
(b)		**Ecological zone**				
Species	***Ace*-1-119S Genotype**	**Mangrove Swamp (N / %)**	**Coastal Savannah (N / %)**	**Deciduous forest (N / %)**	**Rainforest (N / %)**	**Exact p-value**
An. coluzzii	*GG*	42 (42.9)	71 (42.5)	36 (64.3)	241 (91.6)	< 0.001
	GS	48 (48.9)	93 (55.7)	20 (35.7)	22 (8.4)	
	SS	8 (8.2)	3 (1.8)	0 (0)	0 (0)	
An. gambiae s.s.	*GG*	7 (70.0)	46 (43.8)	69 (32.2)	72 (65.4)	< 0.001
	GS	3 (30.0)	43 (41.0)	113 (52.8)	34 (31.0)	
	SS	0 (0)	16 (15.2)	32 (15.0)	4 (3.6)	
(c)		**Settlement**				
Species	**Ace-1-119S Genotype**	**Rural (N / %)**	**Peri-urban (N / %)**	**Urban (N / %)**	**Exact p-value**	
An. coluzzii	*GG*	81 (84.4)	208 (68.2)	101 (55.2)	< 0.001	
	GS	15 (15.6)	87 (28.5)	81 (44.3)		
	SS	0 (0)	10 (3.3)	1 (0.5)		
An. gambiae s.s.	*GG*	73 (51.8)	81 (49.0)	40 (30.1)	<0.001	
	GS	59 (41.8)	66 (40.0)	68 (51.1)		
	SS	9 (6.4)	18 (11.0)	25 (18.8)		

(d)		**Hardy-Weinberg Equilibrium**			
Species	**Genotype**		**Observed**	**Expected**	χ^2	**P-value**
An. coluzzii	*Ace-1*-119S	*GG*	390	397.0	*3.99*	0.046
		GS	183	169.0		
		SS	11	180.0		
An. gambiae s.s.	*Ace-1*-119S	*GG*	194	192.2	*0.14*	0.706
		GS	193	196.5		
		SS	52	50.2		

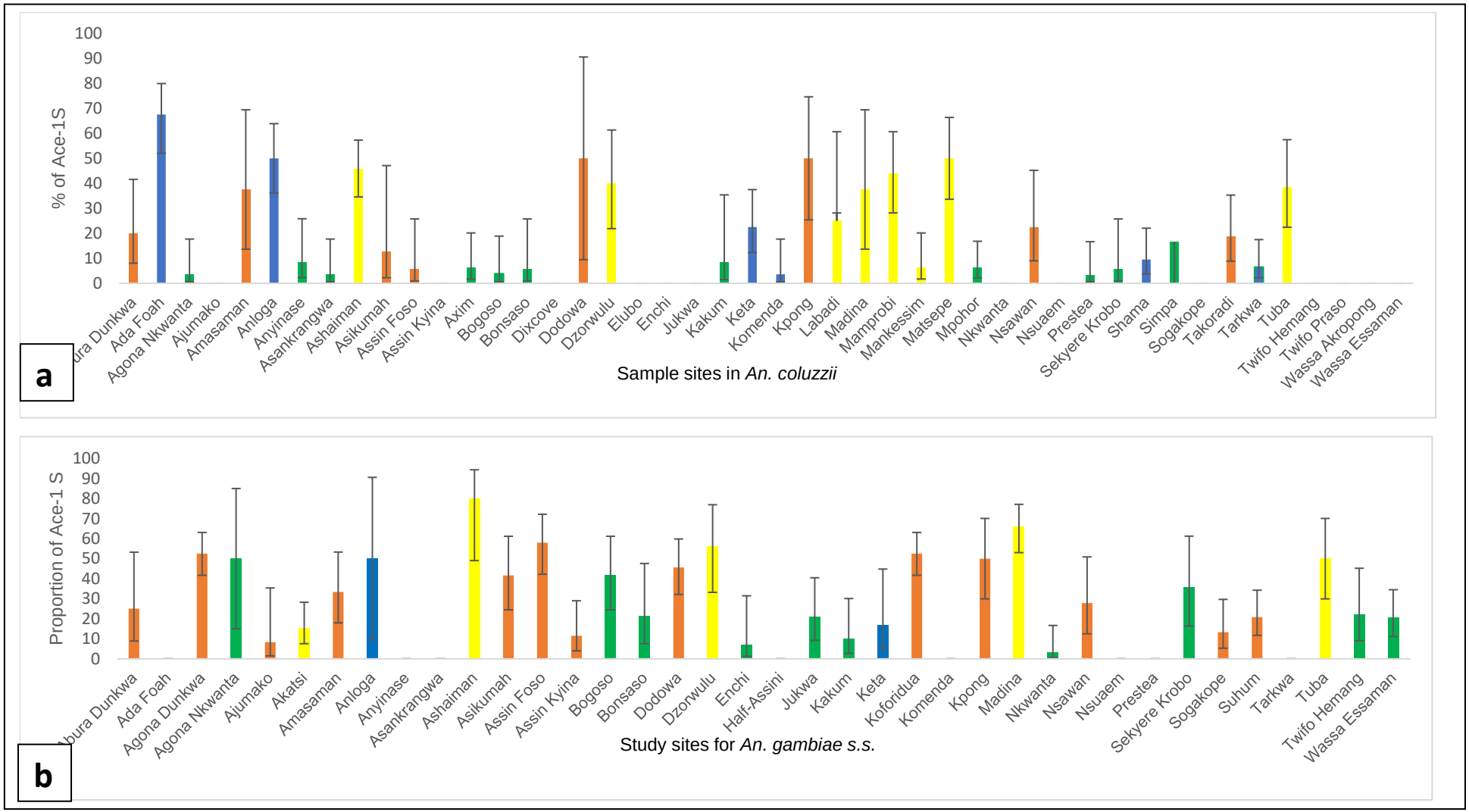


Figure 2.10: Distribution of *Ace-1*-119S allelic frequency with 95% confidence interval (error bars) in (a) *An. coluzzii* and (b) *An. gambiae s.s.* The colour coding of the bar chats indicates the ecological zones; blue = mangrove strand, green = rainforest, yellow = coastal savannah and orange = deciduous forest.

Study site keys

1 = Enchi	8 = Prestea	15 = Agona Nkwanta	22 = Twifo Hemang	29 = Abura Dunkwa	36 = Nsawam	43 = Dodowa	50 = Anloga
2 = Half-Assini	9 = Axim	16 = Dixcove	23 = Sekyere Krobo	30 = Mankessim	37 = Amasaman	44 = Ashaiman	51 = Keta
3 = Elubo	10 = Bogoso	17 = Mpohor	24 = Komenda	31 = Ajumako	38 = Tuba	45 = Kpong	
4 = Asankragua	11 = Tarkwa	18 = Takoradi	25 = Kakum	32 = Asikumah	39 = Mamprobi	46 = Matsepe	
5 = Nkwanta	12 = Bonsaso	19 = Wassa Essaman	26 = Jukwa	33 = Agona Dunkwa	40 = Dzorwulu	47 = Sogakope	
6 = Anyinase	13 = Simpa	20 = Shama	27 = Assin foso	34 = Suhum	41 = Labadi	48 = Ada Foah	
7 = Wassa Akropong	14 = Nsuaem	21 = Twifo Praso	28 = Assin Kyina	35 = Koforidua	42 = Madina	49 = Akatsi	

Figure 2.11: A map showing the distribution of *Ace*-1-119S allelic frequencies and clusters in *An. gambiae* complex among the study sites across southern Ghana.

2.3.4 Relationships among existing species, genotypes (*Vgsc* and *Ace-1*), settlements, ecological zones and land-use types.

To investigate relationships among species, environmental variables and resistance genotypes (*Vgsc*-L1014F, *Vgsc*-N1575Y *Ace-1*-G119S), a principal component analysis was performed. The analysis was limited to two independent composite variables that explained maximal variance in the data set, with which correlations of the original variables was visualised. The model accounted for 54.6% of the total variance with a total Cronbach's alpha value was 0.86, indicating a high level of model reliability. For individual variables, in principal component 1, ecological zone and land-use accounted for the most variance while species and *Vgsc*-1014Y accounted for the most variance in principal component 2 (Figure 2.12). An object plot, which is used to identify outliers in the PCA, showed that the variates are clustered around the origin with no remarkable outliers (Appendix 8).

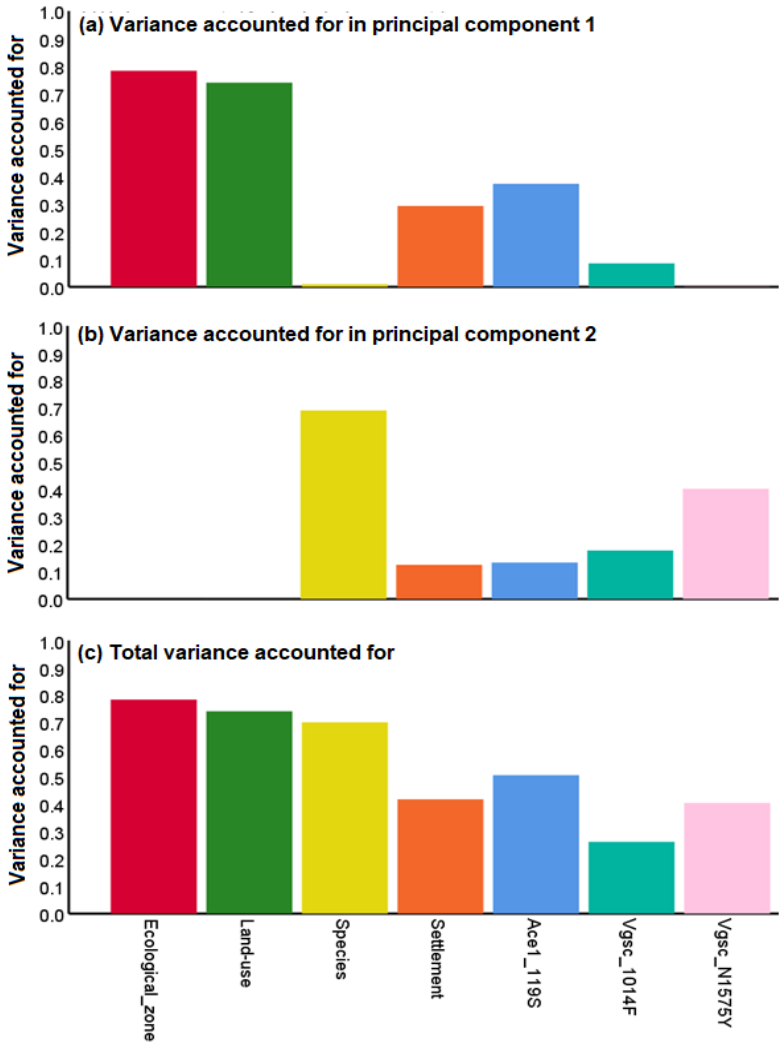


Figure 2.12: The bar graphs showing the variances accounted for by each explanatory variable (a) principal component 1, (b) principal component 2 and (c) the total in the principal component space (the combinations of the two dimensions)

The ecological zone and the land use are strongly correlated but negatively indicating that certain characteristics are lacking in the ecological zone associated with the principal component. Mining and plantations were associated mainly with rainforest, while vegefarm was associated with mangrove swamp and coastal savannah (Figure 2.13). Settlement correlated moderately with ecological zone and land use, with rural areas being closely associated with rainforest, mining and plantation in principal component 1, however urban and peri-urban areas both were associated with deciduous forest in principal component 1 (Figure 2.13). Species accounted for little variance in dimension 1 and so all the assessments were made based on principal component 2 where it counted for relatively high variance. Both *An. gambiae s.s.* and *An. coluzzii* accounted for high variance but they correlated weakly with both ecological zone and land use. For the genotypes, *Ace*-1-119S and *Vgsc*-1014F were not associated with each other as their angle between them was approximately 90^0 (cosine 90^0 = 0) indicating a correlation coefficient close to zero. However, both genotypes correlated moderately with species, and ecological zone and land use. It is noteworthy that *Ace*-1-119S accounted for a higher variance than *Vgsc*-1014F in the model. The relationships between species and genotypes were interpreted based on principal component 2, where species accounted for the most variance. Among *Ace*-1-119S genotypes, *SS* and *GS* were more associated with *An. gambiae s.s.* than with *An. coluzzii* whilst in *Vgsc*-1014F, *LL* and *LF* were more associated with *An. coluzzii* than with *An. gambiae s.s.* (Figure 2.13). The *Vgsc*-1575 was very weakly correlated with the ecological zone and land use but strongly correlated with species (with almost all *YY* and *NY* were associated with *An. gambiae s.s*).

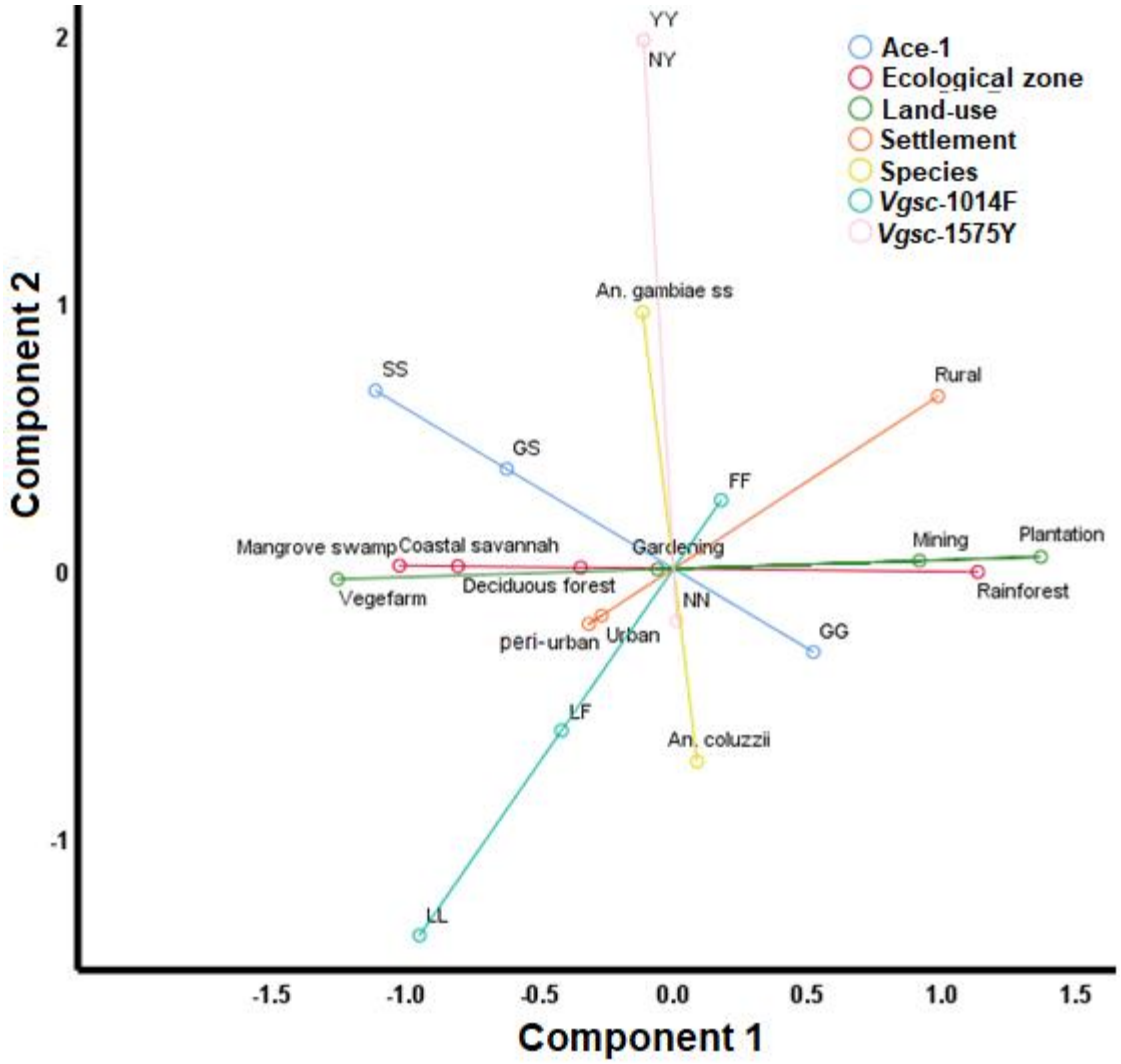


Figure 2.13: The component plot showing the explanatory variables and their factor levels. The lines represent the variables, the length of the line from the origin indicates the variance accounted for, whilst the cosine of the angle between the lines equal to the correlation coefficient. The principal component 1 and 2 accounted for 32.7% and 21.9% variance respectively.

2.3.5 Spatial analysis of *Ace-1*-119S and *Vgsc-1014F*

A simple Mantel test (a test used to correlate two matrices) was performed to determine how well phenotypic resistance to permethrin and fenitrothion and genotypes (*Ace-1*-119S and *Vgsc-1014F*) across populations correlated with geographical distance. Pairwise differences in the proportions of phenotypic resistance between sites were used to create resistance matrices. Phenotypic resistance to permethrin correlated moderately with pairwise geographic distance between sites, whilst the phenotypic resistance to fenitrothion correlated strongly with distance (Table 2.5). The phenotypic resistance to both insecticides remained significantly correlated to geographic distance when the influence of species was controlled

for in the partial Mantel tests. *Ace-1*-119S showed a significant correlation with geographical distance and remained so when species variation was controlled (Table 2.5). *Vgsc-1014F* showed a significant correlation with geographical distance and remained so when species was controlled. Figure 2.14 shows that *Ace*-1-119S exhibits isolation by distance (IBD) in both *An. gambiae s.s.* and *An. coluzzii*. *Vgsc*-1014F showed IBD driven more by *An. coluzzii* but without enough of a signal in *An. gambiae s.s*.

Table 2.5: Simple Mantel tests and partial Mantel tests with correlation coefficient (*R*) and corresponding significant p-values.

(a) Phenotypic resistance				
Parameter	**Phenotypic resistance to permethrin (pairwise %)**		**Phenotypic resistance to fenitrothion (pairwise %)**	
	R	**P-value**	***R***	**P-value**
Distance	0.319	0.001	0.615	< 0.001
Controlling species (% of *An. gambiae s.s.*)	0.283	0.002	0.609	< 0.001

(b) Genotypic resistance				
Parameter	**Correlation with *Ace-1*-119S**		**Correlation with *Vgsc-1014F***	
	R	**P-value**	***R***	**P-value**
Distance	0.475	< 0.001	0.127	0.028
Controlling species (% of *An. gambiae s.s.*)	0.474	< 0.001	0.129	0.025

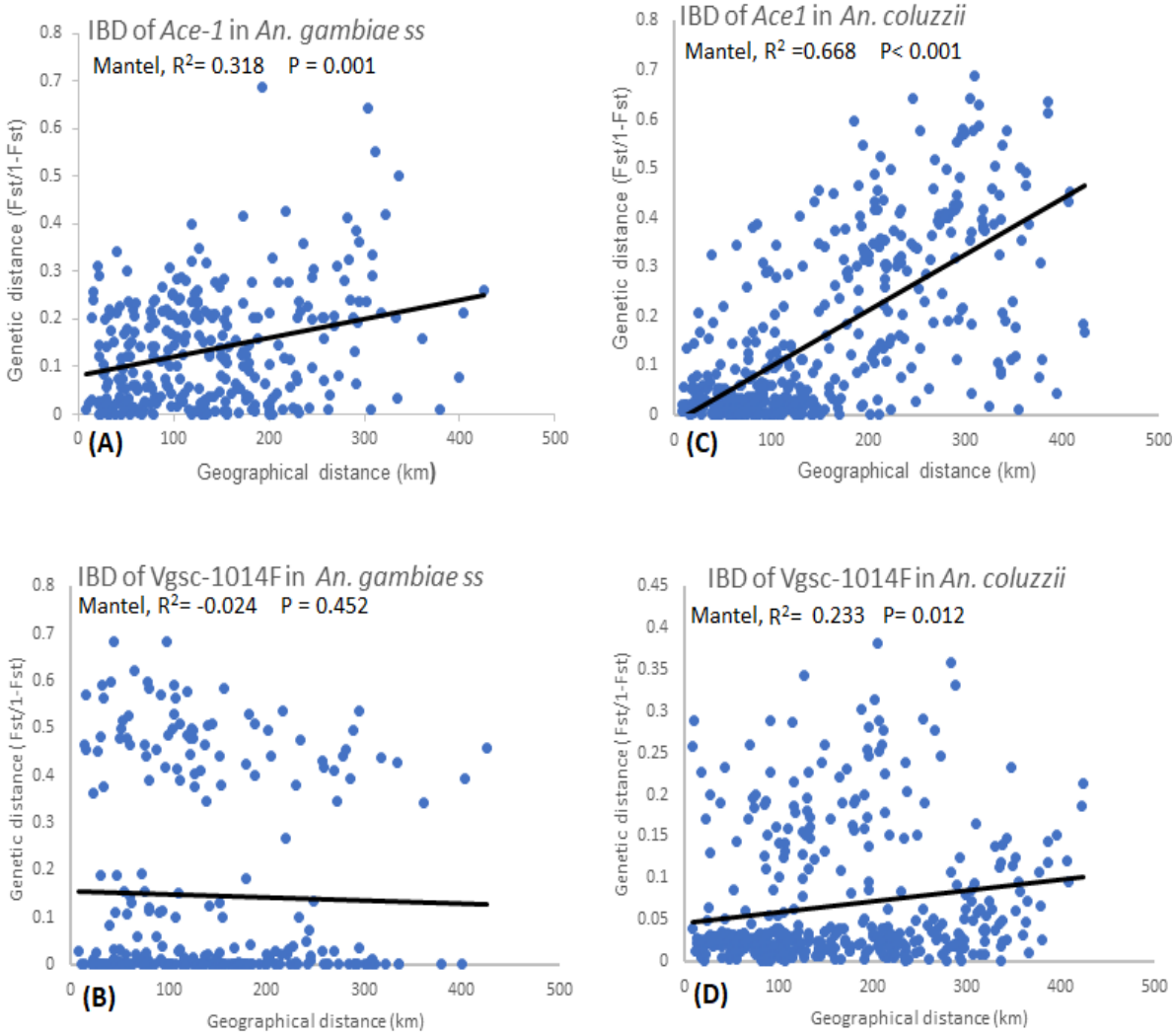


Figure 2.14: The isolation by distance plots with Mantel test correlations between pairwise genetic distances of *Ace-1*-119S and *Vgsc*-1014F and geographical distance in the species pair across southern Ghana.

2.3.6 Model of *Ace-1*-119S and *Vgsc-1014F* with ecological zone, land-use and settlement

Multinomial logistic regression was applied to investigate the explanatory power of species, environmental and land-use practices for *Vgsc L1014F* or *Ace-1 G119S* genotypic variation. For *Vgsc*-1014F, the likelihood ratio tests of the effect of the individual predictors against the constant only model showed that only ecological zone and species made significant contributions to the main model (Table 2.6). The final likelihood ratio tests of the main effect model (i.e. with only ecological zone and species as predictors) ($\chi^2 = 87.2$, df = 8, $p < 0.001$) against the null model were statistically significant indicating that the predictors as a set reliably distinguished between *Vgsc*-1014F genotypes (Table 2.7). The *FF*: *LL* ratio (odds

ratio = 0.14) is significantly lower in mangrove swamp than in rainforest, however, it is not significantly different based on the cumulative impacts from other effect levels.

Table 2.6: The likelihood ratio test of the predictors in the model with their corresponding chi-squares and p-values for *Vgsc*-1014F.

Initial model	**Model Fitting Criteria**	**Likelihood Ratio Tests**		
Effect	**-2 Log Likelihood of Reduced Model**	**Chi-Square**	**df**	**P-value**
Intercept	308.1			
Ecological zone	331.2	23.1	6	< 0.001
Species	342.9	34.7	2	< 0.001
Land-use	320.1	11.9	6	0.063
Settlement	311.0	2.9	4	0.573

Table 2.7: The predictor estimates in terms of odds ratios and 95% confidence interval on *Vgsc*-1014F genotypes using *LL* genotype as a reference category

VGSC_1014F		**X^2**	**df**	**P-value**	**Odds ratio (95% CI)**
LF	**Intercept**	10.9	1	0.001	
	Mangrove swamp	2.7	1	0.133	0.400 (0.121 - 1.321)
	Coastal Savannah	0.4	1	0.543	1.491 (0.412 - 5.404)
	Deciduous forest	2.5	1	0.114	6.301 (0.641 - 61.952)
	***Rainforest**				
	An. coluzzii	0.1	1	0.996	0.997 (0.251 - 3.952)
	****An. gambiae s.s.***				
FF	**Intercept**	43.9	1	< 0.001	
	Mangrove swamp	10.7	1	0.001	0.144 (0.045 - 0.460)
	Coastal Savannah	1.1	1	0.294	0.509 (0.144 - 1.797)
	Deciduous forest	0.3	1	0.605	1.818 (0.189 - 17.518)
	***Rainforest**				
	An. coluzzii	2.2	1	0.140	0.361 (0.093 - 1.397)
	****An. gambiae ss***				

**LL* genotype, Rainforest and **An. gambiae s.s.* were used as reference parameters.

For *Ace-1-119S*, the likelihood ratio tests of the effect of the individual predictors against a constant only (intercept) were all statistically significant (Table 2.8). The final likelihood ratio tests of the main effect model (i.e. only ecological zone, species, settlement and land-use as predictors) (χ^2 = 377.0, df =18, p < 0.001) against the null model were statistically significant indicating that the predictors as a set reliably differentiate between *Ace*-1-119S genotypes.

Table 2.8: The likelihood ratio test of the predictors in the model with their corresponding chi-squares and p-values for *Ace-1*-119S using *GG* genotype as a reference category

Effect	Model Fitting Criteria	Likelihood Ratio Tests		
	-2 Log Likelihood of Reduced Model	χ^2	df	p-value
Intercept	316.006	0	0	.
Ecological zone	350.827	34.821	6	< 0.001
Settlement	345.135	29.129	4	< 0.001
Land-use	425.652	109.646	6	< 0.001
Species	361.509	45.503	2	< 0.001

In the multinomial regression for ecological zones, the *GS*: *GG* ratio is significantly higher in coastal savannah (OR = 2.05) and deciduous forest (OR = 3.82) than in rainforest, however, it is not significantly different in a mangrove swamp (Table 2.9). Similarly, the *SS*: *GG* ratio is significantly higher in the other ecological zones than in Rainforest. For settlement, both *GS*: *GG* and SS: GG ratios are significantly lower in peri-urban areas than in urban areas, however, they are not significantly different in rural areas. For land-use, both *GS*: *GG* and *SS*: *GG* ratios are significantly lower in gardening and plantation than in vegefarm. For species, both *GS*: *GG* and SS: GG ratios are significantly lower in *An. coluzzii* than in *An. gambiae s.s.* (Table 2.9).

Table 2.9: The predictor estimates in terms of odds ratio with 95% confidence interval on *Ace-1*-119S genotypes.

***Ace-1* genotype**		**χ^2**	**df**	**p-value**	**Odd ratio (95% CI)**
GS	Intercept	11.848	1	0.001	
	Mangrove swamp	2.589	1	0.108	1.849 (0.875 - 3.910)
	Coastal savannah	5.183	1	0.023	2.052 (1.105 - 3.810)
	Deciduous forest	23.436	1	< 0.001	3.820 (2.220 - 6.572)
	*Rainforest				
	Rural	0.050	1	0.822	0.939 (0.542 -1.625)
	Peri-urban	20.421	1	< 0.001	0.402 (0.271- 0.597)
	*Urban				
	Mining	20.996	1	< 0.001	0.099 (0.037 - 0.266)
	Gardening	70.702	1	< 0.001	0.135 (0.084 - 0.215)
	Plantation	67.015	1	< 0.001	0.049 (0.024 - 0.101)
	*Vegefarm				
	An. coluzzii	12.580	1	< 0.001	0.529 (0.372 - 0.752)
	**An. gambiae ss*				
SS	Intercept	0.978	1	0.323	
	Mangrove swamp	6.217	1	0.013	11.538 (1.687- 78.888)
	Coastal savannah	3.999	1	0.046	5.830 (1.036 - 32.818)
	Deciduous forest	7.630	1	0.006	9.544 (1.925 - 47.308)
	*Rainforest				
	Rural	0.887	1	0.346	0.615 (0.223 - 1.692)
	Peri-urban	6.300	1	0.012	0.428 (0.221 - 0.830)
	*Urban				
	Mining	0.475	1	0.491	0.439 (0.042 - 4.560)
	Gardening	18.504	1	<0.001	0.179 (0.081 - 0.392)
	Plantation	10.365	1	0.001	0.029 (0.003 - 0.249)
	*Vegefarm				
	An. coluzzii	33.357	1	< 0.001	0.088 (0.038 - 0.200)
	**An. gambiae ss*				

**GG* genotype, rainforest and **An. gambiae s.s.* were used as reference parameters.

2.4 Discussion

2.4.1 Species distribution

Speciation between *An. gambiae s.s.* and *An. coluzzii* is thought to be driven largely by differential larval habitat adaptation (Lehmann and Diabate, 2008b; Gimonneau *et al.*, 2012), a hypothesis that is supported by results in this study. It was found that *An. gambiae s.s*. the population was more common in deciduous forest zones that are characterised by high humidity and transient breeding sites, while *An. coluzzii* was more common in coastal savannah, mangrove swamp and rainforest. This finding is consistent with a previous study in two cities located in the coastal savannah of southern Ghana which reported that *An. coluzzii* are thriving in a relatively dry environment typified by permanent pools that are usually created by anthropogenic activities such as irrigation and building construction (Mattah *et al.*, 2017). The coexistence of *An. coluzzii* and *An. melas*, a brackish species, in five sites within the mangrove swamp is consistent with *An. coluzzii* larvae displaying slightly greater salinity tolerance than *An. gambiae s.s.*, which may allow better exploitation of marginal habitats (Tene Fossog et al., 2015). The predominance of *An. coluzzii* in the rainforest may be an indication of niche expansion (as the sampling was performed during the wet season), or the influence of mining activities on the distribution of mosquitoes. For instance, mining and lumbering are two land-use activities in the rainforest of Ghana, as such the rapid deforestation and numerous illegal small-scale mining activities popularly known as “galamsey” which usually leave behind several abandoned mining pits that become a permanent breeding site of mosquitoes (Emmanuel *et al.*, 2018; Ferring and Hausermann, 2019). *An. coluzzii* may be relatively more likely to survive in such degraded environments if recent reports of adaptation to xenobiotics (Kamdem *et al.*, 2017; Ossè *et al.*, 2019) also apply to the population of western Ghana. It is postulated that the adaptation of Anopheles to various xenobiotics is driving the niche expansion (Ossè *et al.*, 2019). Although both species were found in urban and peri-urban areas, *An. coluzzii* was more common (Table 2.4), confirming recent reports in Ghana (Baffour-Awuah *et al.*, 2016; Mattah *et al.*, 2017). However, historically, contrasting findings were reported in urban suburbs of Accra and Kumasi where *An. gambiae s.s.* was predominant in urban areas (Afrane *et al.*, 2004). This may indicate the possible influence of seasonal variations (Dao *et al.*, 2014; Hidalgo *et al.*, 2018) and/or anthropogenic environmental modifications (Junglen *et al.*, 2009; Nava *et al.*, 2017) on the distribution of mosquito species. Water pollution in urban areas is generally regarded as a principal factor that limits the growth of *Anopheles* larvae, yet several studies have shown that *An. gambiae s.l.* and *An. funestus* are breeding in the polluted urban waters (Antonio-nkondjio *et al.*, 2012; De Silva and Marshall, 2012; Tene Fossog *et al.*, 2013; Kudom *et al.*, 2015; Mattah *et al.*,

2017; Azrag and Mohammed, 2018; Ossè *et al.*, 2019). It has been reported that, within *An. coluzzii*, rural and urban populations are genetically differentiated with selection centred on genes implicated in xenobiotic resistance (Kamdem *et al.*, 2017) and this may be consistent with the hypothesis that anthropogenic-driven selection is the pivotal modulator of ecological divergence between and within species (Kamdem *et al.*, 2012, 2017; Caputo *et al.*, 2014).

The species pair were found to coexist in most sites in southern Ghana, yet only three hybrids (0.3%) were recorded. This low hybridization rate is consistent with previous studies in Ghana where the hybrid frequency of 1.0% (Adasi and Hemingway, 2008) and 0.1% (Essandoh *et al.*, 2013; Clarkson *et al.*, 2014), 0 – 1.4% in Benin (Fassinou *et al.*, 2019), 2.5% in Togo (Amoudji *et al.*, 2019) and 2.1% in Ivory Coast (Fodjo *et al.*, 2018) have been reported, and the phenomenon may be due to assortative mating, larval niche partitioning or selection against hybrids in the wild (Lehmann and Diabate, 2008a). In contrast, a high rate of recombination and heterozygosity between the species pair resulting in a hybrid frequency of 19–24% has been reported in Guinea Bissau (Oliveira *et al.*, 2008; Gordicho *et al.*, 2014). Hence, the extent of isolation between the species pair varies across the species distribution range. Again, it has been shown that extreme asymmetric dominance of one species created by ecological (seasonal) conditions may promote hybridization between the species pair (Niang *et al.*, 2015). However, surges in hybrid detection rate (Lee *et al.*, 2013) are yet to be detected in Ghana despite extensive surveys extending almost 20 years (Yawson *et al.*, 2004) suggesting that they may not be geographically ubiquitous phenomenon.

2.4.2 Permethrin resistance, *Vgsc* (*L1014F*, *N1575Y*) distribution and determinants

Phenotypic resistance of the species pair to pyrethroids was widespread, though with significant variation in prevalence among study sites. The implicated target-site mechanisms are mutations in the *Vgsc* gene (*L1014F*, *N1575Y,* and 13 non-synonymous nucleotide variations including L995F that have been reported recently in *An. gambiae s.s*. and *An. coluzzii* (Clarkson *et al.*, 2018)). The *Vgsc-1014F* allele was found to be widespread in southern Ghana and existed in high frequencies across all sites and these findings are consistent with recent studies in southern Ghana where the average *Vgsc*-1014F for three years was found to be 0.99 (Pwalia *et al.*, 2019) and in Ivory Coast (Fodjo *et al.*, 2018; Zoh *et al.*, 2018), Togo (Amoudji *et al.*, 2019), Benin (Salako *et al.*, 2018) and Burkina Faso (Namountougou *et al.*, 2019) where the *Vgsc*-1014F frequencies range from 0.63 to 1.0. In this study, the *Vgsc-1014F* allele was at a significantly higher frequency in *An. gambiae s.s.* (with many more sites showing fixation) than in *An. coluzzii*, although there was no significant difference between the species pair in the possession of *LF* and *FF* genotypes (Table 2.8).

However, the *Vgsc-1014F* in the species pair is not a universal phenomenon as a report from Togo showed the reverse where the *Vgsc-1014F* frequency in *An. gambiae s.s.* and *An. coluzzii* was found to be 0.77 and 0.84, respectively (Ahadji-dabla *et al.*, 2019). Thus, following introgression and rapid selection the differences in *kdr* frequencies between *An. gambiae s.s.* and *An. coluzzii* in tropical West Africa appear relatively moderate (Lynd *et al.*, 2010).

In our study, a relatively higher *Vsgc-1014F* allelic frequency in rural areas than in urban and peri-urban areas in southern Ghana may be due to rural settlements located mostly in tropical rainforest (Table 2.2) which is characterised by large-scale cocoa, rubber and oil palm plantations that rely heavily on pyrethroids (bifenthrin and lambda-cyhalothrin) for pest controls (Antwi-Agyakwa *et al.*, 2015; Denkyirah *et al.*, 2016; Pesticide Action Network, 2018). The pesticide use for cash crop plantation is likely to heighten selection pressure for *Vgsc*-1014F resistance mutation (Poupardin *et al.*, 2012; Abuelmaali *et al.*, 2013; Nkya *et al.*, 2014; Chouaïbou *et al.*, 2016b). However, *Vgsc*-1014F was also found to be exceptionally high - including allele fixations - in some urban and peri-urban areas with large-scale vegetable farms like Dzorwulu and Ashaiman, and this finding is consistent with what has been reported in Ivory Coast (Zoh *et al.*, 2018), Sudan (Abuelmaali *et al.*, 2013) and Tanzania (Nkya *et al.*, 2014). In each of these diversely located studies, urban and peri-urban agricultural activities were found to be associated with insecticide resistance, although a direct link between agricultural activities and *Vgsc*-1014F is yet to be established. Areas in deciduous forest, in which gardening and small-scale farming are the major land-use types recorded the least *Vsgc-1014F* allelic frequency and may be due to low insecticide usage. In this study, no *Vgsc-1014S* allele was detected in either species, although it has been found in very low frequencies in the populations of species pair in the immediate neighbouring countries of Ghana, i.e. Burkina Faso (Namountougou *et al.*, 2013), Togo (Djouaka *et al.*, 2018), Benin (Djègbè *et al.*, 2014) and Côte d'Ivoire (Chouaïbou et al., 2017). The *V*gsc-1575Y which confers additional resistance to pyrethroids and DDT (Jones *et al.*, 2012) was detected for the first time in Ghana in both species but at very low frequencies, however it is significantly higher in *An. gambiae s.s.* than in *An. coluzzii*. *The Vgsc*-1014F correlated with distance in *An. coluzzii* but not in *An. gambiae s.s.,* however, the exact mechanism for this discrepancy is not known at the moment.

2.4.3 Fenitrothion resistance, *Ace1*-119S distribution and determinants

Resistance of the species pair to fenitrothion was found in a few populations but at a much lower prevalence than that of permethrin. *Ace-1*-119S that confers resistance to CB/OP was detected in both species at varying frequencies among the study sites, but it was

significantly higher in *An. gambiae s.s.* than in *An. coluzzii* even in places where the two species exist in sympatry. However, *Ace-1*-119S in the species pair correlation was moderately significant, suggesting that there is parallel local adaptation as the species pair rarely hybridises. The cause of *Ace-1*-119S frequency discrepancies between the species pair is still unknown, although recent genomic studies via sequencing have shown that the mutation is a recent event that introgressed from *An. gambiae s.s.* to *An. coluzzii* (Grau-Bové *et al.*, 2020). The *Ace-1*-119S genotypes were predominantly heterozygotes (*Ace*-1*GS)* and this may be due to the high fitness cost associated with the possession of *Ace*-1-119S, and so *Ace-1SS* individuals are less likely to survive in the absence of CB/OP, while *Ace*-1*GS* individuals exhibit a better resistance-cost trade-off in both treated and untreated settings and therefore may have a selective advantage in areas with inconsistencies in insecticide treatments (Assogba *et al.*, 2016; Assogba *et al.*, 2018).

The *Ace-1*-119S exhibited strong isolation by distance in both species suggesting that its variance is accounted partly by distance (a non-selective factor). For instance, *Ace-1*-119S was concentrated in the study sites located in the central belt (Figure 2.11) where the predominant land-use activity is vegefarm, and exhibited a clinal reduction with distance towards the eastern and western belts of southern Ghana, confirming the finding of my previous studies based on samples collected eight years earlier Essandoh *et al.,* (2013). Possible explanations are that *Ace*-1-119S might have originated or at least expanded in frequency in the central belt and is still spreading to eastern and western areas, or in the central belt selection pressure is much higher or *Ace-1* duplication is favoured.

The *Ace*-1-119S mutation distribution and variation are also influenced by selective factors such as settlement, ecological zone and land use, with the latter two the key determinants of the distribution of *Ace-1*-119S genotypes (Table 2.10). For instance, *Anopheles* mosquitoes in coastal savannah and deciduous forest are more likely to possess *Ace-1GS* and *Ace-1SS* genotypes than their rainforest counterparts. Mosquitoes in urban settings are much more likely to possess *Ace-1GS* and *Ace-1SS* genotypes than those found in rural and peri-urban settings similar to what has been reported in Ivory Coast where urban areas possessed higher frequencies of *Ace-1GS* and *Ace-1SS* than in rural areas (Zoh *et al.*, 2018). Also, vegefarm areas are more likely to possess *Ace-1GS* and *Ace-1SS* than areas where mining, plantation and gardening are the major land-use activities. Although pesticide usage in cash crop plantations may contribute to the selection pressure for *Ace-1*-119S mutation, it is evident from our studies that the urban agricultural activities have much more impact, supporting the argument for the selection of a high level of *Ace-1*-119S in the south-central belt and the subsequent diffusive spread elsewhere.

Insecticides from the CB and OP classes have been little used in public health in southern Ghana (Figure 2.1), however, it has been reported that the most commonly used agricultural pesticides in Ghana are organophosphate-based herbicides (dimethoate and glyphosate) and carbamate-based fungicide (Mancozeb) (PMI, 2016) with empty bottles of pesticides (e.g. in Appendix 9) found on farms during this study. These agricultural pesticides are likely to elevate the selection pressure for *Ace*-1-119S resistance mutation in *Anopheles* mosquitoes as their usage in numerous small-scale vegetable farms in urban settings is usually intensive and under-regulated (Keiser *et al.*, 2005; Klinkenberg *et al.*, 2008).

2.5 Conclusion

This study shows *Vgsc*-1014F frequencies are very high in both *An*. *gambiae* s.s. and *An*. *coluzzii* across southern Ghana. The *Ace-1*-119S variance across southern Ghana is influenced by geographic distance, settlement and land-use types. The central belt of southern Ghana is characterised by numerous urban vegetable-growing activities that may serve as an *Ace-1*-119S hotspot, and then the mutation spreads gradually towards the western and eastern belts. These results could serve as a basis for designing vector control strategies and insecticides resistance management plans to suit a geographical area taking into consideration the settlement types and the land-use activities. For insecticide resistance management, considering the high level of pyrethroid resistance with concomitant elevated levels of *Vgsc*-1014F frequencies across southern Ghana, the NCMP should continue the use of OP/CB-based insecticides for IRS, but must be monitored regularly for temporal changes. However, in places where *Ace-1*-119 frequencies are high such as the central belt, the Sumishield should be used. Further studies are needed to quantify the source and the scope of CB/OP-based agricultural pesticides that Anopheles mosquitoes are exposed to so as to understand the possible role of agriculture in the selection of *Ace-1*-119S, particularly in urban areas.

Chapter Three: Temporal surveillance of *Ace*-1-based insecticide dynamics in a resistance hotspot in southern Ghana.

Abstract

Variation in mutations affecting the acetylcholinesterase gene (*Ace-1*) target site is a major driver of phenotypic resistance to carbamate (CB) and organophosphate (OP) insecticides in *An. gambiae s.s.* and *An. coluzzii*. In this study, *Ace*-1 diagnostics were used for temporal molecular surveillance as an indicator of possible changes in resistance in Accra, in which there is no use of the CB and OP insecticides for vector control. Between 2002 and 2013, there was a dramatic and unprecedented increase in the frequencies of *Ace*-1-119S alleles and homozygotes in Accra on an exponential trajectory toward fixation; a highly surprising observation because of expected fitness costs of the serine mutant. This study investigated the impacts of this change on phenotypic resistance levels and expanded the time series for *Ace-1* mutations to gain an understanding of the mechanisms underpinning temporal changes.

Prevalence bioassay (exposing mosquitoes to a fixed concentration of an insecticide for a predefined time) and dose-time-response assay (exposing mosquitoes to a fixed concentration of an insecticide for an indefinite amount of time till death of mosquitoes) were performed on adult females to assess the susceptibility and the resistance intensity to bendiocarb/fenitrothion respectively. Species characterizations and *Ace-1* duplication detection were performed using standard PCR. The *Ace*-1 genotyping and *Ace-1* copy number estimates were determined using real-time quantitative PCR. Dose-time-response assays revealed an extreme level of resistance to bendiocarb and fenitrothion but without a significant increase in resistance prevalence to fenitrothion and bendiocarb between 2013 and 2019. The ascent toward fixation halted after 2013, with *Ace*-1-119S frequency stabilising. This cessation occurred due to the co-occurrence of both homogeneous and heterogeneous *Ace-1* duplications, which violate the Mendelian principle. This balance of mutant polymorphisms likely results from different costs and benefits of each, and we show that the multicopy nature of the more resistance-conferring homogeneous duplications involves, probably a wasteful expression of many additional genes within the CNV region. The work shows how molecular surveillance can track changes in resistance in populations missed by standard diagnostic dose assays, highlighting the need for intensive, focal phenotypic investigation. However, the evolution of complex forms of a strongly-selected resistance marker indicates the need to also perform studies regularly to track subtle changes in marker systems.

3.1 Introduction

Diagnosis of insecticide resistance phenotypes in mosquitoes typically relies on bioassays that expose females to a fixed concentration of active ingredient for a predefined time, usually one hour. The proportion of the test cohort surviving after a further 24-hour holding period gives an estimate of the prevalence of resistance in the population. A vector population is regarded as “resistant” if mortality is less than 90%, “suspected resistant” (i.e. requires further confirmation) if mortality is between 90 and 97%, and “susceptible” if mortality is ≥98% (WHO global malaria program, 2012). Prevalence bioassays are useful for detecting resistance regardless of the mechanisms involved and also for detecting new types of resistance emerging in the vector population. However, a diagnostic dose assay alone does not indicate the intensity of resistance phenotype and may mask significant changes in resistance (Toé *et al.*, 2014; Bagi *et al.*, 2015). For instance, dose-response assay studies in southwest Burkina Faso showed a ten-fold increase in resistance in the *An. gambiae s.s.* population yet prevalence assays showed no change in the mortality rate for three years (Toé *et al.*, 2014). Furthermore, there is no direct correlation between laboratory mortality and field efficacy as the diagnostic dosage is set as twice the dosage that induces 100% mortality in insecticide susceptible laboratory strain (WHO, 2016). This implies that phenotypic resistance information provided by bioassays does not link to potential operational control failure (Venter *et al.*, 2017). Moreover, bioassays do not take into consideration the lifetime impact of insecticide exposure as susceptibility testing usually evaluates resistance based on 24-hour mortality outcomes (Viana *et al.*, 2016).

Inspired by increasing resistance and desire for more operationally relevant prevalence assays, WHO established a protocol that provides information on the intensity of phenotypic resistance. This is a revised version of the standard susceptibility diagnostic assay with inclusions of 5X and 10X of the discriminating concentration in a stepwise manner (WHO, 2016). However, this resistance intensity protocol requires a large number of mosquitoes making it practically challenging for widespread monitoring in places where mosquitoes are rare (Weetman and Donnelly, 2015; Donnelly *et al.,* 2016). On a large scale, reliable laboratory facilities for generating bioassay data may be a limiting factor, and comparing data may introduce confounders. The use of molecular diagnostic markers for resistance detection, particularly those that target DNA such as single nucleotide polymorphism (SNP) and copy number variants (CNV) have the potential both to detect resistance at an early stage and provide sensitive tracking of changes within and spread among populations. DNA markers have the additional advantage of being able to use mosquitoes stored for prolonged periods

and are wholly insensitive to environmental conditions. Some molecular markers with substantial predictive power have been deployed in resistance diagnostics. For instance, the glutathione-S-transferase E2 (GSTe2) marker has high diagnostic power in tracking and monitoring DDT and permethrin metabolic resistance in *An. funestus* (Riveron *et al.*, 2014). Again, a molecular marker for cytochrome P450-based resistance (CYP6P9a) has been used to show that pyrethroid resistance in *An. funestus* is reducing the efficacy of LLINs (Weedall *et al.*, 2019; Tchouakui *et al.*, 2020). In *An. gambiae s.l.* resistance to CBs and OPs has been reported across West Africa (Aïkpon *et al.*, 2014; Accrobessy *et al.*, 2017; PMI, 2017; Camara *et al.*, 2018) and it is influenced by a range of factors such as overexpression of detoxification enzymes (Edi *et al.*, 2014; Panini *et al.*, 2016), possession of *Ace-1*-119S mutation and the number and types of copies of the resistant mutation (Djogbénou *et al.*, 2008b; Assogba *et al.*, 2016; Weetman *et al.,* 2018). However, the possession of *Ace-1*-119S has been strongly associated with both bendiocarb and fenitrothion survival with genotypic odds ratios of 15.7 and 28.8 respectively (Essandoh *et al.*,2013), providing a sensitive marker system with very high predictive power for either homozygote genotype. Using such molecular diagnostic markers to detect resistance will enable intervention strategies to be more targeted. However, whilst this work established a clear predictive association between the prevalence of resistance and *Ace*-1-119S frequency, the effect on resistance intensity, which is more relevant to potential operational failures is poorly understood. Copy number variation of *Ace-1*, comprising homogeneous and heterogeneous duplication, has been found in *An. gambiae s.s.* and *An. coluzzii* in several West African countries including Ghana (Djogbénou, Chandre, *et al.*, 2008a; Edi *et al.*, 2014; Djogbénou *et al.*, 2015; Assogba *et al.*, 2016). The heterogeneous duplication involves the combination of two functionally divergent *Ace-1* alleles, *Ace-1*-119G and *Ace-1*-119S. In contrast, the homogeneous duplication consists of identical resistant copies of *Ace-1* (Figure 3.1a). It has also been shown that duplication could involve a chromosomal segment of 203 kilobases comprising the *Ace-1* gene in tandem with 10 genes on the 2R chromosomal arm in both *An. gambiae s.s.* and *An. coluzzii* (Figure 2A) (Assogba *et al.*, 2016). However, in both homogeneous and heterogeneous *Ace-1* duplication, which is far more common in *An. gambiae s.s.* than *An. coluzzii* (Assogba *et al.*, 2018), there are two types: one involving all genes (full duplication), and the other involving internal deletions of the 10 flanking genes (partial duplication) (Assogba *et al.*, 2018) Figure 3.1b. The full duplication appears to be associated with a higher cost than partial duplication but it is unclear if all the 10 genes are expressed.

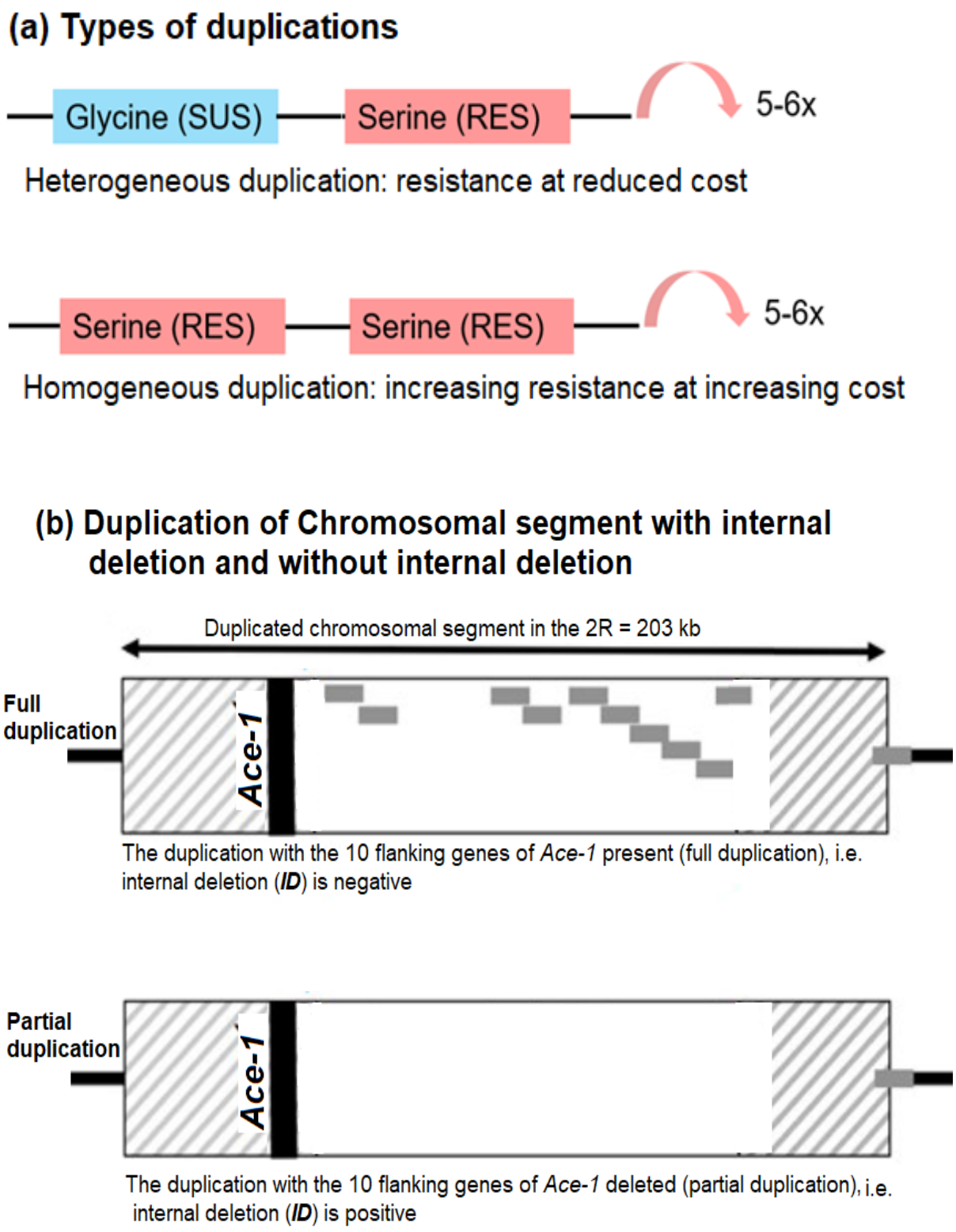


Figure 3.1: The illustrations of different types of (a) *Ace*-1 duplication in *Anopheles gambiae s.s.* (Weetman, Djogbenou and Lucas, 2018) and (b) chromosomal segment duplications with and without internal deletion (***ID***) (Assogba *et al.*, 2018).

In Ghana, resistance to CB and OP insecticides appears to be especially high in the central part of southern Ghana, most notably in the Greater Accra Region, where the highest *Ace-1*-119S frequencies have been reported (Essandoh *et al.*, 2013; Pwalia *et al.*, 2019) and in Chapter 2. This is surprising as these insecticides have seldom been used in public health in southern Ghana and the high fitness cost associated with *Ace*-1-mediated resistance (Djogbénou *et al.*, 2010). It has also been found that *Ace-1* copy number influences resistance directly in *An. gambiae* population because only resistant alleles appear to be duplicated (Edi *et al.*, 2014; Assogba *et al.*, 2016; Assogba *et al.*, 2018).

The work in this chapter was initially motivated by the extension of an *Ace-1* genotyping time series in *An. gambiae s.s* from Madina, southern Ghana (Weetman *et al.*, 2015) to include samples collected in 2011-2013. This revised temporal trend from 2002 to 2013, suggested that the *Ace*-1-119S would rise to fixation in the *An. gambiae s.s.* population (Weetman, unpublished data) (Figure 3.2) and consequently we targeted the Madina population for in-depth phenotypic and genotypic investigations. Results show extreme phenotypes resulting from high *Ace-1* G119S frequencies and copy number variation, with additional investigations to understand costs that may prevent the rise to fixation.

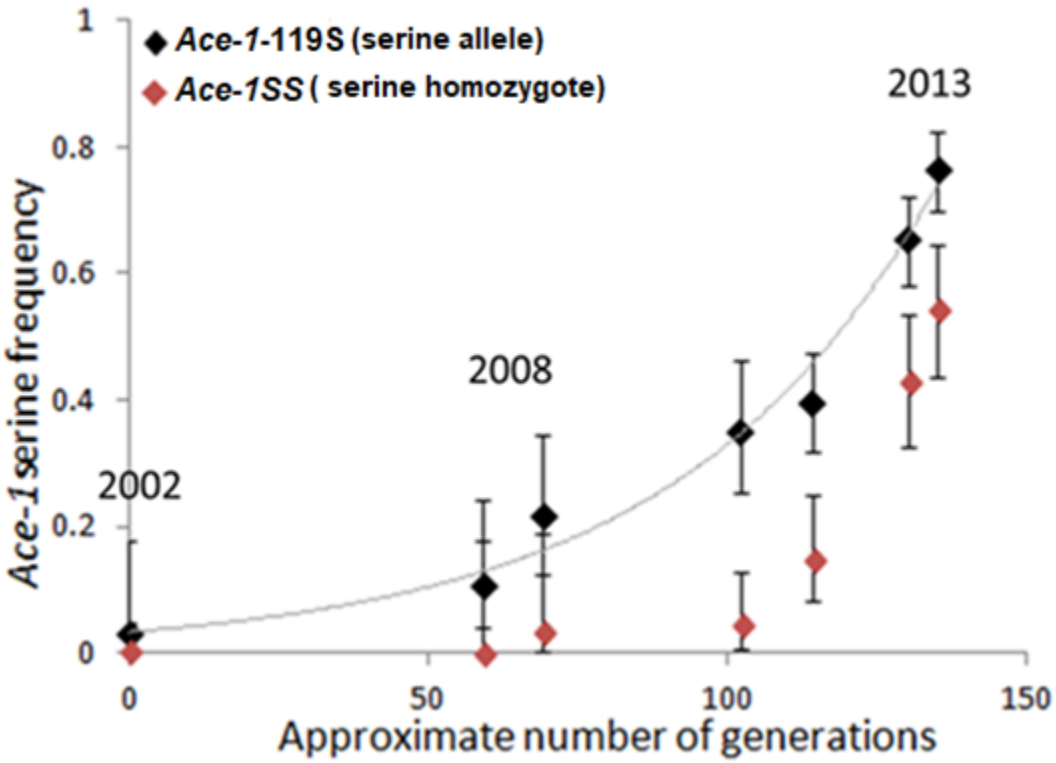


Figure 3.2: A trendline plot showing *Ace*-1-119S frequency against the estimated number of generations of Madina species pair between 2002 and 2013 (Weetman, Essandoh *et al.* unpublished data).

Research questions:

1. What are the levels of *An. gambiae s.l.* resistance to bendiocarb and fenitrothion in Madina?

2. Is the *Ace-1* frequency in *An. gambiae s.s.* moving toward fixation with time considering the current trajectory?

3. Are the heterogeneous and homogeneous duplications operating in the same pattern (distribution, trend and Hardy-Weinberg equilibrium) in both *An. gambiae s.s.* and *An. coluzzii*?

4. What are the copy number variants (CNV) of *Ace-1* in the homozygotes and heterozygotes of *An. gambiae s.s*?

5. The *Ace-1* genotypes are scored on Taqman assay using bidirectional scatter plots of Fam (y-axis for serine) and Hex (x-axis for glycine) signals. What is the relationship between the CNV and Fam/Hex ratio?

3.2 Methods

3.2.1 Sample collections and adult susceptibility tests

Madina (05.668490N, 00.219280W) is a suburb of Accra, which is located within the coastal savannah agroclimatic zone of Ghana (Figure 3.3). Madina is a densely populated urban community with several small-scale vegetable-growing farms comprising backyard gardens and a large plot of land shared by several people used for farming. All *Anopheles* larvae were collected in Madina from 2002 to 2019 were sampled by dipping and raised to adults following a standard insectary protocol. The mosquito samples from 2002 to 2010 (Weetman *et al.*, 2015) and subsequent samples (from 2011 onwards) collected by the author. Standard bioassays were performed on adult mosquitoes to test their susceptibility to bendiocarb (and/or fenitrothion) for 2010, 2011, 2012, 2013 and 2019 samples using WHO test kits, with quantitative phenotyping performed to assess resistance intensity for the same insecticides.

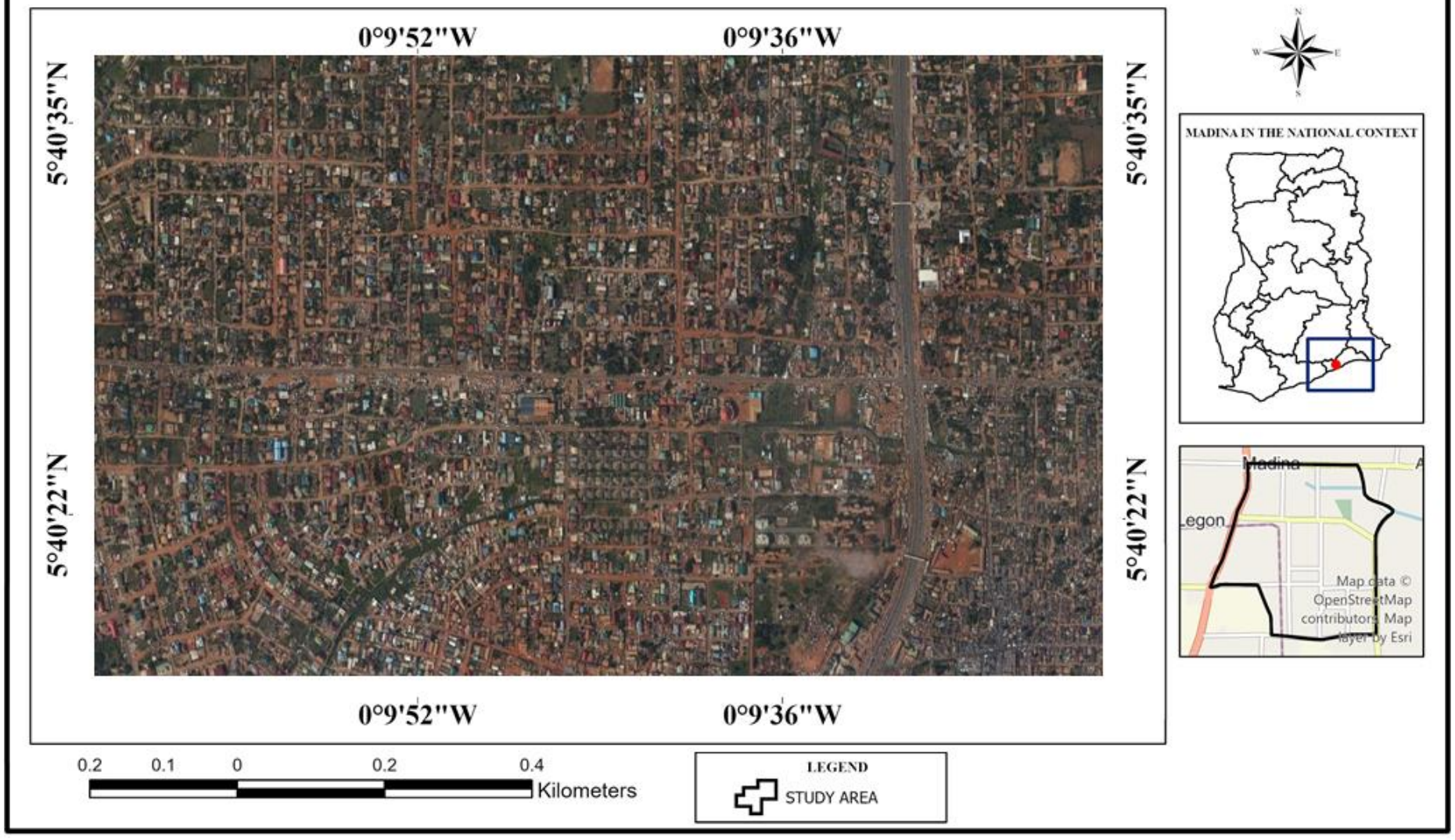


Figure 3.3: Map of Ghana showing the aerial satellite view of Madina with green patches indicating vegefarms and backyard gardens

3.2.2 Species and *Ace-1* genotype diagnostics

Total genomic DNA was extracted individually from a subset of mosquito samples selected randomly from Madina using QIAGEN DNeasy 96 Kits following the manufacturer instructions. Species characterizations into *An. gambiae s.l.* using PCR protocols described by Scott et al., (1993) (Appendix 1), and further characterization into *An. coluzzii* and *An. gambiae s.s* was performed using the SINE PCR protocol described by Santolamazza et al., (2008) (Appendix 2). The mosquitoes were genotyped for the *Ace-1*-G119S mutation (using the fluorescence-based TaqMan assay described by Bass *et.* al., (2009) on an Agilent MxPro 3005 qPCR thermal cycler (Appendix 3). Genotypes (*GG* (homozygous susceptible), *GS* (heterozygous resistant) and *SS* (homozygous resistant)) were scored from bi-directional scattered plots produced by the Agilent MxPro software. The FAM probe detects the mutant allele in amplicons, while the HEX probe detects the wild type susceptible allele.

3.2.3 *Ace-1* duplication detection (*Ace-1* 'duplityping')

Genomic DNA from individual mosquitoes was used for the *Ace-1* duplication (having multiple copies of *Ace-1* alleles) detection PCR based using a protocol described by Assogba *et al.*, (2016). Samples of DNA were PCR-amplified with the primers Agduplispedir2: CTCTTAAGGTGGCGTTGTTCC and AgduplispeRev1: TTCGCACAAAAGGTTGGCA for an initial denaturation step of 3 min at 95°C, followed by thirty-five cycles (95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 min; with a final extension step of 10 min at 72°C (Appendix 3). The PCR products were fractionated on a 2% agarose gel and a band of 460 bp indicates the presences of *Ace-1* duplication, whilst the absence of a band indicates no duplication. DNA from the standard susceptible strain Kisumu was used as a negative (absence of duplication) control (Figure 3.4).

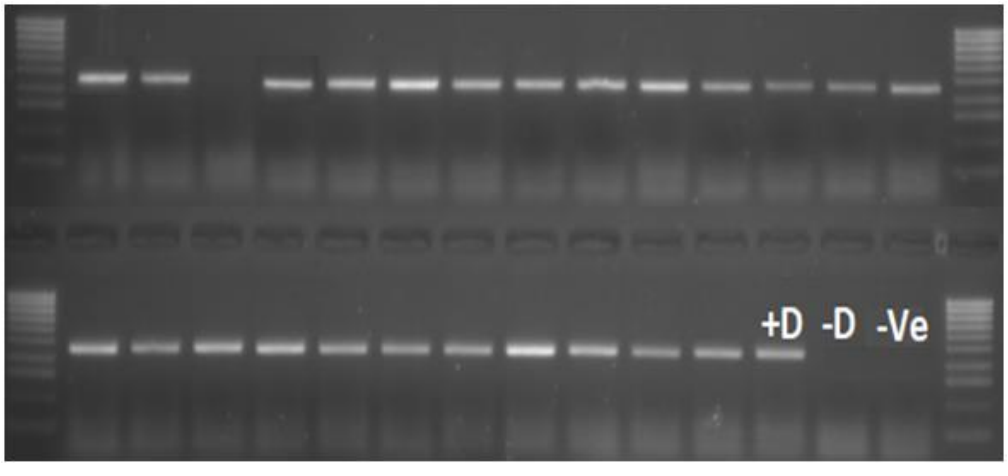


Figure 3.4: PCR gel image showing +D = Duplication positive control for DNA present (460 bp), -D = Kisumu (duplication absent) and -Ve = Negative control for DNA absent.

3.2.4 *Ace-1* copy number estimation in different genotype groups and *Ace-1* genotype calibration

Copy number variation (CNV) of *Ace*-1 has been reported in *An. gambiae s.s*., however, it is not clear whether CNV differs among the *Ace-1* genotypes, and if so, by how many folds change? To investigate the level of copy number variation, real-time quantitative PCRs targeting genomic DNA were performed using two target regions within different exons of the *Ace-1* gene with primers (ACE1_gq_1b and ACE1_gq) and two normalizing genes (CYP4G16 and Elongation Factor (EF) (Edi *et al.*, 2014) with the sequences shown in Appendix 10. A subset of *An. gambiae s.s.* sampled over five years from 2011- 2014 and 2016 from Madina were used for this study, and this is because there were adequate sample sizes for all the three genotypes (*GG*, *GS*, *SS*) in *An. gambiae s.s.* concerning the years under consideration. Thirteen individual samples from each year group consisting of three *Ace-1-SS*, eight *Ace-1-GS* (selected from three distinct clusters of heterozygotes in MXpro bidirectional scattered plot) and two *Ace-1-GG* were used. The qPCR reactions used a total volume of 20.0 µl (ddH_2O = 7.5 µl, SYBR=10.0 µl, primer F = 0.6 µl, primer R = 0.6 µl, and DNA = 1.0 µl) and thermocycling conditions as follow: 95°C for 3min, 40 cycles of 95°C for 10s and 60°C for 10s, 95°C for 1min, 55°C for 30s and 95°C for 30s.Two technical replicates were run for each sample. The gene copy number was estimated relative to Kisumu using the ΔΔCt method described previously by Livak and Schmittgen, (2001).

The emergence of *Ace-1* duplication in *An. gambiae* has made scoring of heterozygous individuals problematic. A study has shown that most *Ace-1-GS* individuals are duplicated and that *Ace*-1-119S mutation exhibits a complex genotype system (*GG*, GS, *G(S)*n, *SS* and (*SS*)*n* where *n* > 1) (Djogbénou *et al.*, 2015). To calibrate the clusters, standardized concentrations

of *Ace-1G* and *Ace-1S* haplotypes (generated through vector cloning by Patricia Pignatelli, LSTM) were mixed at varying ratios (SS, 3S:1G, 2S:1G, 1S:1G, 1S:2G, 1S:3G, GG) and TaqMan assays were performed with 52 samples of known genotypes.

3.2.4 Data analysis

The distributions of species among the years (2011 to 20191) were reported in proportions with 95% confidence intervals and their seasonal variations assessed using the chi-square test. The susceptibility to bendiocarb and fenitrothion were reported as mortality rate with 95% confidence intervals, and the differences among the years were assessed with the Chi-square test. For the 2014 dose-time assay, a dose-time curve was constructed for bendiocarb and fenitrothion, and the median lethal time (LT_{50}) was computed to assess the time taken for half of the exposure mosquitoes to die. A generalised linear model (GLM) with logit function was used to generate the relationship between the sampling year and the mortality rate for both insecticides. The sampling year was used as a predictor whilst the mortality rate used was as an outcome variable. The effects of the predictors were reported as odds ratios with 95% confidence intervals and associated p-values. For *Ace-1*-119S, the differences in the frequencies between the species were assessed with chi-square. A GLM was used to assess the changes in the frequency of *Ace-1*-119S concerning species and sampling time. The differences in the proportion of *Ace-1*-119S genotypes (*GG*, *GS and SS*), duplications and the presence of internal deletion (*ID*) between the species pair and among the years were determined using chi-square. Temporal trend plots for the proportions of *Ace-1*-119S allele and genotypes were constructed to show how they change over the years, and the significance of the change assessed using the chi-square test. The differences between estimated CNVs of adjacent pairs of genotypes were assessed using a t-test.

3.3 Results

3.3.1 Species distribution and phenotypic resistance

Both *An. gambiae s.s.* and *An. coluzzii* were found in sympatry in Madina but in varying proportions among various years (χ^2 =75.9, df = 5, $p < 0.001$) which may reflect seasonal variations (Table 3.1). However, *An. gambiae s.s.* has consistently been the more common species throughout. The species pair showed a high level of phenotypic resistance to bendiocarb and fenitrothion (Table 3.2), with a significant increase in resistance to bendiocarb (χ^2 = 74.1, df = 2, $p < 0.001$) between 2011 and 2013, and to fenitrothion (χ^2 = 79.7, df = 2, *p*

< 0.001) between 2011 and 2014, with a concomitant increase in *Ace-1* -119S frequency (Figure 3.2). To investigate how the unprecedented and unexpected increase in *Ace-1*-119S frequency, extensive collections were made in 2014, and time-dose response bioassays were performed. Over 2000 females were assayed using bendiocarb and fenitrothion. For fenitrothion, a 360 min exposure was required to kill all mosquitoes, with a median lethal time, LT_{50} of 115 min. For bendiocarb, fewer than 20% of mosquitoes were killed even after a 600 min exposure (Figure 3.5). Further recent sampling in 2019 showed that there was a slight but significant decrease in phenotypic resistance of *An. gambiae s.s.* and *An. coluzzii* population to bendiocarb and fenitrothion since 2011, although there is no significant difference among year groups from 2012 to 2019 (Table 3.3).

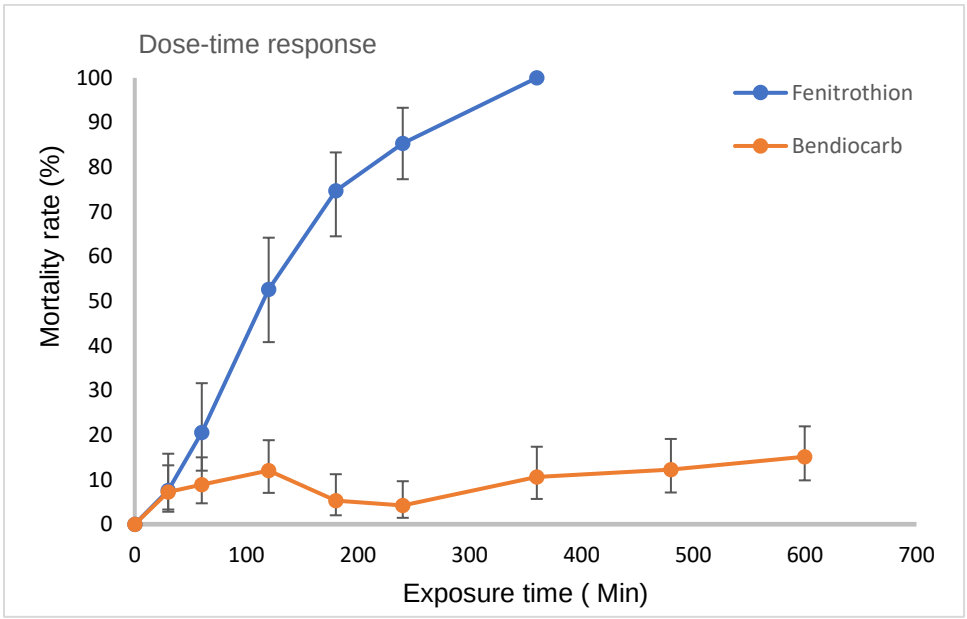


Figure 3.5: Dose-time response of the species pair to fenitrothion and bendiocarb in 2014

Table 3.1: Species distributions in Madina from 2011 to 2019

		The proportion of species (%) with 95 CI				
Year	**Season**	***An. coluzzii***	***An. gambiae s.s.***	X^2	**df**	**P-value**
May, 2011	Mid wet	3.5 (0.7 - 9.9)	96.5 (90.1 - 99.3)	75.9	4	< 0.001
Oct, 2012	Late wet	35.9 (28.0 - 44.4)	64.1 (57.8 - 73.9)			
March, 2013	Early wet	2.1 (0.3 - 7.3)	97.9 (97.7 - 99.7)			
August, 2014	Late wet	12.6 (9.3 - 16.7)	87.4 (83.3 - 90.7)			
August, 2016	Late wet	9.5 (3.9 - 18.5)	90.5 (81.5 - 96.1)			
May, 2019	Mid wet	28.2 (25.3 - 31.4)	71.8 (68.6 - 74.7)			

Table 3.2: The bioassay results from 2011 to 2014 showing the mortality rate (%) with their corresponding 95% confidence interval

Year	Fenitrothion (%)	Bendiocarb (%)
2011	50 (41.6-62.4)	53 (44.0 - 62.6)
2012	37(27.5-47.5)	22 (14.2- 31.1)
2013	not measured	7 (3.4 - 14.6)
2014	19 (12.5 – 27.8)	11 (13.5 – 28.5)
2019	28 (21.1 - 35.9)	25 (18.5 - 32.9)

Table 3.3: A generalized linear model (GLM) showing the changes in the mortality rates of *Anopheles gambiae s.l.* to (a) bendiocarb and (b) fenitrothion between 2011 and 2019 using 2011 as a reference year.

(a) Bendiocarb

Parameter	Odds ratio	95% CI	X^2	df	P-value
(Intercept)	0.1	-0.2 - 0.5	0.5	1	0.462
2019	-2.7	-3.4 - -2.0	54.7	1	< 0.001
2014	-2.2	-2.9 - -1.5	39.6	1	< 0.001
2013	-2.6	-3.4 - -1.8	40.5	1	< 0.001
2012	-1.7	-2.2- -1.1	30.8	1	< 0.001
2011*					

(b) Fenitrothion

Parameter	Odds ratio	95% CI	X^2	df	P-value
(Intercept)	0.7	0.3 - 1.2	11.5	1	0.001
2019	-1.3	-1.9 - -0.8	22.8	1	< 0.001
2014	-2.2	-2.8 - -1.5	42.6	1	< 0.001
2012	-1.2	-1.8 - -0.6	15.5	1	< 0.001
2011*					

*Dead of mosquito is the event *2011 is the reference category

3.3.2 *Ace*-1 genotypic and allelic distribution

Data to 2010 focused solely on *An. gambiae s.s.* but in my collections (2011 onwards) species comparison was possible and the *Ace-1*-119S frequency was significantly higher in *An. gambiae s.s.* than in *An. coluzzii* in all the years under study (χ^2 = 15.1, df =5, p = 0.010). This finding was validated by a generalized linear model (GLM) which shows a strong effect of species and time in the change of *Ace-1*-119S frequency (Table 3.4). However, a significant interaction (species x year) indicated that the change in *Ace-1*-119S frequency over time is less in *An. gambiae s.s.* than in *An. coluzzii* (Table 3.4). Figure 3.6a shows that the significant

species x year interaction (Table 3.4) is driven by the 2019 data point at which point *An. gambiae s.s.* and *An. coluzzii Ace-1*-119 levels are near convergence.

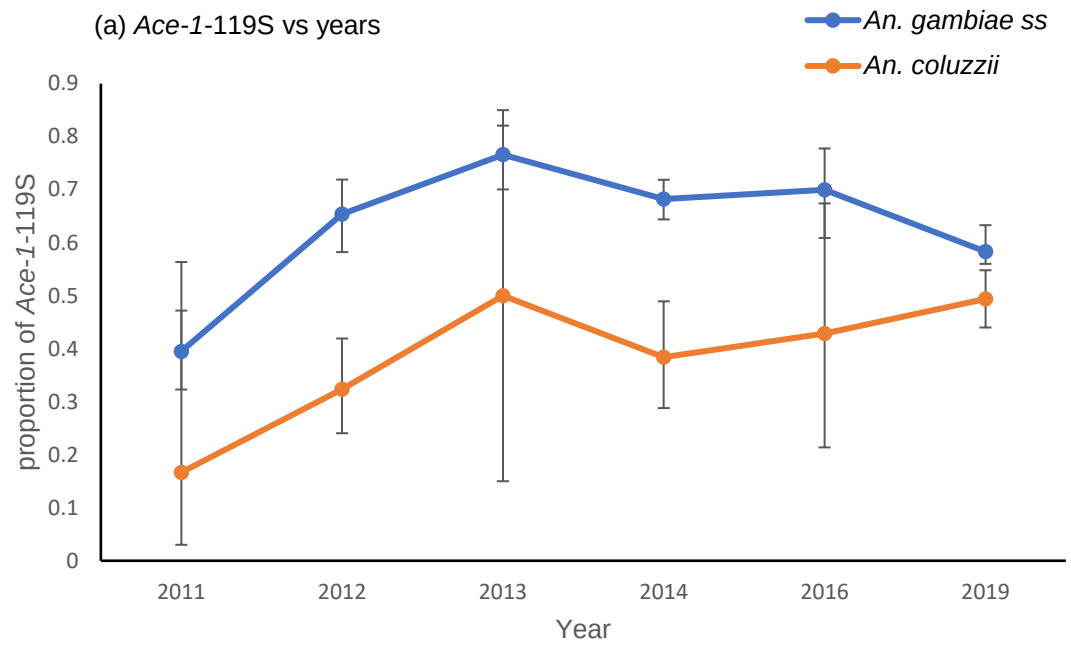
Table 3.4: The generalized linear model (GLM) assessing the change *in Ace-1*-119S frequency concerning species and time with 95% confidence interval (CI).

Parameter	B	95% CI	χ^2	df	p-value
(Intercept)	-1.17	-1.73 - -0.60	16.5	1	< 0.001
An. gambiae s.s. ***An. coluzzii**	1.55	0.93 – 2.16	24.2	1	< 0.001
year	0.19	0.08 – 0.30	11.7	1	0.001
***An. gambiae* s.s. x year** ***An. coluzzii x year**	-0.16	-0.28 - -0.04	6.5	1	0.011

**An. coluzzii* and interaction factor *(*An. coluzzii* x year) were used as reference categories, where the interaction factor is the simultaneous effect of species and the sampling year.

For the temporal trends, the *Ace-1*-119S frequency in *An. gambiae s.s.* peaked in 2013 but surprisingly, given the previous trend, decreased thereafter (Figure 3.6a and Figure 3.7). The *Ace*-1-119S allelic frequency increased significantly from 2011 to 2013 (from 36.7 to 76.6%; χ^2 = 61.6, df = 2, p < 0.001), then there was a significant decrease between 2013 to 2019 (χ^2 = 20.4, df = 3, p < 0.001). For *Ace*-1 genotypes, the *Ace-1SS* in *An. gambiae s.s.* population showed a highly significant increase between 2011 - 2013 (χ^2 = 19.4, df = 2, p < 0.001), and then declined significantly from 2013 – 2019 (χ^2 = 10.3, df = 3, p < 0.016). The *Ace-1GS* decreased gradually but not significantly from 2011 to 2014 (χ^2 = 1.1, df = 3, p = 0.765), but increased significantly from 2014 to 2019 (χ^2 = 9.5, df = 2, p = 0.009). The decrease in the *Ace*-1-119S frequency in 2013 was attributable to a relative decrease in *Ace-1SS* compared to *Ace-1GS* genotypic frequencies (Figure 3.6b).

For *An. coluzzii*, *Ace-1*-119S frequency increased from 2011 to 2013, but not significant (χ^2 = 0.65, df = 2, p = 0. 723), and then decreased from 2013 to 2019, the change was also not significant (χ^2 = 1.3, df = 3, p = 0. 727) (Figure 3.6a). Among *Ace*-1 genotypes, the *Ace-1SS* has been absent in *An. coluzzii* population since 2011, and it was only recorded in 2019 samples at a relatively low frequency (Figure 3.6b). *An. coluzzii Ace-1GS* showed a decrease in frequency from 2011 to 2014, but not significant (χ^2 = 0.8, df = 3, p = 0.839), and then increased from 2014 to 2019, but the change was also not significant (χ^2 = 10.3, df = 3, p < 0.016) (Figure 3.6b). It is noteworthy that *An. coluzzii* allelic and genotypic data must be treated with caution due to small sample sizes in some year groups.



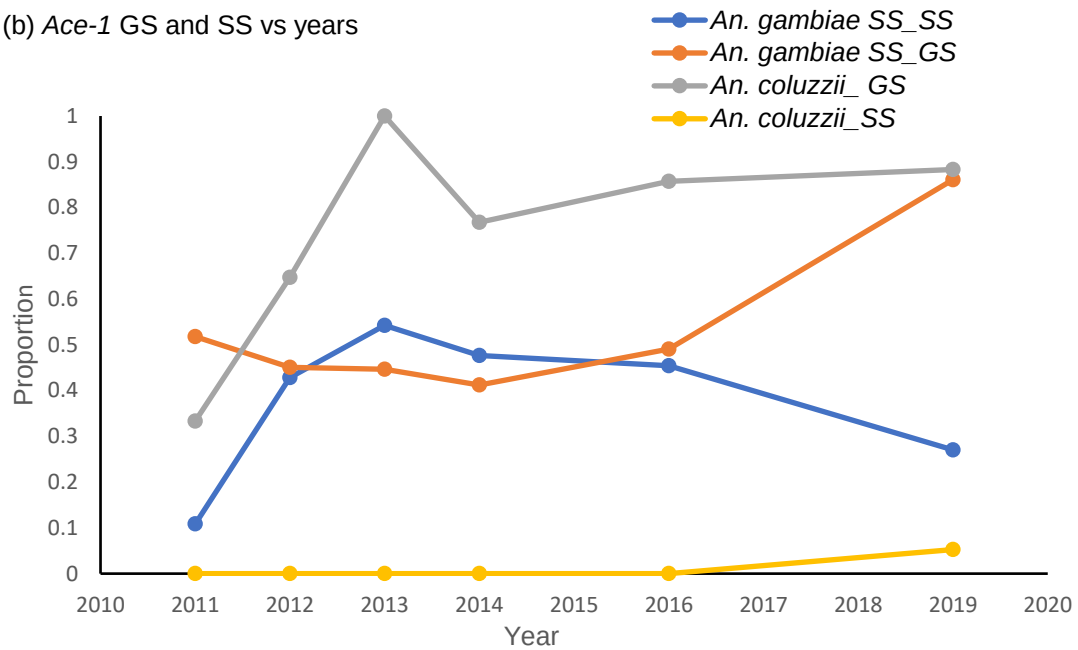


Figure 3.6: The trend plot showing the proportions of (a) *Ace-1*-119S with 95% CI and (b) *Ace-1GS* and *Ace-1SS* in Madina *An. gambiae s.s.* and *An. coluzzii* between 2011 and 2019. Sample sizes are shown in Table 3.5.

Apart from a marginally significant deviation in 2013, *Ace-1* genotypic frequencies in *An. gambiae s.s.* populations were in Hardy-Weinberg expectations (HWE) but in 2019 there was a highly significant deviation, reflecting an excess of heterozygotes (Table 3.5). *An. coluzzii Ace-1* genotypic frequencies showed deviation from HWE in each year where data were available (Table 3.5).

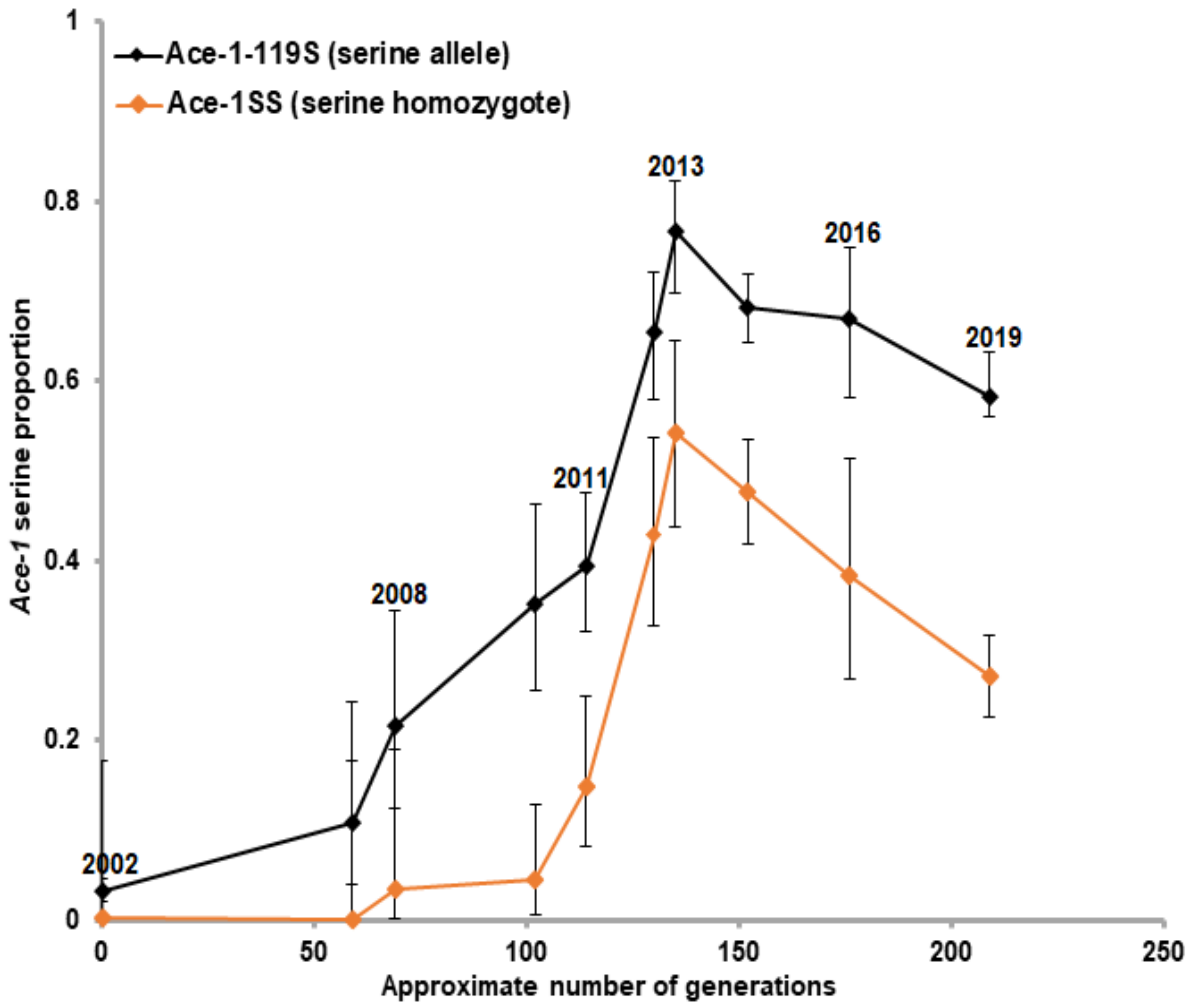


Figure 3.7: Expanded time-series plot showing *Ace*-1-119S (serine allele) and *Ace*-1SS (serine homozygote) frequencies against the estimated number of generations (based on N=12/year assumed) of Madina *An. gambiae s.s.* between 2002 and 2019.

Table 3.5: Frequencies of *Ace-1* genotypes (*SS*, *GS* and *GG*) and alleles (*G*, *S*) with Hardy-Weinberg equilibrium test results in *An. gambiae s.s* .and *An. coluzzii*.

	An. gambiae s.s.					*An. coluzzii*				
	Genotypic frequency			**HWE**	**Allelic frequency**	**Genotypic frequency**			**HWE**	**Allelic frequency**
Year	**SS**	**GS**	**GG**		**S**	**SS**	**GS**	**GG**		**S**
2011	9	43	31	0.297	0.37	0	1	2	ND	0.17
2012	39	41	11	0.964	0.65	0	33	18	< 0.001	0.32
2013	51	42	1	0.017	0.77	0	2	0	ND	0.50
2014	141	122	33	0.398	0.68	0	33	10	< 0.001	0.38
2016	25	27	3	0.211	0.70	0	6	1	ND	0.43
2019	99	229	38	< 0.001	0.58	9	151	11	< 0.001	0.49

*ND = Not determined due to small sample size

3.3.3 *Ace*-1 duplication distributions

A total of 1111 mosquitoes, comprising 872 *An. gambiae s.s.* and 239 *An. coluzzii* were screened for the presence of *Ace*-1 duplication (having multiple copies of the *Ace-1* alleles) using PCRs. The duplication of *Ace-1GS* is termed heterogeneous duplication whilst that of *Ace-1SS* is homogeneous duplication. The *Ace-1* duplication was completely absent in *Ace-1GG* individuals in both *An. gambiae s.s.* and *An. coluzzii*. In *An. gambiae s.s.*, the duplication was detected in 90.0% (95% CI: 88.5 – 93.9%) of *Ace-1GS* and 98.8% (95% CI: 96.7 – 99.7%) of *Ace*-1*SS*, In *An. coluzzii*, the duplication was detected in 87.6% (95% CI: 82.3 – 91.8%) of *Ace-1GS* and all the nine *Ace-1SS* individuals in 2019 (Table 3.6). Whilst the overall number of *Ace-1SS* individuals identified as being non-duplicated was very small (N = 4; 1.2% of total) and might be attributable to experimental error, the PCR results for the duplityping PCR were typically extremely clear and unambiguous (Figure 3.4). Errors are a less probable explanation for the far larger number of *Ace-1GS* individuals indicated as unduplicated (N=63; 9.7% of total). The proportion of unduplicated heterozygotes was higher in *An. coluzzii* than in *An. gambiae s.s.*, and also showed a more pronounced decreasing trend over time, resulting in a significant species x year interaction term (Table 3.7).

Table 3.6: Distribution of *Ace-1* duplication in Madina *An. gambiae s.s.* and *An. coluzzii* stratified according to *Ace-1* genotypes and sampling years. The frequencies are expressed in whole numbers with their corresponding percentages in brackets.

Genotype	Years			
	2011			
	An. gambiae s.s.		*An. coluzzii*	
	Duplicated (N/%)	Unduplicated (N/%)	Duplicated (N/%)	Unduplicated (N/%)
GG	0 (0)	31 (100)	0 (0)	0 (0)
GS	41 (95.3)	2 (4.7)	2 (66.7)	1 (33.3)
SS	9 (100)	0 (0)	0 (0)	0 (0)
Genotype	2012			
	An. gambiae s.s.		*An. coluzzii*	
	Duplicated (N/%)	unduplicated	Duplicated	unduplicated
GG	0 (0)	9 (100)	0 (0)	10 (100)
GS	30 (96.8)	1 (3.2)	15 (71.4)	6 (28.6)
SS	24 (100)	0 (0)	0 (0)	0 (0)

	2013			
Genotype	***An. gambiae s.s.***		***An. coluzzii***	
	Duplicated	**unduplicated**	**Duplicated**	**unduplicated**
GG	0 (0)	1 (100)	0 (0)	0 (0)
GS	38 (90.5)	4 (9.5)	1 (50)	1 (50.0)
SS	51 (100)	0 (0)	0 (0)	0 (0)
Genotype	**2014**			
	An. gambiae s.s.		***An. coluzzii***	
	Duplicated	**unduplicated**	**Duplicated**	**unduplicated**
GG	0 (0)	21 (100)	0 (0)	7 (100)
GS	66 (86.8)	10 (13.2)	18 (81.8)	4 (18.2)
SS	119 (97.5)	3 (2.5)	0 (0)	0 (0)
Genotype	**2016**			
	An. gambiae s.s.		***An. coluzzii***	
	Duplicated	**unduplicated**	**Duplicated**	**unduplicated**
GG	0 (0)	2 (100)	0 (0)	0 (0)
GS	22 (88.0)	3 (12)	2 (66.7)	1 (33.3)
SS	18 (94.7)	1 (5.3)	0 (0)	0 (0)
Genotype	**2019**			
	An. gambiae s.s.		***An. coluzzii***	
	D	**N**	**D**	**N**
GG	0 (0)	38 (100)	0 (0)	11 (100)
GS	211 (92.1)	18 (7.9)	139 (92.1)	12 (7.9)
SS	99 (100)	0 (0)	9 (100)	0 (0)

Table 3.7: The generalized linear model (GLM) assessing the change in proportions of non-duplicated heterozygotes (*Ace-1GS*) by species and sampling time.

Parameter	B	95% CI	X^2	df	p-value
(Intercept)	-0.03	-1.23 – 1.18	0.00	1	0.967
An. gambiae s.s. ***An. coluzzii**	-2.61	-4.16 - -1.05	10.81	1	0.001
year	-0.39	-0.64 - -0.15	9.94	1	0.002
An. gambiae s.s **x year** ****An. coluzzii*** **x year**	0.45	0.13 – 0.76	7.83	1	0.005

* *An. coluzzii* and interaction *(*An. coluzzii* x year) were used as reference categories

3.3.4 *Ace-1* Copy number variation and *Ace-1* genotype calibration

Whilst the duplityping PCR indicates the presence of a CNV, it gives no information on the number of duplicated copies. A qPCR was used to estimate how the copy number of *Ace-1* corresponds with genotype (including heterozygote) subdivisions scored from a Taqman scatterplot (Figure 3.8). Fourteen specimens of Madina *An. gambiae s.s.* (comprising three representatives of *Ace-1SS*, nine *Ace-1GS,* and two *Ace-1GG*) from each year cohort from 2011 to 2016 were used to estimate the average *Ace-1* copy number relative to (unduplicated) Kisumu. The copy number of *Ace-1* follows a clear progression from *Ace-1SS* > *Ace-1GS*_High > *Ace-1GS*_Middle > *Ace-1GS*_Low > *Ace-1GG* (Figure 3.9). For instance, the estimated copy number of *Ace*-1 alleles in 119SS individuals was significantly higher than that of 119GS_High individuals (t = 5.8, df = 14, $p < 0.001$), while *Ace-1GS*_Low individuals possessed significantly higher copy numbers of *Ace*-1 alleles than *Ace*-1*GG* (t = 5.6, df = 9, $p < 0.001$). This result suggests that because most serine alleles are duplicated, the allele balance ratio of serine/glycine gives a useful proxy for the number of serine alleles.

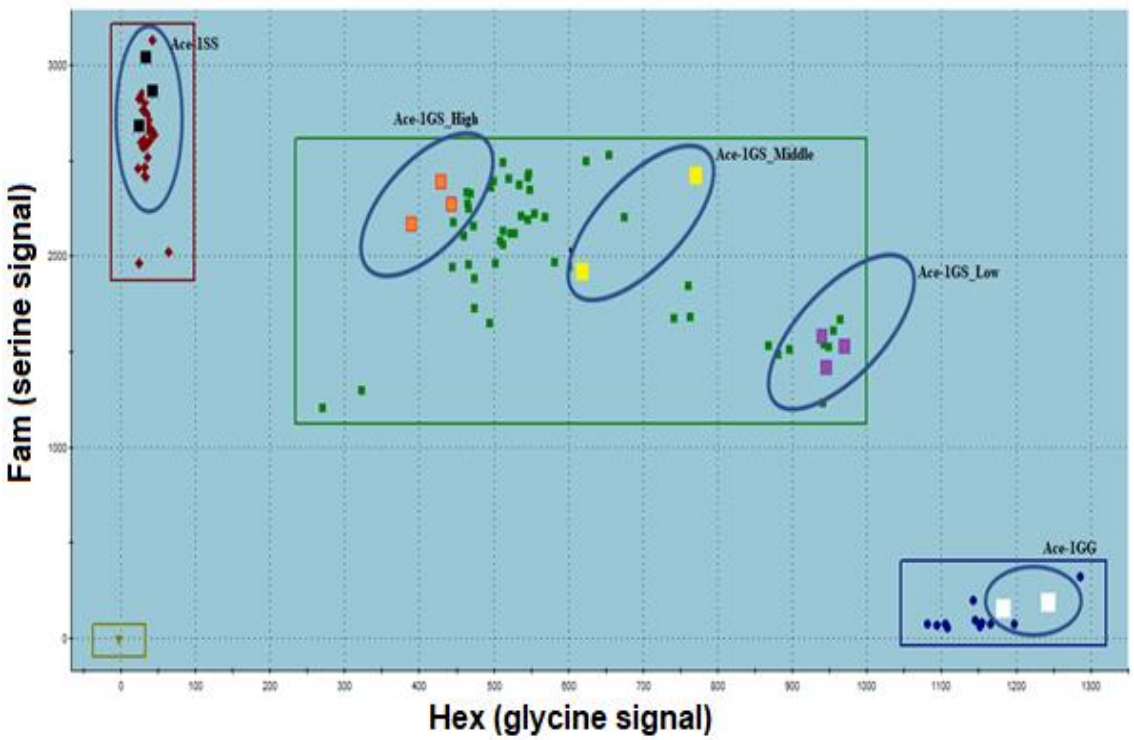


Figure 3.8: TaqMan MXpro bidirectional scattered plot showing 13 *An. gambiae s.s.* samples selected from *Ace*-1 genotype clusters of Madina 2014 for copy number variation analysis. Coloured square dots are control genotypes (of known G119S status). The samples in the red, green, blue and yellow rectangular boxes represent individuals scored as homozygous resistant (*Ace-1*SS), heterozygous resistant (*Ace-1*GS), homozygous susceptible (*Ace-1*GG) and blank control respectively. The selected samples in the blue circles were used for *Ace-1* copy number estimations.

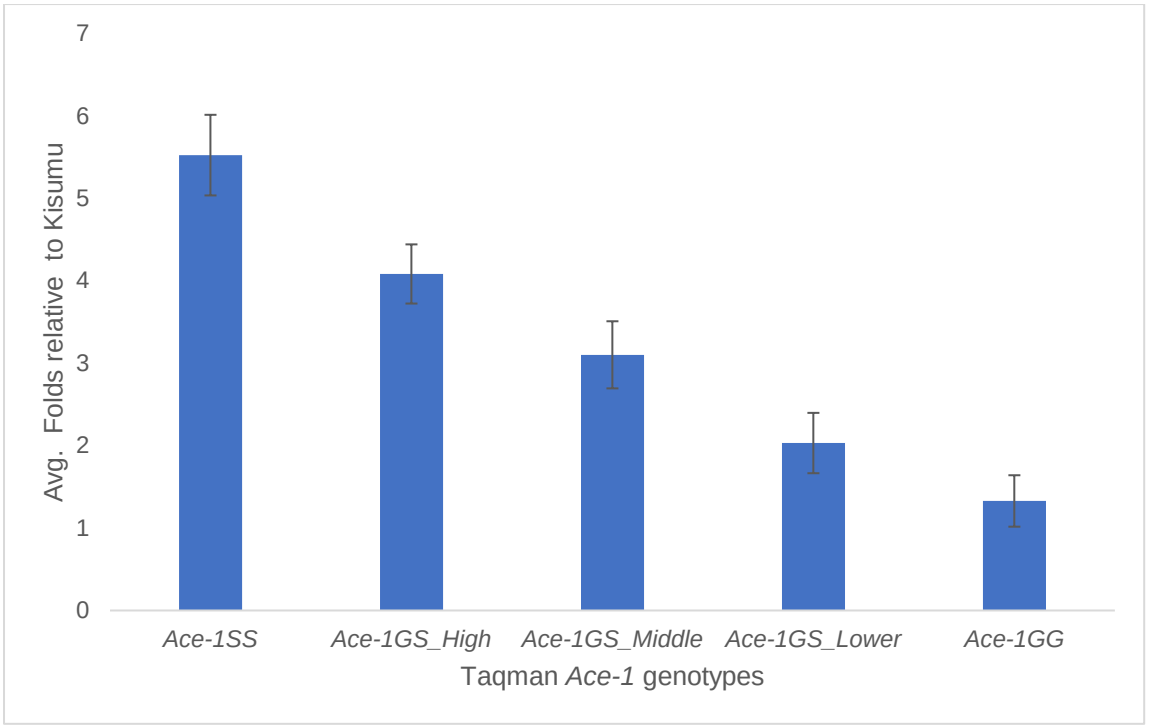


Figure 3.9: Plot of average fold change (ΔΔCt ±SEM) of *Ace-1GG*, *Ace-1GS* and *Ace-1SS* relative to Kisumu. The fold change indicates the number of copies of *Ace-1* present in the specimens compared to Kisumu (which serves as a reference). The *Ace1SS* showed a higher copy number of Ace-1 than *Ace-1GS*, and *Ace-1GS* in turn showed a higher copy number than *Ace-1GG*. The *Ace-1GG* copy number is approximately equal to Kisumu (fold-change ~ 1). The three forms of heterozygote (*Ace*-1*GS*_high, *Ace-1GS*_Middle and *Ace-1GS*_Lower) correspond to the relative signals of Fam (serine) and Hex (glycine) in the Mxpro (Taqman) bidirectional scatter shown in Figure 3.8.

The calibration analysis focused on the three clone-generated copy numbers of *Ace-1* heterozygotes (*GGS, GS* and *GSS*) because *Ace-1* homozygotes (*GG* and *SS*) are readily differentiated. A plot of the average of Fam/Hex against laboratory-cloned genotypes (*GGS, GS* and *GSS*) was generated (Figure 3.10) and then was used to characterise 36 *Ace-1* heterozygotes into *Ace-1* copy number groups (*GGS, GS* and *GSS*). The plot showed that *Ace-1GSS* separation worked well but not the *Ace-1GS* from *Ace-1GGS* and 10 heterozygotes were characterised as *Ace-1GGS* and 23 as *Ace-1GSS*.

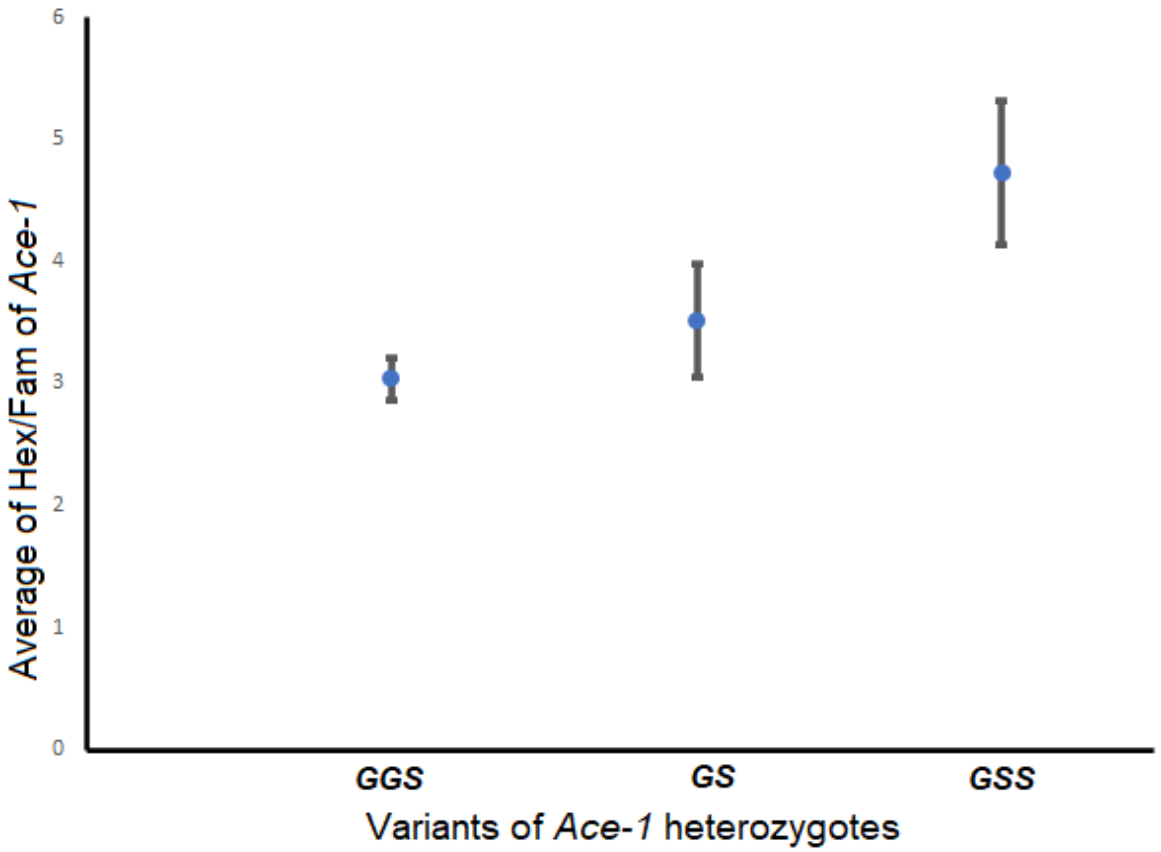


Figure 3.10: A plot of average Fam/Hex of *Ace-1* versus *Ace-1S* heterozygotes with their 95% confidence interval.

3.4 Discussion

The results revealed that *An. coluzzii* and *An. gambiae s.s* are highly resistant to bendiocarb and fenitrothion, however, between 2013 and 2019, there was no significant increase in phenotypic resistance. The rise of *Ace-1*-119S toward fixation halted after 2013 and stabilised. Our study also revealed that most of the *Ace-1*-119S alleles are duplicated given rise to the co-existence of heterogeneous and homogeneous *Ace-1* duplication in the species pair. However, the temporal dynamics of the *Ace*-1 duplication types are operating differently in *An. coluzzii* and *An. gambiae s.s*.

Discriminatory bioassays have been useful tools in detecting and comparing resistance between sites (Badolo *et al.*, 2012), however, as resistance becomes established in vector populations, diagnostic techniques that are flexible enough to follow the evolution and capable of detecting subtle changes in resistance are required to provide more information on the epidemiological impact of resistance (Weetman and Donnelly, 2015). This study harnesses the molecular marker, *Ace-1*, as a diagnostic tool in assessing the temporal

variations in resistance of *An. gambiae s.s.* and *An. coluzzii* to carbamate and organophosphate insecticides.

An. gambiae s.s. and *An. coluzzii* populations in Madina exhibited a high prevalence of phenotypic resistance to both bendiocarb and fenitrothion from 2011 to 2019. However, the degree of resistance prevalence fluctuated among the years under study which may be due to seasonal variations in the species composition as *Ace*-1-119S conferring resistance to CB and OP was found to be markedly higher in *An. gambiae* s.s. than in *An. coluzzii*. There was a dramatic increase in phenotypic resistance between 2011 and 2013 for bendiocarb and between 2011 and 2012 for fenitrothion (Table 3.2 and Table 3.3) which also corresponded with an unprecedented rise in *Ace*-1-119S allelic frequency to fixation (Figure 3.2). These findings necessitated an in-depth investigation to determine the resistance level and give better insight into potential operational impacts. A dose-time response in 2014 showed an extreme level of resistance of *An. gambiae s.s.* and *An. coluzzii* to both bendiocarb and fenitrothion, as the LT50 of almost 2.0 hours for fenitrothion, and 10-hour exposure to bendiocarb recorded mortality less than 20%. This finding confirms that a lot of mosquitoes are required to investigate the resistance level via bioassays.

The *Ace-1* serine frequency is a very sensitive marker for phenotypic resistance to bendiocarb and fenitrothion (Essandoh *et al.*, 2013) and other organophosphates (Edi *et al.*, 2014; Grau-Bové *et al.*, 2020). Contrary to prior expectations, the *Ace*-1-119S allelic frequency peaked in 2013 and declined gradually thereafter in *An. gambiae s.s*. The reason for this observation is that the existence of heterogeneous and homogeneous duplications of *Ace*-1 within *An. gambiae s.s.* populations exhibit different/competing dynamics in terms of costs and benefits appear to be leading to the establishment of a semi-stable equilibrium between the two duplications, suggesting that the selection environment is probably heterogeneous. Thus, the selection of different alleles with different fitness-trade-offs may result from the variation in vector control practices creating a mosaic environmental spectrum from untreated to intensely treated areas (Assogba *et al.*, 2016). This hypothesis follows from evolutionary theory and has comparable support in *Cx. pipiens* (Milesi *et al.*, 2017), in which only heterogeneous duplications exist, but with varying numbers of 119S to 119G. However, successional dynamics between different genotypes are thought to have been driven by mosaic CB/OP insecticide control practices (Labbé *et al.*, 2007; Milesi *et al.*, 2017).

Ace-1-119S is significantly higher in *An. gambiae s.s.* than in *An. coluzzii* and confirms findings of previous studies (Essandoh *et al.,* 2013) and this may be due to differential selection pressure experienced by the species pair probably in their larval stage. For instance, *An. coluzzii* occupy more complex environments where insecticidal pressure is likely to act

alongside multiple other sources of selection, as such *An. coluzzii* may use more resource to develop adaptations to other xenobiotics in addition to CB/OP (Kamdem *et al.*, 2017; Ossè *et al.*, 2019). In *An. gambiae s.s.,* the *Ace-1* genotypes mainly showed conformity to Hardy-Weinberg equilibrium presumably because they have both homogeneous and heterogeneous duplications, which can serve as 'regular' genotypes, and this observation is consistent with the findings in Ivory Coast where both duplications exist in *An. gambiae s.s.* population (Assogba *et al.*, 2016). However, there is a recent increase in heterogeneous duplications creating a substantial-frequency shift that drove the *An. gambiae s.s.* population out of HWE. The rapid rise of homogeneous duplications followed by a balancing rise of the heterogeneous duplications may be an indication of changes in selection pressure favouring the latter.

The *Ace-1* homozygous duplication is selectively advantageous in high OP/CB treated areas whilst the heterogeneous duplication is favoured in areas characterized by a mosaic of treated and untreated OP/CM. Hence, the coexistence of the two duplication types conferring different phenotypes (i.e. local selection) varying frequencies show inconsistencies in OP/CB treatment in Madina over the years under consideration. It is likely that heterogeneous duplication may prevail over homogeneous duplication with time if the inconsistent treatment continues. For instance, in southern France, the shift from the intense to the moderate treatment of OP caused *Ester-2 t*o prevail over *Ester* alleles conferring resistance to esterase in *Cx. pipiens* indicating selection acting at the local level (Labbe *et al.*, 2005).

An. coluzzii, on the other hand, possesses almost entirely heterogeneous duplications, which with sufficient sample size clearly show HWE deviation. The excess of heterozygotes found in the Madina *An. coluzzii* population is similar to the findings reported among *An. coluzzii* population in Tiassale, Ivory Coast (Edi *et al.*, 2012, 2014) where the deficit of *Ace-1*-119S homozygotes is attributed to the establishment of permanent heterozygosity due to the inability of heterogeneous duplications to sort out freely into *Ace*-1-119G and *Ace*-1-119S alleles (Djogbénou *et al.*, 2010).The recent appearance of homogeneous duplications, at relatively low frequency, in *An. coluzzii* may suggest that the homogeneous duplication originated from *An. gambiae s.s.* and has recently been introgressed into *An. coluzzii* population in Madina (Grau-Bové *et al.*, 2020). This finding confirms the study in Ivory Coast where only 2% of *An. coluzzii* showed homogeneous duplication (Assogba *et al.*, 2016). The predominance of heterogeneous duplication in *An. coluzzii* also explains why the *Ace*-1-119S frequency in *An. coluzzii* is less than that of *An. gambiae s.s.*, since the theoretical maximum frequency of *Ace*-1-119S in heterogeneous duplication is 50%. Addition, homogeneous duplication exhibits selective advantage in areas with high levels of OP/CM treatment, hence its recent emergence in *An. coluzzii.*

Although it has been shown that heterozygotes with more *Ace*-1-119S copies exhibit higher resistance (Edi *et al.*, 2014), it is not clear whether the number of *Ace*-1-119S copies in *An. gambiae s.s.* heterozygotes are higher than in *An. coluzzii* or vice versa. It is, however, expected that an equivalent number of *Ace*-1-119S copies produces the same resistance in each species and so further studies are required to clarify this. No duplication was detected in *Ace-1GG* individuals in either species and this finding is similar to what has been reported among the populations of *An. gambiae s.s.* and *An. coluzzii* from Burkina Faso, Togo, Benin and Ivory Coast (Assogba *et al.*, 2016, 2018), however, duplications have been reported in *Ace-1GG* in *An. gambiae s.s*. in Ghana though they are very rare (Weetman *et al.*, 2015; Grau-Bové *et al.*, 2020). The duplicated *Ace-1GG* are fully susceptible to CB/OP (Weetman *et al.*, 2015), however other phenotypic consequences (in terms of fitness cost) associated with duplication are yet to be explored. The higher proportion of unduplicated heterozygotes in *An. coluzzii* than in *An. gambiae s.s.*, and also a remarkable declining trend over time in *An. coluzzii* may suggest that the unduplicated state was the original form but has recently been almost competitively replaced by the duplicated form. This may explain why *Ace-1* duplication and *Ace-1*-119S frequencies are higher in *An. gambiae s.s.* than in *An. coluzzii*. Again, a decline in the suboptimal genotype is expected to occur more in *An. coluzzii* as they are still catching up to *An. gambiae s.s* in *Ace-1* evolution. Our result clearly shows that the shifting *Ace*-1 duplication type dynamics are operating in the opposite in each species – presumably heading towards an equilibrium that may or may not be the same for each species depending on the strength and consistency of insecticidal selection pressures they are experiencing. It also worth noting that due to the complexity of the *Ace*-1 locus, the HWE deviation is not the useful indicator for *Ace*-1 duplication that was suggested previously (Labbé, Berthomieu, *et al.*, 2007; Djogbénou *et al.*, 2009).

Both homogeneous and heterogeneous *Ace-1* duplications have also been reported in *An. gambiae s.s* and *An. coluzzii* populations in Benin, Togo and Burkina Faso, and they may have been selected to influence carbamate (and /or organophosphate) resistance via higher copy numbers of *Ace-1* alleles (Djogbénou *et al.*, 2015; Assogba *et al.*, 2016). This hypothesis has been confirmed by a recent genomic study which has shown that the number of serine alleles (*Ace-1*-119S) is a key determinant of PM resistance in *An. gambiae s.s.* and *An. coluzzii* (Grau-Bové *et al.*, 2020). Previous studies have found that additional copies of *Ace-1* alleles lack sequence variations indicating that the duplication is a very recent event (Djogbénou *et al.*, 2008a; Essandoh *et al.*, 2013).

Copy number estimates of *Ace-1* alleles in Madina *An. gambiae s.s. Ace-1SS* was a five-fold increase relative to Kisumu while *Ace-1GG* was found to be the same as Kisumu (one-fold). However, in the heterozygotes, the copy number estimations were found to be four-

folds, three folds and two-fold increase relative to Kisumu in *Ace-1*GS-High, *Ace-1*GS-Middle and *Ace-1*GS-low respectively. This indicates that the copy number of *Ace-1* alleles in heterozygotes is quite variable and it also explains the wide degree of dispersion in their genotype display in bidirectional scatter plot (Figure 3.8). It is therefore postulated that various forms of heterogeneous duplication may be selected in environments with a mosaic of treated and untreated areas as they exhibit phenotypic resemblance to standard heterozygote, with an intermediate level of resistance and lower fitness cost (Assogba *et al.*, 2016). Moreover, heterogeneous duplication has a selective advantage over a standard heterozygote in that it leads to the fixation of heterozygosity (Milesi *et al.*, 2017).

The scoring of *Ace-1* genotypes with TaqMan MxPro bidirectional plot is usually problematic, particularly for heterozygotes, due to the *Ace-1* duplication in *An. gambiae s.s.* and *An. coluzzii* and so calibration is necessary to investigate whether the various forms of heterogeneous duplication could be determined with TaqMan genotyping where the Fam/Hex ratio could be used as a proxy for the relative resistance allele frequency. Our results suggested that the *Ace-1*GSS separates well from *Ace-1*GS and *Ace-1*GGS, but less well from *Ace-1*GS from *Ace-1*GGS. The Taqman *Ace-1* genotyping assay is noisy in distinguishing between *Ace-1GG* and *Ace-1GS* and may not be accurate for individuals, but maybe useful for comparing groups.

3.5 Conclusion

The emergence and spread of *Ace-1*-based carbamate and organophosphate resistance via duplicated resistant alleles are a considerable threat to malaria control using any insecticides within these classes and limit the insecticide options for resistance management strategies such as rotations. Ghana NMCP and Anglogold Malaria Control Unit have been carrying out IRS with Actellic in the three northern regions of Ghana since 2014, and currently, Sumishield (Clothianidin) is being piloted for IRS in selected communities in Ghana. As such, IRS scaled up is likely to be performed by NMCP in a rotation programme of Actellic (OP) and later Sumishield (Clothianidin), and this will require empirical data to serve as a reference guide so that control programmes will be tailored to epidemiological settings. Developing techniques to accurately quantify the amount of CB, OP and even Clothianidin in the environment before the insecticides are recommended for public usage in the various epidemiological regions is very difficult and so *Ace*-1 may provide a more feasible option as a proxy marker for total environmental selection pressure. This implies that using Taqman assay and PCR to determine the frequencies of *Ace-1* genotypes and duplications in mosquito populations in a particular region can provide substantial information on the intensity of CB and OP usage.

Chapter Four: Age-specific costs of pirimiphos-methyl resistance in *Anopheles gambiae*.

Abstract

Age is a key parameter in resistance monitoring since it impacts a wide range of physiological processes in mosquitoes including insecticide susceptibility. Pyrethroid and DDT resistance is ubiquitous, yet the IRS and LLIN are still effective in controlling *An. gambiae s.l.* in most populations and it may be partly due to older adult females, the most important epidemiological stage of the vector, still being susceptible. The age-specific resistance is, however, not a universal phenomenon and may depend on insecticide class and resistance mechanism. The *Ace-1*- mediated resistance is associated with different variants (*Ace-1* G119S genotypes, duplication and internal deletion) with each possessing different fitness costs. However, it is currently unknown whether these multi-level resistant costs remain constant with mosquito age and how they influence the phenotypic outcome of CB and OP exposure. To bridge this knowledge gap, the susceptibility of *An. gambiae s.s.* and *An. coluzzii* from Madina, Ghana, to pirimiphos-methyl (PM) was performed by assaying different ages, ranging from 3 to 27 days old. Species, *Ace-1* genotypes and *Ace-1* duplication (a full-length duplication or one with internal deletion *(ID*) were typed, and age were assessed as predictors of the survival of mosquitoes to PM exposure. Metabolic enzyme mediation of PM resistance was investigated by performing susceptibility tests to PM with prior exposure to piperonyl butoxide (PBO) synergist, a blocker of metabolic enzymes. The cost of possession of *Ace-1* resistant mutation, duplication and *ID* on mosquito longevity was also investigated by genotyping insecticide-unexposed females as they died naturally at different ages. There were marked differences in susceptibility among 3, 9 and 18-day old cohorts, however, there was no consistent age-specific increase (or decrease) in mortality irrespective of dose level or species upon PM exposure. In both *An. gambiae s.s.* and *An. coluzzii*, it was also found that *Ace-1SS* individuals (homozygous resistant) are more likely to survive PM exposure than *Ace-1GS* individuals, but amongst these heterozygotes, those exhibiting internal deletion were more likely to survive PM exposure than those with the full-length duplication. The synergist PBO significantly increased the survival of mosquitoes to PM, suggesting that P450-involvement in the metabolic conversion of PM to its activated oxon form is more important than metabolic detoxification. The daily mortality rate of unexposed mosquitoes in the laboratory showed no significant difference in the survival rate of *Ace-1GS* and *Ace-1SS* in both *An. gambiae s.s.* and *An. coluzzii*. The mosquitoes possessing chromosomal segment duplication have a significantly higher survival rate than those without. Further studies are required to investigate the susceptibility of blood-fed females to PM exposure and also on metabolomics to investigate the role of various P450s in PM metabolism.

4.0 Introduction

Insecticide application for malaria vector control through LLINs and IRS has contributed significantly towards malaria reduction in Sub-Saharan African (Bhatt *et al.*, 2015). Each targets the adult anopheline mosquito which is the most epidemiologically important life stage and impacts the vector population by reducing the density and the life span. This alters the age structure of the vector population such that fewer mosquitoes live long enough to become infective with *Plasmodium* (Garrett-Jones and Grab, 1964). However, the development and spread of resistance to all the four major insecticide classes (DDT, pyrethroids, carbamates (CBs) and organophosphates (OPs) deployed in public health are a great threat to sustained control and may reverse the fragile gains made over the years. For instance, *An. gambiae s.s.* and *An. coluzzii* resistance to pyrethroids is very common with the most common target-site mechanism, *Vgsc*-1014F, approaching fixation in many vector populations in West Africa (Hien *et al.*, 2017; Zoh *et al.*, 2018; Ahadji-dabla *et al.*, 2019; Pwalia *et al.*, 2019). Patches of the carbamate (CB) and organophosphate (OP) resistance have also been reported in *An. gambiae s.l.* across West Africa (Essandoh *et al.*, 2013; Aizoun *et al.*, 2014; Camara *et al.*, 2018) with an implication of multiple mechanisms such as *Ace*-1-119S, *Ace*-1 duplication and, for carbamates, the elevation of *CYP6* P450s (Edi *et al.*, 2014).

Resistant mosquitoes are expected to survive longer on average than their susceptible counterparts in insecticide-treated settings, and so they are more likely to live long enough to impact malaria transmission. However, there have been contrasting findings on the impact of insecticide resistance on malaria transmission. For instance, it has been shown that resistance would increase the incidence of malaria due to an increased mosquito tolerance (White *et al.*, 2011), whilst studies in East Africa have reported that LLIN is still effective in preventing malaria despite the existence of multiple insecticide resistance mechanisms in the vector populations (Lindblade *et al.*, 2015; Ochomo *et al.*, 2017). A recent WHO-coordinated multi-country observational cohort study in Asia and Africa showed that LLIN is effective in lowering infection prevalence in users and no evidence of an association between insecticide resistance and infection prevalence (Kleinschmidt *et al.*, 2018). Although several studies have established a link between insecticide resistance and malaria prevalence (Kigozi *et al.*, 2012; Coleman *et al.*, 2017; Protopopoff *et al.*, 2018), the impacts of resistance have not been as widespread as might be expected, which may suggest that the association is much more complex than previously thought as other factors such as the age of mosquitoes influence resistance.

Insecticide resistance has been found to decline as mosquitoes age, this implies that older mosquitoes that have lived long enough to be capable of transmitting parasites are more likely to be killed on encountering insecticides (Lines and Nassor, 1991; Hodjati and Curtis, 1999; Chouaibou *et al.*, 2012; Jones *et al.*, 2012). The decline in mosquito insecticide tolerance with increasing age may partly explain the continued effectiveness of LLIN and IRS even in resistance areas. Results across studies are summarised in Table 4.1. For instance, the mortality rate was found to be significantly higher to deltamethrin and bendiocarb in older (17-19-day old) than in younger (3-5-day old) *An. gambiae s.l.* in both laboratory and field-based experiments (Jones *et al.*, 2012). Susceptibility to lambda-cyhalothrin in *An. funestus* also increases with age (Hunt *et al.*, 2005). Similarly, susceptibility to bendiocarb, deltamethrin and DDT in 14-day old *An. gambiae* and *Aedes aegypti* was found to be higher than younger mosquitoes (Rajatileka *et al.*, 2011). Recent a study in Kenya has reported that 14 –16 days old *An. gambiae s.s.* and *An. arabiensis* exhibited reduced phenotypic resistance to deltamethrin compared to the 2–5 days old counterparts (Machani *et al.*, 2019). The decline in insecticide resistance with mosquito age is hypothesised to be caused partly by the reduction in the expression levels of detoxifying enzymes in older mosquitoes, thereby making it difficult for older mosquitoes to bear the fitness cost associated with resistant alleles (Rowland and Hemingway, 1987). For example, detoxification of malathion in *An. stephensi* and *An. gambiae s.l.* decreases as mosquitoes age, with mosquitoes that are malathion-resistant at emergence becoming increasingly susceptible with age (Rowland and Hemingway, 1987). It has also been reported that the activity levels of GSTe2 (overexpression of which is implicated in DDT resistance) decrease with age in both *Anopheles and Aedes* (Ranson *et al.*, 2001; Lumjuan *et al.*, 2005).

Conversely, some studies have also shown no significant increase in the susceptibility to insecticide exposure with age, and in some cases, the susceptibility even decreases with age (Table 4.1). For instance, in *An. gambiae s.s.* strains from Western Kenya, the susceptibility of 3-day females to DDT was found to be significantly higher than that of 14 -day old, but there was no significant difference in susceptibility between the age groups for bendiocarb and deltamethrin exposures (Rajatileka *et al.*, 2011), and this may suggest that the specific insecticide, or combination of population/strain and insecticide, may play a role in the age-specific decrease/increase in susceptibility.

Mutation of acetylcholinesterase (the target for organophosphate and carbamate insecticides) in *Anopheles gambiae* involves a single point mutation - G119S - and copy number variation of the gene plus a 203 kb surrounding areas of the genome (Assogba *et al.*, 2016). Alone, the resistant serine allele carries a heavy energetic cost, and would rarely be selected as a homozygote even though it confers strong resistance to both organophosphate

and carbamate insecticides (Alout *et al.*, 2008). However, duplication of the gene has affected this cost/benefit trade-off, and all resistant alleles now appear to be present as multiple copies (Assogba *et al.*, 2016; Assogba *et al.*, 2018). These take two forms: (1) heterogeneous duplications which pair a resistant and a susceptible allele together in tandem on the same chromosome, which reduces the resistance cost because glycine alleles are fully functional; (2) homogeneous duplications where resistant serine alleles are copied *n* times, with both resistance and cost increasing with the number of copies of the serine allele (Weetman, Djogbenou and Lucas, 2018). An additional cost of resistance may be incurred according to the surrounding genomic area duplicated along with the resistant allele. In one kind of the duplication, *Ace-1* and a surrounding area including 10 genes occur, whereas, in the other, only *Ace*-1 and some non-coding flanking region are copied (Figure 3.1b). This second, reduced-size copy is expected to be less costly than the first because of reduced costs of cell replication and unnecessary gene expression (Assogba *et al.*, 2018).

Table 4.1: Studies on the effect of age on the susceptibility of *An. gambiae s.l.* and *An. funestus* to insecticides**.** Only studies where bioassays were performed with non-blood-fed mosquitoes were included in this list.

Species of mosquitoes	Site of studies or where the mosquito strains come from	The insecticide used for bioassay	Age groups	Susceptibility conclusion	Insecticide mechanism(s)	Authors and year
An. gambiae s.s. (96%) and An. arabiensis (2.75%)	Western Kenya	Deltamethrin	2–5 days and 14–16 days old	Significantly higher susceptibility in the older mosquitoes	The enzyme activity of monooxygenases, GSTs and β-esterases were significantly higher in younger mosquitoes.	(Machani *et al.*, 2019)
An. arabiensis	Northern Tanzania	Deltamethrin, permethrin, lambda-cyhalothrin, bendiocarb	2, 3, 5, and 10 days old	For pyrethroids, significantly higher susceptibility in the older mosquitoes. For bendiocarb, there was no significant difference in susceptibility.		(Mbepera *et al.*, 2017)
An. coluzzii	Tiassalé , Côte d'Ivoire	Deltamethrin, permethrin, and propoxur	1, 2, 3, 5 and 10 days	For the pyrethroids and propoxur, susceptibility increases with age.		(Chouaibou *et al.*, 2012)
An. gambiae s.s.	south-west Burkina Faso	Deltamethrin	3-5 day and 17-19 day old	Significantly higher susceptibility in older mosquitoes.		(Jones *et al.*, 2012)
An. gambiae s.s.	Zanzibar (Tanzania),	DDT ,bendiocarb and deltamethrin	3-day and 14-day old	DDT, significantly higher susceptibility in older mosquitoes. Bendiocarb, no significant difference in susceptibility. Deltamethrin, no significant difference in susceptibility.	Significant higher expression of GSTe2 in 3-day than in 14-day old	(Rajatileka *et al.*, 2011)

	Western Kenya	DDT ,bendiocarb and deltamethrin	3-day and 14-day old	DDT, significantly higher susceptibility in younger mosquitoes. Bendiocarb and deltamethrin, no significant difference in susceptibility.	No significant difference in the expression of GSTe2 between the two age cohorts	
	Akron (Benin)	DDT ,bendiocarb and deltamethrin	3-day and 14-day old	DDT, significantly higher susceptibility in older mosquitoes. Bendiocarb, no significant difference in susceptibility. Deltamethrin, significantly higher susceptibility in older mosquitoes.	Significant higher expression of GSTe2 in 3-day than in 14-day old	
An. funestus	southern Mozambique	Permethrin	3, 5, 10, 14, 20, and 30 days old	The susceptibility increases with age	The P450 gene over-expression remains constant throughout the life span of the mosquitoes.	(Christian *et al.*, 2011)
An. gambiae s.l.	Zanzibar, Tanzania	DDT	1 day and 12-14 days old	significantly higher susceptibility in older mosquitoes		(Line and Nassor, 1991)

To effectively manage insecticide resistance and sustain the long-term efficacy of vector control, there is a need for regular monitoring. According to the guidelines developed by the WHO to monitor insecticide resistance, it is recommended that 3-5-day old non-blood fed female mosquitoes are used for susceptibility bioassay test. This standardised protocol facilitates comparison between different tests, which is necessary when monitoring for spatial or longitudinal variations in resistance in the field (Rajatileka *et al.*, 2011). However, the mosquitoes within this recommended age range cannot transmit *Plasmodium* parasites. Adult anopheline mosquitoes also experience a high daily mortality rate such that only a small proportion of the population live long enough to transmit malaria and so tools that reduce the longevity of the mosquito will impact the vectorial capacity significantly (Cook, *et al.*, 2008; Cook and Sinkins, 2010). Age is an important parameter to be considered in resistance monitoring since it impacts a wide range of physiological processes in mosquitoes including immunity, susceptibility to infections, flight, reproductive success and insecticide susceptibility (Hillyer *et al.*, 2005; Castillo, Robertson and Strand, 2006; Cornet, Gandon and Rivero, 2013; Pigeault *et al.*, 2015). Although most previous studies have reported increased susceptibility as mosquitoes age, the mechanisms for this are not clear, though some cost of resistance is likely to be involved, which older mosquitoes are less able to bear (for example they may express lower levels of detoxifying enzymes). If an important resistance mechanism has alleles that confer different costs, it might be expected that this will impact the age versus insecticide susceptibility relationship. *The Ace-1*- mediated resistance is associated with a range of costs; from (i) low (Glycine) to (ii) moderate (heterogeneous Glycine+Serine duplication) to (iii) high (homogeneous Serine+Serine with only *Ace*-1 duplicated) to (iv) potentially very high (homogeneous Serine+Serine with Ace-1 plus 10 surrounding genes duplicated). Whether these multi-level costs for *Ace-1*-based resistance remain constant with mosquito age is unknown, but this is important to identify the impact of the resistance mechanism on malaria transmission, which relies on older mosquitoes. Again, although IRS is particularly essential for malaria control by vector intervention, no study has yet been performed on the effect of age on mortality for PM. In this study, I proposed to test whether *Ace*-1 mediated resistance changes with age in adult female *An. gambiae s.s.* and *An. coluzzii* from Madina, a suburb of Accra where our previous studies have demonstrated very high levels of *Ace*-1 based resistance.

Research questions:

1. What is the current susceptibility status of *An. gambiae s.l.* to bendiocarb, fenitrothion, malathion, deltamethrin and PM using the WHO recommended age (3-5 day old)?

2. Does an increase in age make mosquitoes more susceptible to PM exposure?

3. Do the possession of *Ace*-1 119S, duplication and internal deletion influence the survival of mosquito to PM exposure as mosquitoes age?

4. Does susceptibility to PM exposure increase with metabolic enzyme inhibition using piperonyl butoxide (PBO) synergist?

5. What is the cost of possessing *Ace*-1 resistant mutation, duplication and internal deletion in the absence of treatment on the longevity of adult female mosquitoes?

4.2 Methodology

4.2.1 Sample collections, maintenance and adult susceptibility tests

Anopheles larvae were collected from a variety of habitats in the suburb of Madina such as pools, roadside puddles, drainage channels and irrigations fields, by the dipping method using hand-held ladles between January and May 2019. The larvae were transported a short distance to CSIR- Water Research Institute Laboratory in Accra.

The larvae were kept in white plastic trays and covered with nets and fed with Tetramin fish food. Pupae were collected daily in a beaker and placed in mosquito cages and date recorded. When the adults emerged, the males were removed from the cages to avoid mating. Adult female mosquitoes were maintained on a 10% sugar solution. To assess the effect of age on the phenotypic resistance *of An. gambiae s.l.,* bioassay tests were performed by exposing mosquitoes of varying age cohorts (3, 6, 9, 12, 15, 18, 21, 24 or 27 days) to 0.25% of pirimiphos-methyl (PM) WHO papers for either 30 minutes or 60 minutes and the ambient temperature and humidity were recorded. Testing was performed at both short and standard times because it was expected that only a few mosquitoes would survive the standard exposure time, and this may not allow the detection of a significant age-specific change in mortality. A small subset of the 3-day and 6-day old mosquito cohorts were used to assess the effect of age on higher resistance level by exposing them to 1.25% (5X) of PM for 30 minutes and 60 minutes. Additional bioassay tests were performed with batches of 3-day and 6-day old female mosquitoes exposed for 60 min to 4% piperonyl butoxide (PBO) synergist, prior to PM exposure, to find out whether susceptibility will be enhanced with metabolic enzyme inhibition. To investigate the cost of possessing *Ace*-1-119S, duplication and internal deletion on the longevity of *An. gambiae s.l.,* two cages, A and B, containing 238 and 244 female mosquitoes respectively from the same cohort were set aside and the daily mortality rate of unexposed mosquitoes in the laboratory setting monitored. Then genotyping was done to establish the relationship between the possession of *Ace-1* and mosquito lifespan.

4.2.2 Species and *Ace-1*-119S diagnostics

Total genomic DNA was extracted individually from assayed mosquitoes from each age cohort for both 30-min-PM and 60-min-PM using Nexttec Kits following the manufacturer instructions. An average of 56 mosquitoes was selected randomly from the bioassay outcome, and to account for heterogeneity within the population, the number of “Dead” and “Live” mosquitoes were selected proportional to the sample size. Preliminary studies showed that *An. coluzzii* makes up about 30% of *An. gambiae s.l.* in Madina and so the 56-sample size was chosen to enable enough samples for pairwise species comparison of age, susceptibility and genotypes in *An. coluzzii* and *An. gambiae s.s*. However, the sample size was increased for cohorts when there were an over-representation of one species in the 56 diagnosed mosquitoes. The species characterizations into *An. coluzzii* and *An. gambiae s.s.* was performed using the PCR protocol described by Santolamazza *et al.*, (2008). The mosquitoes were genotyped for the *Ace-1*-119S using the TaqMan allelic discrimination (fluorescence-based) assay described by Bass *et al.*, (2010).

4.2.3 *Ace-1* duplication (‘duplityping’) and internal deletion (*ID*) detection

Genomic DNA from individual mosquitoes was used for the *Ace-1* duplication detection PCR described by Assogba *et al.*, (2016). The *Ace-1* duplicated individuals were further characterized into “full duplication” (duplicated 203-kb chromosomal segment comprising *Ace*-1 gene in tandem with 10 genes present) and “partial duplication” (duplicated chromosomal segment has *Ace-1* flanking genes deleted) using a PCR protocol described by Assogba *et al.*, (2018).

4.2.4 Analysis

The data on bioassay mortality rate in percentages with 95% confidence interval. Phenotypic data were analysed using Generalized Linear Models using a binomial logistic regression to model the dependence of “Live” and “Dead” on the explanatory variables age at exposure (3, 6, 9, etc days), *Ace*-1 genotype (*GG, GS,* and *SS*), *Ace*-1 duplitype (presence, absence) and species (*An. gambiae s.s*, *An. coluzzii*). The significance of each of the explanatory variables was assessed using a Wald χ^2 test. The Nagelkerke R^2 was used to assess how useful the explanatory variables are in predicting the response variable. The effect of PBO on the survival of mosquito following PM exposure was assessed using a generalised linear model with binary logit function, and the goodness of fit of the model was determined

using the omnibus χ^2 test ($p < 0.05$ indicating significant difference). Survival analysis was performed to investigate the time to death of mosquitoes and identify the cumulative effects of *Ace*-1 genotypes, chromosomal segment duplication and internal deletion (*ID*) status on the daily survival of *An. gambiae s.s.* and *An. coluzzii*. Kaplan-Meier survival curves were plotted using cumulative survival data, according to species, *Ace*-1-119S genotypes, chromosomal segment duplication and *ID* status. The comparison of the survival curves was done using a log-rank test, with $p < 0.05$ indicating a significant difference in the population survival curves. Cox's proportional hazards regression was used to model the survival times of mosquitoes using *Ace*-1-119S genotypes, duplication status and *ID* status as predictors. In this model, the response variable is the hazard risk, i.e., the probability of a mosquito dying at a given point in time. The time estimates of the predictors were reported as odds (risk) ratios with 95% confidence intervals and associated p-values.

4.3 Results

4.3.1 Phenotypic Resistance and Species diagnostics

Bioassay tests were performed by exposing 3-5-day old (the WHO-recommended age) *An. gambiae s.l.* females to determine their susceptibility to the standard doses of malathion, deltamethrin, fenitrothion, bendiocarb and PM. The results showed a high phenotypic resistance to all the five insecticides, with the lowest mortality rate recorded for bendiocarb, 7 % (95%CI: 3.7 -12.6) while the highest was for PM, 69% (95%CI: 58.0 – 77.8) (Table 4.2).

Table 4.2: The mortality rate of *An. gambiae s.l.* to three insecticide classes after standard bioassay for 60-minute insecticide exposure

Insecticide	Age (days)	Dead	Live	Total	Temp (^{0}C)	Humidity (%)	Mortality rate (%) with 95% CI
Malathion	3-5	47	64	111	25	82	42 (33.0 -52.1)
Deltamethrin	3-5	84	168	252	26	85	33 (27.5 – 39.5)
Fenitrothion	3-5	55	98	153	25	86	36 (28.4 – 44.1)
Bendiocarb	3-5	11	141	152	26	85	7 (3.7 – 12.6)
PM	3-5	63	29	92	25	87	69 (58.0 – 77.8)

To investigate the age-specificity of PM mortality, 848 mosquitoes of varying ages (from 3-day to 27-day old) were exposed to 0.25% PM for both 30 min and 60 min. An average of 56 assayed mosquitoes from each age cohort for both 30-min-PM and 60-min-PM were selected randomly for species diagnostics, and to account for heterogeneity within the population, the number of “Dead” and “Live” mosquitoes were selected proportional to the sample size. Of 864 mosquitoes characterized as members of *An. gambiae* complex, 244 (28.2%, 95%CI: 25.3- 31.4%) were *An. coluzzii* while *An. gambiae s.s.* recorded 620 (71.8%,

95%CI: 68.6 - 74.7%) (Table 4.3). The generalized linear model (Table 4.4) shows that following mosquito exposure to PM, there is a strong effect of species and age in the death of mosquitoes. For both 30-min-PM and 60-min-PM exposure, there is more death in *An. coluzzii* than in *An. gambiae s.s*. For age factor, in 30-min-PM exposure, the mortality of mosquitoes increases marginally with age, while in 60-min-PM, the mortality decreases with age, and there is also evidence of the significant effect of species x age interaction for 60-min-exposure (Figure 4.1). However, it is noteworthy that the results for the age-mortality relationship following PM exposure are inconsistent.

Table 4.3: Samples of age-specific assays characterized into species with their corresponding mortality rates. The number of samples used is put in a bracket.

	30 min-PM exposure		**60 min-PM exposure**	
Age cohort (days)	*An. coluzzii*	*An. gambiae s.s.*	*An. coluzzii*	*An. gambiae s.s.*
	Mortality (%)	Mortality (%)	Mortality (%)	Mortality (%)
3	50.0 (14)	31.0 (42)	90.0 (20)	52.8 (36)
6	40.0 (20)	39.0 (36)	-	-
9	56.3 (16)	42.5 (40)	85.0 (20)	70.0 (60)
12	50.0 (16)	25.0 (40)	78.3 (23)	82.9 (41)
15	70.0 (10)	26.1 (46)	75.0 (12)	64.3 (28)
18	100.0 (12)	81.8 (44)	68.0 (25)	66.0 (47)
21	50.0 (16)	35.9 (39)	-	-
24	46.7 (15)	42.9 (42)	46.2 (13)	51.4 (35)
27	75.0 (12)	56.8 (44)	-	-

The dash (-) indicates no bioassay was performed

Table 4.4: The generalized linear model showing the death of mosquitoes following PM exposure by species and age

(a) 30 min-PM exposure					
Parameter	**B**	**95% CI**	**χ^2**	**df**	**p-value**
(Intercept)	-0.24	-0.72 - 0.24	0.9	1	0.331
An. gambiae s.s	-0.63	-1.04 - -0.23	9.3	1	0.002
****An. coluzzii***					
Age	0.04	0.01 - 0.06	9.9	1	0.002

(b) 60 min-PM exposure					
Parameter	**B**	**95% CI**	**χ^2**	**df**	**p-value**
(Intercept)	2.68	1.46 - 3.90	18.7	1	< 0.001
An. gambiae s.s.	-1.89	-3.24 - -0.53	7.4	1	0.006
****An. coluzzii***					
Age	-0.11	-0.19 - -0.04	8.5	1	0.004
***An. gambiae s.*s x Age**	0.10	0.01 – 0.19	5.3	1	0.021
****An. coluzzii* x Age**					

* *An. coluzzii* and interaction *(*An. coluzzii* x age) were used as reference categories. Event = death

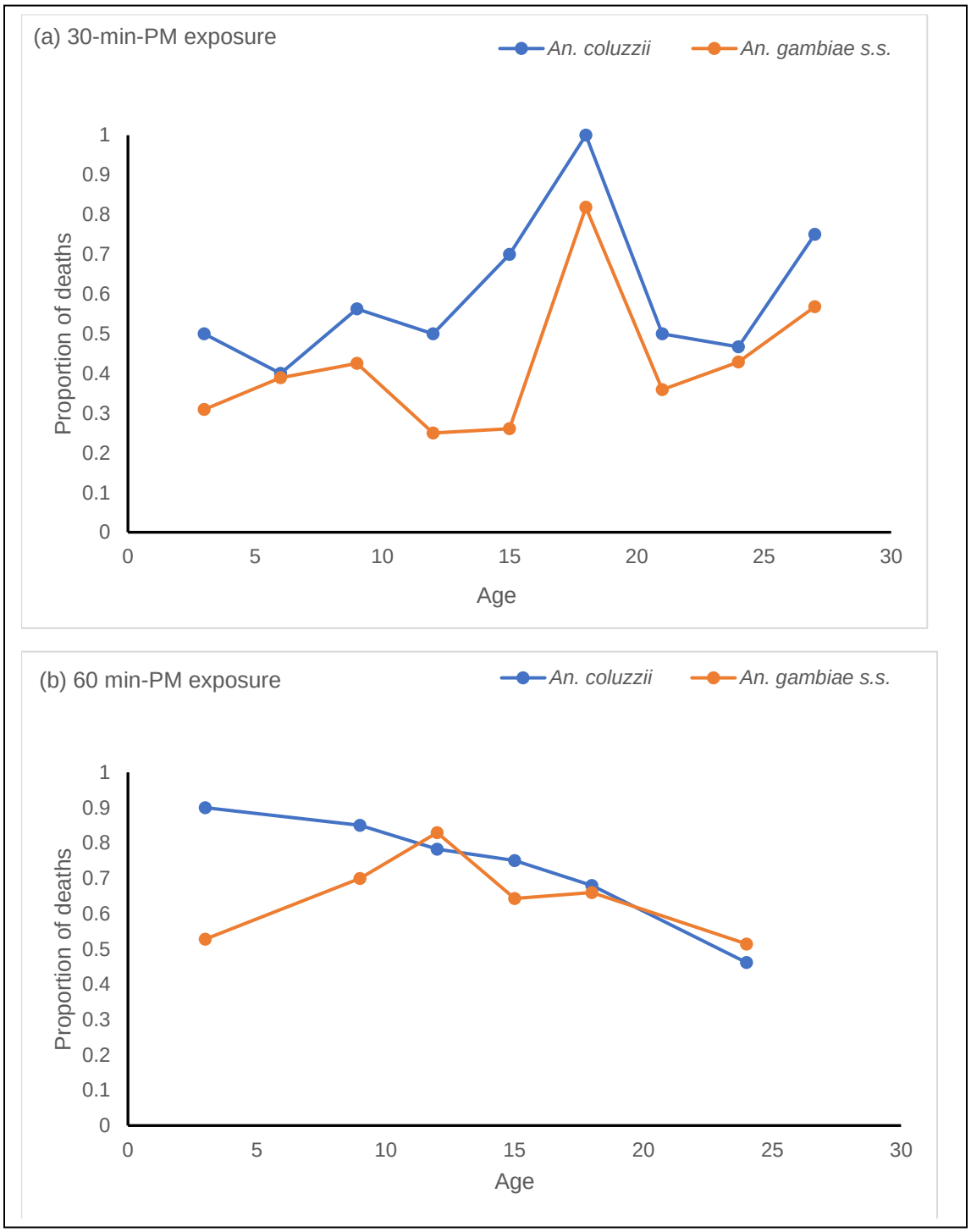


Figure 4.1: Proportion of death of mosquitoes with age following (a) 30-min-PM exposure and (b) 60-min-PM exposure

4.3.2 *Ace*-1 genotyping

In *An. coluzzii,* the *Ace-1-119S* genotypic frequencies for *Ace*-1*GG*, *Ace*-1*GS* and *Ace*-1*SS* were found to be 4% (95%CI: 2.0- 7.4%), 86% (95%CI: 80.6–89.8%) and 10% (95%CI: 6.7-14.8%) respectively. In *An. gambiae s.s.,* the *Ace-1-119S* genotypic frequencies for *Ace*-1*GG*, *Ace*-1*GS* and *Ace*-1*SS* were found to be 4% (95%CI: 2.9- 6.3%), 64% (95%CI: 601–67.8%) and 32% (95%CI: 28.0-35.4%) respectively. The *Ace-1* genotypic frequencies differed significantly between the species pair (χ^2 = 43.0, df = 2, p < 0.001), though heterozygotes were the most common genotype in each species. Similarly, *Ace-1*-119S allelic frequency was significantly higher in *An. gambiae s.s.* (64%, 95%CI: 60.9 - 66.3%) than in *An. coluzzii* (53%, 95%CI: 48.5 – 57.6%), (χ^2 = 16.3, df = 1, p < 0.001). However, there was no significant variation in *Ace*-1-119S frequency with age in each of the species pair (Table 4.5).

Table 4.5: The generalized linear model showing the variation of *Ace-1* frequency with age in the species pair.

Species	Parameter	B	95% CI	X^2	df	P-value
An. coluzzii	(Intercept)	-0.041	-0.51- 0.42	0.03	1	0.863
	Age	0.015	-0.01 – 0.04	1.07	1	0.301
An. gambiae s.s.	(Intercept)	0.620	0.34 – 0.90	18.75	1	< 0.001
	Age	0.001	-0.02 -0.02	0.02	1	0.879

*Events: *Ace-1*-119S frequency

Of 864 adult female mosquitoes, *Ace*-1 duplication was detected in 821 (95%) while absent in 43 (5%) individuals. In both species, the duplication was absent in all *Ace*-1*GG* individuals but present in all *Ace*-1*SS* individuals (Table 4.6 and Table 4.7). The proportions of *Ace*-1*GS* individuals with *Ace*-1 duplication in *An. gambiae s.s.* and *An*. *coluzzii* were 98.5% (95% CI: 96.7 – 99.4%) and 100.0% (95% CI: 98.3 – 100.0%) respectively. The 821 individuals with duplicated chromosomal segment were further screened for the presence/absence of internal deletion (*ID*) of flanking genes of *Ace*-1 locus. For duplicated *Ace*-1*GS*, the frequencies of *ID* presence in *An*. *coluzzii* and An *An. gambiae s.s.* were found to be 59% (95% CI: 52.3 – 66.1%) and 84% (95% CI: 80.1– 87.6%) respectively, (χ^2 = 45.3, df = 1, p < 0.001). For duplicated *Ace*-1*SS*, the frequencies of *ID* presence in *An*. *coluzzii* and in *An. gambiae s.s.* were found to be 88% (95% CI: 68.8 – 97.5%) and 99% (95% CI: 97.2 – 100%), (Fisher's exact, p = 0.005) (Table 4.8). In both species, the frequency of *ID* presence in *Ace-1SS* is significantly higher than in *Ace-1GS* (Table 4.8). The result shows that *ID* presence in duplicated resistant individuals is very common in the Madina population.

Table 4.6: The mortality rate (%) of 30-PM-exposed mosquitoes for different age cohorts and their distributions in terms of species, *Ace*-1 genotypes, *Ace-1* duplication and internal deletion.

(a) Species vs Ace-1 genotypes vs 30-min-PM mortality vs mosquito age						
	An. coluzzii			***An. gambiae s.s***		
Genotype	***GG***	***GS***	***SS***	***GG***	***GS***	***SS***
Age cohort	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**
3	100	46.2	-	100	30.4	0
6	100	38.9	0	100	44.8	0
9	-	64.3	0	100	50	10
12	-	53.3)	0	100	36.4	0
15	-	70	-	-	36.7	6.3
18	-	100	-	-	96.4	56.3
21	-	53.8	33.3	-	52.6	20
24	-	50	0	-	54.5	0
27	100	70	100	100	58.1	40

(b) Species vs *Ace-1* genotypes vs 30-min-PM mortality rate vs duplication							
	Species	***An. coluzzii***			***An. gambiae s.s***		
	Genotype	**GG**	**GS**	**SS**	**GG**	**GS**	**SS**
	Mortality	**%**	**%**	**%**	**%**	**%**	**%**
Duplication status	**Present**	-	58.8	22.2	-	51.7	16.4
	Absent	100	-	-	100	66.7	-

(c) Species vs *Ace-1* genotypes vs 30-min-PM mortality rate vs internal deletion							
	Species	***An. coluzzii***			***An. gambiae s.s***		
	Genotype	**GG**	**GS**	**SS**	**GG**	**GS**	**SS**
	Mortality	**%**	**%**	**%**	**%**	**%**	**%**
Internal deletion	**Present**	100	82.1	50.0	100	87.5	-
	Absent	-	47.5	14.3	-	44.8	16.4

The dash (-) indicates no sample was recorded

Table 4.7: The distribution of species, *Ace*-1 genotypes, 60-PM-exposed phenotypes against (a) mosquito age, (b) *Ace-1* duplication (c) internal deletion (*ID*).

(a) Species vs *Ace-1* genotypes vs 60-min-PM mortality vs mosquito age

	An. coluzzii			***An. gambiae s.s***		
Genotype	***GG***	***GS***	***SS***	***GG***	***GS***	***SS***
Age cohort	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**	**Mortality (%)**
3	100	94.1	0.0	100	75.0	0
9	100	87.5	66.7	100	90.0	26.3
12	100	75.0	75.0	100	92.6	54.5
15	-	75.0	-	-	75.0	50.0
18	100	68.2	50.0	100	88.5	18.8
24	-	71.4	16.7	100	57.1	27.3

(b) Species vs *Ace-1* genotypes vs 60-min-PM mortality rate vs duplication

	Species	***An. coluzzii***			***An. gambiae s.s***		
	Genotype	***GG***	***GS***	***SS***	***GG***	***GS***	***SS***
	Mortality	**%**	**%**	**%**	**%**	**%**	**%**
Duplication status	**Present**	-	78.9	43.8	-	82.1	28.8
	Absent	100	-	-	100	66.7	-

(c) Species vs *Ace-1* genotypes vs 60-min-PM mortality vs internal deletion

	Species	***An. coluzzii***			***An. gambiae s.s***		
	Genotype	***GG***	***GS***	***SS***	***GG***	***GS***	***SS***
	Mortality	**%**	**%**	**%**	**%**	**%**	**%**
Internal deletion	**Present**	100	97.8	0.0	100	89.3	100
	Absent	-	59.1	46.7	-	80.2	27.8

The dash (-) indicates no sample was recorded

Table 4.8: Distribution of internal deletion (*ID*) among the mosquitoes with the duplicated chromosomal segment in both *An. gambiae s.s.* and *An. coluzzii*.

Species	Internal deletion (*ID*) of flanking of genes of *Ace-1*	*Ace-1*-119S genotypes with Duplication			Fisher's exact P-value
		GS	*SS*	Total	
An. coluzzii	Absent	85	3	88	0.004
	Present	124	22	146	
An. gambiae s.s.	Absent	62	1	63	< 0.001
	Present	329	195	524	

4.3.3 Factors affecting mosquito survival following pirimiphos methyl exposure

A binary logistic regression analysis was performed to assess whether age, species, *Ace*-1-119S genotypes, *Ace*-1 duplication and internal deletion could predict PM phenotypic resistance. For 30-min-PM analysis *Ace*-1 genotype, internal deletion and mosquito age made a significant contribution to the model (Table 4.9). *An. gambiae s.s.* survived marginally better than *An. coluzzii* following 30-min PM exposure but not statistically significant, suggesting that the observed survival difference is attributable to other significant factors (*Ace-1* genotype or/and internal deletion) in the model which also differs between species (Table 4.9). The interaction factors (species x genotypes, species x *ID,* and genotype x ***ID)*** neither made significant contributions nor improved the overall outcome of the model, and so they were not incorporated in the final model. There is a significant decrease in the survival of mosquitoes with age given that the other variables in the model are held constant (Table 4.9). However, within each species, it was observed that the effect of age on the survival of mosquitoes following 30-min PM exposure was significant in *An. gambiae s.s.* but not in *An. coluzzii* (Table 4.10).

Table 4.9: The predictors of survival of 30 min-PM exposure with the corresponding estimates in odds ratios. The *Ace-1GS*, *Ace-1-ID*-Absent and *An. coluzzii* were set as reference categories for *Ace-1* genotypes, *Ace-,* internal deletion *(ID)* and Species respectively

Predictors	coefficient	S.E.	χ^2	P-value	Odds ratio	95% CI for Odds ratio
Constant	0.64	0.30	4.6	**0.032**	1.90	
Age	-0.04	0.01	10.1	**0.002**	0.96	0.93 - 0.98
Ace-1(SS)	1.54	0.28	31.1	**< 0.001**	4.60	2.71 - 7.97
Ace1_ID (Present)	1.84	0.35	28.2	**< 0.001**	6.30	3.19 - 12.38
Species (*An. gam ss*)	0.06	0.24	0.1	**0.794**	1.10	0.66- 1.71

**Ace-1 GS*, *ID*-absent and *An. coluzzii* were used as reference categories for genotypes, *ID* and species respectively.

*All *Ace-1GG* individuals were phenotypically “Dead”, and so they were not included in the model. The *Ace-1* duplication was not included in the model because almost all *Ace-1GS* and *Ace-1SS* individuals were duplicated.

Table 4.10: A GLM showing the effect of age on the survival of *An. coluzzii* and *An. gambiae s.s* after 30 min-PM exposure

Species	Parameter	B	95% CI	X^2	df	P-value
	(Intercept)	0.30	-0.45 – 1.04	0.6	1	0.434
An. coluzzii	Age	-0.04	-0.08 – 0.01	2.6	1	0.105
An. gambiae s.s.	(Intercept)	1.16	0.65 – 1.66	20.0	1	< 0.001
	Age	-0.05	-0.08 - -0.02	10.8	1	0.001

*Dependent Variable: Survival *Model: (Intercept), Age

In common with the analysis for 30-min exposure, the 60-min-PM analysis showed significant *Ace*-1 genotype and internal deletion effect, and once again species was not a significant factor (Table 4.11). However, here genotype x species interaction made a significant contribution to the model, suggesting inconsistency in the effect of genotypes on survival following 60-min PM exposure between the species (Figure 4.2). For a given age, the odds of surviving to 60-min-PM exposure is significantly higher in *Ace-1SS* individuals than in *Ace-1GS* individuals. Holding other variables constant, the odds of surviving in *Ace*-1 duplicated mosquitoes with *ID* is significantly higher than mosquitoes without *ID*. *An. gambiae s.s.* survival following 60-min PM exposure is not significantly different from *An. coluzzii* based on other effect levels. The age of mosquitos is not significant when the effects of other factors were accounted for in the model (Table 4.11). However, age alone as a predictor of survival had a significant effect on *An. coluzzii* (with PM survival increasing marginally with age), but not in *An. gambiae s.s.* (Table 4.12).

Table 4.11: The predictors of survival of 60-min-PM exposure with the corresponding estimates in odds ratios

Predictors	B	S.E.	χ^2	p-value	Odds ratio (95% CI)
Constant	-1.61	0.41	15.1	< 0.001	
Ace-1(SS)	1.58	0.34	21.3	< 0.001	4.8 (2.5 - 9.5)
Ace1_ID (Present)	2.07	0.57	13.3	< 0.001	7.9 (2.6 - 24.1)
Species (*An. gambiae s.s.*)	-0.05	0.35	0.1	0.89	1.0 (0.5 - 1.9)
Age	0.03	0.02	1.8	0.179	1.0 (1.0 - 1.1)
Ace-1 (SS) x Species (*An. gambiae s.s.*)	1.41	0.68	4.3	0.038	4.1 (1.1 - 15.6

*The *Ace*-1GS, *Ace*-1-ID-Absent and *An. coluzzii* were set as reference categories for *Ace*-1 genotypes, *Ace*-1 ID and Species respectively

*Ace-1 duplication did not appear in the final model because almost all *GS* and *SS* individuals were duplicated.

*All Ace-1GG individuals were phenotypically “Dead”, and so they were not included in the mode

Table 4.12: The GLM showing the effect of age on the survival of *An. coluzzii* and *An. gambiae s.s* after 60 min-PM exposure

Species	Parameter	B	95% CI	X^2	df	p-value
An. coluzzii	(Intercept)	-2.52	-3.74 - -1.3	16.5	1	0.000
	Age	0.11	0.03 – 0.18	7.6	1	0.006
An. gambiae s.s	(Intercept)	-0.80	-1.43 - -0.18	6.3	1	0.012
	Age	0.02	-0.02 – 0.06	0.7	1	0.396

*Dependent variable: survival

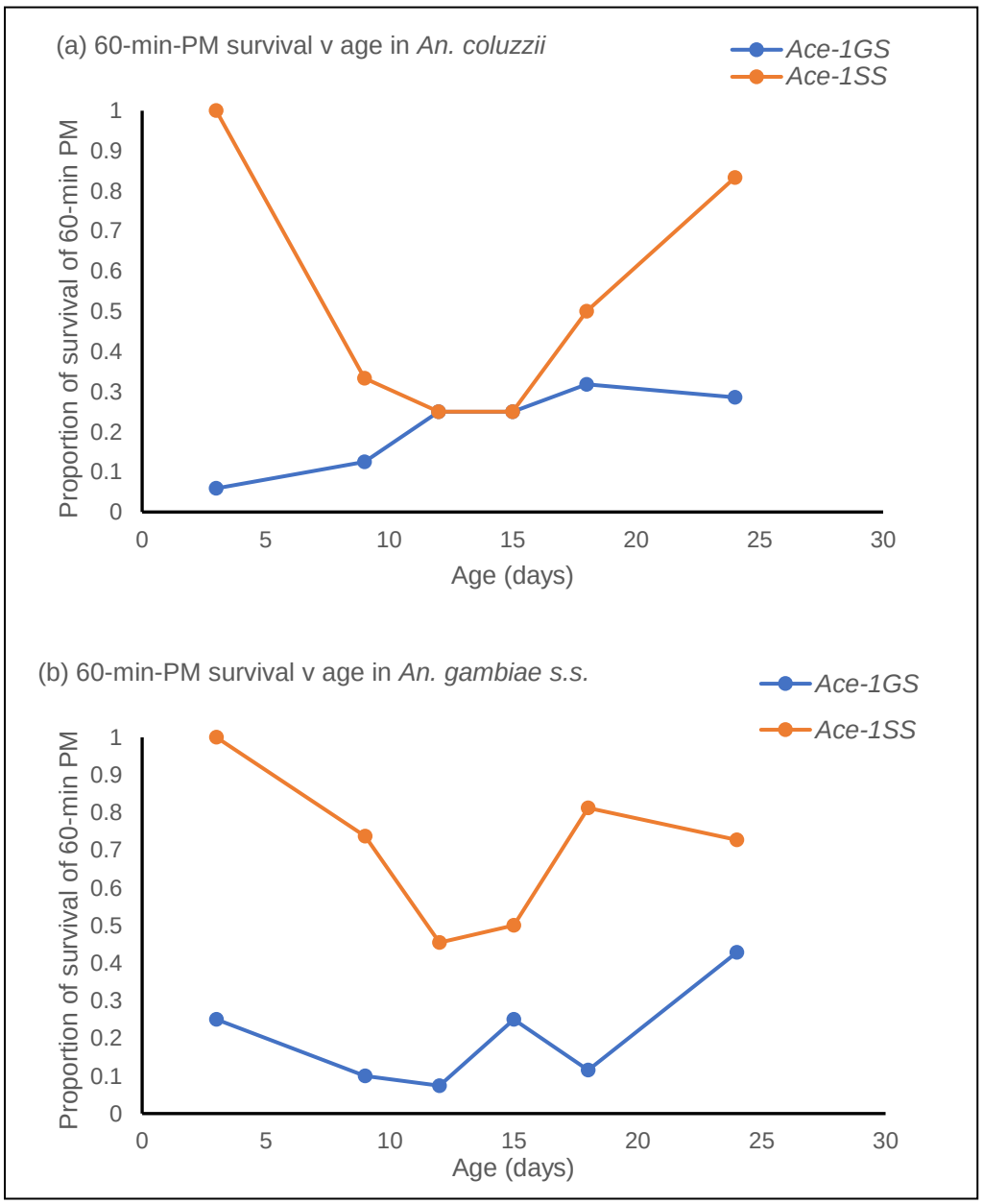


Figure 4.2: The survival of *Ace-1GS* and *Ace-1SS* individuals with age following 60-min-PM exposure in (a) *An. coluzzii* and (b) *An. gambiae s.s*.

To assess the possible role of metabolic enzymes in the phenotypic resistance as mosquitoes age, a synergist assay with PBO was performed (Table 4:13 and Figure 4.3). The PBO-present is associated with significantly lower mortality than PBO-absent. The age of mosquitos is not significant when the effects of other factors were accounted for in the model. However, age alone as a predictor, mortality increased marginally with age (Table 4.11b). The interaction PBO-presence x age made a significant contribution to the model, which may suggest that the role of metabolic enzymes in the survival of mosquitoes following 60-min exposure may depend on the age of the mosquito. It noteworthy that age is not a very useful predictor in this model considering the inconsistencies in the bioassay results (Figure 4.3 and Appendix 10).

Table 4.13: A generalized linear model comparing the mortality of 60min-PM exposure and 60min-PBO +60min PM exposure

(a) A model with PBO and Age as predictor variables

Parameter	B	Lower	X^2	df	P-value
(Intercept)	0.85	0.31 – 1.40	9.4	1	0.002
PBO-present *PBO-absent	-1.71	-2.41 - -1.0	22.6	1	< 0.001
Age	0.03	-0.03 – 0.08	0.9	1	0.339
PBO-present * Age *PBO-absent * Age	0.09	0.03 – 0.16	8.1	1	0.004

(b) A model with only Age as a predictor variable

Parameter	B	Lower	X^2	df	P-value
(Intercept)	-0.10	-0.42 – 0.23	0.3	1	0.561
Age	0.08	0.05 – 0.11	25.2	1	0.000

*PBO-absent and *(PBO-absent x Age) are used as reference categories

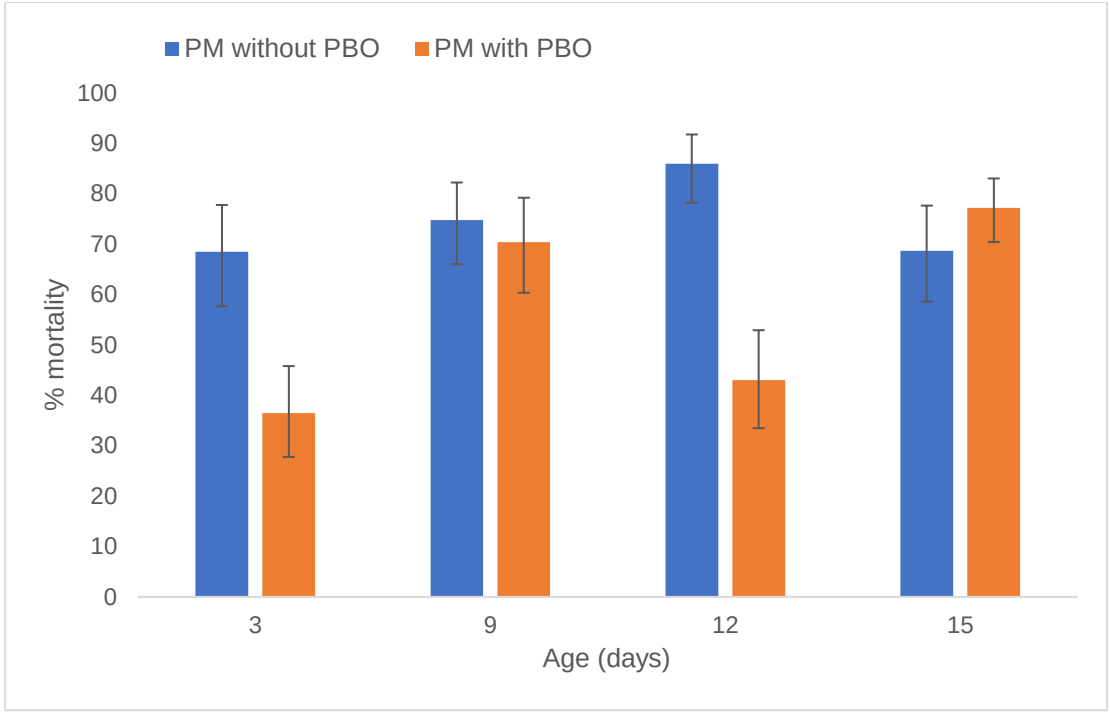


Figure 4.3: Mortality rates with 95% confidence interval of *Anopheles gambiae s.l.* after 24-hour recovery of 60-min PM exposure (blue bars) and 60-min PBO + 60-min PM exposure (red bars).

4.3.4 Determinants of mosquito mortality in the laboratory setting

A total of 481 *An. gambiae s.l.* were used to investigate the daily mortality rate of mosquitoes in the laboratory setting. Kaplan-Meier curves were used to analyse the time-to-death of unexposed mosquitoes and compare the mortality rate of *An. gambiae s.s.* (330) and *An. coluzzii* (151), the *Ace*-1 genotypes, mosquitoes with and without the *Ace*-1 duplication and internal deletion (*ID*) of the duplicated region. From the survival-time plot (Figure 4.4), *An. gambiae s.s.* has a significantly higher survival rate than *An. coluzzii* (log-rank test, $\chi^2 = 24.15$, df =1, $p < 0.001$). For *Ace*-1 genotypes, in order not to bias the analysis, *Ace-1GG* were excluded due to its small sample size in *An. coluzzii*. In both species, *Ace-1SS* showed slightly but not significantly higher survival than *Ace-1GS* (Figure 4.5). Focusing on the duplicated *Ace-1GS* individuals, which showed relatively large numbers of individuals *ID*-present and *ID*-absent individuals in both species, there was no significant difference in the survival of mosquitoes with or without *ID* in either species.

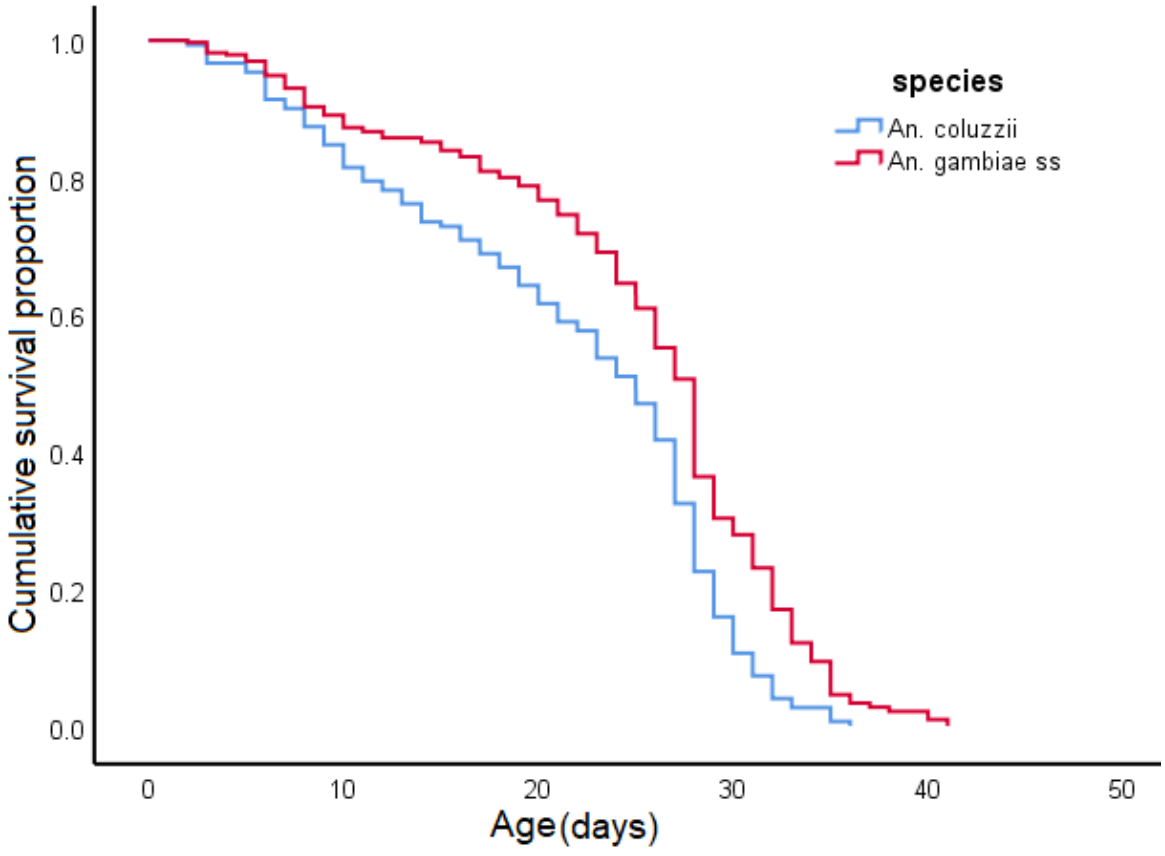
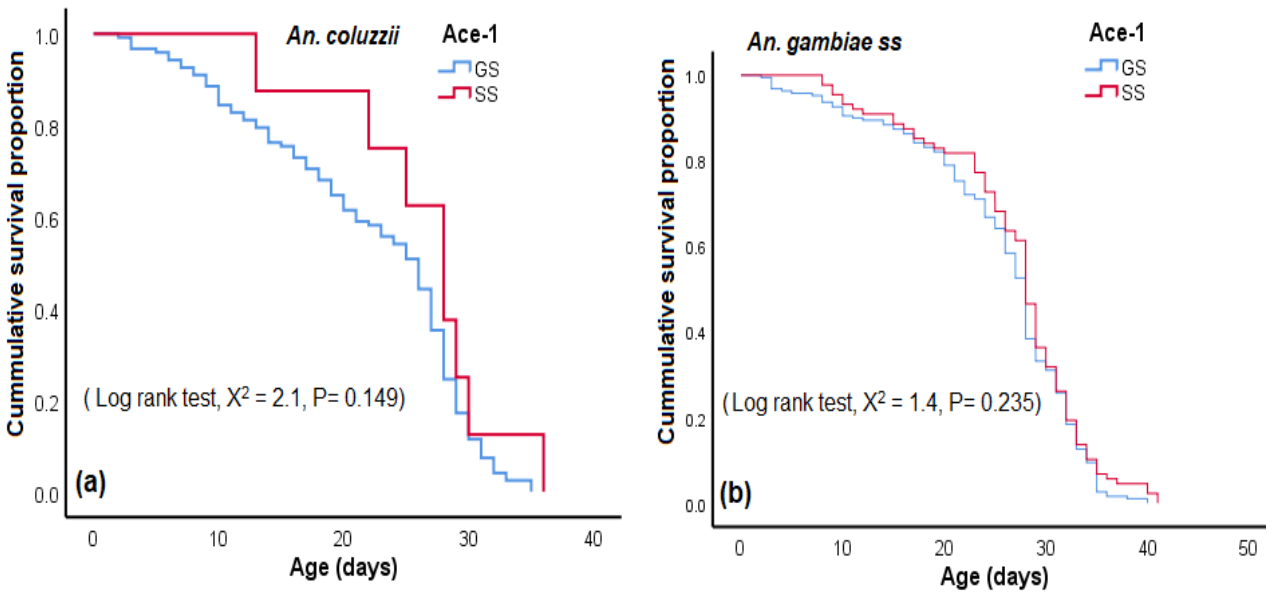


Figure 4.4: The Kaplan- Meier plot demonstrating the probability of survival of *An. gambiae s.s.* and *An. coluzzii* with time. The steps represent the deaths of mosquitoes.



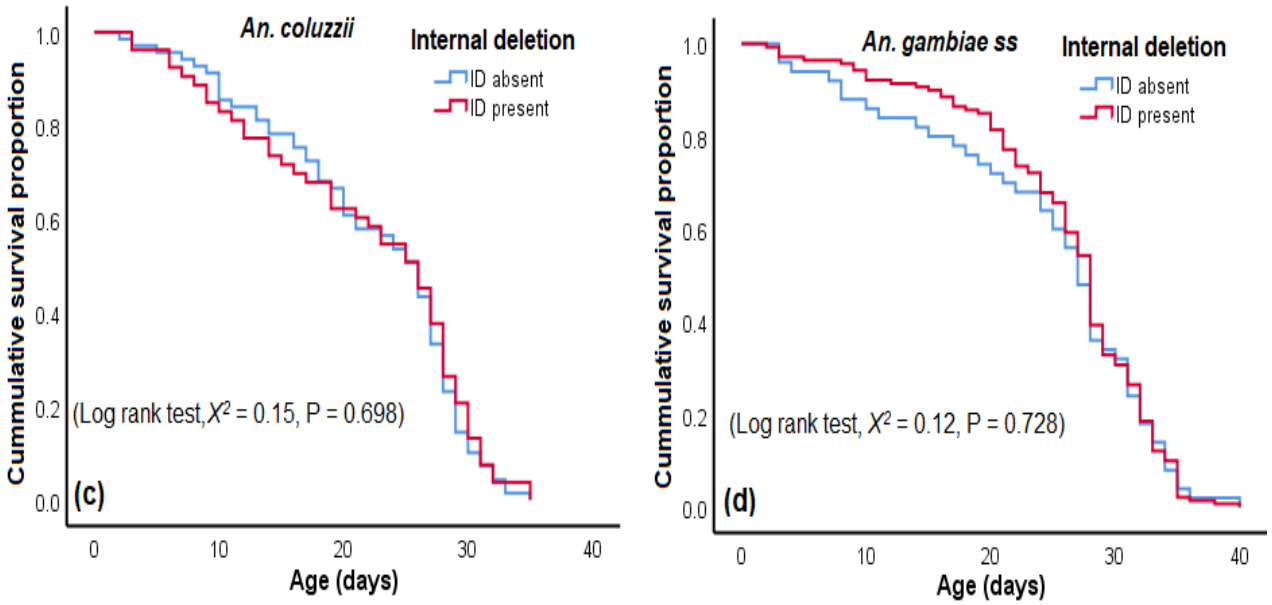


Figure 4.5: The Kaplan-Meier plots showing the probability of survival of *Ace-1 GS* and Ace-1 SS in (a) *An. coluzzii*, and (b) *An. gambiae s.s.* , and the survival of *ID* absent and *ID* present in (c) *An. coluzzii*, and (d) *An. gambiae s.s.*

The Cox's proportional hazards model was used to test the difference between survival times of species, *Ace*-1 genotypes, chromosomal segment duplication status and internal deletion (*ID*) simultaneously. In this model, the response variable is the hazard risk, i.e. the probability of a mosquito dying at a given point in time. The Omnibus test shows the overall model was significant (χ^2 = 32.4, df = 4, p < 0.001). Species and *Ace*-1 duplication made a significant contribution to the model. The interaction did not make a significant contribution to the model. The odds ratio for species suggests that the risk of death for *An. gambiae s.s.* significantly less than that for *An. coluzzii* (Table 4.14). Again, the risk of deaths in individual mosquitoes with *Ace-1* duplication is significantly less than that for individuals without the duplication.

Table 4.14: Cox's proportional hazards analysis for mortality

Parameter	B	χ^2	df	p-value	Odds ratio	95.0% CI for Exp(B)	
						Lower	Upper
species (*An. gambiae s.s*)	-0.37	11.1	1	0.001	0.69	0.55	0.86
Dup_ace-1 (Dup-present)	-0.56	7.5	1	0.006	0.57	0.38	0.85
Ace-1S (SS)	-0.16	1.5	1	0.216	0.85	0.67	1.10
Del_ace1 (Del-present)	-0.12	1.0	1	0.309	0.89	0.70	1.12

**An. coluzzii*, Duplication-absent, *Ace-1-GS* and *ID*-absent were set as reference categories.

4.4 Discussion

The study seeks to investigate the possible association between the age of mosquitoes and pirimiphos-methyl resistance and to provide insights into how *Ace*-1-mediated resistance changes as adult female *An. gambiae s.l.* age. The study utilised bioassay tests to assess the phenotypic resistance of mosquitoes of varying age cohorts to PM and identify the underlying mechanisms that drive the resistance. Results showed a marginal increase in susceptibility to 30-min-PM, but a decrease in susceptibility to 60-min-PM exposure with an increased in age. However, the age-susceptibility relationship following PM exposure is not consistent rather than systematic. However, the possession of at least one resistance *Ace-1* allele enhanced the survival of mosquitoes to PM exposure and this finding is similar to what has been reported in a recent study (Grau-Bové *et al.*, 2020). For a given age cohort, the synergist PBO appears to enhance the survival of mosquitoes. A further study was performed to investigate the cost of possessing different resistant genotypes in the species pair by monitoring the daily mortality rate of unexposed mosquitoes in the laboratory setting.

The phenotypic resistance of *An. gambiae s.s.* and *An. coluzzii* in Madina to pyrethroids, CBs and OPs were quite high with the prevalence varying markedly among the various insecticide classes. This result is similar to the findings of a recent study in Opeibea, a suburb of Accra 13 km away from Madina, where a high resistance prevalence of *An. gambiae s.l.* to deltamethrin, bendiocarb and PM was reported (Pwalia *et al.*, 2019). For the effect of age on susceptibility, the 30 min PM exposure showed a marginal decrease in the survival of mosquitoes. However, for the standard 60 min PM exposure, the results showed no significant difference in the susceptibility of *An. gambiae s.l.* to PM with age. On the contrary, the susceptibility of *An. gambiae s.l.* to DDT and deltamethrin increases with age which was mainly attributed to the decrease in the expression of insecticide detoxification genes (Rajatileka *et al.,* 2011; Machani *et al.*, 2019).The susceptibility of *An. gambiae s.l.* to CB propoxur has also been found to increase with age (Chouaibou *et al.*, 2012). For the few studies that used malathion and bendiocarb, no valid conclusions on the susceptibility with age was made as mosquitoes were fully susceptible even at lower exposure time (Rajatileka *et al.*, 2011; Chouaibou *et al.*, 2012; Mbepera *et al.*, 2017). This may suggest that there is a possible role of the insecticide mode of action or mechanism specificity in the susceptibility of mosquitoes. Again, several previous studies usually used two age groups (e.g., 3 – 5 day and 15 -17 day) (Table 4.1) either due to logistic or rarity of mosquitoes and so no information is provided on the intervening age groups and this may lead to false-positive conclusions. This is because in our results, although there was no consistent trend in the PM susceptibility with age, there was significant variability among age groups. For instance, 18-day old mosquitoes

were significantly more susceptible than 9-day old (χ^2 = 4.2, df = 1, p = 0.040), and the 9-day old mosquitoes were, in turn, more susceptible than 3-day old counterparts (χ^2 = 35.9, df = 1, p < 0.001), and so just relying on these three age cohorts could have resulted in an erroneous conclusion. Furthermore, different populations or strains of the same species of mosquitoes may respond differently to insecticide treatment. For instance, in *An. gambiae s.s.* strain from western Kenya, 14-old females were more tolerant to DDT than 3-day old counterparts, while in *An. gambiae s.s.* strain from Akron, Benin, 14-day old females were more susceptible to DDT than 3-day old (Rajatileka *et al.,* 2011). As such the effect of age on the survival of mosquitoes following insecticide exposure is not a universal phenomenon and may depend on the type of insecticide, the mechanism specificity and the population/strain of the mosquitoes.

An. gambiae s.s. was more common than *An. coluzzii* although they were found in sympatry. There was marginal but significantly higher susceptibility to PM exposure in *An. coluzzii* than in *An. gambiae s.s*. A similar finding has been observed in Gambia where *An. gambiae s.s.* and *An. coluzzii* from the same village differ in their susceptibility to DDT and deltamethrin (Opondo *et al.*, 2016). For a given age, mosquitoes possessing *Ace-1SS* were more likely to survive the PM exposure than *Ace-1*GS, and this may indicate that in CB/OP treated areas, *Ace-1*SS individuals have a higher fitness advantage than *Ace-1*GS individuals (Djogbénou *et al.*, 2009). Although species alone could not reliably predict the phenotypic status of the mosquitoes following PM exposure, the significant interaction found between species and *Ace-1* genotypes may suggest that some latent factors are disproportionally distributed between the species and among the *Ace-1* genotypes that are likely to influence the survival mosquitoes to PM exposure.

The *Ace-1* duplication frequency was surprisingly high in the species pairs in Madina, and PCR diagnostics were even repeated to confirm the findings, although it could not reliably predict the phenotypic status following PM. However, the presence/absence of internal deletion (*ID*) within the duplicated chromosomal segment influenced the survival to PM exposure significantly as mosquitoes possessing *ID* are more likely to survive PM exposure (Table 4.7 and 4.9). The presence of *ID* was quite common in both species (Table 4.8) although it was found to be significantly higher in *An. gambiae s.s.* than in *An. coluzzii*. Previous studies have reported the presence of *ID* in *An. coluzzii* and *An. gambiae s.s.* populations from Togo, Benin and Cote D'Ivoire (Assogba *et al.*, 2018). A recent study has shown that *Ace-1* genotype proportion and copy number are the principal predictors of PM survival (Grau-Bové *et al.*, 2020), and this may be the case for *Ace-1GS* individuals. This is because *Ace-1SS* individuals survive well with PM exposure, however, the response of *Ace-1GS* is very mixed, and so it is likely that the *ID* is the latent variable associated with *Ace-1*

duplication (higher copy number) probably to offset the cost of having more copies (Assogba *et al.*, 2018).

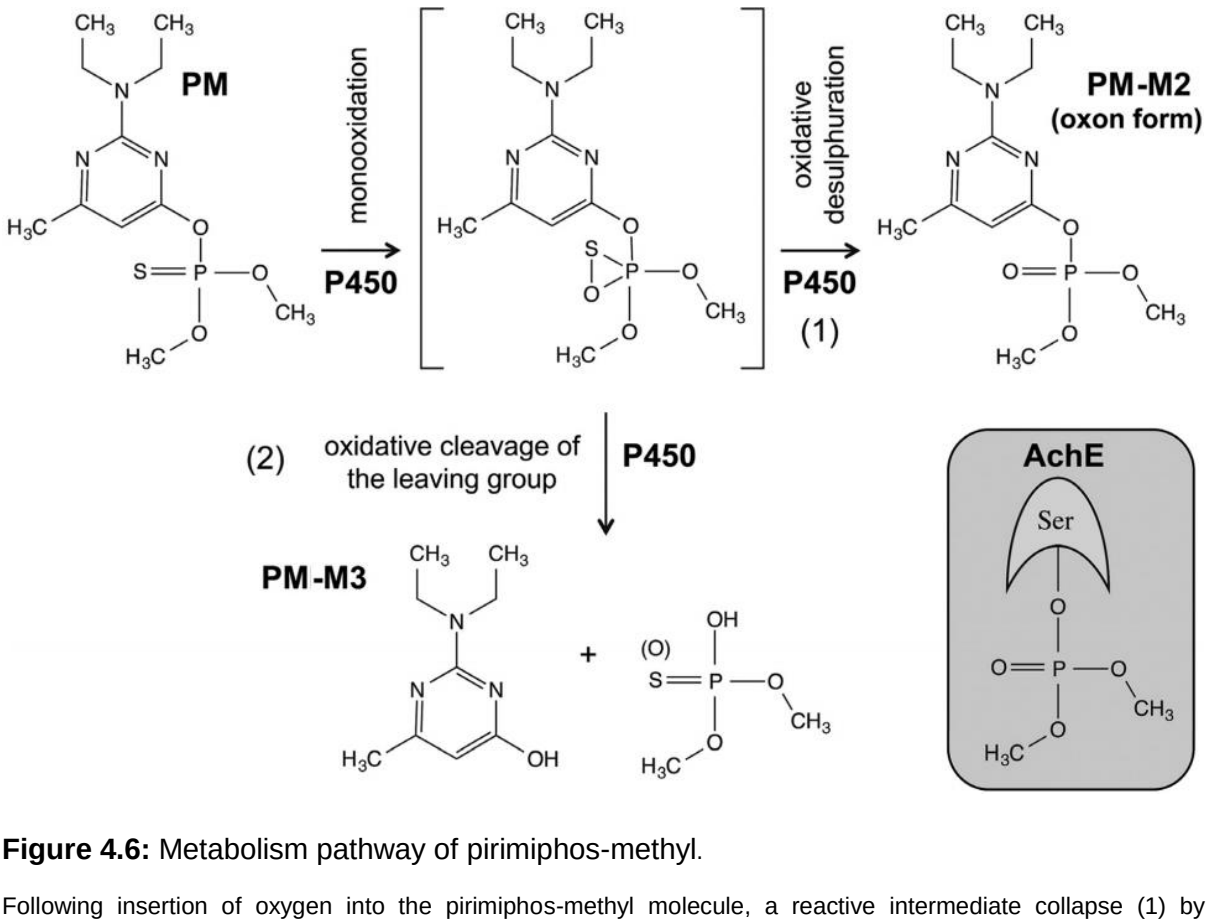


Figure 4.6: Metabolism pathway of pirimiphos-methyl.

Following insertion of oxygen into the pirimiphos-methyl molecule, a reactive intermediate collapse (1) by desulfuration, or (2) by cleavage of the ester linkage leading to PM-M2 and PM-M3 respectively. Alternative routes of P450 metabolism of pirimiphos-methyl that can produce other metabolites are not shown. The oxon form inhibits the AchE by forming a covalent bond with a serine residue at the active site. PM, pirimiphos methyl; PM-M2, pirimiphos-methyl; PM-M3, 2-diethylamino-6-hydroxyl-4-methylpyrimidine and AchE, acetylcholinesterase (Yunta *et al.*, 2019)**.**

Interestingly, rather than increasing susceptibility, pre-exposure to the synergist PBO significantly enhanced the survival of PM-exposed mosquitoes. It has been shown that PM requires various types of P450s for the activation of the insecticide into the active toxic pirimiphos-methyl-oxon as well as the inactive product, 2-diethylamino-6-hydroxy-4-methylpyrimidine (Figure 4.6). The pre-exposure of mosquitoes to PBO results in varying degree of inhibition of the P450s which could cross-react other P450s and may increase or decrease the insecticidal effect of PM insecticidal activity depending on the relative rates of production of the active pirimiphos-methyl-oxon and inactive 2-diethylamino-6-hydroxy-4-

methylpyrimidine (Yunta *et al.*, 2016, 2019). As such, the balance between the two pathways and the exact P450 genes involved are likely to influence the response to PM exposure.

The daily mortality rate of unexposed mosquitoes in the laboratory setting showed that *An. gambiae s.s.* has a significantly higher survival rate than *An. coluzzii.* However, there was no significant difference in the survival rate of *Ace-1GS* and *Ace-1SS* individuals in both species. The comparison on survival rate between resistance genotypes and susceptible ones were not performed because only a few individuals were found to be susceptible. Surprisingly, mosquitoes possessing *Ace-1* duplication exhibited a significantly higher survival rate than those without.

4.5 Conclusion

Our studies found no evidence of an increase in the susceptibility of *An. gambiae s.l.* to PM exposure with age, suggesting that the increase in susceptibility to insecticides in older mosquitoes is not a universal phenomenon and may depend on the insecticide/mechanism specificity and the strain or population of the mosquito. In both *An. gambiae s.s.* and *An. coluzzii*, it was found that *Ace-1SS* individuals are more likely to survive PM exposure than *Ace-1GS* individuals, and also the mosquitoes with internal deletion are more likely to survive PM exposure than those without the internal deletion. Further studies are required to investigate the susceptibility of blood-fed females to PM exposure since anopheline mosquitoes that most like to transmit malaria are the blood-fed ones and also blood meal has been found to transiently reduce the susceptibility of mosquitoes to insecticides (Machani *et al.*, 2019). The synergist PBO appears to enhance the survival of mosquitoes and may be due to the mechanisms by which PM operates and so studies on metabolomics will be imperative to investigate the role of various P450s in PM metabolism as mosquito age. For the cost of possessing *Ace-1S* on longevity, there was no evidence of a difference in the survival rate of *Ace-1GS* and *Ace-1SS* in both *An. gambiae s.s.* and *An. coluzzii,* but the mosquitoes with chromosomal segment duplication showed a slightly higher survival rate than those without.

Chapter Five: Genome-wide analysis of population structure and its determinants in *Anopheles gambiae s.s.* and *An. coluzzii* across southern Ghana.

Abstract

Knowledge of genetic diversity and population structure of *An. gambiae s.s. and An. coluzzii*, the principal malaria vectors in West African, is important to help target vector intervention strategies and to evaluate patterns of resistance and potential for further spread. *Anopheles gambiae s.s.* and *An. coluzzii* with known species identification and resistance marker genotypes were chosen from 34 sites spanning the four ecological zones, and land-use types were genotyped using low-coverage (notionally 5-10X) sequencing. This yielded a total of approximately 10,000 biallelic single nucleotide polymorphisms (SNP), after removal of markers from the Y- and unknown chromosomes and quality control filtering to remove SNPs exhibiting extreme F_{IS} values. Focusing on SNPs from chromosome 3, which lacks common inversion polymorphisms, the analysis showed a low level of genetic relatedness among mosquitoes even though larval sampling was performed within a 3.0-km radius in each study site. There was low genetic differentiation and no population structure within of *An. coluzzii* and *An. gambiae s.s.* across southern Ghana, with almost no evidence of recent introgression between the species pair. *An. coluzzii* showed isolation by distance at a local sampling scale, suggesting there is distance-limited dispersal reflecting contemporary gene flow. In *An. gambiae s.s.*, showed no isolation by distance, even smaller geographical distance of separation, suggesting the observed low differentiation is due to historical patterns rather than contemporary gene flow. Our results also showed that distance is not the primary cause of *Ace-1* differentiation, but rather habitat differentiation which correlates with distance. Apart from selection pressure, the rise and spread of insecticide resistance loci such as *Ace-1*-119S and *Vgsc*-1014F within mosquito populations will depend on the distance and habitat differentiation. Also, in an attempt to use genetically modified mosquitoes to control vector populations in Ghana, the released genetic material is likely to spread rapidly within *An. coluzzii* local populations, but not clear for *An. gambiae s.s*.

5.1 Introduction

Insecticide resistance can be heterogeneous even across relatively small distances (Sarpong *et al.,* 2015; Kisinza *et al.*, 2017; Egyir *et al.*, 2019; Oumbouke *et al.*, 2020). For instance, in southern-eastern Tanzania, *Anopheles arabiensis* showed significant variation in the susceptibility to DDT, pyrethroids and carbamate in three neighbouring villages that are just 4-9 km apart (Matowo *et al.*, 2017). Again, a recent fine-scale study in Bouaké, Ivory Coast, has reported a significant variation in the susceptibility of *An. coluzzii and An. gambiae s.s* to the four main insecticide classes among 40 villages that are a few km to a few tens of apart (Oumbouke *et al.*, 2020). This resistance heterogeneity may reflect the variation in selection pressure exerted directly by insecticides or indirectly by xenobiotics (Nkya *et al.*, 2013, 2014) and/or mosquito population structure across diverse habitat types (Ng'Habi *et al.*, 2011; Gélin *et al.*, 2016; Kaddumukasa *et al.*, 2020).

To develop effective tools to make vector intervention strategies more targeted, there is a need to understand the vector population structure, distribution, movement patterns and connectivity across various landscapes (Saarman *et al.*, 2018). This information is needed to facilitate the tracking and prediction of the spread of genes of interest, such as those conferring insecticide resistance (Lehmann *et al.*, 2003). These require studies that blend genetic variations, spatial patterns and ecological processes which the field of landscape genetics has emerged to address (Manel and Segelbacher, 2009; Manel and Holderegger, 2013). Landscape genetics focuses on the "research that explicitly quantifies the effects of landscape composition, configuration (spatial elements) and matrix quality (space separating species' habitat) on gene flow and spatial genetic variation (Storfer *et al.*, 2007).

Landscape genetics has traditionally been deployed by conservation biologists to identify vulnerable animal populations and corridors to promote gene flow between isolated subpopulations (Storfer *et al.*, 2010). However, landscape approaches are now increasingly utilized in public health, e.g. to identify potential hotspots for health interventions and containment of disease (Hemming-Schroeder *et al.*, 2018) and to investigate the determinants of patterning, and spread of mutations of medical importance including drug or insecticide resistance loci. For instance, landscape genetic analyses have been employed to inform the design and implementation of control activities of tsetse flies (*Glossina fuscipes fuscipes*) in Uganda (Opiro *et al.*, 2017; Saarman *et al.*, 2018), and the triatomines *Rhodnius ecuadoriensis* in Ecuador (Hernandez-Castro *et al.*, 2017) and *Triatoma infestans* in Argentina (Piccinali and Gürtler, 2015). Using landscape analyses, human transport along rivers was identified as the main cause of the spatial spread of *Aedes aegypti* in the Peruvian Amazon (Guagliardo *et al.*, 2019). In São Paulo, Brazil, invasive patterns, expansion and local

adaptation of *Ae. albopictus* was investigated using landscape genetic analyses (Multini *et al.*, 2019). In southern China, using landscape genetics, the patchy distribution knockdown resistance mutations in *An. sinensis* was attributed to the local selection and frequent migrations among populations (Chang *et al.*, 2016).

Studies have identified subdivisions within *An. gambiae s.s.* and *An. coluzzii* populations that are attributed to distance, major geographical features (Lehmann *et al.*, 2000), habitat (Lanzaro and Yoosook, 2013) and seasonality (Midega *et al.*, 2008). For instance, in Kenya, the Rift Valley complex serves as a barrier to gene flow in *An. gambiae s.s.* with differentiated populations on either side of the barrier (Lehmann *et al.*, 2000). A recent study in Lake Victoria, Uganda, has revealed that *An. gambiae s.s.* on the Ssese Islands (60 km from the mainland) are genetically distinct from their mainland counterparts (Lukindu *et al.*, 2018). Again, a macrogeographic population structure study in 12 African countries revealed that *An. coluzzii* is subdivided into distinct west, central, and southern African genetic clusters that correspond to the central African rainforest belt and northern and southern savannah biomes, indicating that restrictions to gene flow are associated with the transitions between the biomes (Pinto *et al.*, 2013). In Ghana, genetic discontinuities have been observed to commensurate with forest-savannah transitions regardless of the species (Yawson *et al.*, 2007). In Cameroon, it has been found that population differentiations within both *An. coluzzii* and *An. gambiae s.s.* correspond to rural-urban divisions (Kamdem *et al.*, 2017). Moreover, *An. gambiae* have a genome that is characterised by multiple polymorphic paracentric chromosomal inversions that are non-random in their distribution throughout the geographical ranges of *An. gambiae* (Coluzz, 1984; Lee *et al.*, 2009; Lanzaro and Yoosook, 2013), and these chromosomal inversions promote local adaptation and ecological divergence (Sharakhov *et al.*, 2006; Ayala *et al.*, 2011; Ayala, Ullastres and González, 2014; Cheng *et al.*, 2018).

Studies on vector population structure and genetic diversity hinge on the use of molecular markers that may provide data to analyse the sources and movements of the vectors (Wei *et al.*, 2019). In *An. gambiae*, relatively small panels microsatellite markers have been used for almost all population genetic studies to date, which may not accurately reflect genome-wide diversity (Väli *et al.*, 2008; Glover *et al.*, 2010; Fischer *et al.*, 2017). To gain improved insights into population structure, particularly at fine scales, it is imperative to apply larger numbers of markers distributed genome-wide. Single nucleotide polymorphisms (SNPs) are far more common in the genome than microsatellites and, if large numbers are screened, are potentially more informative than microsatellites (Daw, Heath and Lu, 2005; Ball *et al.*, 2010). For identification of populations exhibiting spatial demographic independence, thus, those likely to respond independently to control, but experiencing substantial gene flow many

loci are needed (Waples, 1998). The development of a large number of SNP markers for amplification using array ('chip') methodologies is time-consuming and expensive (Weetman *et al.*, 2010; Fu *et al.*, 2017). However, these limitations have been overcome by the development of high-throughput genotyping-by-sequencing techniques, restriction site-associated DNA (RAD), that enables quick detection of SNPs across the entire genome of the focal organism (Wang et al 2012). The RAD-based approaches can generate thousands of SNP genotypes required for linkage mapping and profiling genetic variation in natural populations (Wang *et al.*, 2012). For instance, in Guangzhou, China, double-digest RAD has been used to identify the drivers of gene flow among urban populations of the *Ae. albopictus* (Schmidt *et al.*, 2017). Again, in East Africa, 2b-RAD has been used to show an extensive shared polymorphism but contrasting demographic histories among *An. gambiae s.s*, *An. arabiensis* and *An. merus* population (O'Loughlin *et al.*, 2014). The RAD-based approaches are useful in tracking population differentiation (Barley *et al.*, 2015; Szulkin *et al.*, 2016), however, the high DNA-volume requirement has far limited their applications to insects with large body sizes (Fu *et al.*, 2017).

A recently-developed genotyping technology, Nextera-tagmented reductively-amplified DNA (NextRAD), operates by fragmenting and ligating adaptor sequences to the genome via engineered transposons, and it does use restriction enzymes to reduce the complexity of the genome (Nextera DNA Prep Reference Guide (Illumina, 2016). It requires a very small quantity of DNA, i.e. less than 50 ng (Russello *et al.*, 2015) thereby overcoming the high DNA-volume requirement of RAD, and allowing the generation of sequence data from small insects that were not possible with the RAD applications. The NextRAD has successfully been applied to study genetic diversity and population structure in cassava whitefly (*Bemisia tabaci*) (Wosula *et al.*, 2017), a potato psyllid (*Bactericera cockerelli*) (Fu *et al.*, 2017) and a mosquito species (*An. darlingi*) (Emerson *et al.*, 2015). More recently, it has been used to reveal the existence of population differentiation in life-history traits of *An. darlingi* in response to temperature (Chu *et al.*, 2020).

Traditionally, whole-genome sequencing (WGS) requires loading a single sample onto a lane, and sequencing is performed at an average coverage depth of 20X, 30X, 50X etc. and has been used in several genome-wide association studies (GWAS) in Aedes (Schmidt *et al.*, 2017) and Anopheles (The Anopheles gambiae 1000 Genomes Consortium, 2016, 2020). High-coverage sequencing remains the gold standard for detecting a very rare variant and also in studies where high confidence in genotypes of individuals is a requisite (Li *et al.*, 2011; Buerkle and Gompert, 2013; Malmberg *et al.*, 2018). However, sequencing a large number of individuals becomes challenging due to high cost (Li *et al.*, 2011; Martin *et al.*, 2020). On the contrary, low-coverage WGS involves loading more samples into a lane (each is barcoded so

that data from individuals can be identified out of the mix) and sequencing performed at a reduced coverage for each sample. Recent studies have shown that low-coverage WGS (≥ 4X) using population-specific haplotypes for imputation is an efficient practical alternative to high-coverage WGS (Li *et al.*, 2011; Gilly *et al.*, 2019; Martin *et al.*, 2020) at a cheaper cost. In this study, the NextRAD technique performed the sequencing with an average depth of coverage of 10X. However, the drawback is that read coverage is never uniform and varies among individuals, and when all reads are lined up only the parts of the genome which are covered in the majority of individuals provide data. Using SNPs generated by low-coverage WGS NextRAD technique together with the ecological variable will be used to resolve how patterns of gene flow are influenced by ecological features and how resistance mutations may spread, within an all-encompassing goal of obtaining information for the design of control programmes that reduce the impact of insecticide resistance.

The rapid pace of urbanization and agricultural activities present pollution and insecticidal pressures across sub-Saharan Africa, which may select for locally adapted, differentiated *An. gambiae* populations (Kamdem *et al.*, 2012, 2017). Knowledge of how such discrete populations are connected is essential in identifying how niche adaptation may occur in response to anthropogenic changes, and also for understanding the spread of insecticide resistance. For instance, the selection of *Ace-1-119S* in West Africa is likely to occur at the aquatic larval stage via agricultural insecticide application because these insecticide classes have only recently been used for IRS. Though affected by the number of copies, and whether these shown internal deletions (Assogba *et al.*, 2016; Assogba *et al.*, 2018), possession of the *Ace-1*-119S allele alone is relatively costly in the absence of insecticides (Djogbénou et al., 2010). Agriculture may thus create habitat patches beyond which migration of resistant alleles into untreated areas is selectively unfavourable. In this study, we aimed to quantify the fine-scale population structure of *An. gambiae s.s and An. coluzzii* across southern Ghana to understand how genetic barriers and gene flow may determine the current and future geographical spread of *Ace-1*-mediated insecticide resistance. The population genetic analysis focused mainly on chromosome 3 markers because of the different signal that recombinational variation gives on the other chromosomes (The Anopheles gambiae 1000 Genomes Consortium, 2016, 2020) (The Anopheles gambiae 1000 Genomes Consortium, 2016, 2020). Using a low coverage sequence approach providing almost 10,000 SNP markers in over 300 samples, we seek to address the following research questions

1. What is the population structure exhibited by *An. gambiae s.s.* and *An. coluzzii* populations in southern Ghana and is there evidence of barriers to gene flow?
2. How genetic differentiation is affected by geographical distance, and habitat variations?

3. Do loci, especially *Ace-1* G119S, show evidence of limitation by gene flow and/or habitat?

5.2 Methods

5.2.1 Study sites and mosquito samples

The study involves the use of few mosquitoes from many sites, which provide greater power for landscape genetic analysis (Manel *et al.*, 2003; Willing, Dreyer and Oosterhout, 2012). Mosquitoes were taken from a subsample of the collections described in Chapter 2, with 34 study sites spanning the four major agro-climatic zones in southern Ghana (Figure 2.4). The sampled larvae were collected between April 2016 and October 2017 and raised to adults following the standard insectary protocols. Genomic DNA was extracted individually from each mosquito, and species and *Ace-1* genotype characterizations were performed. Eight mosquito samples - of known species and *Ace-1* genotypes - were picked purposefully to be approximately proportional to the relative frequency of the genotypes from each site, and in study sites where both *An. coluzzii* and *An. gambiae s.s.* coexist, 16 samples (comprising eight of each species) were used (Figure 5.1).

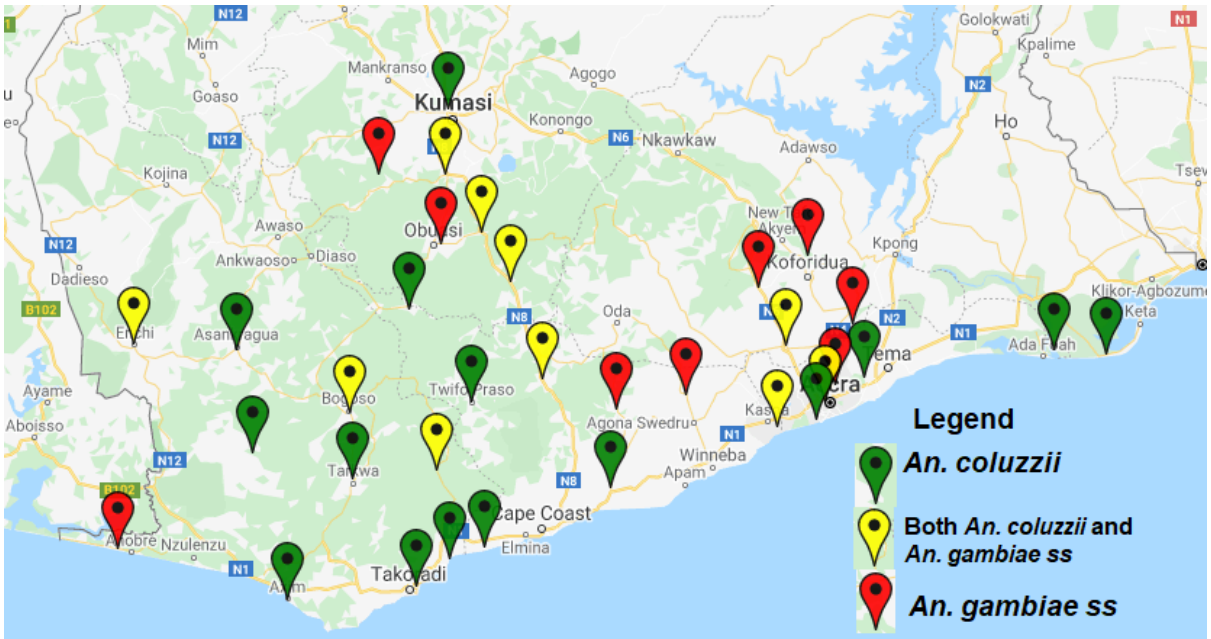


Figure 5.1: Map showing 34 study sites where mosquito samples were collected.

5.2.2 DNA quantification and RNAse Treatment Protocol

A volume of 99-µl of AE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used to dilute 1- µl of DNA of each mosquito sample in each well of an elution plate except. A serial dilution (0, 1:5, 1:10, 1:20, 1:100) of a standard lambda DNA was used to set up a standard curve. The mixture was incubated at room temperature for five minutes and the fluorescence-absorbance of the samples was measured using DNA using a spectro-microplate reader with wavelength excitation = 480 nm and emission= 520 nm. The average absorbance value of the blanks is subtracted from that of each of the samples. The absorbance values of the standard lambda DNA were used to generate a standard curve of fluorescence. Then using the curve, the concentrations of the standard lambda DNA and the volume of extracted, the DNA concentrations of the samples were estimated (Figure 5.2).

(A) Raw absorbance values

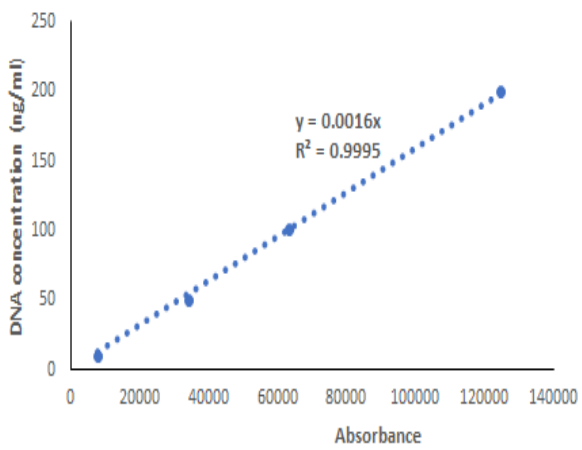
sample	1	2	3	4	5
A	5398.7	3302.7	10307.0	2223.0	124939.0
B	2005.0	4347.7	7574.0	3397.3	63314.3
C	5115.7	7040.0	2367.3	2785.3	34276.7
D	5325.7	2470.7	2908.7	3463.0	7842.3
E	7146.7	3699.7	9199.7	3524.7	1772.7
F	3403.0	3425.0	2395.3	2159.0	1678.7
G	8663.7	4840.0	7149.0	2974.3	1686.3
H	9868.7	8137.7	9037.7	6873.3	1631.7
			Avg absorbance of blanks =		1692.3

(B) Blank reduction

Sample	1	2	3	4	5
A	3706.3	1610.3	8614.7	530.7	123246.7
B	312.7	2655.3	5881.7	1705.0	61622.0
C	3423.3	5347.7	675.0	1093.0	32584.3
D	3633.3	778.3	1216.3	1770.7	6150.0
E	5454.3	2007.3	7507.3	1832.3	80.3
F	1710.7	1732.7	703.0	466.7	-13.7
G	6971.3	3147.7	5456.7	1282.0	-6.0
H	8176.3	6445.3	7345.3	5181.0	-60.7

The red and green hightlights are the standard DNA and the blanks respectively

(C) Standard curve



(D) Concentration of DNA (ng/ul)

Sample	1	2	3	4
A	1.2	0.5	2.8	0.2
B	0.1	0.8	1.9	0.5
C	1.1	1.7	0.2	0.3
D	1.2	0.2	0.4	0.6
E	1.7	0.6	2.4	0.6
F	0.5	0.6	0.2	0.1
G	2.2	1.0	1.7	0.4
H	2.6	2.1	2.4	1.7

Figure 5.2: The illustration of the steps in the estimation of DNA concentration of samples selected for whole-genome sequence

The DNA samples were further treated with RNase following the manufacturer's protocols to remove RNA contamination. The products were incubated at home temperature for 30 min and then run electrophoresis with 2.0% gel agars stained with Peqgreen dye for 45 min. The presence of a band under UV was regarded as a good quality DNA sample for sequencing. The DNA samples were stored in dry ice and sent to SNPsaurus, Oregon, USA, for (low-coverage) genome sequencing (approximately 5-10X).

5.2.3 Quality control and data analysis

2.2.3.1 Read Quality checks

The raw reads in variant call format (VCF) from SNPsaurus sequencing were checked to select SNPs with good quality reads using a Phred score of 30. A Phred score of 30 indicates the probability that a given base is called incorrectly by the sequencer is 1 in 1000 times. The reads were then aligned to the *Anopheles gambiae* PEST (chromosomes) reference genome and samples with bacterial contamination and very low read coverage were removed. An admixture analysis was performed to assign samples to *An. gambiae s.s.* and *An. coluzzii*, identify hybrids and confirm PCR-species diagnostics using Bayesian Analysis of Population Structure (BAPS) v 6 (Corander *et al.*, 2013). The analysis was performed with pre-defined cluster mode and prior indices of clusters assigned 3, indicating three populations (i.e. *An. gambiae s.s.*, *An. coluzzii* and hybrid). Samples with admixtures (hybrids) were not incorporated in the subsequent analyses.

5.2.3.2 Missing data, Hard-Weinberg Equilibrium (HWE), minor allele frequency (MAF) filtering and Linkage disequilibrium (LD)-based pruning

Marker loci with at most 7% missing data were retained for further analysis (Anderson *et al.*, 2010). Haploview software was used to compute the pairwise LD among all markers, HWE and MAF for each marker. Markers with a low MAF tend to give limited information, and may also lead to incorrect estimates of population structures (Ahrens *et al.*, 2018; Linck and Battey, 2019). The MAF threshold was set at 0.01. Markers displaying extreme deviation from HWE evident as significant after Bonferroni correction (here corrected $\alpha = 0.000005$). To avoid strongly non-independent markers in the dataset, LD was computed and regions with elevated LD were removed, then the retained regions were pruned such that no pair of SNPs within 50kb window exhibited LD at a level of $R^2 > 0.2$) using Plink (Purcell *et al.*, 2007). In this

scenario, when R^2 > 0.2 for a pair of SNP markers, the marker with higher missing data, or lower MAF is removed.

5.2.3.3 Relatedness analysis

Understanding the degree of relatedness between individuals is important in population genetic analyses and inclusion of very closely related individuals may bias the studies as there will be over representation of genotypes within families (Anderson *et al.*, 2010). A metric used to identify related individuals is identity by descent (IBD), which is a degree of recent shared ancestry between pair of individuals computed from the genetic markers by maximum likelihood estimate (MLE) (Milligan, 2010) using MLrelate software (Kalinowski *et al*., 2006). The MLE has a lower mean square error and performs well with a relatively small sample size (Milligan, 2010). When two Individual samples are exhibiting high levels of relatedness, one of them is removed. This analysis is particularly important here because mosquito larval samples were used and a previous study had shown that larvae collected from pools within a 3-km radius are highly related (Lehmann *et al.*, 2003). A Grubb's outlier test was performed to identify populations exhibiting an extremely high level of relatedness ($p < 0.05$, indicating a significant outlier).

5.2.3.4 Genetic diversity and differentiation

To investigate the population diversity and differentiation among mosquito species the chromosome 3L and 3R, which are selectively neutral (no inversions) (The Anopheles gambiae 1000 Genomes Consortium, 2020), were used. To measure genetic diversity, GenAlex v6 and Genetic Data Analysis (GDA) software were used to compute the total number of alleles (Na), the effective number of alleles (Ne) (it is a basic measure of genetic variation which a reciprocal of homozygosity and it describes the number of alleles that would be expected in a locus in each population (Tajima *et al.*, 1994; Bhandari *et al.* 2017), Shannon's information index (I) (it allows for the discrimination of the various levels among populations with the same number of alleles (Sherwin *et al.*, 2017; Konopiński, 2020), observed heterozygosity (Ho), expected heterozygosity (He), and the within- and among-individual inbreeding coefficients F_{IS} and F_{ST}.

5.2.3.5 Population structure analysis

A linear transformation of Nei's genetic distance was performed via covariance matrix with data standardization. A principal coordinate analysis (PCoA) was conducted to identify populations with genetically similar individuals using GenAlex v 6 (Peakall and Smouse, 2006). The plotting was done only on the eigenvalue of the first two axes that accounted for the most cumulative variance. The computerised Nei's genetic distance was also used to construct a dendrogram, which is a maximum likelihood and hierarchical method tree that shows a phylogenetic relationship existing among populations. The dendrogram was constructed by the unweighted pair group method (UPGMA) using a PhyML online tool (Guindon and Gascuel, 2003). The Nei's genetic distance has been shown to exhibit a higher efficiency in phylogeny construction compared to the standard genetic distance, F_{ST} (Takezaki and Nei, 2008).

Cluster analysis was conducted with BAPS software v6 (Corander *et al.*, 2013) that applies a Bayesian stochastic partition approach to identify the number of clusters within the populations of each species pair. With SNPs data converted into Genepop format, the analysis was run with default settings and with the maximum number of clusters (*K*) set at multiple levels up to *K* = 20 in every case. The BAPS was used owing to greater speed and it is also useful to investigate a much larger range of possible values of *K*. However, where no clustering was found, cluster analysis was also conducted using STRUCTURE software to validate the results. STRUCTURE is a Bayesian-algorithm based cluster analysis that software applies Markov Chain Monte Carlo (MCMC) (Pritchard *et al.,* 2000). Four iterations were conducted for each inferred *K* (where *K* is the number of clusters) which ranged from 1 to 5. For each run, 10000 repetitions of burn-in and 20000 MCMC repetitions. The optimal value of *K* is estimated based on post hoc analyses using ln Pr(X|K) method software (Pritchard *et al.,* 2000).

5.2.3.6 Spatial Analysis

Genetic differentiation (distance) between each pair of populations (F_{ST}) was computed using Genepop software v 4.7 (Rousset, 2019), using the formula below:

$$F_{st} = \frac{H_T - H_S}{H_T}$$

Where H_T is the expected heterozygosity in the total population and H_s the mean expected heterozygosity within subpopulations.

For the geographical distance between each pair of populations, the GPS coordinates were used to compute using Geographic Distance Matrix Generator v 3 software (Ersts, 2011). Mantel tests were then performed to assess the spatial dependence averaged over all distances by correlating the genetic and the geographical distance matrices via permutation of 10000 using ZT software (Bonnet and Van De Peer, 2002). For habitat matrix, pairwise sites with similar ecological zones were coded as 1, while dissimilar pairwise sites were coded as 0. Correlations were also performed between genetic distance and habitat similarity matrix, and then a partial Mantel test performed between genetic distance and habitat while controlling the effect of geographic distance. To test the changes in correlation at different distances to identify spatial structure at a fine-scale, a Mantel correlogram was used to generate a series of estimated correlation (***r***) computed for specified successive distance classes and the significance was determined after sequential Bonferroni correction (α/n, where α = critical value, 0.05, and n = ranked class i.e. 1, 2, 3,4,5). A multivariate Mantel correlogram was constructed by plotting genetic distance (F_{ST}) with multiple non-overlapping and contiguous distant classes. A distogram was generated to assess the spatial patterns in genetic structure by plotting the average F_{ST} against the average geographic distance within each defined distance class.

5.2.3.7 Outlier detection analysis

With no evidence of population structure and weak significant differentiation within the populations of each of the species pair from the analysis of large numbers of SNPs, an outlier detection analysis was performed on unpruned data to identify extreme loci to look for differentiation. The Bayescan software v 2.1 (Foll, 2012) was used to investigate loci that are

likely to be under selection at the population-level, habitat-level and land-use level based on genetic differentiation (F_{ST}). Bayescan implements a reversible-jump MCMC algorithm to estimate the posterior probability that each locus is subject to selection and it is very efficient in detecting a high percentage of true selective loci under a fully neutral model (Pérez-Figueroa *et al.*, 2010). The analysis was performed on each species by the computation run using thinning interval =10, pilot runs = 20, pilot run length = 5000, burn in = 50000 and a prior odd = 10 (Figure 5.3).

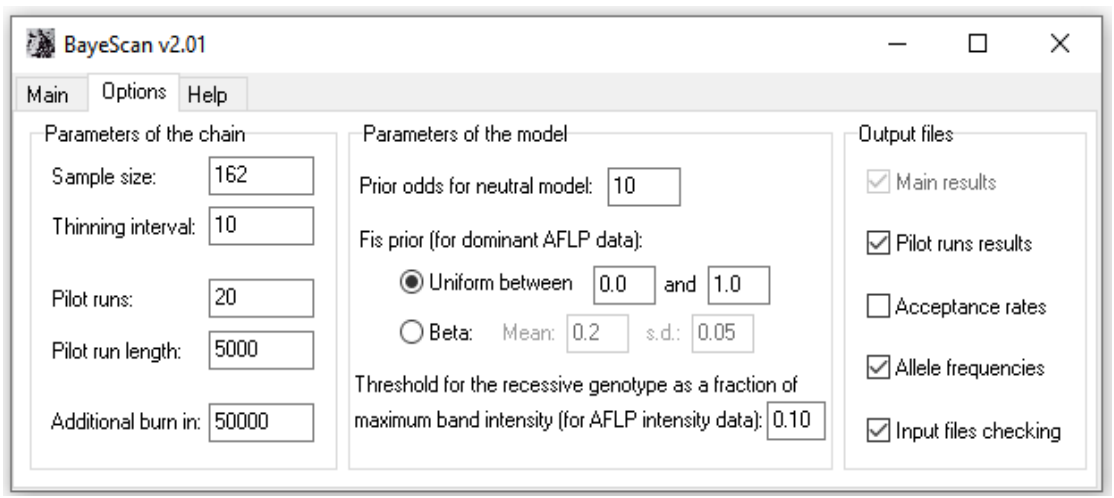


Figure 5.3: The Bayescan v 2.1 showing the input parameters for performing a reversible-jump MCMC.

For each SNP marker, Bayescan computed a posterior probability (*P*), posterior odds (the ratio of posterior probabilities expressed in logarithm to base 10), the estimated alpha coefficient indicating the strength and direction of selection (+ve alpha = diversifying selection and -ve alpha = purifying selection) and the F_{ST} averaged over populations. The decision on locus outlier was made based on posterior odds ($\log_{10}$ PO), with a locus considered to be a significant outlier if $\log_{10}$PO > 0.5. A plot of F_{ST} against log10 (PO) with a vertical line showing the critical point of $\log_{10}$PO = 0.5 was used to identify loci under selection.

5.3 Results

5.3.1 Marker and sample quality control and initial investigations

Following removal of contaminated samples and low-quality reads, chromosomes 2, 3 and X exhibited over 10000 SNPs common to all samples (Table 5.1). Samples with >20% missing data were excluded, resulting in 320 individual samples. As an initial investigation of the

performance of the SNPs, we used X chromosome SNPs (which are expected to show the strongest signal of interspecific differentiation within the *An. gambiae* complex) in a clustering analysis in BAPS. The best solution identified two clusters corresponding perfectly to *An. gambiae s.s.*, *N* = 162 and *An. coluzzii, N* = 153, also including eight samples showing evidence of significant, though relatively low, genomic admixture (Figure 5.4). The distribution of differentiation along the X chromosome between species (i.e., clusters) was investigated by computing F_{ST} for each SNP. Plotted against the position on the chromosome (Figure 5.5), differentiation showed the general profile expected for *An. gambiae s.s.* vs *An. coluzzii* in Ghana (where interspecific introgression appears rare) with the highest differentiation in the latter regions, especially toward the centromere (e.g. Clarkson et al. 2014). Before proceeding to further analyses, filtering was performed within each species to remove SNPs deviating from the specified thresholds for missing data, MAF, and HWE (Table 5.2).

Table 5.1: The data summary showing (a) the number of SNPs per chromosome and (b) the number of samples based on filtering criteria

a) Chromosome	Number of mapped, polymorphic SNPs pre-filtering	Number of SNPs after initial quality control filtering
2L	1944	1753
2R	2724	2486
3L	1464	1320
3L	2064	1832
X	3194	2875
Total	11365	10266

b) Species	Number of samples sent for sequence	Number of samples after removal ≥ 20% missing data	Number of samples After removing admixtures
An. coluzzii	205	168	162
An. gambiae s.s.	182	155	153
Total	384	323	315

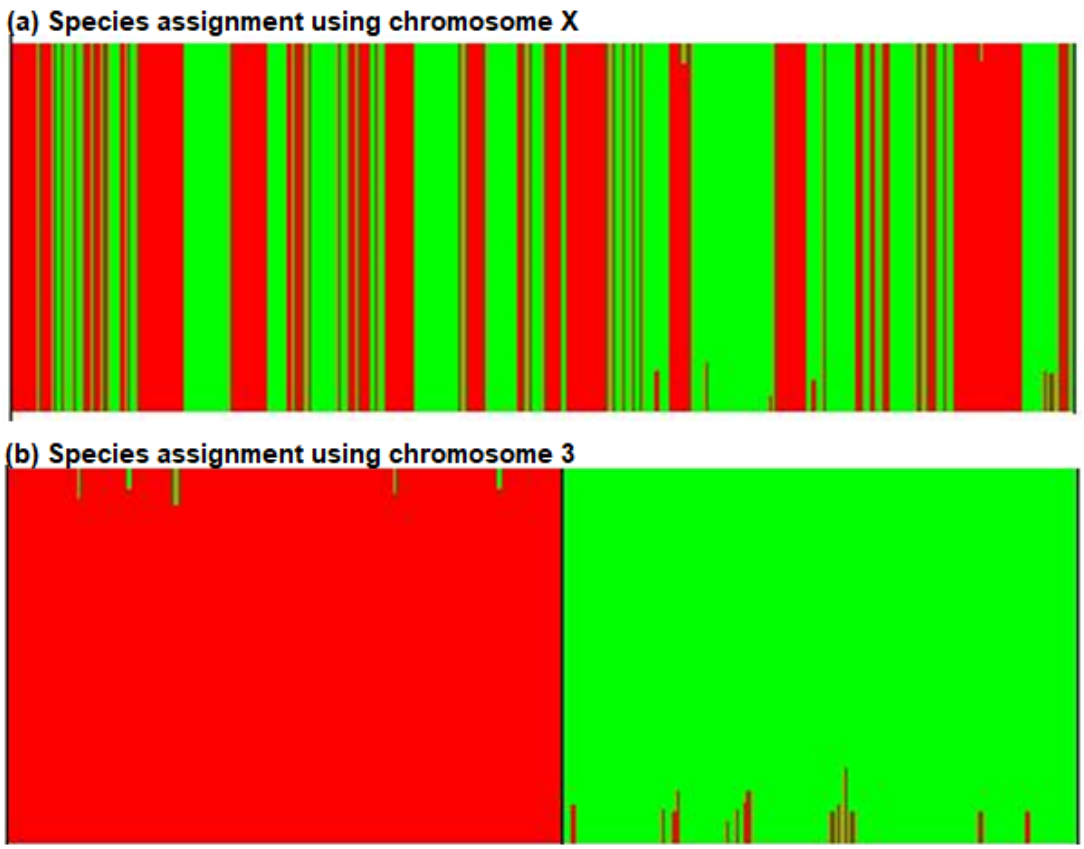


Figure 5.4: Cluster assignment of species from at *K* = 2 using (a) Chromosome X and (b) chromosome 3. The red corresponds to *An. coluzzii* while the green corresponds to *An. gambiae s.s.* The short projections are the admixtures that indicate hybridizations.

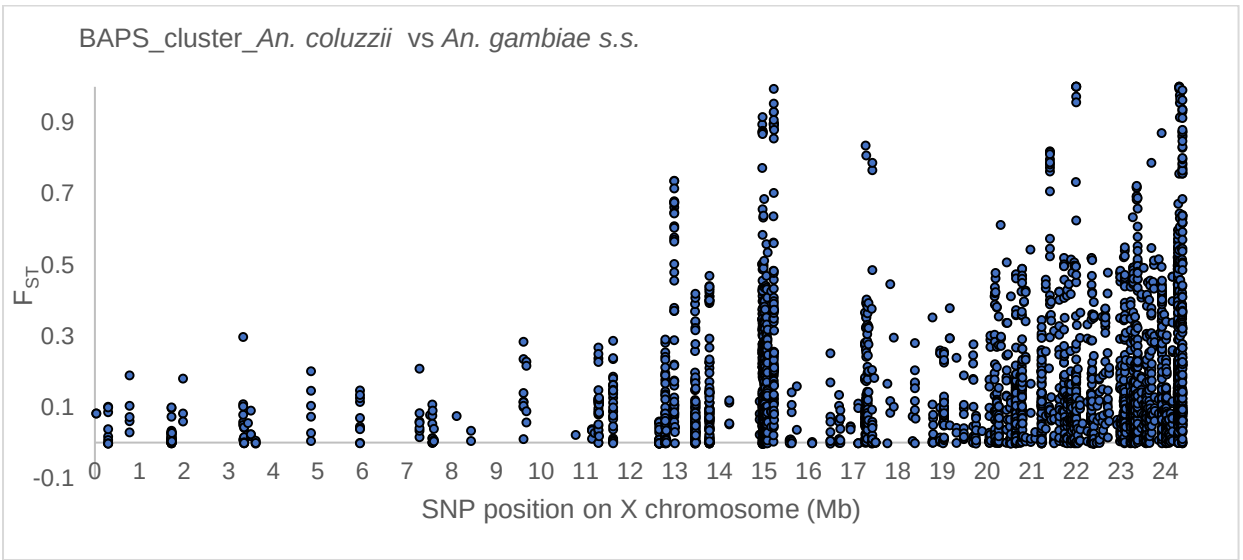


Figure 5.5: A plot of SNP loci F_{ST} against the loci position showing the profile of *An. gambiae s.s*. versus *An. coluzzii***.** The loci distribution pattern for the pairwise comparison of the F_{ST} between the species is similar to Clarkson et al (2014).

Table 5.2: The data summary showing the number of SNPs per chromosome and based on filtering criteria in the species pair.

Species	Chromosome	Missing data (>7%)	MAF (< 0.01)	HWE (< 0.000001)
An. coluzzii	2L	1650	1650	1300
	2R	2088	1678	1303
	3L	954	762	560
	3R	1424	1212	937
	X	2367	1749	1302
An. gambiae s.s.	2L	1498	1260	946
	2R	2188	1841	1369
	3L	1196	971	759
	3R	1628	1399	847
	X	2493	1948	1408

5.3.2 Linkage disequilibrium of the loci in each species

A plot of missing data against locus position showed that SNP distribution was non-uniform along all the chromosomes with markers tending to occur in clumps (Appendix 9), raising the possibility of more markers in linkage disequilibrium (LD) than might normally be expected in *An. gambiae* s.s. low-LD genome (Neafsey *et al.*, 2010). The LD between adjacent loci on each chromosome for each species was determined to identify potentially non-independent markers for removal prior to subsequent analyses. The LD was visualised using decay plots which are scattered plots of LD coefficient R^2 versus physical distances between all loci pairs along the chromosomes for each species (Figure 5.6 and 5.7). As expected, SNP data appeared to show a rapid decay of LD with physical distance in both species. After pruning, where one of the two markers with higher missing data or lower minor allele frequency is removed), no pair of SNPs within the 50kb window exhibited LD at a level of $R^2 > 0.2$, the proportion of remaining loci are shown in Figure 5.8.

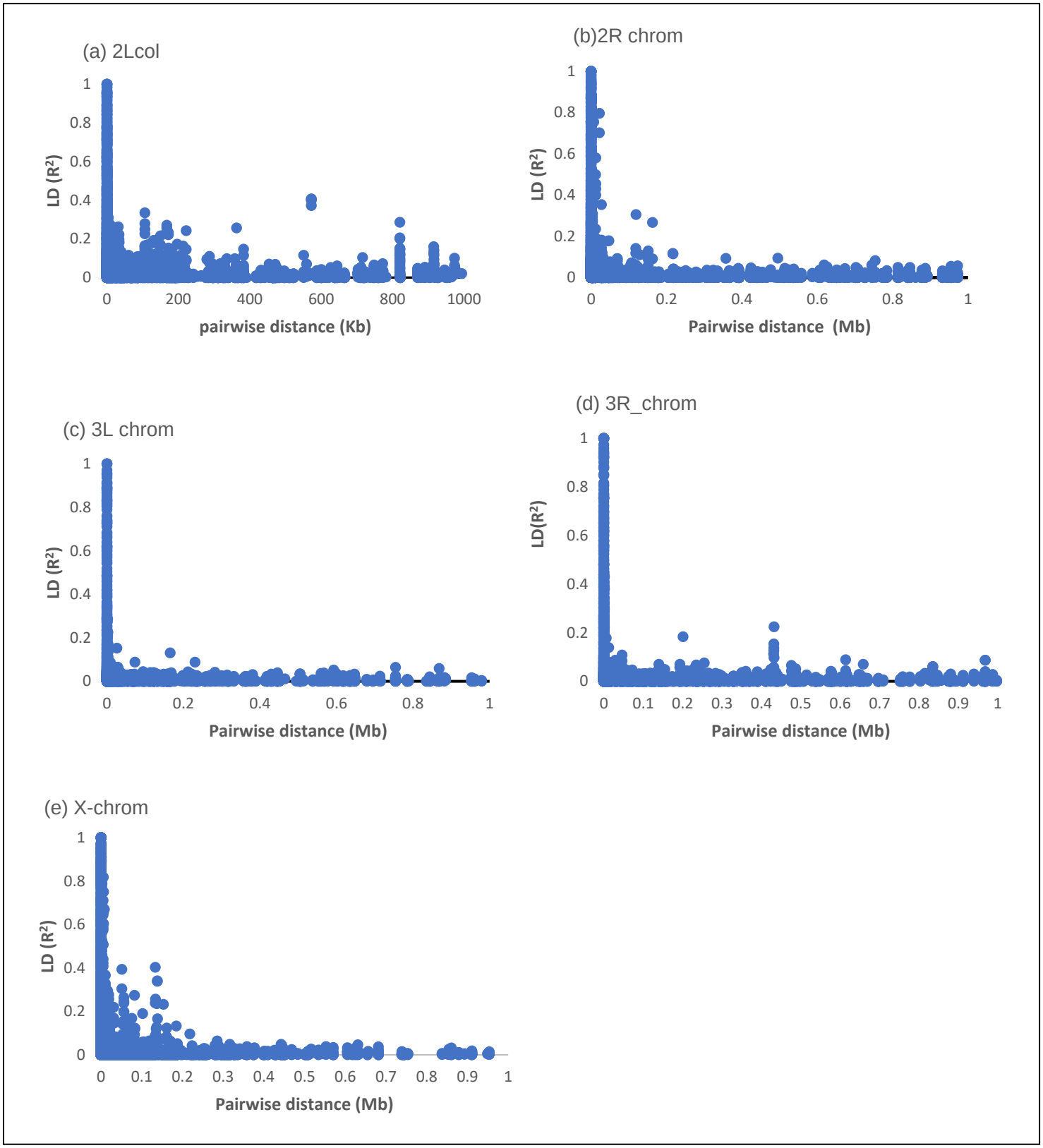


Figure 5.6: The plots of LD decay over distance for five chromosomes in *An. coluzzii*. Each data point represents a pairwise measure of LD coefficient, R^2, as a function of physical distance in bases.

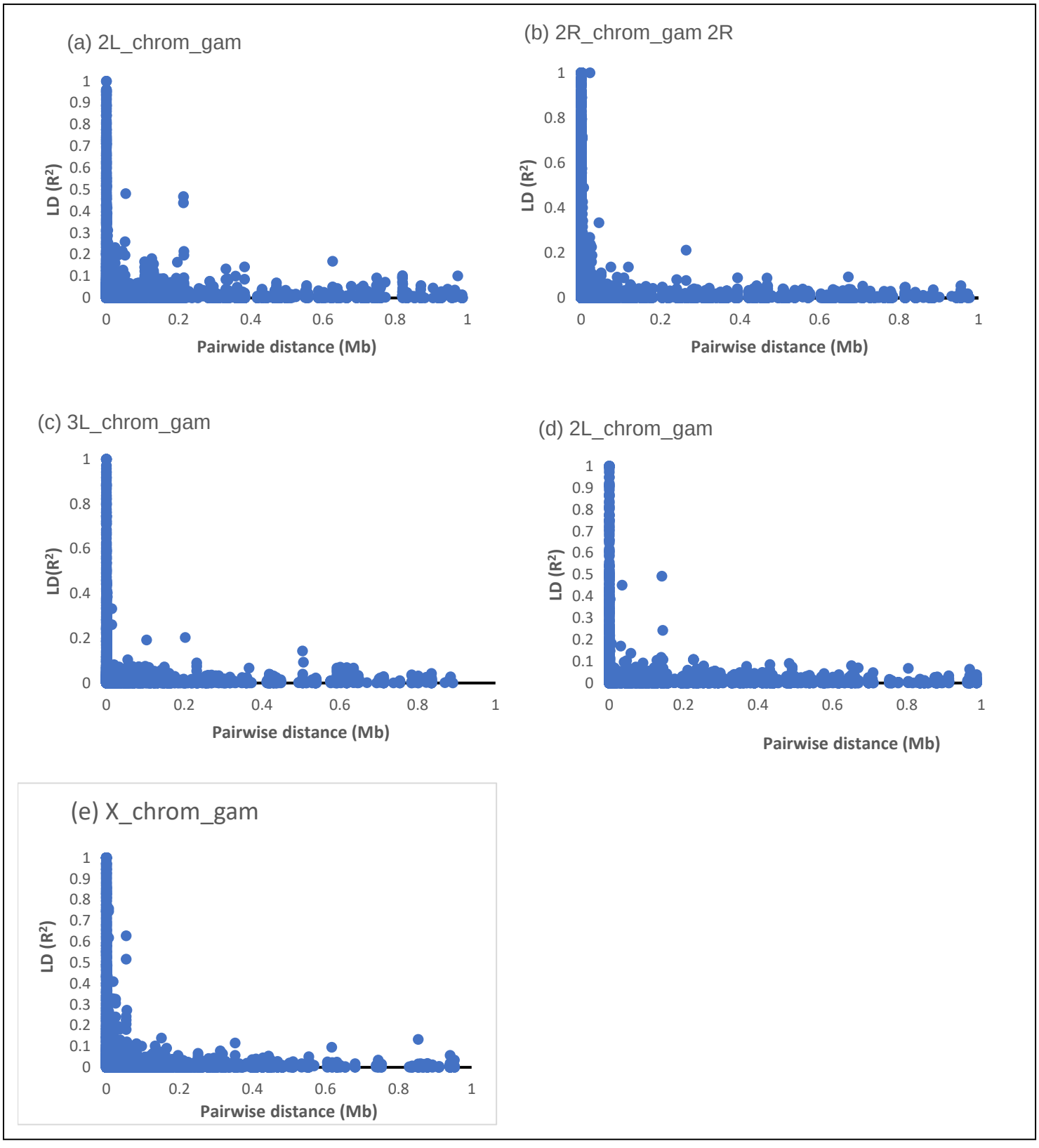


Figure 5.7: The plots of LD decay over distance for five chromosomes in *An. gambiae s.s*. Each data point represents a pairwise measure of LD coefficient, R^2, as a function of physical distance in bases.

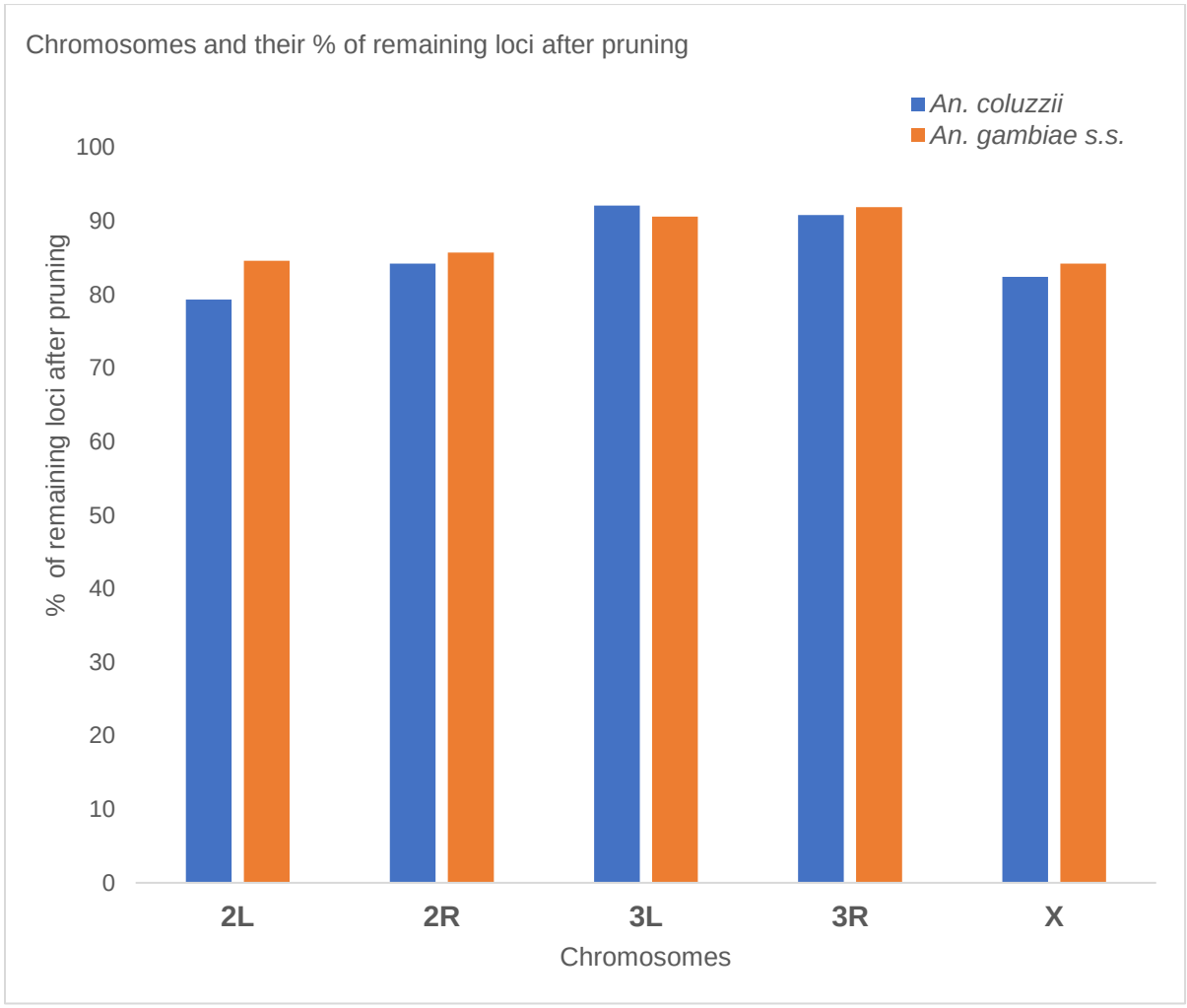


Figure 5.8: The percentage of remaining loci after LD pruning 50 kb window exhibited LD at a level of $R^2 > 0.2$ with 50kb window**.** The pruning was performed such that when two loci within 50 kb window have LD coefficient greater than 0.2, the one with the higher missing data or lower minor allele frequency is removed.

5.3.3 Relatedness analysis

The relatedness between individual pairs of mosquitoes was estimated using chromosome 3 SNPs for both *An. coluzzii* and *An. gambiae s.s*. The MLE estimation showed that the median percentage relatedness for *An. coluzzii* and *An. gambiae s.s.* were 3.6% and 6.5 % respectively, indicating that the great majority of individual mosquitoes are not siblings. However, there were a few outlier collections with higher levels of relatedness among individuals in both *An. coluzzii* and *An. gambiae s.s.* (Table 5.3). To reduce non-independence (arising from siblings) in the dataset one of the pairs of individuals with full-sib (0.5) relatedness was removed for further analysis.

Table 5.3: The estimation of relatedness by maximum likelihood using MLrelate software for all populations in both (a) *An. coluzzii* and (b) *An. gambiae s.s*. The FS = full sibling, HS = half-sib and U = unrelated and percentage of relatedness (FS + HS/Total * 100).

(a) *An. coluzzii* relatedness estimation

Population	individuals	pairwise comparison	FS	HS	U	%	sig. outlier
Ada Foah	7	21	0	2	19	9.5	
Assin Fosu	7	21	0	0	21	0	
Anhwiankwanta	4	6	0	0	6	0	
Asankrangwa	5	10	0	0	10	0	
Ashaiman	6	15	0	0	15	0	
Asokwa	7	21	0	2	19	9.5	
Axim	8	28	0	1	27	3.6	
Bantama	4	6	0	1	5	16.7	
Bogoso	8	28	1	3	24	14.3	
Dunkwa-on-Offin	5	10	0	0	10	0	
Dzorwulu	8	28	0	0	28	0	
Enchi	9	36	0	1	35	2.8	
Komenda	8	28	0	1	27	3.6	
Mamprobi	7	21	0	1	20	4.8	
Mankessim	7	21	0	0	21	0	
Manso-Nkwanta	7	21	0	0	21	0	
New-Edubiase	6	15	0	0	15	0	
Nsawan	8	28	0	7	21	25	< 0.001
Shama	6	15	0	0	15	0	
Takoradi	8	28	0	0	28	0	
Tarkwa	6	15	0	0	15	0	
Twi Praso	8	28	0	0	28	0	
Tuba	7	21	0	0	28	0	
Wassa Essaman	5	10	0	0	10	0	

(b) *An. gambiae s.s.* relatedness estimation

POP	individuals	pairwise comparison	FS	HS	U	%	sig. outlier
Assin Fosu	8	28	0	0	28	0	
Agona Dunkwa	4	6	0	0	6	0	
Akatsi	5	10	0	0	10	0	
Anhwiankwanta	5	10	0	0	10	0	
Asikumah	7	21	1	2	18	14.3	
Asokwa	7	21	0	1	20	4.8	
Bogoso	8	28	0	2	26	7.1	
Dodowa	8	28	0	1	27	3.6	
Dzorwulu	7	21	0	1	20	4.8	
Enchi	6	15	0	3	12	20	
Half Assini	6	15	0	0	15	0	
Koforidua	8	28	2	11	15	46.4	< 0.001
Madina	19	171	0	2	169	1.2	
New Eduabiase	9	36	0	1	35	2.8	
Nkwanta	8	28	0	1	27	3.6	
Nsawan	8	28	2	4	22	21.4	
Obuasi	15	105	0	1	104	1	
Suhum	7	21	0	0	21	0	
Tuba	5	10	0	0	6	0	
Wassa Essuman	4	6	0	0	6	0	

5.3.4 Genetic diversity

The sequencing revealed 1606 SNPs in *An. gambiae s.s.* and 1497 SNPs in *An. coluzzii*; all SNPs were polymorphic (and with MAF > 0.01 within each species dataset). In *An. coluzzii*, the overall proportion of polymorphic loci was 67% (95% CI, 64.4 – 69.3%), ranging from 52% to 75% in the Anhwiankwanta and Enchi samples respectively (Figure 5.9a). In *An. gambiae s.s.*, the overall proportion of polymorphic loci was 63% (95% CI, 60.7 – 65.5%), ranging from 51.2% in Tuba to 87.5% in Madina (Figure 5.9b). The proportion of polymorphic loci in *An. gambiae s.s.* was marginally lower than in *An. coluzzii* (Mann Whitney, U = 131, p = 0.016). These findings indicate that there is a considerable level of distinctiveness existing among the *An. gambiae s.s.* populations.

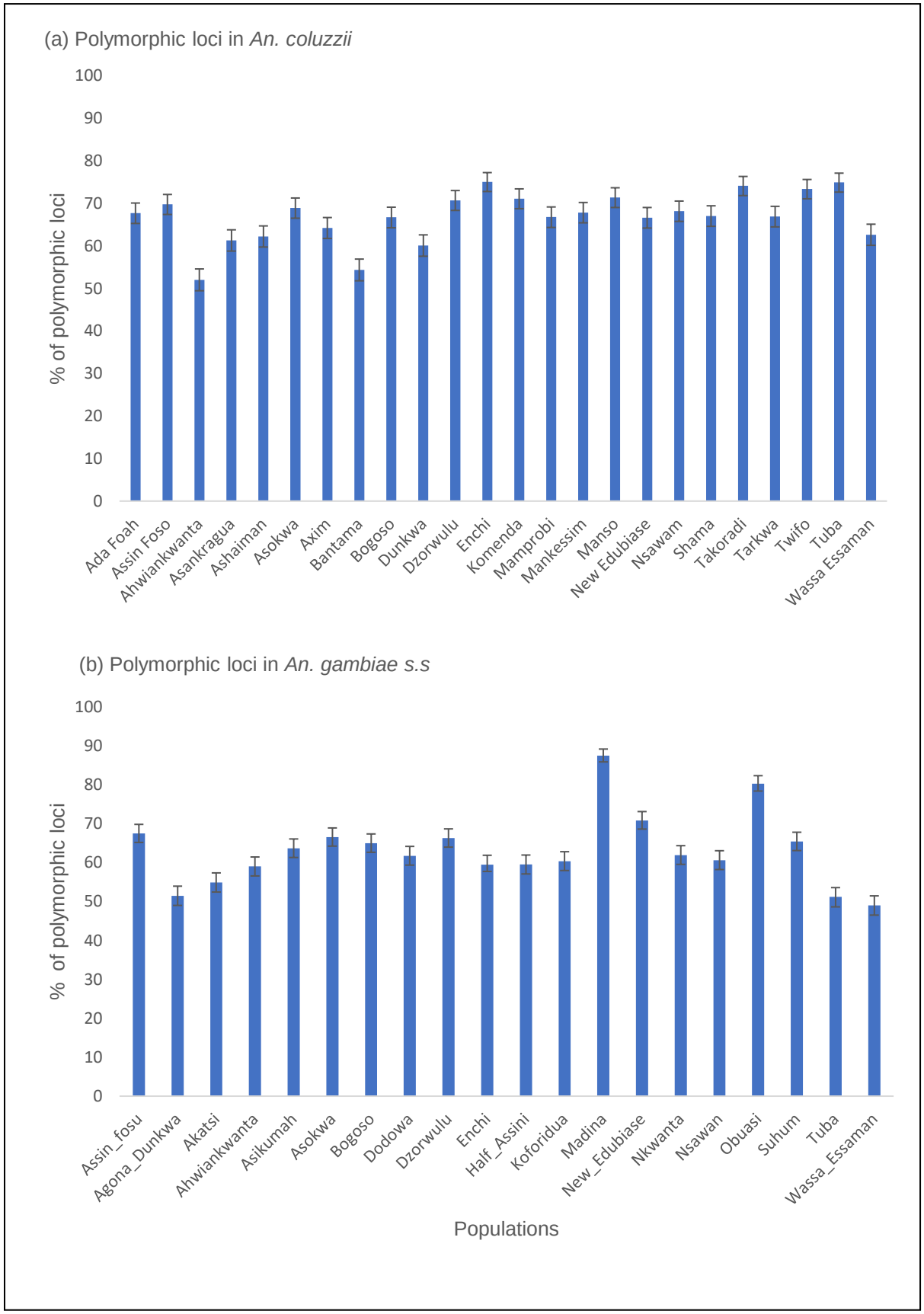


Figure 5.9: The distribution of polymorphic loci in the population of (a) *An. coluzzii* and (b) *An. gambiae s.s.*

Table 5.4 shows the mean number of different alleles (*Na*) among the populations in *An. coluzzii* was 1.67 (±0.002) ranging from 1.52 in Anhwiankwanta to 1.74 Enchi. The mean number of different alleles (Na) among the populations in *An. gambiae s.s.* was 1.63 (±0.003), ranging from 1.51 in Tuba to 1.88 in Madina. For the number of effective alleles (Ne), the average among *An. coluzzii* populations was 1.24 (±0.001), ranging from 1.22 to 1.25, while in *An. gambiae s.s.* the average among the populations was 1.26 (±0.002), ranging from 1.25 to 1.28. For Shannon's index (I), the average among *An. coluzzii* populations was 0.27 (±0.001), ranging from 0.25 to 0.28, while in among *An. gambiae s.s.* populations, the average was 0.27 (±0.001), ranging from 0.25 to 0.31. The allelic richness ranged from 1.52 in Anhwiankwanta to 1.75 in Tuba and Enchi for *An. coluzzii* populations, while in *An. gambiae s.s.*, it ranged from 1.27 in Wassa Essaman to 1.87 in Madina.

For the observed heterozygosity (*Ho*), it ranges from 0.19 in Axim to 0.22 in Nsawam with an average of 0.21 among *An. coluzzii* populations. Among *An. gambiae s.s.* populations, the observed heterozygosity ranges from 0.21 of Wassa Essaman to 0.24 of Anhwiankwanta with an average of 0.22. The observed heterozygosity among *An. gambiae s.s.* populations was significantly different from that *An. coluzzii* ($U = 6.5$, $p < 0.0001$). For the expected heterozygosity (He), which is the measure of genetic variability, it ranges from 0.16 in Axim to 0.19 in Wassa Essaman with an average of 0.18 for *An. coluzzii* populations. For *An. gambiae s.s.* populations, expected heterozygosity ranges from 0.17 in Nsawam to 0.20 in Tuba with an average of 0.18. However, the difference between expected heterozygosity among *An. coluzzii* and *An. gambiae s.s.* populations was not statistically significant ($U = 179.5$, $p = 0.159$). The Wilcoxon signed-rank test showed that, in both *An. coluzzii* ($p < 0.001$) and *An. gambiae s.s* ($p < 0.001$), the observed heterozygosity was significantly higher than expected heterozygosity among all the populations, thereby generating a negative inbreeding coefficient (F_{IS}), indicating excess outbreeding among the populations.

Table 5.4: Genetic diversities of 24 *An. coluzzii and 20 An. gambiae s.s. populations* were determined based on 1497 SNPs and 1606 SNPs respectively across southern Ghana. The values shown are the number of different alleles (*Na*), number of effective alleles (*Ne*), Shannon's information index (*I*), the allelic richness (*Ar*), the observed heterozygosity (*Ho*), expected heterozygosity (*He*) and inbreeding coefficient (F_{IS})

(a) An. coluzzii

Population	Na	Ne	I	Ar	He	Ho	F_{IS}
Ada Foah	1.68	1.24	0.27	1.68	0.18	0.21	-0.17
Assin Foso	1.70	1.23	0.27	1.70	0.18	0.20	-0.16
Anhwiankwanta	1.52	1.24	0.25	1.52	0.18	0.20	-0.13
Asankragua	1.61	1.24	0.27	1.61	0.19	0.21	-0.15
Ashaiman	1.62	1.24	0.26	1.62	0.18	0.20	-0.17
Asokwa	1.69	1.24	0.28	1.69	0.18	0.21	-0.13
Axim	1.64	1.22	0.25	1.64	0.16	0.19	-0.18
Bantama	1.54	1.25	0.26	1.54	0.19	0.21	-0.18
Bogoso	1.67	1.24	0.27	1.67	0.17	0.20	-0.18
Dunkwa	1.60	1.24	0.26	1.60	0.18	0.21	-0.16
Dzorwulu	1.71	1.25	0.28	1.71	0.18	0.21	-0.18
Enchi	1.75	1.24	0.28	1.75	0.18	0.21	-0.17
Komenda	1.71	1.24	0.28	1.71	0.18	0.21	-0.16
Mamprobi	1.67	1.23	0.26	1.67	0.17	0.20	-0.15
Mankessim	1.68	1.24	0.27	1.68	0.18	0.20	-0.17
Manso Nkwanta	1.71	1.25	0.28	1.71	0.19	0.21	-0.14
New Edubiase	1.67	1.23	0.27	1.67	0.18	0.20	-0.14
Nsawam	1.68	1.25	0.27	1.68	0.18	0.22	-0.22
Shama	1.67	1.25	0.28	1.67	0.19	0.21	-0.16
Takoradi	1.74	1.25	0.29	1.74	0.18	0.21	-0.16
Tarkwa	1.67	1.25	0.28	1.67	0.19	0.21	-0.16
Twifo	1.73	1.24	0.28	1.73	0.18	0.21	-0.14
Tuba	1.72	1.25	0.28	1.75	0.19	0.21	-0.14
Wassa Essaman	1.67	1.25	0.28	1.63	0.19	0.21	-0.14
Mean	1.67	1.24	0.27	1.67	0.18	0.21	-0.16

(b) An. gambiae s.s.

Pop	Na	Ne	I	Ar	He	Ho	f
Assin_fosu	1.68	1.26	0.28	1.67	0.18	0.23	-0.25
Agona_Dunkwa	1.52	1.26	0.26	1.51	0.19	0.23	-0.24
Akatsi	1.55	1.26	0.26	1.55	0.18	0.22	-0.26
Anhwiankwanta	1.59	1.28	0.28	1.59	0.20	0.24	-0.23
Asikumah	1.64	1.27	0.28	1.64	0.19	0.23	-0.25
Asokwa	1.67	1.27	0.28	1.67	0.19	0.23	-0.22
Bogoso	1.65	1.26	0.27	1.65	0.18	0.23	-0.28
Dodowa	1.62	1.25	0.26	1.62	0.17	0.21	-0.28
Dzorwulu	1.66	1.26	0.28	1.66	0.19	0.22	-0.22
Enchi	1.60	1.26	0.26	1.59	0.18	0.23	-0.26
Half_Assini	1.60	1.25	0.26	1.59	0.18	0.22	-0.25
Koforidua	1.60	1.25	0.26	1.60	0.17	0.22	-0.30
Madina	1.88	1.27	0.31	1.87	0.19	0.23	-0.23
New_Edubiase	1.71	1.25	0.28	1.71	0.18	0.22	-0.20
Nkwanta	1.62	1.25	0.26	1.62	0.17	0.22	-0.29
Nsawan	1.61	1.25	0.26	1.61	0.17	0.22	-0.29
Obuasi	1.80	1.26	0.29	1.80	0.18	0.22	-0.22
Suhum	1.65	1.26	0.28	1.65	0.19	0.22	-0.22
Tuba	1.51	1.26	0.25	1.52	0.20	0.22	-0.16
Wassa Essaman	1.49	1.25	0.25	1.49	0.19	0.21	-0.17
Mean	1.63	1.26	0.27	0.63	0.18	0.22	-0.24

5.3.4 Population differentiation and structure

The Analysis of molecular variance (AMOVA) (Table 5.5) for both *An. coluzzii* and *An. gambiae s.s. revealed* that most of the variation occurred within the individual mosquitoes, with the percentage variance of 87 % and 97% respectively. There was a low variance among populations in both species corresponding with low F_{ST} (F_{ST} in *An. coluzzii* and *An. gambiae s.s.* are 0.01 and 0.02 respectively) indicating low overall genetic differentiation among populations.

Table 5.5: An AMOVA for *An. coluzzii* and *An. gambiae s.s*. populations

Species	Source of variation	df	MS	Estimated variance.	%	P-value
An. coluzzii	Among Pops	23	218.18	2.01	1%	0.002
	Among Indiv	138	191.11	20.93	12%	
	Within Indiv	162	149.24	149.24	87%	
An. gambiae s.s.	Among Pops	19	226.62	3.2	2%	0.016
	Among Indiv	133	178.26	2.14	1%	
	Within Indiv	153	173.99	173.99	97%	

The values shown are degrees of freedom (df), mean squared deviations (MS), estimated variance, and percentage of variance partitioning, F_{ST} (the proportion of the total genetic variance contained in the subpopulation, it ranges from 0 to 1, high values show a considerable degree of differentiation among populations) and the corresponding p-value (with < 0.05, indicating significant difference).

Principal coordinates analysis (PCoA) was used to visualise differentiation among the mosquito populations (Figure 5.10). The cumulative variance accounted for by the two dimensions in *An. coluzzii* and *An. gambiae s.s.* were 14% and 21% respectively. To explore the genetic relationships of *An. coluzzii* and *An. gambiae s.s*, the computed Nei's genetic distances between populations were subjected to UPGMA cluster analysis (Figure 5.11a and Figure 5.12a). In both species seven subgroups with a coefficient of 0.002 indicating very weak clustering. The genetic trees did not conform to the two-dimensional plots of PCoA, however, the populations appear to correspond to spatial habitat types as populations with similar vegetation cover tends to be in proximity on the genetic trees (Figure 5.11b and Figure 5.12b) indicating the habitat influence in the distribution of mosquitoes. A cluster analysis was performed with a distance-based (maximum likelihood and hierarchical) method and a dendrogram was constructed by the Unweighted Pair-Group method (UPGMA) using a PhyML online tool (Guindon and Gascuel, 2003).

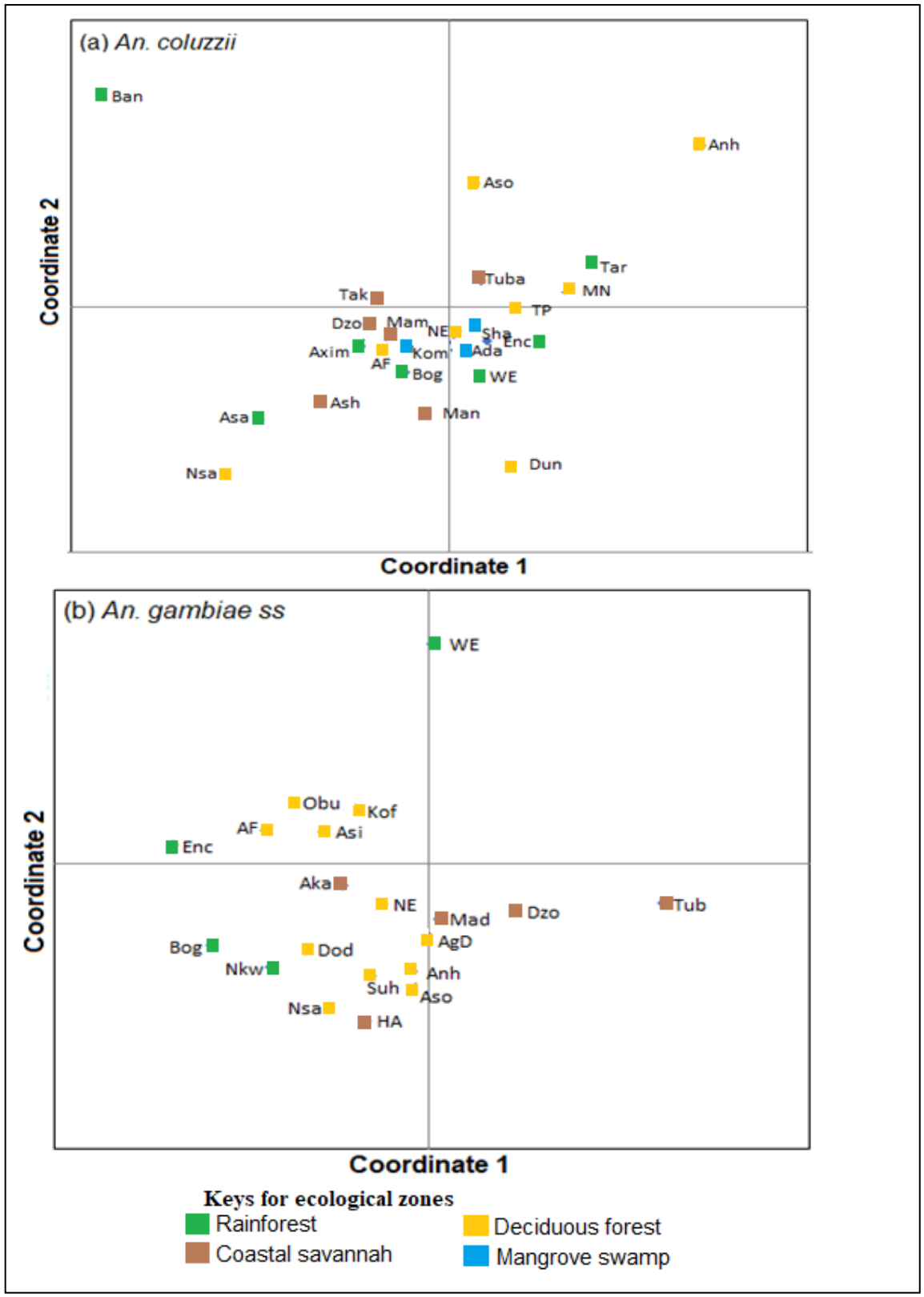


Figure 5.10: A PCoA scatter diagram showing the distributions of populations according to their ecological zones (a) *An. coluzzii* and (b) *An. gambiae s.s.* based on Nei's genetic distance

To explore the phylogenetic relationships among the populations of *An. coluzzii* and *An. gambiae s.s.*, the Nei's genetic distances between populations were subjected to UPGMA cluster analysis (Figure 5.11a and Figure 5.12a). In both species seven subgroups with a coefficient of 0.002 indicating very weak clustering among the populations. The genetic trees did not conform to the two-dimensional plots of PCoA, however, the populations appear to correspond to spatial habitat types as populations with similar ecological zones tend to be in proximity in the genetic trees (Figure 5.11b and Figure 5.12b). This observation may suggest that habitat types are likely to influence the distribution of mosquitoes.

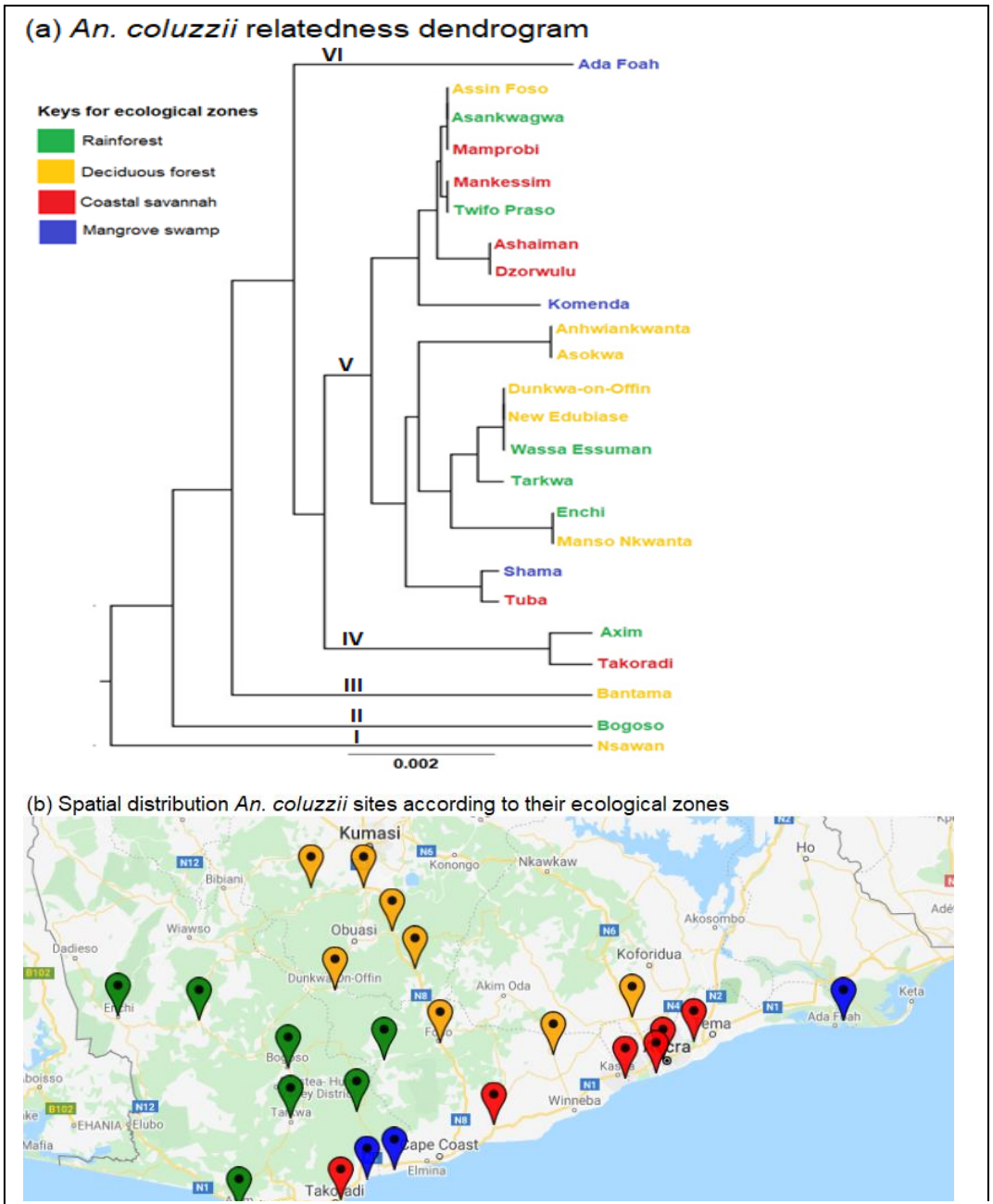


Figure 5.11: (a) Hierarchical cluster analysis using UPGMA of Nei's pairwise genetic distances among 24 populations of *An. coluzzii* and (b) the spatial distribution of the *An. coluzzii* populations according to their ecological zones overlaid on vegetation cover

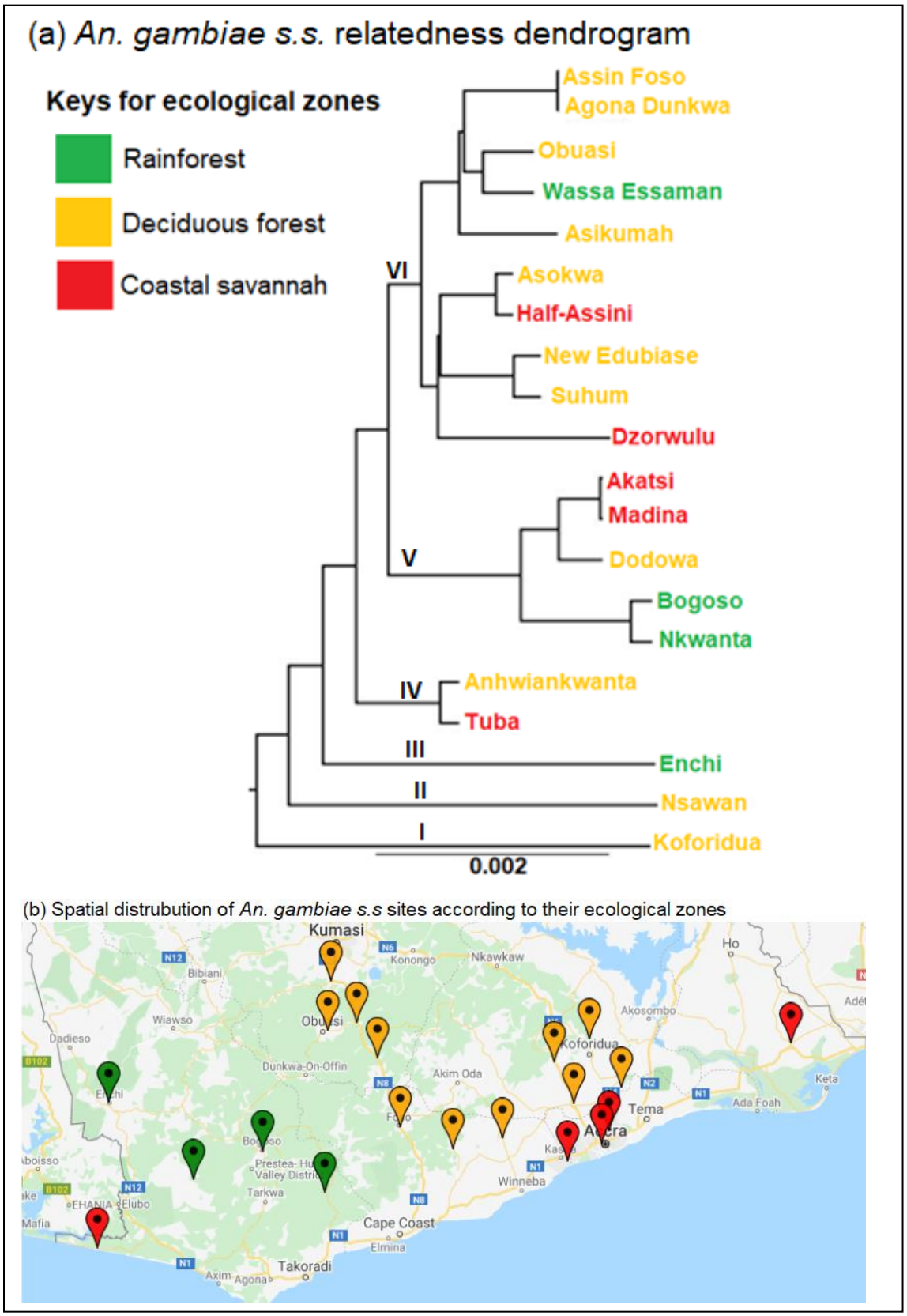


Figure 5.12: (a) Hierarchical cluster analysis using UPGMA of Nei's pairwise genetic distances among 20 populations of *An. gambiae s.s.* and (b) spatial distribution *of An. gambiae s.s.* according to their ecological zones populations overlaid on vegetation cover.

To identify the number of distinct groups existing within *An. coluzzii* and *An. gambiae s.s* populations, clustering analysis was performed using STRUCTURE and BAPS software on combined chromosome 3 i.e. (3L+3R). For both *An. coluzzii* and *An. gambiae s.s*, the best (i.e. the maximum likelihood of data of LnP(K)) clustering solution for both software occurred at K = 1, indicating no clustering (Figure 5.13).

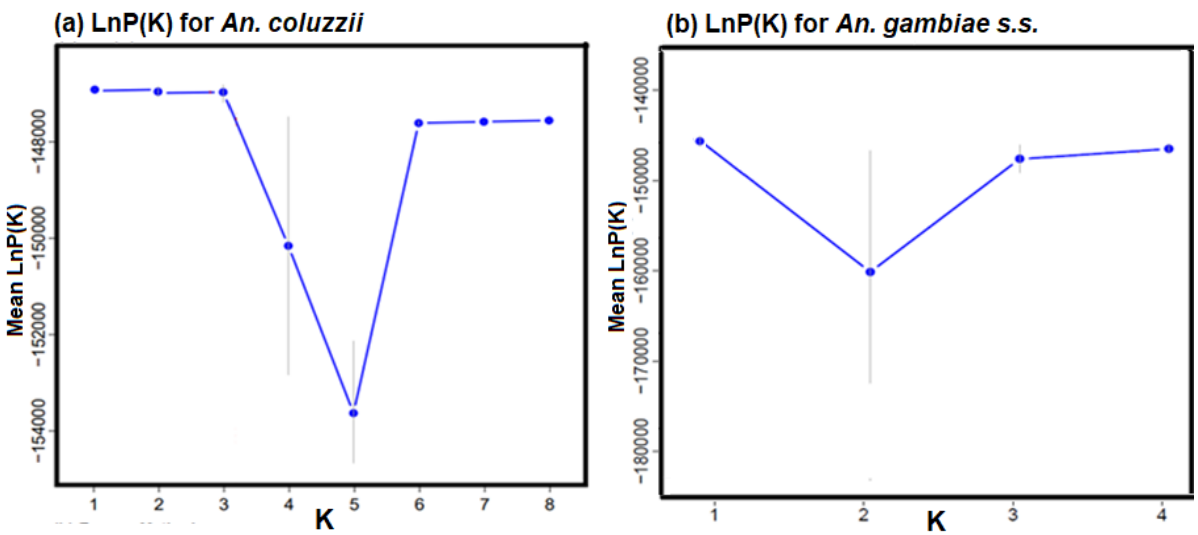


Figure 5.13: The post hoc analyses of STRUCTURE results to determine the number of clusters (a) *An. coluzzii* and (b) *An. gambiae s.s*. based on SNPs of chromosome 3 showing the LnP(K) for the mean maximum likelihood occurring at $K = 1$.

5.3.5 Isolation by distance analysis

Whilst population structure in the form of distinct clusters was not evident, the significant differentiation among populations within each species indicates that populations are not panmictic. To investigate possible indicators of distance-limited dispersal, isolation by distance was investigated using the chromosome 3 dataset, via Mantel test analysis. A Mantel test between the pairwise matrices of F_{ST} and geographic distance of all populations revealed a low but marginally significant correlation ($r = 0.23$, $p = 0.045$) in *An. coluzzii*, indicating weak isolation by distance (Figure 5.14). Variation in correlation at a smaller geographical scale was assessed with a multivariate Mantel correlogram. The correlogram is a plot between the Mantel correlation coefficients (r) and the upper limits of the specified successive geographic distance classes (d). The Mantel coefficient ($\boldsymbol{r}$) is generated via a correlation between a matrix of dummy variables (with specified pairwise distance class coded as 1 and all the other distance classes are coded as 0) and genetic distance matrix (F_{ST}) using ZL software. A distogram is a plot between the mean F_{ST} of specified successive geographic distance classes and the upper limits of the distance classes.

For *An. coluzzii*, the correlogram (Figure 5.15a) shows a highly significant genetic similarity among mosquito populations up to 70 km. However, no Mantel correlations were significant at any larger distance classes, though F_{ST} increased slightly in the larger distance classes (Figure 5.15b), consistent with the results from the overall Mantel test (Figure 5.14). Overall, these results indicate weak isolation by distance in *An. coluzzii*, driven primarily by variation between the most local scale of sampling and larger scales.

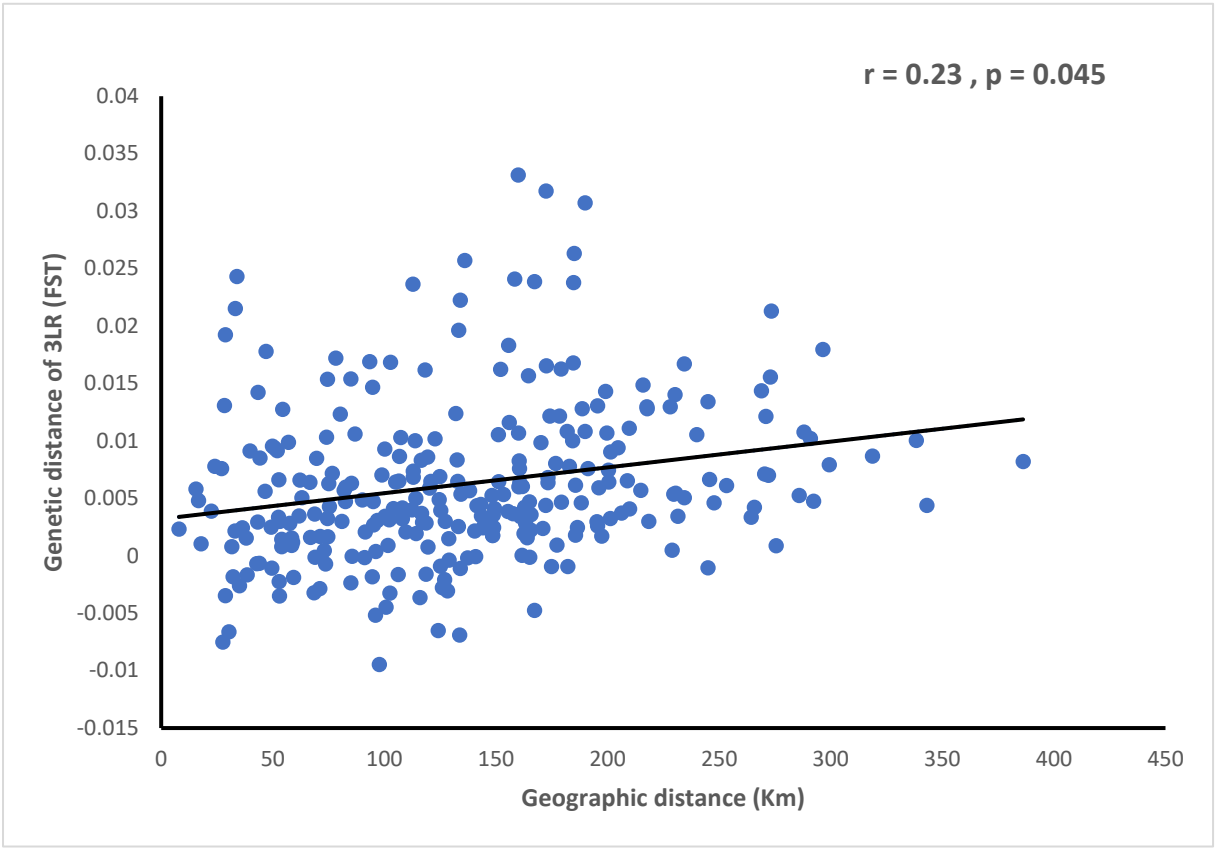


Figure 5.14: Relationship between 3LR genetic distance (pairwise F_{ST}) and geographic distances (r = 0.23, p = 0.045) for the 24 populations of *An. coluzzii* across southern Ghana.

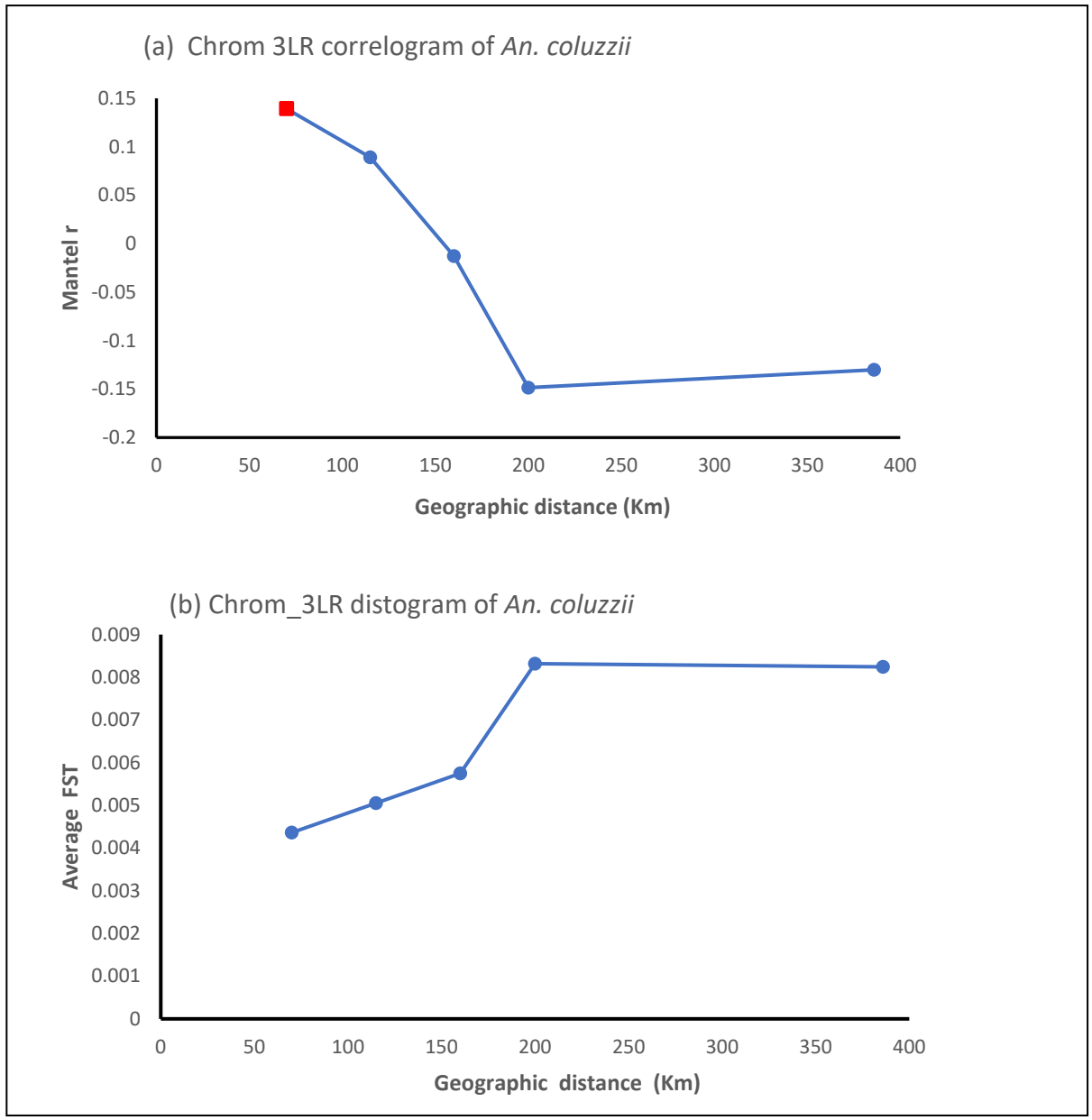


Figure 5.15: (a) Construction of a Mantel correlogram for a genetic similarity matrix of 24 *An. coluzzii* populations based on chromosome 3. The red circle in the correlogram indicates a significant Mantel test after Sequential Bonferroni correction. (b) The distogram showing the average genetic differentiation of geographic distance classes in *An. coluzzii*.

For *An. gambiae s.s.*, a Mantel test between the pairwise matrices of F_{ST} and geographic distance of all populations revealed no significant correlation (r = 0.06, p = 0.308), indicating no isolation by distance (Figure 5.16). The correlogram (Figure 5.17a) shows no significant genetic similarity among mosquito populations even on a smaller geographical scale, with the distogram showing no consistent trend in the change in differentiation with distance (Figure 5.17b). Overall, our results indicate no isolation by distance in *An. gambiae s.s*.

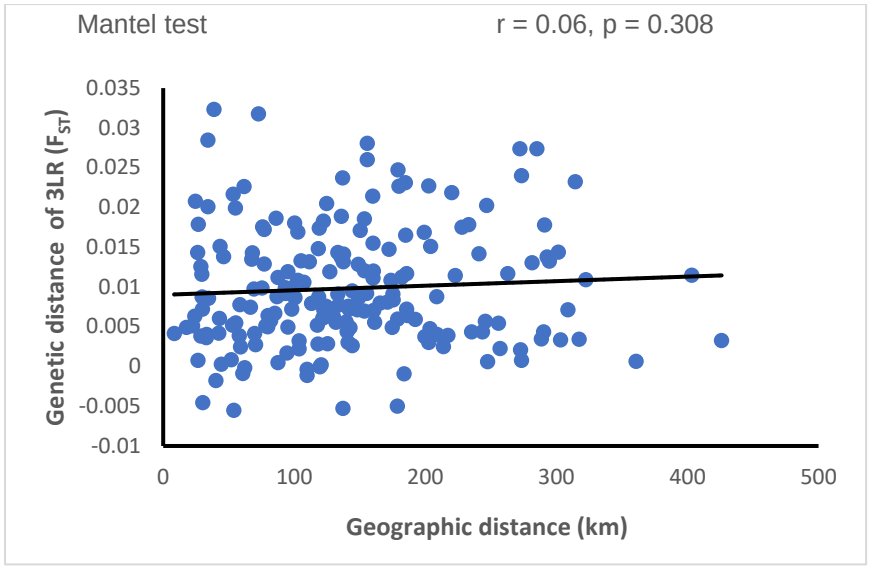


Figure 5.16: Relationship between 3LR genetic distance (pairwise F_{ST}) and geographic distances (r = 0.23, p = 0.045) for the 24 populations of *An. gambiae s.s.* across southern Ghana.

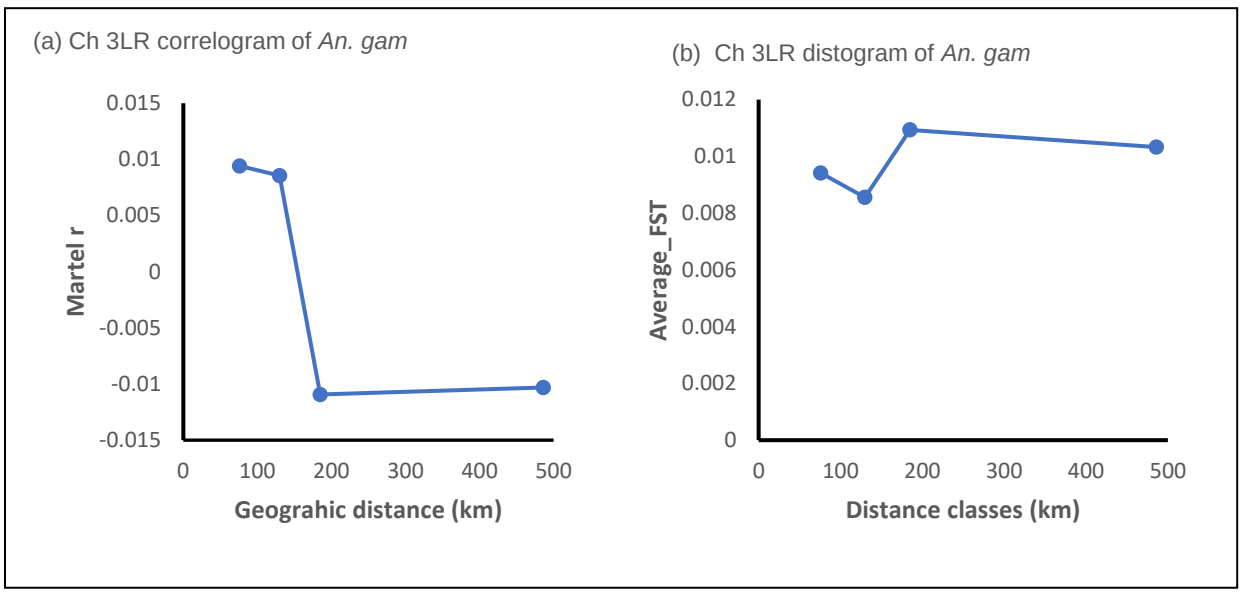


Figure 5.17: (a) Construction of a Mantel correlogram for a genetic similarity matrix of chromosome 3 for 20 *An. gambiae s.s.* populations with geographic distance with no significant Mantel test after sequential Bonferroni correction. (b) The distogram showing the average genetic differentiation of geographic distance classes in *An. gambiae s.s*.

5.3.6 Adaptive determinants of isolation by distance and population structure

With weak evidence of isolation by distance in *An. coluzzii* and no evidence of isolation by distance in *An. gambiae s.s.*, the possible effect of habitat on genetic differentiation was

investigated using chromosome 3. A correlation between the pairwise habitat (ecological zone) similarity matrices and the pairwise matrices of F_{ST} of all populations revealed a marginally significant association (p = 0.045) in *An. coluzzii*, indicating a weak effect of habitat in genetic differentiation. For *An. gambiae s.s.* mosquitoes, there was no significant correlation (p = 0.235) between pairwise habitat similarity matrices and the pairwise matrices of F_{ST} of all populations.

5.3.7 The association of adaptive chromosomes 2L, 2R and X with geographic distance

The analyses were repeated for chromosomes 2L, 2R and X, which owing to restrictions on recombination e.g. via adaptive polymorphic inversions may deviate from neutral expectations to a much greater degree than chromosome 3. For *An. coluzzii*, 2L and 2R genetic distances showed moderate while X showed a strong significant correlation with geographic distance (Table 5.6), indicating that the chromosomes correlate with geographic distance. The 2L, 2R and X genetic distances were significantly correlated with the habitat similarity matrix (Figure 5.18a, Figure 5.19a and Figure 5.20a). The correlograms of 2L, 2R and X (Figure 5.18b, Figure 5.19b and Figure 5.20b) shows a very weak but highly significant genetic similarity among mosquito populations up to 115 km in each of the chromosomes. The distograms (Figure 5.18c, Figure 5.19c and Figure 5.20c) show a consistent increase in genetic differentiation of 2L, 2R and X with increasing geographic distance in *An. coluzzii*. For *An. gambiae s.s.*, 2L, 2R and X genetic distances showed no significant correlation with geographic distance (Table 5.6), indicating that the chromosomes exhibited no isolation by distance. The 2L genetic distances were significantly correlated with the habitat similarity matrix, while the 2R and X showed no significant correlation with habitat.

Table 5.6: The Mantel tests showing the correlation *(r*) and the corresponding p-values the genetic distances of Chromosome 2L, 2R and X and the geographic distance and habitat similarity matrix among *An. coluzzii* and *An. gambiae s.s.* populations.

	Correlating parameters	*An. coluzzii*		*An. gambiae s.s.*	
Chromosome		**r**	**p**	**r**	**p**
2L	2L_FST v Geo_dist	0.41	< 0.001	0.06	0.307
	2L_FST v Habitat	NA	0.017	NA	0.023
2R	2R_FST v Geo_dist	0.31	0.004	0.08	0.242
	2R_FST v Habitat	NA	0.019	NA	0.491
X	X_FST v Geo_dist	0.6	< 0.001	0.07	0.284
	X_FST v Habitat	NA	0.006	NA	0.328

*NA = not applicable

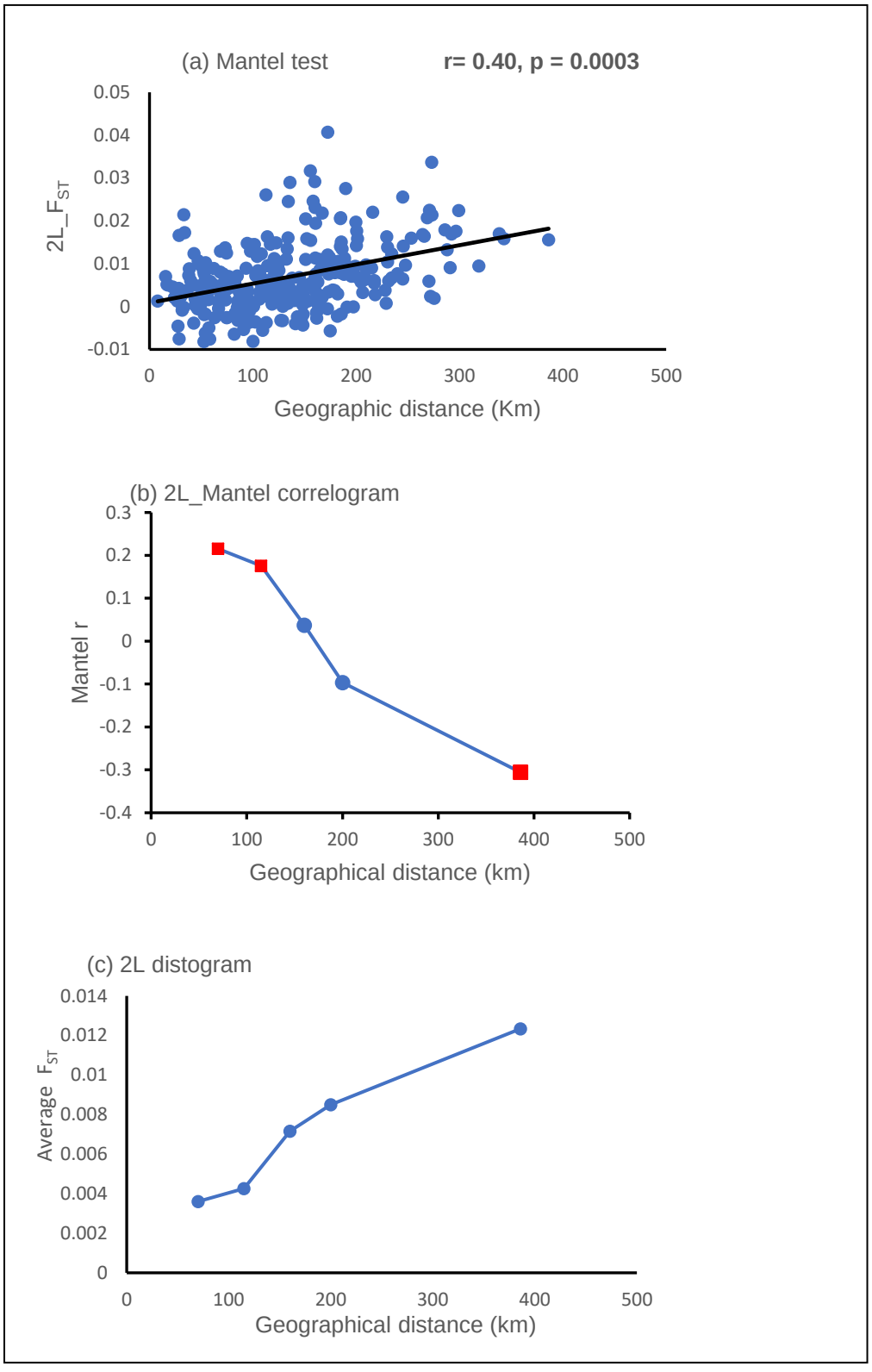


Figure 5.18: The correlations of chromosome 2L in *An. coluzzii* : (a) the Mantel test between the 2R genetic differentiation (F_{ST}) and geographic distance showing the correlation coefficient and p-values (b) a correlogram for 2L genetic similarity matrix of 24 *An. coluzzii* populations with the red square in the correlogram indicating significant Mantel test after sequential Bonferroni correction, and (c) a distogram showing an increased in genetic differentiation of 2L with geographic distance in *An. coluzzii*.

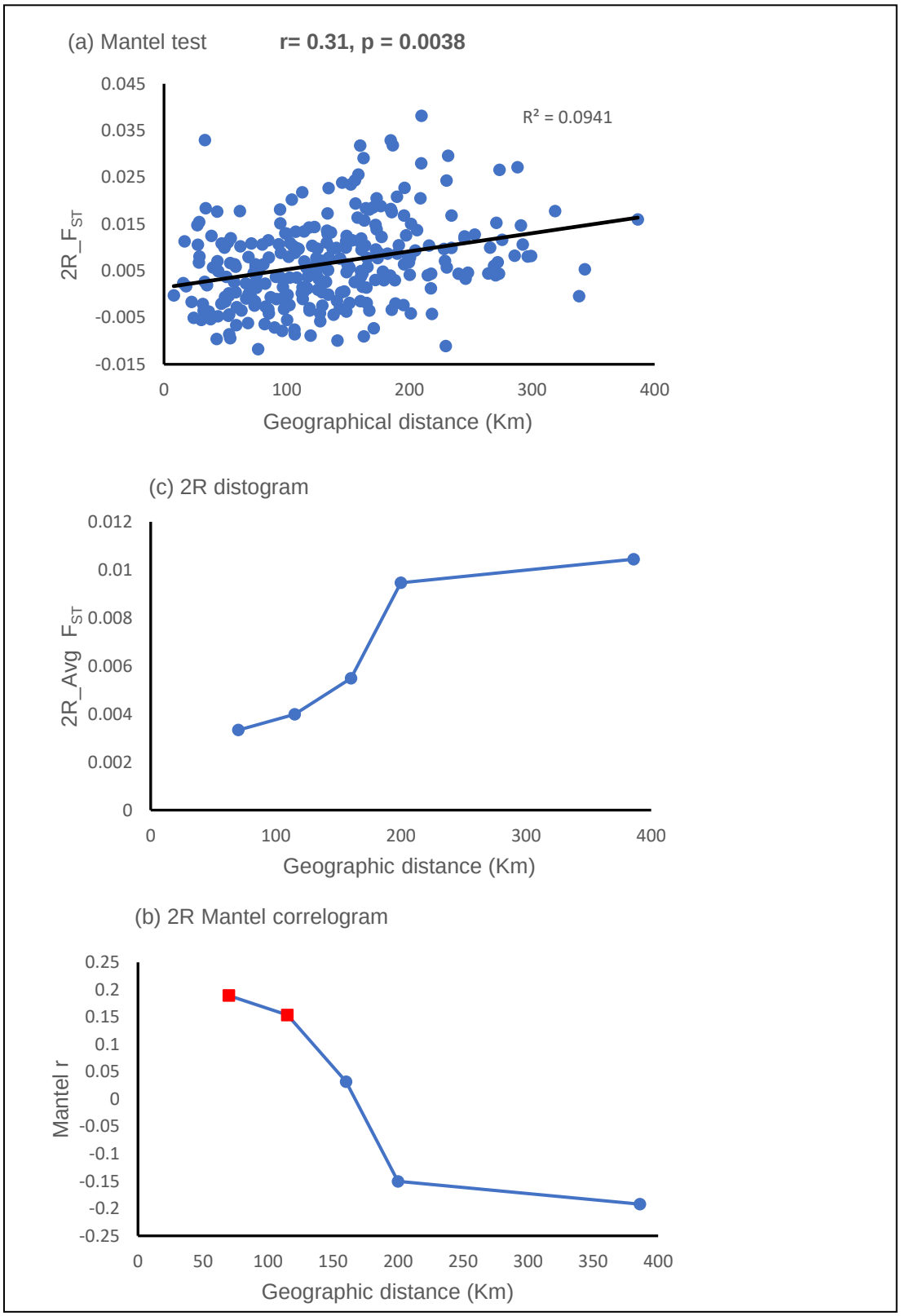


Figure 5.19: The correlations of chromosome 2R in *An. coluzzii* : (a) the Mantel test between the 2R genetic differentiation (F_{ST}) and geographic distance showing the correlation coefficient and p-values (b) a correlogram for 2R genetic similarity matrix of 24 *An. coluzzii* populations with the red square in the correlogram indicating significant Mantel test after sequential Bonferroni correction, and (c) a distogram showing an increased in genetic differentiation of 2R with geographic distance in *An. coluzzii*.

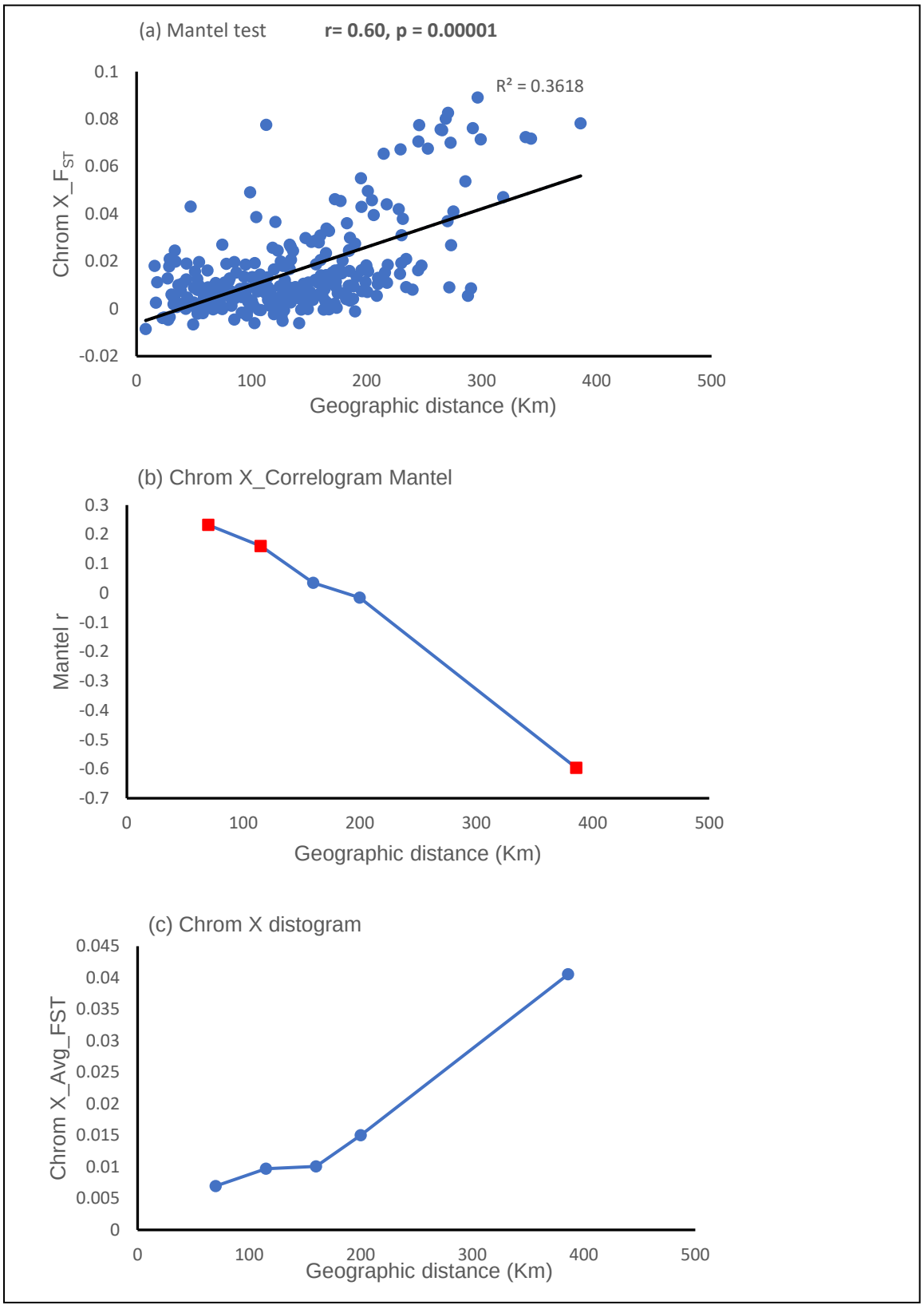


Figure 5.20: The correlations of chromosome X in *An. coluzzii* : (a) the Mantel test between the X genetic differentiation (F_{ST}) and geographic distance showing the correlation coefficient and p-values (b) a correlogram for X genetic similarity matrix of 24 *An. coluzzii* populations with the red square in the correlogram indicating significant Mantel test after sequential Bonferroni correction, and (c) a distogram showing an increased in genetic differentiation of X with geographic distance in *An. coluzzii*.

5.3.7 Outlier detection analysis

With no evidence of population structure, and weak but significant differentiation among populations within each species, an outlier detection analysis was performed to identify extreme loci that are likely to be under selection. A Bayescan analysis at the population-level, habitat-level (rainforest, deciduous forest, coastal savannah and mangrove swamp) and land-use (plantation, vegefarm, gardening and mining) level for each species was performed on unpruned SNP data using $\log_{10}PO > 0.5$ as a threshold. For *An. coluzzii* chromosome X, the number of loci identified putatively under diversifying selection (positive alpha values) at the population-level, habitat-level and land-use level are 31, 28 and 22 respectively. Almost all the loci under selection were located around 15 Mb as shown for population-level in Figure 5.21 with comparable loci in land-use (Appendix 12) and habitat (Appendix 13) level analysis. Eighteen loci under selection were common among population, habitat and land use (Figure 5.22). However, no loci were identified as significant on any other chromosome for population (Appendix 14) and habitat (Appendix 15).

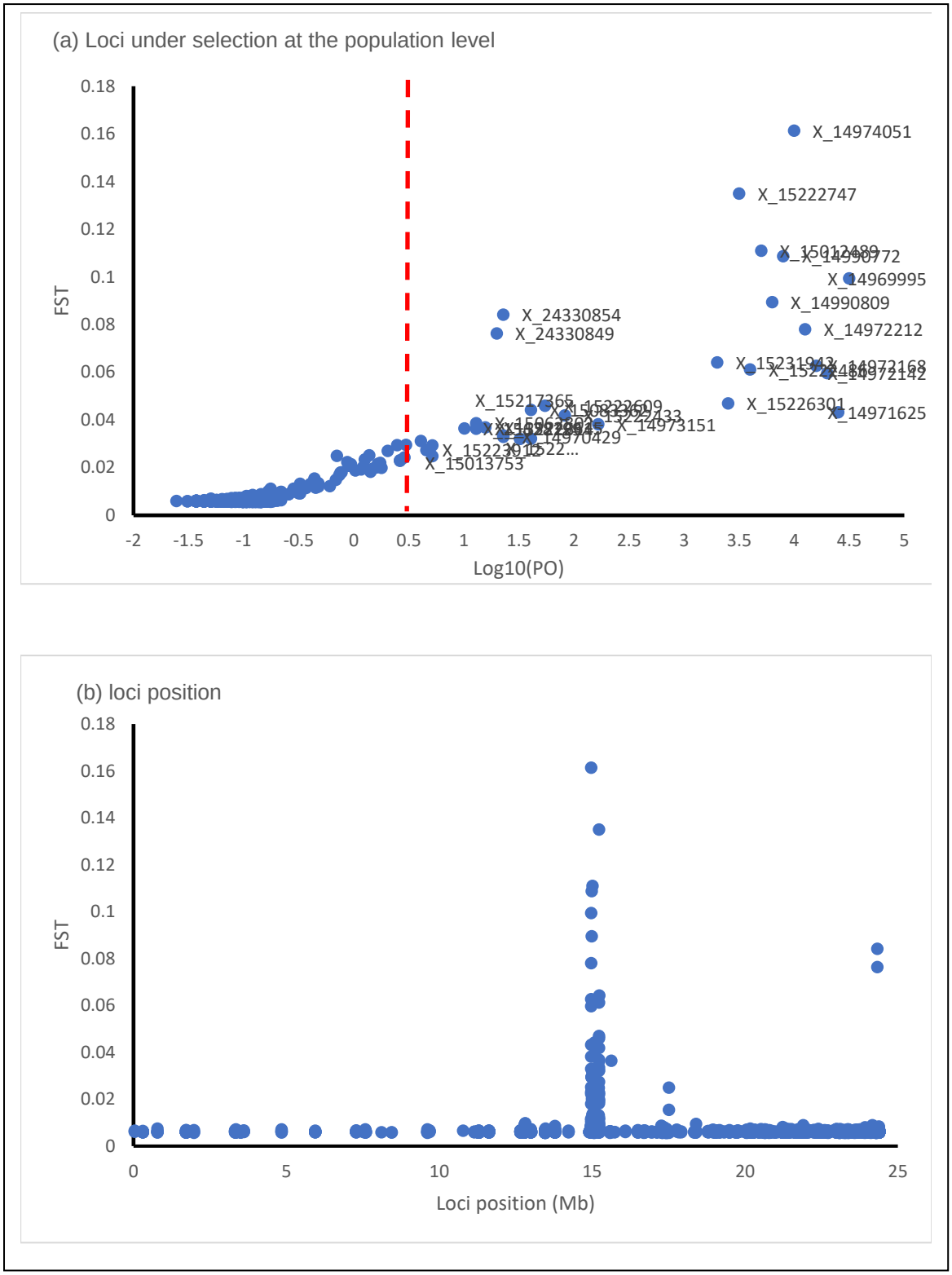


Figure 5.21: The detection of outlier X chromosome SNPs in *An. coluzzii* using Bayescan**:** (a) The Log10 (PO) are shown on the x-axis and F_{ST} values of Chromosome X for 24 populations on the y-axis with the red vertical line indicating the selection threshold. Thirty-one SNPs were identified under selection (b) The plot of SNPs F_{ST} and loci position along Chromosome X for populations, showing the loci under selection projected above the background.

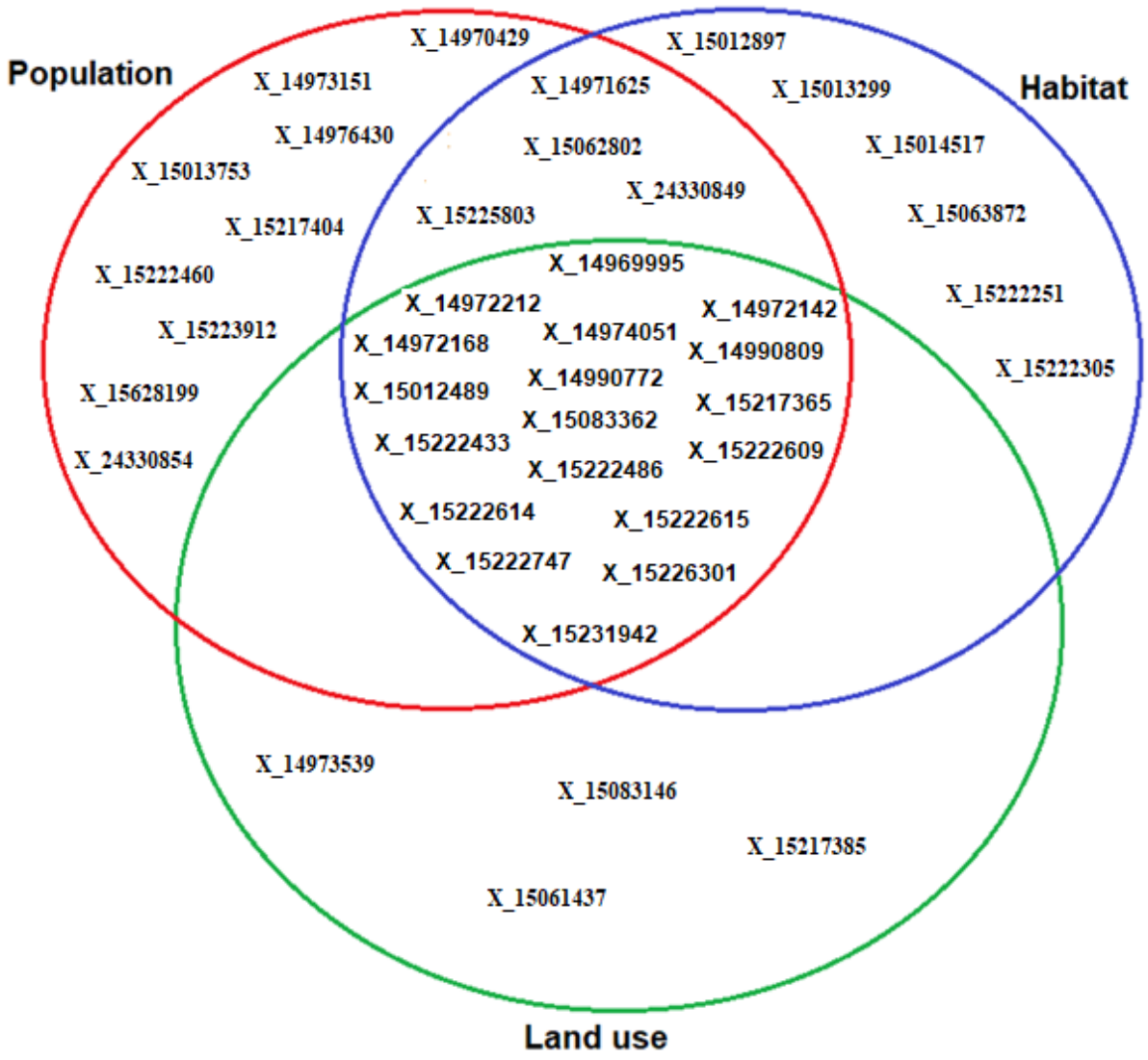


Figure 5.22: The pie chart showing the distribution of chromosome X SNPs under selection among populations, major habitat types and land use in *An. coluzzii* with 18 common SNPs existing among the three factors.

In *An. gambiae s.s.*, SNPs detected as putatively under diversifying selection at the population level were found on all chromosome arms, though fewer were identified when using habitat and land use as the units of comparison (Table 5.7). Twenty-four out of the 26 outlier SNPs of the X chromosomes in the population-level analysis were all located around 15 megabases (Figure 5.23). In 2L, 2R, 3L and 3R chromosomes, fewer loci were identified under selection in the population level (Table 5.7 and Figure 5.24). For habitat-level analysis, five SNPs and one SNP were identified as outliers in 2L and X chromosomes respectively (Appendix 16). For land-use-level analysis, 2L and X chromosomes recorded seven and two outlier SNPs respectively (Appendix 17). No outlier SNPs were found among habitat and land-use levels of analyses for 2R, 3L and 3R chromosome in *An. gambiae s.s*.

Table 5.7: Loci identified putatively under selection in all the chromosomes of *An. gambiae s.s* at the population, habitat and land-use level. A further transcriptomic will be required to identify the functions of these loci.

Chromosome	Bayescan analysis level		
	Population	Habitat	Land-use
2L	2L_13407857 2L_1589120	2L_13412660 2L_13403515 2L_13407857 2L_13426869 2L_13426992	2L_13407857 2L_1589120 2L_13403515 2L_13426992 2L_13422324 2L_13426869 2L_13417022
2R	2R_57968712 2R_57969323 2R_57968667 2R_57969321 2R_57978742	NL	NL
3L	3L_20275056 3L_563269 3L_658419	NL	NL
3R	3R_21055640	NL	NL
X	X_14971307, X_14971798 X_14971374, X_15217404 X_14971379, X_15222251 X_14973468, X_14974051 X_14974107, X_15226255 X_15222257, X_24285221 X_14971376, X_14970936 X_14973151, X_15223640 X_15222564, X_15222971 X_15223713, X_15226249 X_14972211, X_15084277 X_14974049, X_14971959 X_15222208, X_17913704	X_15222251	X_15222257 X_14971307

*NL = no loci found under selection

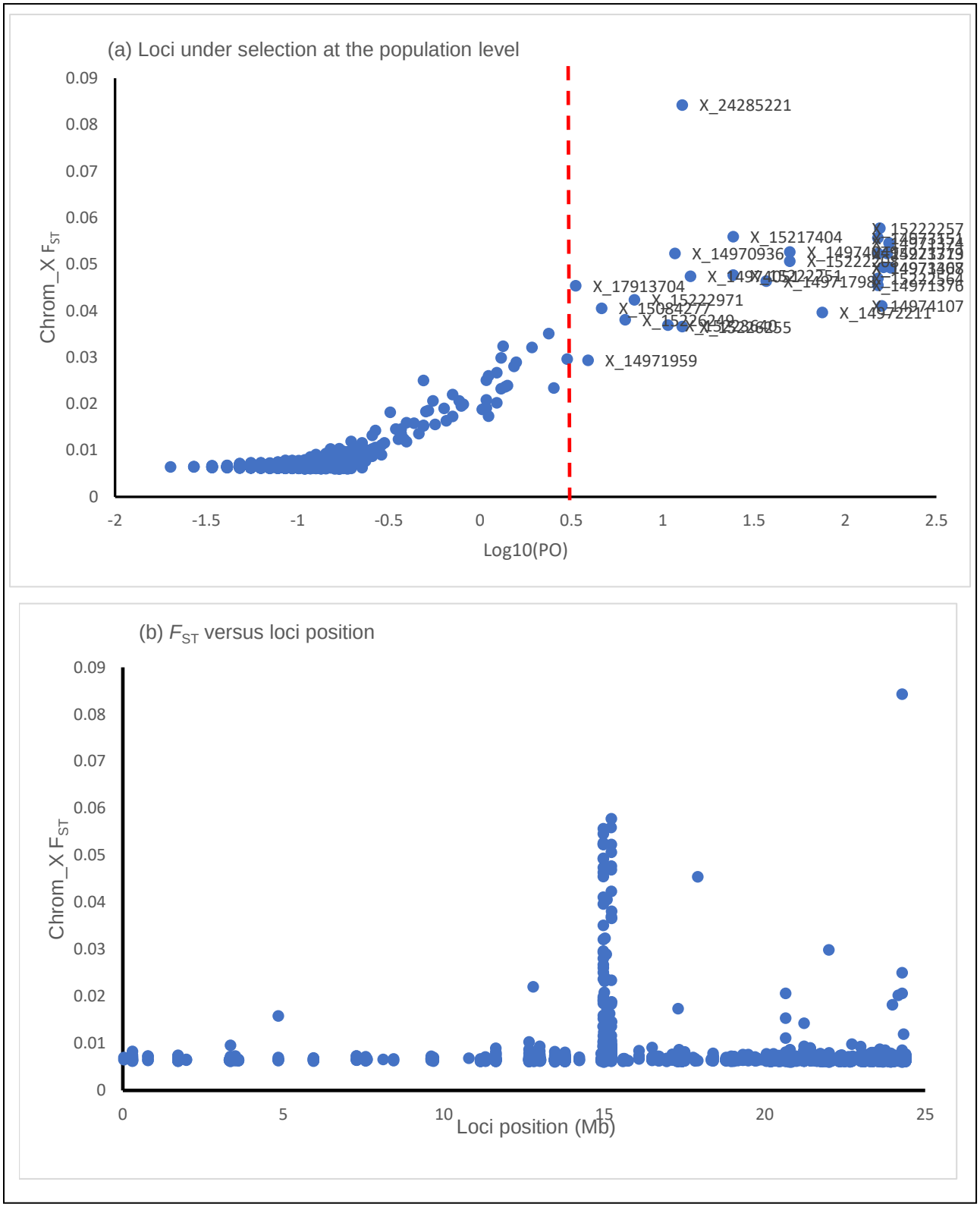


Figure 5.23: The detection of outlier X chromosome SNPs in *An. gambiae s.s* using Bayescan: (a) The Log10 (PO) are shown on the x-axis and F_{ST} values of Chromosome X for 20 populations on the y-axis with the red vertical line indicating the selection threshold. Twenty-six SNPs were identified under selection (b) The plot of SNPs F_{ST} and loci position along Chromosome X for populations showing the loci under selection projected above the background.

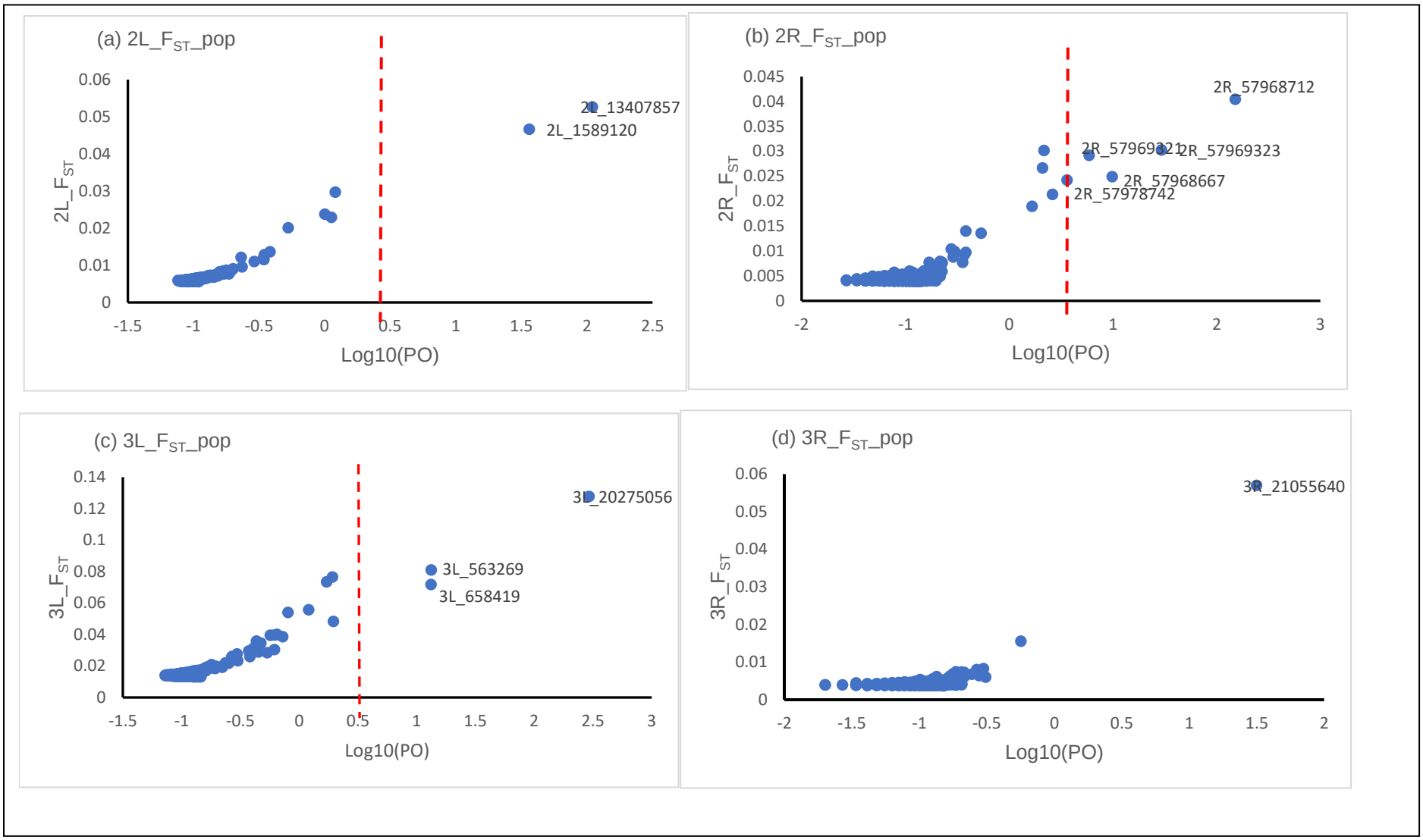


Figure 5.24: The detection of outlier SNPs in *An. gambiae s.s* population in (a) 2L chromosome (b) 2R chromosome (c) 3L Chromosome and (d) 3R chromosome. The Log10 (PO) on the x-axis and FST values of Chromosomes for *An. gambiae s.s.* populations across southern Ghana on the y-axis with the red vertical line indicating the selection threshold

The outlier SNPs on chromosome X common to both *An. coluzzii* and *An. gambiae s.s.* populations were identified in the PEST genome of vectorbase using the range (14969995 - 15231942 bp) of the loci identified putatively under selection as input for the BIOMART search tool (Appendix 18). Six protein-coding genes were located within the region (Table 5.8) however their functions (including that of their paralogues and orthologues of *An. melas* and *An. quadriannulatus*) are yet to be described.

Table 5.8: The genes that correspond to 18 loci that are undergoing diversifying selection in around 15 Mbp of the chromosome X in both *An. gambiae s.s.* and *An. coluzzii*.

Gene stable ID	Transcript stable ID	Gene start (bp)
AGAP000817	AGAP000817-RA	15003958
AGAP000816	AGAP000816-RA	15007941
AGAP013474	AGAP013474-RA	15013481
AGAP013173	AGAP013173-RA	15053437
AGAP013424	AGAP013424-RA	15086694
AGAP012997	AGAP012997-RA	15160656

5.4 Discussion

The study seeks to use chromosome 3 markers to investigate population structure and genetic differentiation in *An. gambiae s.s.* and *An. coluzzii* and how they are influenced by geographic distance and habitat variations. The kinship analysis showed that the vast majority of larvae sampled from multiple breeding sites were not siblings. There was no distinct population structure, however, there was weak but significant differentiation among populations within each species. *An. coluzzii* population exhibited weak isolation by distance, while *An. gambiae s.s* showed no evidence of isolation by distance. Few loci around 15 Mb of Chromosome X showed high levels of differentiation among the populations and habitat types within each of the species.

5.4.1 Genetic relatedness and genetic diversity

Genetic relatedness within the populations of each species was generally low, although the mosquitoes were sampled within an average distance of 3 km in each study site. This finding is contrary to what has been reported previously where the highest level of relatedness was observed among *An. gambiae s.s*. collected within a radius of 3 km (Lehmann *et al.*, 2003). However, recent studies among *An. arabiensis* in Kenya have shown that the genetic relatedness depends on the larval abundance within the breeding sites where mosquitoes were sampled (Odero *et al.*, 2019), which may be influenced by the oviposition behaviour of mosquitoes and the physiochemical properties of the breeding sites (Okal *et al.*, 2015; Eneh *et al.*, 2019).

Chromosome X showed a strong signal of interspecies differentiation to confirm the species characterization by PCR into *An. coluzzii* and *An. gambiae s.s*., and few species were identified with admixtures. Even in chromosome 3 which does not have any significant barriers to recombination, there was very little evidence of recent introgression as no individual showed >20% admixture (Figure 5.4), indicating a low level of introgression. This in turn explains the ongoing speciation as the divergence between the two species are driven by assortative mating in their sympatric range (Costantini *et al.*, 2009; Marsden *et al.*, 2011). This finding is consistent with previous studies in southern Ghana where very few admixtures were found between the species pair (Clarkson *et al.*, 2014). With very low levels of hybridization between the two species in southern Ghana, diversity and structure analyses were performed separately for each species. The proportion of polymorphic loci was quite variable among the populations of *An. coluzzii* and *An. gambiae s.s.*, but significantly higher in *An. coluzzii* than in *An. gambiae s.s.* In each of the *An. coluzzii* and *An. gambiae s.s.* populations, the number of effective alleles (Ne) was lower than the number of different alleles (Na) indicating imbalanced allelic frequencies (a phenomenon where the genome has more copies of some genes than other genes) in the populations. The expected heterozygosity did not differ substantially between *An. coluzzii* and *An. gambiae s.s*. However, in all the populations of both species, the observed heterozygosity was significantly higher than the expected heterozygosity suggesting excessive outbreeding. This observation may explain the imbalanced allelic frequencies in the populations, and it is also interesting that even within populations with a relatively high number of individuals being full-sib and half-sib such as Nsawan *An. coluzzii* population and Koforidua *An. gambiae s.s.* population (Table 5.5) where allele imbalance is expected to have resulted from the sampled populations represented by progenies of few females (Latreille *et al.*, 2019), but instead was due to excessive outbreeding. The allelic

imbalance due to outbreeding has also been reported in mouse (Pinter *et al.*, 2015) and bovine mammals (Guillocheau *et al.*, 2019).

5.4.2 Population structure and spatial analysis

The analysis of molecular variance revealed very low differentiation within populations of *An. coluzzii* and *An. gambiae s.s.* despite variability in habitat types and land utilization. This may suggest frequent genetic exchange between different populations across southern Ghana. This finding is consistent with previous studies performed in East Africa using mtDNA and microsatellite loci where weak differentiation (F_{ST} = 0.02) was reported in *An. gambiae s.s.* population (Lehmann *et al.*, 2000). Similarly, a study using restriction-site associated DNA sequencing to genotype genome-wide SNPs in wild-caught *An. gambiae s.s.* in East Africa (Kilifi in Kenya and Muheza in Tanzania separated by 190 km) also reported a weak differentiation (F_{ST} = 0.04) (O'Loughlin *et al.*, 2014). However, a recent genome-wide SNP study in Cameroon where substantial population differentiation was reported within *An. coluzzii* (Kamdem *et al.*, 2017), but weak in *An. gambiae s.s*.

The component analyses using Nei's genetic distances showed no distinct groupings within the populations of each of the species. However, *An. coluzzii* populations from Bantama and Anhwiankwanta, and *An. gambiae s.s.* populations from Wassa Essaman and Tuba appeared the most differentiated from the majority of the populations which may be due to a natural break in gene flow. The cluster analysis revealed no evidence of structure within both *An. coluzzii* and *An. gambiae s.s.* populations, implying that the populations are in panmixia. This finding is supported by the low level of differentiations existing among mosquito populations (Table 5.8), which suggests that there is no restriction to gene flow. One possible explanation of this finding is that there is no major landscape feature across southern Ghana acting as a barrier to gene flow like the Great Rift Valley complex which divides east and west Kenya (Kamau *et al.*, 1998; Lehmann *et al.*, 2000). Another possible explanation is that both species dwell among human habitation, and might have experienced extensive population and range expansion as humans spread. This expansion might have left a genetic signal that is masking population structure (Besansky *et al.*, 1997; Donnelly, Licht and Lehmann, 2001). Our finding is similar to a microsatellite study in Tanzania where no population structure was found in *An. gambiae s.s* (Maliti *et al.*, 2014), but contrary to the finding in Cameroon where population structure has been reported within *An. coluzzii* (Kamdem *et al.*, 2017).

An. coluzzii populations exhibited isolation by distance (IBD) with a marginal significance which indicates that the weak genetic differentiation is structured in geographic

space. However, the Mantel correlograms show a significant IBD at short range, thus, at a local scale (70 km), there is evidence of distance-limited dispersal. This pattern is consistent with the situation shown in Case IV in Hutchison and Templeton (1999) (Figure 5.25), where migration-drift equilibrium is reached only at local scales according to the stepping-stone model of regional population structure. In this model, natural populations are influenced by gene flow and drift such that there is a substantial migration occurring in the adjacent populations, and so F_{ST} gives a useful index of gene flow. However, beyond this locale scale, it is difficult to know whether further patterning is evident due to a lack of equilibrium. A historical signal of expansion or population connectivity may stop IBD from showing up over the full sampling range.

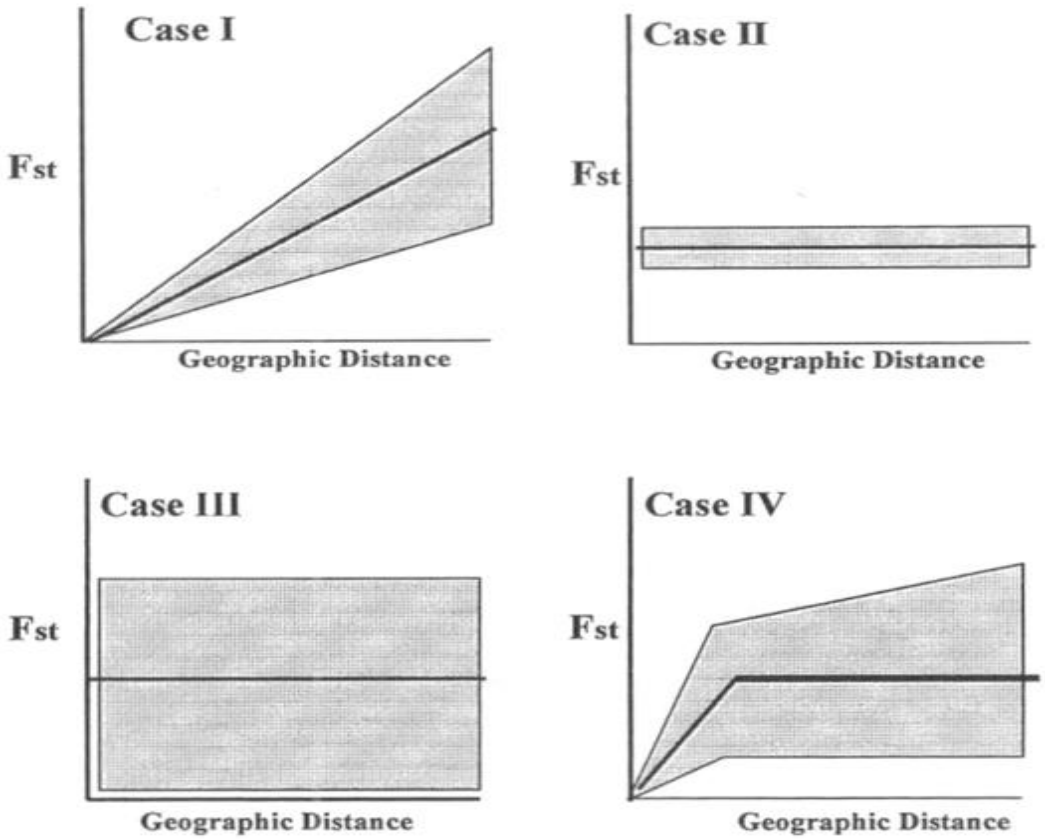


Figure 5.25: Graphs showing hypothetical associations between genetic distance (FST) and geographic distance for four scenarios: (I) regional equilibrium between gene flow and drift (II) lack of regional equilibrium with gene flow much more influential than drift (III) lack of regional equilibrium with gene flow much more influential than drift (IV) lack of regional equilibrium where gene flow and drift affect regional population structure differently depending on the scale; with effect gene flow at shorter distances and drift more influential at greater distances of geographic separation (Hutchison and Templeton, 1999).

Although this study is at a fine geographical scale, the finding is consistent with the recent genome-wide SNP studies across West Africa where there is evidence of isolation by distance, suggesting a low effective migration rate within *An. coluzzii* as the cause of the observed pattern (The Anopheles gambiae 1000 Genomes Consortium, 2020). There was a marginal association between genetic distance and habitat which suggests that mosquitoes adapted to local conditions may be more genetically differentiated from mosquitoes in populations with different conditions. Similar findings have been reported in Cameroon where *An. coluzzii* populations are thriving in local habitats (Simard *et al.*, 2009) and this phenomenon is hypothesized to facilitate niche expansion (Kamdem *et al.,* 2017).

An. gambiae s.s., on the other hand, showed weak population differentiation with no evidence of isolation by distance (i.e. no relationship between genetic distance (F_{ST}) and geographic distance) and did not show migration-drift equilibrium at even a smaller geographic distance of separation. This finding is consistent with previous studies in southern Ghana using microsatellite where the effect of geographical distance on the genetic differentiation patterns was found to be negligible (Yawson *et al.*, 2007). This implies that *An. gambiae s.s.* over its range across southern Ghana comprises populations that exhibit continuous and active allele exchange at a substantial magnitude to prevent them from diverging genetically. Similarly, recent genome-wide studies across West Africa at a large geographical scale reported no population structure among *An. gambiae s.s.* individuals from Guinea, Burkina Faso, Ghana and Cameroon (The *Anopheles gambiae* 1000 Genomes Consortium, 2020), suggesting there is a high rate of effective migration among *An. gambiae s.s.* individuals. However, considering the dispersal capabilities of *An. gambiae s.s.*, i.e. a maximum flight range of 7 km (Touré *et al.*, 1998), then lack of genetic differentiation between populations of more than 400 km apart might have resulted from contemporary gene flow among populations due to active migration and passive transportation through anthropogenic activities (Lanzaro and Tripet, 2003). Another possible explanation is that *An. gambiae s.s.* dispersal may be in the range of tens to hundreds of kilometres from the indirect estimate (Carnahan *et al.*, 2002) aided by environmental factors such as wind movement (Huestis *et al.*, 2019) thereby increasing genetic connections among populations. For instance, the flight range may increase when females fail to find a suitable location for oviposition or blood-meals (Delatte *et al.*, 2013), which probably aided by the wind. Again, it could mean that the populations are genetically similar because they were historically so and the genomes still exhibit this or because of very strong continuity of populations.

In *An. gambiae s.s.*, the contemporary panmixia restricts genetic drift thereby constraining the establishment of genetic structure. The genetic distance showed no correlation with habitat, suggesting that habitat did not account for the differentiation among

An. gambiae s.s. The study showed different patterns of distance-limited dispersal and differentiations existing among *An. coluzzii* and *An. gambiae s.s.* populations based on markers of chromosome 3. Similarly, two macrogeographical studies across Africa using microsatellites (Pinto *et al.*, 2013) and SNPs (The Anopheles gambiae 1000 Genomes Consortium, 2020) have also reported greater isolation by distance and population differentiation in *An. coluzzii* than in *An. gambiae s.s*. particularly in West Africa. One possible explanation is that *An. coluzzii* are more likely to adapt to different local ecological niches (Kamdem *et al.*, 2012, 2017; Pinto *et al.*, 2013). It is also postulated that there is a lower effective migration rate in *An. coluzzii* than in *An. gambiae s.s*., thereby limiting gene flow among *An. coluzzii* populations across a wide geographical range with diverse habitat types (The *Anopheles gambiae* 1000 Genomes Consortium, 2020).

5.4.3: Weak population differentiation and absence of population structure and their implications on the distribution of resistance alleles

In both *An. coluzzii* and *An. gambiae s.s*, high levels of genetic diversity and weak population differentiation, and absence of population structure indicate high gene flow (Vandewoestijne and Van Dyck, 2010). Therefore, in theory, the selection of resistance alleles such as *Ace-1*-G119S and *Vgsc*-L1014F mutations are likely to spread unhindered if their selective pressures (insecticide treatments) persist in the environment. For instance, *Ace-1*-G119S mutation and *Ace-1* duplications have been selected in OP/CB resistance in several places in West Africa (Djogbénou et al., 2008; Essandoh *et al.*, 2013; Camara *et al.,* 2018). It appears de novo emergence at the local populations is accounting for this phenomenon considering the geographical distances between the populations, however, haplotype analysis has shown that *Ace-1*-119S has a common ancestral origin in both West African (Djogbénou *et al.,* 2008; Essandoh *et al.,* 2013; Weetman *et al.*, 2015), suggesting an unimpeded gene flow and a selective sweep (global selection). Furthermore, in Central Africa, decades of studies had not found *Ace-1*-G119S mutation in *An. coluzzii* and *An. gambiae s.s* populations (Antonio-Nkondjio *et al*., 2011, 2016), however, it has recently been reported in Cameroon and phylogenetic studies have shown that it was introduced to Central Africa (Elanga-Ndille *et al.,* 2019) through gene flow.

Insecticide application is usually non-uniform across a wide geographic landscape, especially in most parts of Africa where treatment is inconsistent, coupled with high fitness cost associated with resistant alleles may suggest that migration of resistant mosquitoes beyond treated areas is difficult. For instance, the main source of *Ace-1* selection come from

agriculture (Antonio-Nkondjio *et al*., 2016) and a gradual decline in *Ace-1* frequencies from agricultural areas creating a cline has been observed in Ghana (Figure 2.11), highlighting a step-by-step migration of resistant alleles in response to spatial treatment gradient.

In *An. coluzzii*, though there is no clear population structure, the presence of isolation by distance and significant correlogram at 70km suggests that there is progressive genetic differentiation from one population to another (Vandewoestijne and Van Dyck, 2010) creating local adaptation which has been reported among several *An. coluzzii* populations (Simard *et al.*, 2009; Kamdem *et al.,* 2017). The local adaptation implies that selected resistance alleles are likely to spread across slowly across the heterogeneous landscapes, and this may explain why the *Ace-1*-G119S mutations and *Ace-1* duplications are significantly higher in *An. gambiae s.s.* than in *An. coluzzii* in almost all areas where the two species dwell in sympatry.

5.4.4 Spatial analysis for adaptive markers

In both *An. coluzzii* and *An. gambiae s.s*, *Ace-1*-119S exhibited no association with a genetic distance of chromosome 3 but showed strong isolation by geographic distance, which may suggest that distance is not the primary cause of *Ace-1* differentiation, but rather because of habitat differentiation which correlates with distance. The Chromosome 2L, 2R and X SNPs showed strong isolation by distance in *An. coluzzii*, but only showed genetic similarities among *An. coluzzii* mosquito populations up to 115 km. Their genetic distance showed significant association with habitat, suggesting that habitat-specific factors account for their differentiation. It is noteworthy that our finding is not definite as the mosquito sampling was not evenly distributed among the habitat types, and therefore a fine-scale study with uniform sampling distribution will be required. In *An. gambiae s.s.*, for chromosomes 2L was significantly associated with habitat.

5.4.5 Identification of loci under selection

A large number of SNPs may provide much power in detecting evidence for local adaptation from outlier loci that exhibit exceptionally high differentiation among populations (Hauser & Seeb, 2008). In both species, at least 18 loci in X chromosomes were identified as outliers undergoing diversifying selection for population, habitat or land-use level. The diversifying selection is a type of selection where the extreme traits are favoured over the intermediate traits. Diversifying selection increases genetic variance and drives speciation (Barton, 2010). The detection of these loci in both species in terms of population, habitat and

land-use with a high level of differentiation against their background genome may support the notion that they are truly under selection, and may not be the result of isolation by distance (Meirmans, 2012; Lotterhos and Whitlock, 2014). This finding supports the identification of chromosome X as the primary candidate of the genomic island of speciation between *An. gambiae s.s.* and *An. coluzzii* (Aboagye-Antwi *et al.*, 2015; Caputo *et al.*, 2016). The outlier loci showed relatively high differentiation and they are located around 15 Mbp, however, their adaptive significance is currently unknown as a BLAST search in Vectorbase identified six genes in the coding region that are yet to be described. Further studies are therefore required to determine the exact role of these genes in local adaptation.

5.5 Conclusion

This study deals with the population differentiation and structure of *An. gambiae s.s.* and *An. coluzzii* populations using genome-wide SNP markers on a fine-scaled sampling across southern Ghana. This study provides essential information to predict the spread of resistance alleles among *An. gambiae s.s.* and *An. coluzzii* populations. It appeared that habitat differentiation influences mosquito distribution and differentiation, and so further studies will be required to investigate the association between habitat-specific factors and population differentiation. Again, this study focused only on southern Ghana due to time constraint, it will be prudent to conduct similar work in the middle and the northern belts of Ghana to obtain the nationwide picture of population structure and differentiation among mosquitoes to guide vector interventions across varied geographic landscapes.

Chapter Six: General discussion and conclusions

6.1 Summary

Malaria control through vector intervention across sub-Saharan Africa relies mainly on the use of insecticides for indoor residual spraying (IRS) and treatment of bednet (ITN) and more recently long-lasting insecticide-treated bednet (LLIN) and ITN with PBO synergist. These insecticide-based tools have successfully been used to reduce malaria cases and deaths since 2000 (WHO, 2020). However, insecticide resistance limits the use of chemical formulations for vector control as it reduces the efficacy of IRS and ITN, and threatens to derail the gains made. The emergence of resistance mutations in anopheline mosquitoes is directly through selection pressure exerted by insecticides, yet it is modulated by several physiological, genetic and ecological factors (Belinato and Martins, 2016). In West Africa, the widespread pyrethroid and DDT resistance and the patches of the carbamate (CB)/organophosphate (OP) resistance in the principal malaria vectors, *An. coluzzii* and *An. gambiae s.s.*, have necessitated a study to identify and understand the drivers of resistance so as to provide information to guide vector control and the subsequent resistance management. In this study, the Chapter 2 focused on the spatial distribution of the target-site resistant mechanisms (Ace-1-119S and Vgsc -1014F) and their environmental drivers, the Chapter 3 focused on the use of molecular surveillance to track changes in resistance in populations, Chapter 4 focused on age-specific cost of resistance and finally Chapter 5 used genome-wide markers to determine population differentiation and structure.

6.2 Key and novel findings

In Chapter 2, *An. coluzzii* and *An. gambiae s.s.* are two main anopheline species in southern Ghana, but they show varying proportions among the study sites and ecological zones (mangrove swamp, coastal savannah, deciduous forest and rainforest). *An. coluzzii* was a more predominant species with evidence of thriving in degraded environments (such as “galamsey” pits) and brackish settings, confirming the exploitation of marginal habitats by *An. coluzzii* for niche expansion (Tene Fossog *et al.*, 2015; Kamdem *et al.*, 2017). A very few hybrids were found in this study, and considering the trend of decline of hybrids over the years (Essandoh *et al.*, 2013; Clarkson *et al.*, 2014) coupled with few admixtures from the population genetic analysis indicate very little introgression between the species fuelling speciation.

Phenotypic resistance of *An. coluzzii* and *An. gambiae s.s.* to pyrethroids (permethrin and deltamethrin) was quite high and widespread in southern Ghana, the prevalence however varies significantly among study sites. For CB bendiocarb and OP fenitrothion, phenotypic

resistance was generally low but locally high in some sites, particularly, those located in the central-eastern belt of southern Ghana where numerous commercial vegetable-growing activities are carried out, which may suggest a high selection pressure exerted via the use of agricultural pesticides. Phenotypic resistance to CB and OP was significantly lower than that of pyrethroids. However, the mosquitoes were markedly more resistant to CBs than to OPs even though they have the same mode of action in terms of the target site, and this may be due to the differences in the insecticide specificity and pharmacodynamic interactions at the target site.

The main implicated target-site resistance mechanism of pyrethroids, *Vgsc*-L1014F, was very high and even fixed in several populations across southern Ghana in both species. There was also the first report of *Vgsc*-N1575Y, which operates in synergy with *Vgsc*-L1014F to enhance pyrethroid resistance (Jones *et al.*, 2012), within the species pair in Ghana. The *Vgsc*-1014F frequencies were particularly high in urban and peri-urban areas with large-scale vegetable farms, while the least *Vgsc*-1014F frequencies were also associated with deciduous forest where domestic gardening was the major land-use activity.

For CB/OP, the *Ace-1*-G119S and its copy number variations (CNVs) were the main implicated target-site resistant mechanisms. The *Ace-1*-119S frequencies are relatively low with patchy spatial distributions compared to *Vgsc*-1014F. The *Ace-1*-119S genotypes were predominantly heterozygotes (*Ace*-1*GS)* and this may be due to the high fitness cost associated with the possession of *Ace*-1-119S. The *Ace-1*-119S was quite ubiquitous in the central belt that is characterised by numerous urban agricultural activities, but the frequency declined clinally towards the western and the eastern belts, which may suggest there is variation in the selection pressures in these regions. It appears that ecological zones and land-use activities are principal determinants of the distribution of *Ace-1*-119S genotypes. The frequencies of *Vgsc*-L1014F and *Ace-1-119S* were significantly higher in *An. gambiae s.s.* than in *An. coluzzii* even in sites where the species exist in sympatry, and this may be due to the onset of the resistant alleles in the *An. gambiae s.s.* before they introgressed into *An. coluzzii*.

In chapter 3, the results showed that the *Ace-1*-119S frequency is a very sensitive marker for phenotypic resistance to CB and OP. Most of the *Ace-1*-119S alleles are duplicated creating the co-existence of heterogeneous and homogeneous *Ace-1* duplications, which are exhibiting competing dynamics in terms of costs and benefits and appeared to be leading to the establishment of a semi-stable equilibrium between the two duplications. However, the temporal dynamics of the *Ace-1* duplication types are operating in opposite ways in each species. In *An. gambiae s.s.,* both homogeneous and heterogeneous duplications serve as

'regular' genotypes, but the rapid rise of homogeneous duplications accompanied by a balancing rise of the heterogeneous duplications may indicate a change in selection pressure favouring the thrive of the latter. In *An. coluzzii*, duplication was mainly heterogeneous, with homogeneous type appearing recently at relatively low frequency. There was a high proportion of unduplicated heterozygotes in *An. coluzzii*, but the frequency is declining markedly over time. Our results also suggest that the *Ace-1*-119S allele and duplication might have originated in *An. gambiae s.s.* and introgressed into *An. coluzzii*, and this may explain why Ace-1-119S is significantly higher in *An. gambiae s.s.* than in *An. coluzzii*. This is interesting because Chapter 2 results showed a very low level of hybridization, yet the introgression of Ace-1 duplication appears to be a recent event.

In Chapter 4, we assessed how phenotypic resistance to pirimiphos methyl (PM) changes with age, and how the association is mediated by the possession of *Ace*-1 resistance alleles. There was no consistent trend in the age-susceptibility relationship following PM exposure. This is because *Ace-1SS* individuals survive well with PM exposure, however, the response of *Ace-1GS* is very mixed, and so it is likely that the *ID* is the latent variable associated with *Ace-1* duplication (higher copy number) probably to offset the cost of having more copies (Assogba *et al.*, 2018). Unexpectedly, the synergist PBO appears to enhance the survival of mosquitoes following PM exposure which may be due to the balancing effect between the two P450 metabolic pathways involved. In tandem with other similar studies (Rajatileka, Burhani and Ranson, 2011; Jones *et al.*, 2012; Mbepera *et al.*, 2017; Machani *et al.*, 2019), the effect of age on the survival of mosquitoes following insecticide exposure is not a universal phenomenon and may depend on the type insecticide, the mechanism specificity and the population/strain of the mosquitoes. For unexposed mosquitoes, there was no evidence of a difference in the survival rate of *Ace-1GS* and *Ace-1SS* in each species, but the mosquitoes with *Ace-1* duplication showed a slightly higher survival rate than those without.

In Chapter 5, our results showed that genetic relatedness was generally low within a 3.0-km radius and may be partly due to high outbreeding among mosquitoes found in this study. There was low population differentiation and no evidence of population structure within each species, but the patterns differ between the two species. *An. coluzzii* populations showed evidence of distance-limited dispersal at the local scale and a strong association of adaptive markers with habitat, suggesting the adaptation of the mosquito to diverse local ecological niches is restricting gene flow in a large geographical range. In *An. gambiae s.s.*, there was no evidence of distance-limited dispersal even at a local scale, as such the local

level of differentiation may be due to gene flow via passive and active migration facilitated by human transportation and wind action.

6.3 Implication for vector control

To sustain an effective malaria control by vector intervention, the knowledge and understanding of the mosquito population differentiation and structure are essential in identifying and tracking the spread of insecticide resistance. In this study, *An. gambiae s.s* and *An. coluzzii* showed high phenotypic resistance to pyrethroid in southern Ghana with concomitant widespread Vgsc-1014F resistance mutation, and this is a great threat to vector control particularly the use of ITN which relies solely on pyrethroids. To sustain the effectiveness of IRS and ITN, there is a need to increase the use of other insecticides with different modes of actions in rotation or mosaic pattern.

Carbamates and organophosphate are alternatives, however *An. gambiae s.s* and *An. coluzzii* resistance to CB bendiocarb and OP fenitrothion is very high in the central belt, and temporary and spatial trends show the resistance is spreading towards the western and eastern belts. Resistance to OP pirimiphos methyl (PM), which has recently been introduced into public health for IRS, is relatively low, however, PM must not be used in conjunction with PBO synergist since it appears to enhance the survival of mosquitoes instead. The high level of CB/OP resistance corresponds to a high proportion of *Ace-1*-119S and the evolution of heterogeneous and homogeneous duplications of *Ace*-1. These duplications with varying cost-benefit cause mosquitoes to thrive in the environmental mosaic of treated and untreated areas created by inconsistent vector control practices. These diverse target-site resistance mechanisms are worrying as they pose a threat to vector control via the IRS.

Our study also showed that ecological zones and land-use types influence the distribution of mosquito species and resistant loci, which implies that vector intervention tools must consider environmental factors before embarking on large-scale control programmes. Although accurate quantification of insecticides in the environment exerting selection pressure is difficult, however sensitive resistance markers, such as *Ace-1*, can be used as a proxy to determine the environmental selection pressure before insecticide recommendation is made. For instance, *An. coluzzii* are expanding their ecological niches by thriving in marginal and disturbed breeding sites such as abandoned mining pits (e.g. galamsey pits) and brackish pools. It is believed that the expansion is modulated by adaptation to xenobiotics (Kamdem *et al.*, 2017) which in the long term may result in the creation of genetically distinct groups or

vector systems. Again, these local adaptations may imply that malaria vector control tools including insecticide applications are less likely to work uniformly in a wide geographical range.

In this study, few admixtures found among the species suggest that there is marginal but appreciable genetic exchange between the species pair which may facilitate the introgression of resistance alleles. The low genetic differentiation and no structure coupled with no major spatial barrier to gene flow within *An. gambiae s.s.* and *An. coluzzii* populations in southern Ghana suggest that insecticide resistance loci such as *Ace-1*-119S and *Vgsc*-1014F are likely to spread readily once they arise in the population, although it will depend on the distance and habitat differentiation, the strength, timing or spatial distribution of selective pressures, or differences in fitness costs in the absence of positive selection (The Anopheles gambiae 1000 Genomes Consortium, 2020).

Furthermore, our results showed that in an attempt to use genetically modified mosquitoes to control vector populations in Ghana, the released genetic material is likely to spread rapidly within *An. coluzzii* local populations. However, the spread of genetic material within *An. gambiae s.s.* is not clear as there was no evidence of contemporary gene flow, and so the low differentiation across the geographic range under consideration observed from this study may be the result of historic signals. Again, the outbreeding nature, low genetic differential and weak structure of the species pair may suggest a confluence of individuals from different geographical sources, which may result in polymorphism in the susceptibility to plasmodium, vector competence or resistance to insecticides (Ayala *et al.*, 2020), which in turn could influence the effectiveness of insecticide treatment and malaria control at large.

6.4 Limitations and recommendations for future work

The study focused on southern Ghana, as such it is recommended that similar studies should be performed in the middle and northern Ghana to obtain the nationwide picture of phenotypic resistance of Anopheles mosquitoes to pyrethroids, CB and OP and their underlying target-site resistance mechanisms. In this study, it was found unexpectedly that *An. coluzzii* is thriving in abandoned mining pits (“galamsey” sites) which are usually polluted with heavy metals (e.g. mercury) used for small-scale mining. It is, therefore, necessary to perform further studies on the physicochemical properties of the “galamsey” pits and their relationship with mosquito density.

A recent study showed there is a slight decrease in the prevalence of phenotypic resistance of *An. gambiae s.s.* and *An. coluzzii* population to bendiocarb but remain almost

the same for fenitrothion since 2014. However, it is not clear whether it translates into a reduction in the level of resistance. There were not enough mosquitoes in 2019 sampling to explore this, and so it recommended a further dose-response need to be performed for the insecticides to find out how/if the level of resistance changes as *Ace-1S* frequency and *Ace-1* duplication type decline. The *Ace-1* full duplication (with no internal deletion) appears to be associated with higher fitness cost than partial duplication (with an internal deletion of 10 flanking genes), but it is not clear whether all the 10 genes in the full duplication are expressed. It is therefore recommended that further investigations into the copy number variants of the flanking genes and their expression levels must be performed in the future.

Most studies have reported an increase in susceptibility of mosquitoes to insecticides with age, however, for PM, susceptibility with age showed inconsistent trends. Again, PM with PBO synergist enhanced the survival of mosquitoes. The exact underlying metabolic mechanisms responsible for these anomalies are not yet known, and it is therefore recommended that further studies on metabolomics need to be performed to investigate the role of various enzymes (particularly P450s) in the PM metabolism as mosquitoes age.

With all conventional SNP markers from this study showing a simple isolation-by-distance pattern without any clear population structure and local adaptation, there is a need for further studies using outlier loci to investigate whether there is any possible differentiation at a coarse-scale level. Again, an advanced spatial analysis using a database populated with topographical, climatic and land-use variables to establish grids of environmental variation for subsequent integration with genetic data. This information will enable the prediction of patterns of insecticide resistance based on ecological and environmental variables.

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Appendix

Appendix 1: The PCR Protocol for the characterization of *Anopheles gambiae sensu lato*

Reagent	Amount (µl)		Thermocycler conditions
Buffer (10x)	2.5		Initial 95ºC for 3 minutes
dNTP (10mM)	0.5		95 ºC for 30 seconds
ddH_2O	13.773		

Taq DNA polymerase (5U/ µl)	0.175		50ºC for 30 seconds 30 cycles 72ºC for 30 seconds 72ºC for 10 minutes
Quadrimaculatus Primer (10mM)	4.469		
Gambiae Primer (10mM)	0.624		
Universal Primer (10mM)	1.248		
Arabiensis Primer (10mM)	0.463		
Melas Primer (10mM)	1.248		
Mosquito leg	-		
Total volume	25		

Primer QD: 5'- TCC TGA GGA GGC TGC ATT TT -3' 319 bp

Primer GA: 5'- CTG GTT TGG TCG GCA CGT TT -3' 390 bp

Primer UN: 5'- GTG TGC CCC TTC CTC GAT GT -3' 468 bp

Primer AR: 5'- AAG TGT CCT TCT CCA TCC TA -3' 315 bp

Primer ME: 5'- TGA CCA ACC CAC TCC CTT GA -3' 464 bp

Appendix 2: The PCR protocol of *An. coluzzii* and *An. gambiae s.s.*diagnostics

Reagent	Amount (µl)		Thermocycler conditions
Buffer (10x)	2.5		Initial 95ºC for 3 minutes 95 ºC for 30 seconds 54ºC for 30 seconds } 35 cycles 72ºC for 60 seconds 72ºC for 10 minutes
dNTP (10mM)	0.5		
ddH_2O	18.8		
Taq DNA polymerase (5U/ µl)	0.2		
Dup-for (10mM)	1.0		
Dup-rev (10mM)	1.0		
DNA template	1		
Total volume	25		

Appendix 3: The PCR protocol of *Ace-1* duplication diagnostics

Reagent	Amount (µl)		Thermocycler conditions
Buffer (10x)	2.5		Initial 95ºC for 3 minutes 95 ºC for 30 seconds 60ºC for 30 seconds } 35 cycles 72ºC for 60 seconds 72ºC for 10 minutes
dNTP (10mM)	0.5		
ddH_2O	18.8		
Taq DNA polymerase (5U/ µl)	0.2		
Dup-for (10mM)	1.0		
Dup-rev (10mM)	1.0		
DNA template	1		
Total volume	25		

Appendix 4: The TaqMan assay protocol for *Ace-1/Vgsc* genotyping

Reagent	Volume (µl)		Thermocycler conditions
Sensimix	5.0		Hold: 95ºC for 10min 95 ºC for 10 seconds } 40cycles 60ºC for 45 seconds
Probe	0.125		
ddH_2O	3.875		
DNA template	1		
Total volume	10		

Appendix 5: Mortality rate of *An. gambiae s.l.* to five insecticides using the standard WHO discriminating concentrations.

		Mortality rate after 24-hour recovery period (% (N))				
Site	Date	Permethrin	Fenitrothion	Bendiocarb	DDT	Deltamethrin
Tuba	11/2016	36.0 (89)	72.4 (76)	X	X	X
Ashaiman	09/2016	12.0 (108)	45.8 (107)	9.2 (109)	1.9 (107)	X
Dzorwulu	11/2016	0.0 (103)	35.3 (116)	21.4 (84)	X	X
Keta	09/2016	55.0 (111)	98.1 (106)	62.9 (105)	39.1 (110)	62.0 (121)
Twifo Praso	08/2016	23.8 (101)	98.9 (90)	X	X	X
Ajumako	08/2016	11.8 (102)	X	X	X	X
Assin Fosu	08/2016	11.2 (98)	82.5 (97)	X	X	X
Takoradi	08/2016	2.6 (192)	92.2 (206)	X	12.5 (96)	X
Axim	06/2016	20.6 (107)	100.0 (104)	X	X	X
Shama	06/2016	14.1 (99)	98.1 (104)	X	X	X
Half-Assini	06/2016	31.7 (63)	100.0 (95)	X	X	X
Dixcove	06/2016	11.8 (93)	96.2 (104)	X	X	X
Mpohor	06/2016	15.7 (89)	95.2 (42)	X	X	X
Tarkwa	07/2016	50.0 (90)	X	X	X	X
Nkwanta	07/2016	60.6 (94)	X	X	X	X

*__X__ = Bioassay was not performed due to the small mosquito number.

*(__N__)= Number of mosquitoes assayed.

Appendix 6: Distribution of *VGSC-1014F* genotypes according to samples with allelic frequencies

	***VGSC*-1014F Genotypes**				
Site	**FF**	**LF**	**LL**	**Total**	**Ace-1-119S%**
Abura Dunkwa	12	4	0	16	87.5
Ada Foah	13	4	4	21	71.4
Agona Dunkwa	24	16	0	40	80.0
Agona Nkwanta	15	1	0	16	96.9

Ajumako	2	14	0	16	56.3
Akatsi	0	23	0	23	50.0
Amasaman	0	16	0	16	50.0
Anloga	13	10	1	24	75.0
Anyinase	12	2	0	14	92.9
Asankrangwa	8	5	3	16	65.6
Ashaiman	27	11	2	40	81.3
Asikumah	0	16	0	16	50.0
Assin Foso	26	2	0	28	96.4
Assin Kyina	14	2	0	16	93.8
Axim	12	4	0	16	87.5
Bogoso	21	4	0	25	92.0
Bonsaso	14	2	0	16	93.8
Dixcove	19	4	1	24	87.5
Dodowa	24	0	0	24	100.0
Dzorwulu	16	2	0	18	94.4
Elubo	11	1	0	12	95.8
Enchi	20	2	0	22	95.5
Half-Assini	16	0	0	16	100.0
Jukwa	14	1	1	16	90.6
Kakum	13	3	0	16	90.6
Keta	8	16	2	26	61.5
Koforidua	40	0	0	40	100.0
Komenda	12	3	1	16	84.4
Kpong	13	2	1	16	87.5
Labadi	13	3	0	16	90.6
Madina	29	3	0	32	95.3
Mamprobi	1	15	0	16	53.1
Mankessim	13	3	0	16	90.6
Matsepe	11	3	2	16	78.1
Mpohor	18	6	0	24	87.5
Nkwanta	15	0	1	16	93.8
Nsawan	13	5	0	18	86.1
Nsuaem	12	3	0	15	90.0
Prestea	10	6	0	16	81.3
Sekyere Krobo	14	2	0	16	93.8
Shama	15	6	0	21	85.7
Simpa	11	4	0	15	86.7
Sogakope	15	1	0	16	96.9
Suhum	24	0	0	24	100.0
Takoradi	11	5	0	16	84.4
Tarkwa	19	5	0	24	89.6
Tuba	5	18	0	23	60.9
Twifo Hemang	15	1	0	16	96.9
Twifo Praso	12	4	0	16	87.5
Wassa Akropong	13	3	0	16	90.6

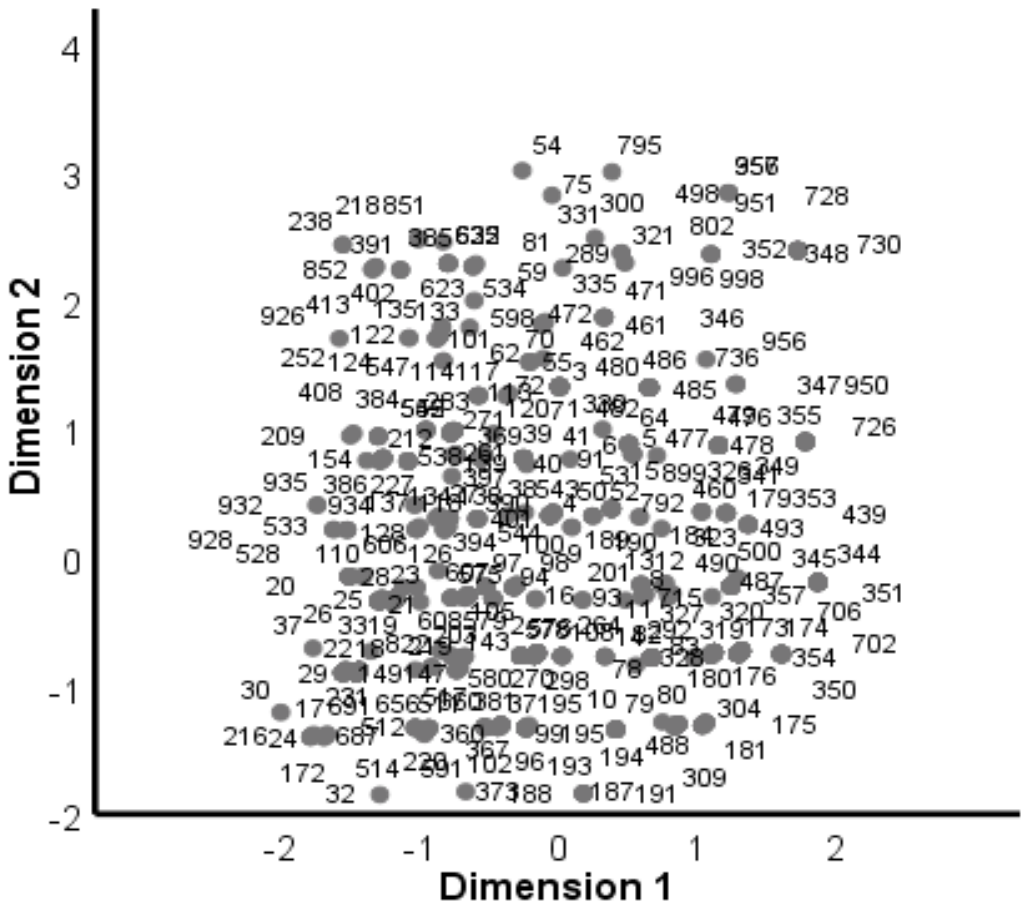
Wassa Essaman	28	2	0	30	96.7
Grand Total	736	268	19	1023	85.0

Appendix 7: Distribution of *Ace*-1-119S genotypes according to samples with allelic frequencies

	***Ace-1*-119S genotypes**				
Site	***GG***	***GS***	***SS***	**Total**	***Ace-1*-119S%**
Abura Dunkwa	9	7	0	16	21.9
Ada Foah	2	11	8	21	64.3
Agona Dunkwa	5	28	7	40	52.5
Agona Nkwanta	13	3	0	16	9.4
Ajumako	15	1	0	16	3.1
Akatsi	16	7	0	23	15.2
Amasaman	5	11	0	16	34.4
Anloga	0	24	0	24	50.0
Anyinase	12	2	0	14	7.1
Asankrangwa	15	1	0	16	3.1
Ashaiman	5	30	5	40	50.0
Asikumah	7	7	2	16	34.4
Assin Foso	9	15	4	28	41.1
Assin Kyina	13	3	0	16	9.4
Axim	14	2	0	16	6.3
Bogoso	16	7	2	25	22.0
Bonsaso	13	2	1	16	12.5
Dixcove	24	0	0	24	0.0
Dodowa	7	12	5	24	45.8
Dzorwulu	2	15	1	18	47.2
Elubo	12	0	0	12	0.0
Enchi	21	1	0	22	2.3
Half-Assini	16	0	0	16	0.0
Jukwa	11	5	0	16	15.6
Kakum	13	3	0	16	9.4
Keta	15	11	0	26	21.2
Koforidua	8	22	10	40	52.5
Komenda	15	1	0	16	3.1
Kpong	2	12	2	16	50.0
Labadi	9	6	1	16	25.0
Madina	2	20	10	32	62.5
Mamprobi	2	14	0	16	43.8
Mankessim	14	2	0	16	6.3
Matsepe	0	16	0	16	50.0
Mpohor	21	3	0	24	6.3
Nkwanta	15	1	0	16	3.1

Nsawan	9	9	0	18	25.0
Nsuaem	15	0	0	15	0.0
Prestea	15	1	0	16	3.1
Sekyere Krobo	11	4	1	16	18.8
Shama	17	4	0	21	9.5
Simpa	10	5	0	15	16.7
Sogakope	12	4	0	16	12.5
Suhum	16	6	2	24	20.8
Takoradi	10	6	0	16	18.8
Tarkwa	21	3	0	24	6.3
Tuba	5	16	2	23	43.5
Twifo Hemang	12	4	0	16	12.5
Twifo Praso	16	0	0	16	0.0
Wassa Akropong	16	0	0	16	0.0
Wassa Essaman	21	9	0	30	15.0
Grand Total	584	376	63	1023	24.5

Appendix 8: The plots of objects in principal component space. The plot shows how the variates are distributed in space.

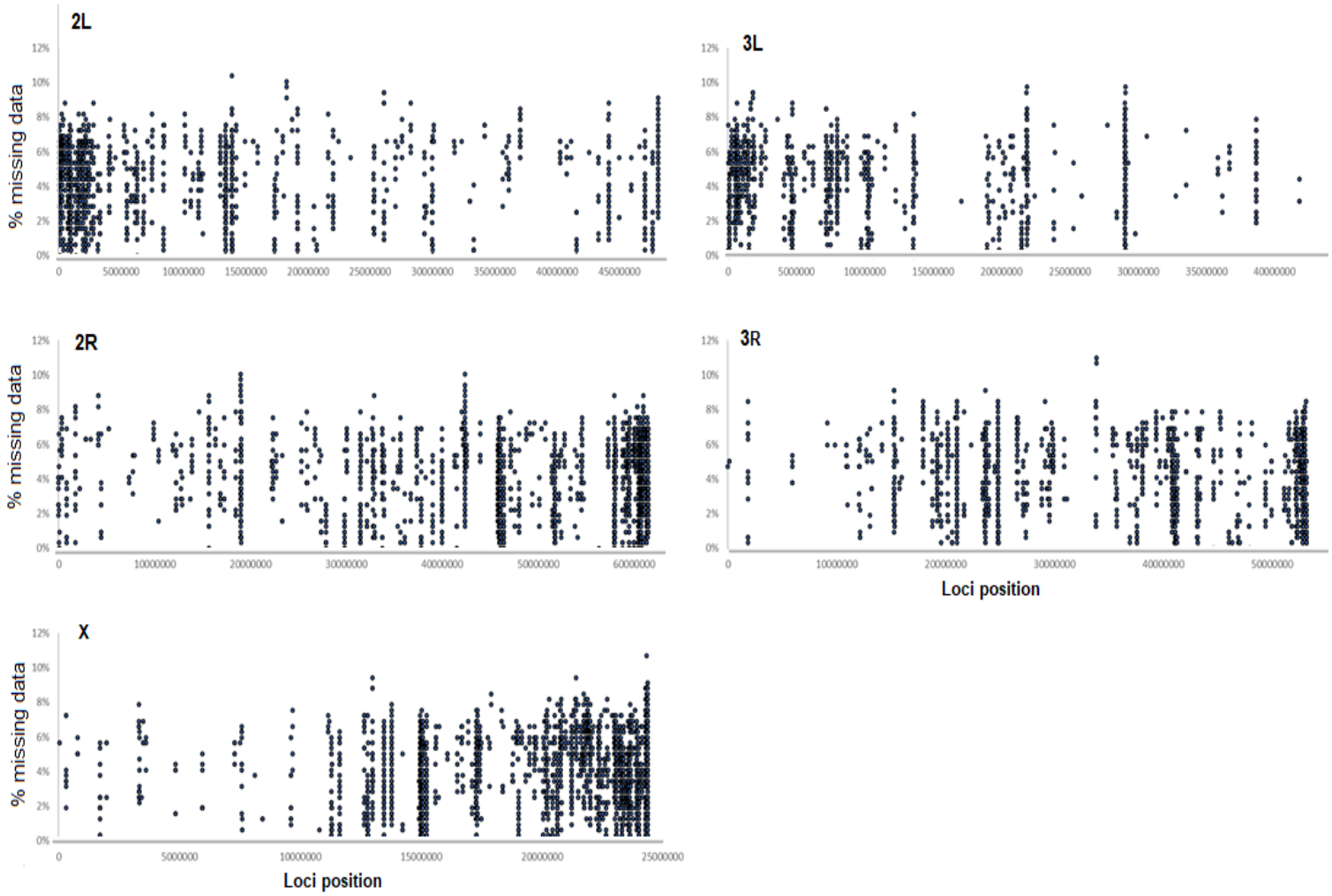


Appendix 9: Image of an empty bottle of Glyphosate, an organophosphate-based herbicide, found on the vegetable farm in Dzorwulu.

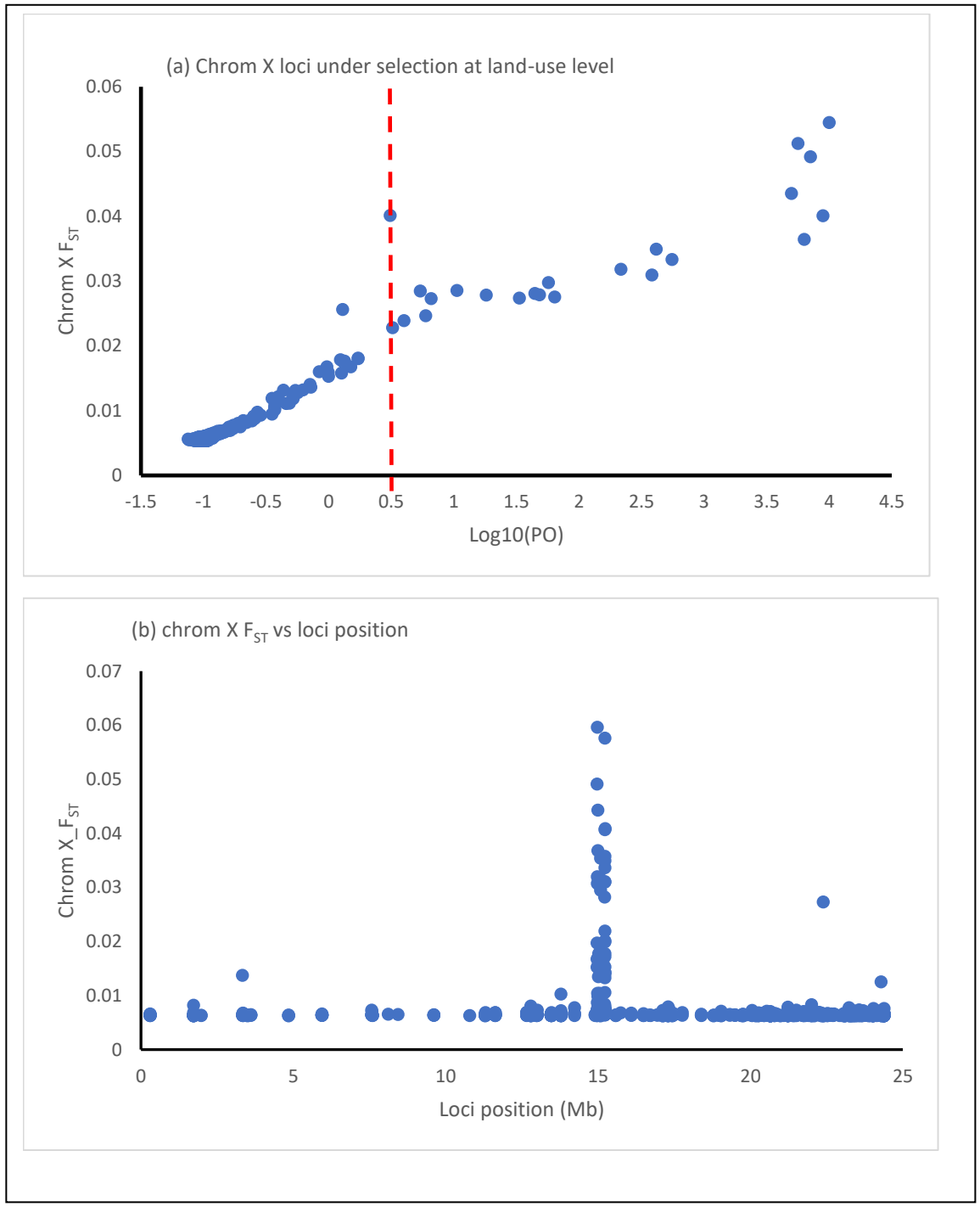
Appendix 10: The phenotypic status of *Anopheles gambiae s.l.* per a WHO test tube for 60 min-PM exposure and 60 min-PBO + 60 min PM exposure

60 MIN PM ONLY				60 MIN PBO + 60 MIN PM			
AGE	Dead	Live	Total	Age	Dead	Live	Total
3	12	17	29	3	14	8	22
3	10	19	29	3	16	7	23
3	10	20	30	3	18	6	24
3	11	19	30	3	15	8	23
9	16	8	24	9	22	8	30
9	17	9	26	9	23	8	31
9	18	5	23	9	25	5	30
9	18	7	25	9	19	9	28
12	13	13	26	12	26	4	30
12	11	16	27	12	24	2	26
12	12	15	27	12	23	5	28
12	10	17	27	12	25	5	30
15	21	5	26	15	18	7	25
15	22	3	25	15	17	7	24
15	19	7	26	15	16	8	24
15	20	7	27	15	17	9	26
15	20	7	27				
15	21	6	27				
15	19	7	26				

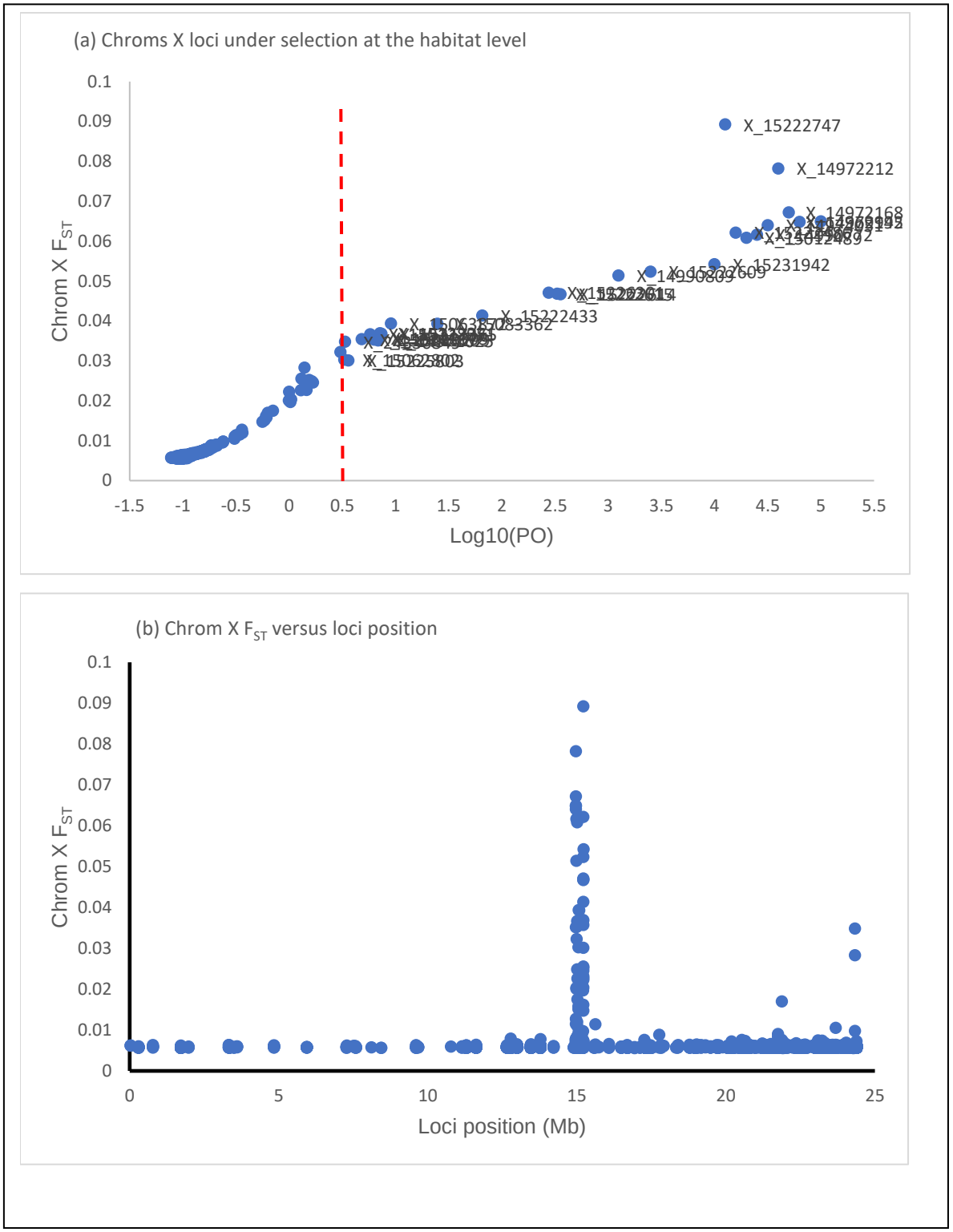
Appendix 11: The plots of missing SNPs against the loci position along chromosomes. The plots show that the SNPs were not evenly distributed along the chromosomes.



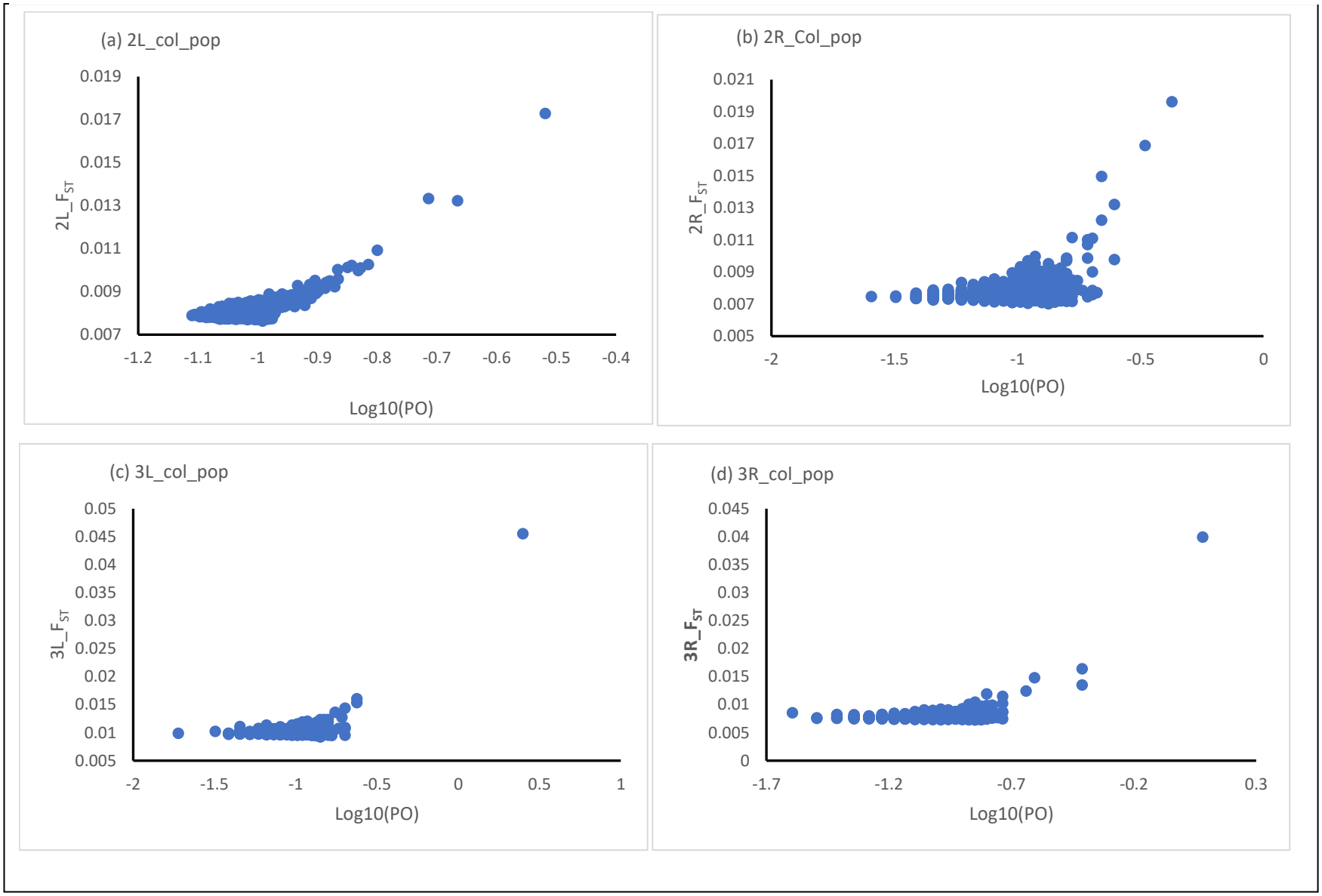
Appendix 12: The detection of outlier X chromosome SNPs in *An. coluzzii* for land-use using Bayescan. **(**a) The Log10 (PO) are shown on the x-axis and FST values of Chromosome X for four major land-use types across southern Ghana on the y-axis with the red vertical line indicating the selection threshold. Eighteen SNPs were identified under selection (b) The plot of SNPs FST and loci position along Chromosome X for land- use.



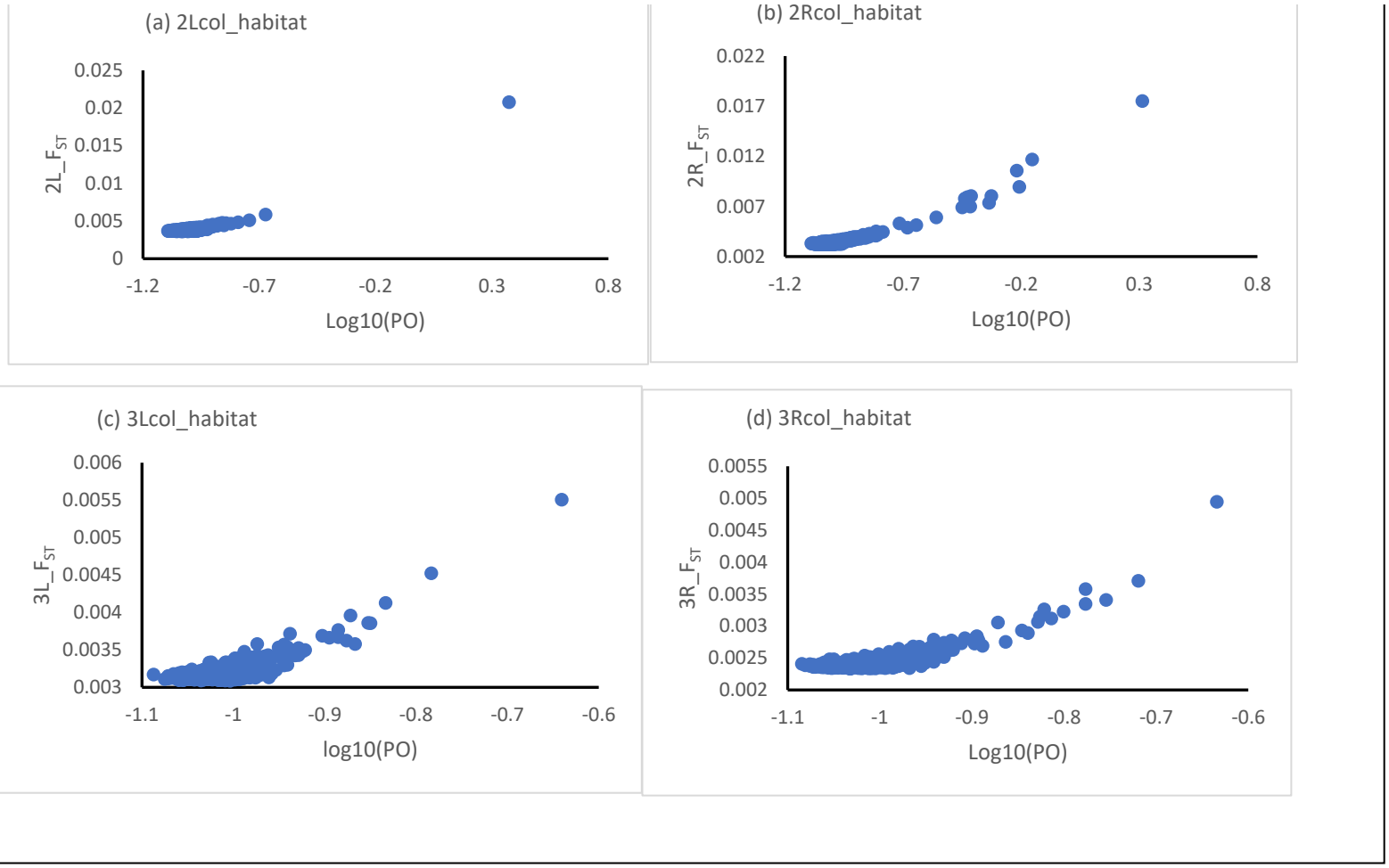
Appendix 13: The detection of outlier X chromosome SNPs in *An. coluzzii* for habitat using Bayescan. (a) The Log10 (PO) are shown on the x-axis and F_{ST} values of Chromosome X for four major habitat types across southern Ghana on the y-axis with the red vertical line indicating the selection threshold. Twenty-three SNPs were identified under selection (b) The plot of SNPs F_{ST} and loci position along Chromosome X for habitat



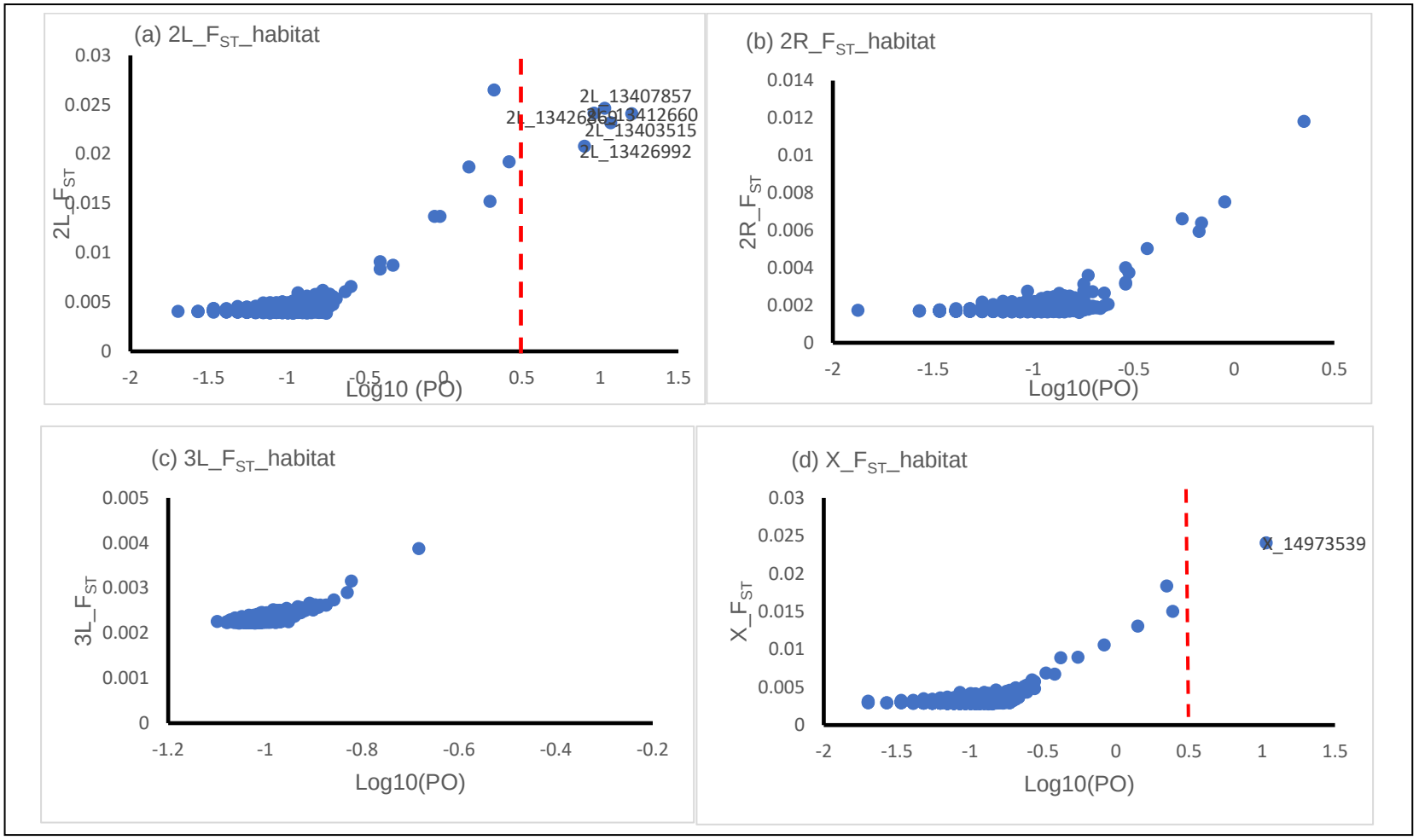
Appendix 14: The detection of outlier loci in 2L, 2R, 3L and 3R chromosomes for population level in *An. coluzzii* using Bayescan :
The Log10 (PO) are shown on the x-axis and F_{ST} values of Chromosome 2L, 2R,3L and 3R for 24 populations on the y-axis. No outlier SNPs were found.



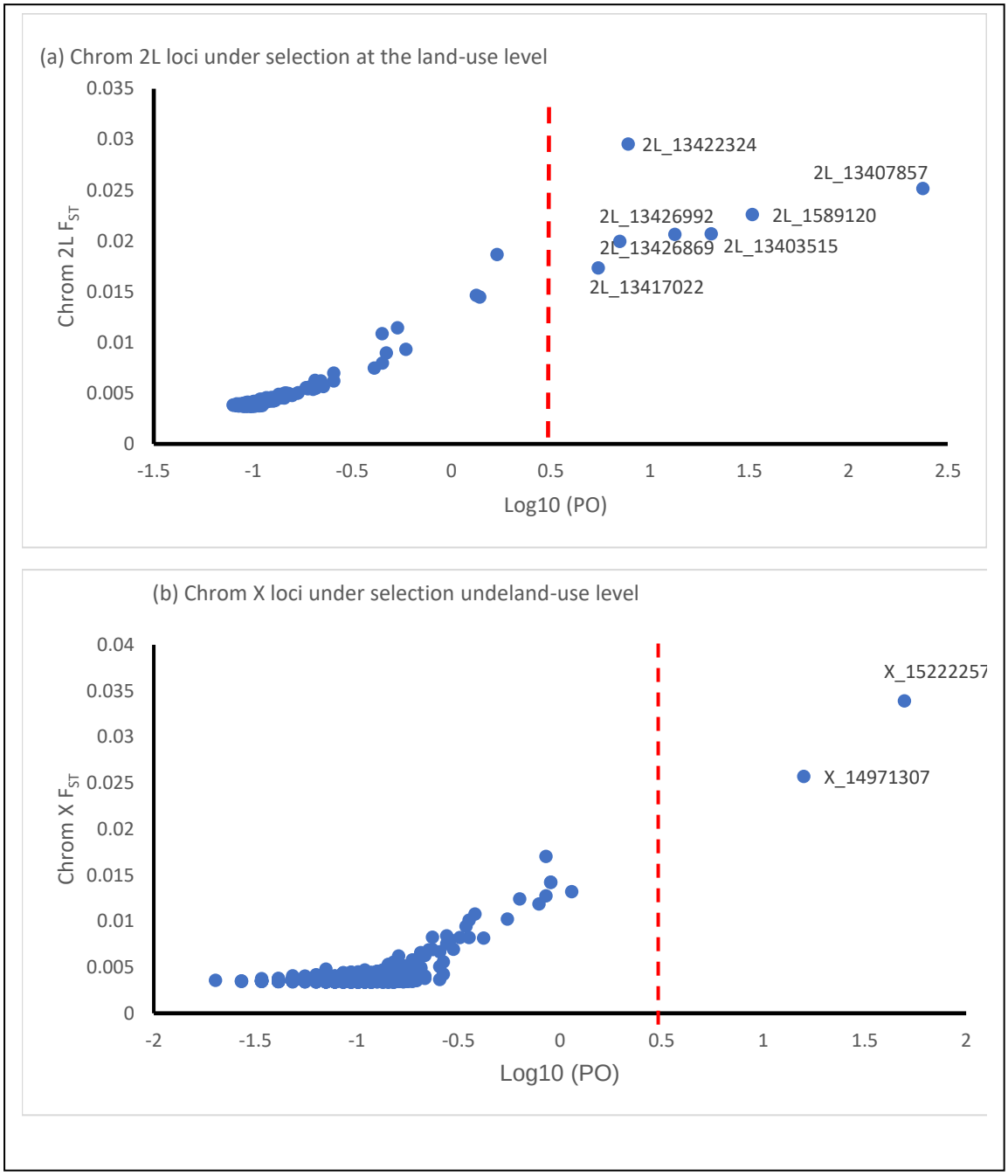
Appendix 15: The detection of outlier loci in 2L, 2R, 3L and 3R chromosomes for habitat level in *An. coluzzii* using Bayescan : The Log10 (PO) are shown on the x-axis and F_{ST} values of Chromosome 2L, 2R,3L and 3R for four major habitat types on the y-axis. No outlier SNPs were found.



Appendix 16: The detection of outlier SNPs in *An. gambiae s.s.* at habitat level: (a) 2L chromosome (b) 2R chromosome (c) 3R Chromosome and (d) X chromosome. The Log10 (PO) on the x-axis and F_{ST} values of Chromosomes for *An. gambiae s.s.* habitat types across southern Ghana on the y-axis with the red vertical line indicating the selection threshold. The specific outlier loci are labelled on each plot.



Appendix 17: The detection of outlier SNPs in *An. gambiae s.s.* at land-use level analysis**:** (a) 2L chromosome and (b) X chromosome. The Log10 (PO) on the x-axis and F_{ST} values of Chromosomes for *An. gambiae s.s.* land-use types across southern Ghana on the y-axis with the red vertical line indicating the selection threshold. The specific outlier loci are labelled on each plot.



Appendix 18: A region of chromosome X in *An. gambiae* (*An. coluzzii and An. gambiae s.s.*) where outlier SNPs correspond to six protein-coding genes.