KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

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DEPARTMENT OF THEORETICAL AND APPLIED BIOLOGY

STUDIES ON HYBRIDIZATION, GENE FLOW AND RESISTANCE STATUS OF M AND S MOLECULAR FORMS OF *ANOPHELES GAMBIAE S.S*

BY

JOSEPH CHABI (BSc. Hons.)

JUNE 2015

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By

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A Thesis submitted to the Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology in partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHILOSOPHY

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DECLARATION AND CERTIFICATION

I hereby declare that this submission is my own work towards the Master of Philosophy (MPhil.) degree, and that, to the best of my knowledge it contains no material previously published by another person or material which has been accepted for the award of any other university degree, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to my family, especially my wife Rosalie Yao Ahou and my daughter Audrey Merlène CHABI in compensation for all my absence at home and your support. Find in this work the outcome of all the hard time you had. I also dedicate to my colleagues of Vestergaard-NMIMR Vector Labs for their permanent support and encouragement. Thank you all for your immense contributions to the success of this project.

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LIST OF ABBREVIATIONS

µl: Micro litre µM:

Micro molar

°C: degree Celsius

An: *Anopheles*

Ace-1: Acetylcholinesterase

CTAB: cetyl trimethyl ammonium bromide

DNA: Deoxyribonucleic Acid dNTP:

Deoxynucleotide triphosphate

DDT: Dichlorodiphenyltrichloroethane

EDTA: Disodium ethylene diamine tetraacetate

HCl: Hydrogen chloride

IGS: Intergenic spacer

IRAC: Insecticide Resistance Action Committee

IRM: Insecticide resistance management

ITN: Insecticidal treated net

IVM: Integrated vector management

Kdr: Knock down resistance

LLIN: Long lasting insecticide treated net

LSTM: Liverpool School of Tropical Medicine

MgCl2: Magnesium chloride

NaCl: Sodium chloride

NMIMR: Noguchi Memorial Institute for Medical Research

OP: Organophosphate PCR:

Polymerase Chain Reaction

rDNA: Ribosomal DNA

RFLP: Restriction fragment length polymorphism

SINE: Short interspersed element

S.L: Sensu lato

S.S: Sensu stricto

UV: Ultra-violet

WHO: World Health Organization

ABSTRACT

Anopheles gambiae s.s is the most efficient malaria vector in Africa, particularly in sub-Sahara African countries. *An. gambiae s.s* is composed of two sibling species differentiated by the chromosomal form and named M and S molecular forms. New advances in complete mapping of the distribution of the vector have recently encountered the gene flow between them. Though the two forms have just been considered as different species and named *An. coluzzii* and *An. gambiae s.s* for M and S form respectively, yet M/S hybrid specimen have been found in some West Africa countries.

This study was designed to investigate in the laboratory the hybridization between the two species, the resistance mechanisms of the hybrid mosquitoes and its probable impact on the vector control management. Three different mosquito colonies, including an *An. gambiae s.s* M form resistant to pyrethroid and DDT, *An. gambiae s.s* S form resistant to all classes of insecticides and *An. gambiae s.s* S form susceptible to all insecticides were used to establish a hybrid mosquito colony. A slight mass crossing was done by inversely crossing 10 males and 10 females of each form. Resistance status and mechanisms of the progenies were determined using WHO susceptibility test and PCR methods respectively. An average of 50 males and 50 females were analyzed per crossing and per generation. The results showed that 100% of the females of the first generation of each crossing was M/S hybrid irrespective of the origin of the parent. Also all the males of each crossing inherited the female parent form, but none was found to be hybrid. Furthermore, all the hybrids showed similar DNA sequences whatever the crossing and the generation analyzed. In addition the progeny of the two resistant strains crossed recorded 66% and 80% *kdr* frequencies according to the form of male or female and higher than the resistant and susceptible hybrid progeny, yielding 50% *kdr* frequency. The *ace-1* genotype was about 50% for all the hybrid progenies with a slight increment for the female M and male S forms progeny (52%). But no significant difference was observed among the *ace-1* frequencies of the three M/S hybrids progenies.

This study confirmed the hybridization of the M and S molecular form of *An. gambiae s.s* as reported, and also showed the resistance mechanisms of the hybrids according to the parent resistance status. However, additional investigations need to be undertaken to fully characterize the M/S resistance mechanisms and furthermore, their susceptibility to malaria parasites

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Introduction

Malaria remains a major public health burden causing the death of more than 600,000 people each year, mostly in children under the age of five, and affecting those living in the poorest countries(WHO, 2011). Currently, the major obstacles to malaria control and elimination are the absence of a protective vaccine, the spread of parasites resistant to anti-malarial drugs and the resistance of mosquitoes to insecticides (Yassine and Osta, 2010). Controlling mosquito vectors is fundamental to reduce mosquito-borne diseases by targeting and reducing vectorial capacity and hence the transmission. Vector control through the use of chemicals for mosquito bed nets and indoor residual spraying is still the cornerstone of malaria prevention (WHO, 2011). Unfortunately, the extensive use of insecticides since the 1950s has led to the development of insecticide resistance worldwide, which represents a major challenge to the use of insecticides for vector control. Strategies to prevent or delay and/or control the spread of insecticide resistance in natural mosquito populations continue to be a challenge faced by many national malaria control programmes (NMCPs).

The various mechanisms that enable insects to resist the action of insecticides can be grouped into four distinct categories including metabolic resistance, target-site resistance, reduce penetration and behavioral avoidance (IRAC, 2011). It is obvious that insecticide resistance in malaria vectors is increasing worldwide due to the increasing selection pressure on mosquito populations caused by the presence of urban, domestic and/or agricultural pollutants in the environment (Nkya *et al*., 2013). Transversal and longitudinal monitoring surveys are essential to address the spatio-temporal

changes in resistance (dynamic) and to design appropriate strategies for a better control of resistant malaria vector populations worldwide.

Among the global vectors of human malaria, the most important species belong to the *An. gambiae* complex, including *An. gambiae s.s.*, which is the primary vector responsible for malaria transmission in sub-Saharan Africa. *Anopheles gambiae* s.s. consists of two genetically distinct species known as *An. gambiae* M and S molecular forms and recently considered as two different species and named *An. coluzzii* and *An. gambiae s.s Giles* respectively (here after considered as *An. gambiae* M and S molecular forms for the purpose of the project)

In Africa, the *L1014F kdr* mutation responsible for DDT and pyrethroid resistance is mostly widespread and predominant in the *An. gambiae* molecular S form compared to the M form (Ranson *et al*., 2011), except in some countries such as Benin (Yadouleton *et al*., 2010), Equatorial Guinea (Sharp *et al.,* 2007) and Niger (Czeher *et al.,* 2008). Some authors suggested that the *kdr* alleles may have arisen from at least four independent mutation events in the *An. gambiae* S-form (Yadouleton *et al*., 2010). Regarding the M form, it is not clear whether the *kdr* mutation resulted from an introgression from the S form only (della Torre *et al*., 2001, Weill *et al*., 2000) and/or from independent mutation events, as recently suggested in a study conducted in Bioko, Equatorial Guinea (Reimer *et al*., 2005). The second mutation, a leucine-serine substitution at the same codon (*L1014S*), was identified first in a colony of *An. gambiae s.l.* from Kenya. This substitution has been lately reported in Burundi, Cameroon, Gabon, Equatorial Guinea, Uganda, Republic of Congo and Angola, mainly in co-occurrence with the *1014F kdr* allele. Although some authors have reported that the *1014S* allele may confer lower level of pyrethroid resistance than the *1014F* allele (Reimer *et al*., 2008), its spread from eastern to central Africa and more recently to West Africa (Badolo *et al*., 2012) suggests a survival advantage of

mosquitoes sharing this mutation in the presence of pyrethroid insecticides. So far, the *L1014S* allele has always been detected in the S molecular form (Santolamazza *et al*., 2008) but recent findings showed the occurrence of the *1014S* allele in the M form in Equatorial Guinea (Ridl *et al*., 2008) and Cameroon (Reimer *et al*., 2008). In these two countries, the *1014S* allele was present either at very low frequencies, alone or associated with the *1014F* allele.

In most African countries, the two molecular forms of *An. gambiae* s.s are sympatric or occur alone in their site, and/or found separately according to the season. Recently, hybrid specimens have been reported in several parts of Africa.

Understanding the relationship between the two molecular forms has been the focus of ongoing research efforts. The S form has the broadest distribution occurring throughout sub-Saharan Africa, whereas the M form occurs throughout West and parts of Central Africa (della Torre *et al*., 2005). Although the M and S forms are largely reproductively isolated in most places where they occur together, this has been shown not to be true everywhere. Hybridization between forms occurs at low frequency (~1%) in Mali (Wondji *et al*., 2005) and reproductive isolation between M and S forms appears to be complete in Cameroon (Caputo *et al*., 2008). In Gambia, M/S hybrids were identified from a number of sites at frequencies as high as 16.7% of the *An. gambiae* (Oliveira *et al*., 2008). In Guinea-Bissau, hybrids were recovered in over 20% of the individuals assayed and recently, more than 40% were observed in the same country and in Senegal (Marsden *et al*., 2011, Nwakanma *et al*., 2013, Riehle *et al*., 2011). These results suggest that linkage between the M and S alleles and those genes that directly affect reproductive isolation has broken down in a much broader geographic area than previously thought.

Therefore, the notion of *An. gambiae s.s* M form and *An. gambiae s.s* S form largely reproductively isolated (incipient species) and also that hybridization only occurs in the "FarWest" region of Africa (Caputo *et al.*, 2011) might be an oversimplification.

1.2 Rationale of the study

Malaria being a major vector-borne disease is still affecting and causing the death of 300-600 million of people every year, mostly among children under the age of 5 years (WHO, 2011). All the approaches to control either the vector or the parasite have faced challenges in terms of effectiveness. Several resistance mechanisms occurring in the vector have driven the failure of most of the control measures. From the use of DDT in the 1950¨s as indoor residual spraying insecticide to ITN and recently LLINs including combination of insecticides, the negative impact of the resistance mechanisms has yielded the need to understand vector behavior before any undertaking any control measures. Among this, the hybridization occurring in *An. gambiae s.s* has brought special attention to include that in the vector studies.

The potential impact of increasing of hybridization between *An. gambiae* s.s. M and S molecular forms has not yet been fully explored. This trend could bring to a certain extent, some challenges in terms of malaria vector control strategies and efforts to address insecticide resistance.

This study considered the most important vector species in the transmission of malaria in subSaharan Africa countries, *An. gambiae s.s* M and S molecular forms. The study also investigated the hybridization between them currently to improve upon our knowledge on how the new species may respond to insecticide testing.

1.3 Objectives

1.3.1 Aim of the study

The main objective of the study was to:

Assess the hybridization, gene flow and susceptibility status of M, S and M/S molecular forms of *Anopheles gambiae sensu stricto*

1.3.2 Specific objectives

The set objectives were to:

I. Assess the generational development of M/S hybrid strain of *An. gambiae s.s*

II. conduct susceptibility tests on parents and progenies to determine resistant status of the insects to various insecticides

III. determine the resistance *kdr* and *ace-1* genotypes of the M/S hybrid mosquitoes

IV. Assess the gene flow between M/S hybrid, M and S molecular forms

CHAPTER TWO

LITERATURE REVIEW

2.1. An. gambiae complex

Until recently, *An. gambiae* was considered a complex of at least seven sibling species that show varying degrees of behavioral and ecological differences (Coluzzi, 1984; Davidson, 1964; White 1974, 1985). Recently, *An. gambiae sensu stricto* which is a complex of two different molecular forms were considered as two different species and named as *An. coluzzii* and *An. gambiae Giles*, resulting on 8 different sibling species in the *An. gambiae* complex. *Anopheles coluzzii*, formerly known as *Anopheles gambiae* M molecular form, was defined as a separate species in 2013 (Coetzee, 2013) and *An. gambiae* S form as *An. gambiae s.s Giles*.

Four of the sibling species are adapted to freshwater breeding sites: *An. gambiae s.s Giles* and *An. coluzzii* predominate in humid areas and are generally highly anthropophilic (della Torre *et al*., 2001), *An. arabiensis* Patton extends into drier Savanna areas, more zoophilic and exophilic and *An. quadriannulatus* Theobald is zoophilic, comprising allopatric taxa in Ethiopia and southern Africa (Hunt *et al*., 1998). Two saltwater tolerant species *An. melas* Theobald of West Africa and *An. merus* Donitz of East Africa are generally more exophagic and zoophilic and thus less efficient vectors than *An. gambiae s.s*. The sixth species, *An. bwambae White*, occurs around hot springs in Uganda (Gillies, 1968). *An. melas* and *An. merus* have distinct geographical distributions while sympatry between them and the others or among the others is of common occurrence (Bryan *et al*., 1982; Coetzee *et al*., 2000, Lindsay *et al*., 1998, White 1974; 1985).

Various degrees of genetic incompatibility, mainly expressed as hybrid male sterility, have been shown to exist between six species of the complex. Studies of the inversions which occur on the polytene chromosomes in the ovarian nurse cells of adult females as well as on the salivary glands of stage larvae have largely confirmed these differences (Coluzzi, 1984). Intraspecific polymorphism in these paracentric inversions has been shown among most of the sibling species with *An. gambiae s.s*.

and *An. arabiensis* having the most (Bryan *et al*., 1982; Bryan *et al*., 1987; Coluzzi, 1984; Coluzzi *et al*., 1979, Toure *et al*., 1994; 1998). Within *An. gambiae s.s.* the different forms were named as "Forest", "Bamako", "Bissau", "Mopti" and "Savannah" chromosomal forms and it has been reported that the frequency of their inversions vary with changes in environmental conditions suggesting some sort of climatic adaptation (Coluzzi *et al*., 1979, Thomson *et al*., 1997). Molecular forms of *An. gambiae s.s.* have been described (Favia et al. 1997) but these did not conform with the defined chromosomal types (della Torre *et al*., 2001, Gentile *et al*., 2001).

2.2. Life cycle of *An. gambiae s.l*

The adult female *Anopheles* requires at least one blood meal before she can oviposit. After a blood meal, she must find a suitable site for laying her developed eggs. Eggs are deposited singly on the surface of water at various breeding sites including shallow sunlit pools, borrow pits, drains, car tracks, foot prints near water holes, rice fields, irrigation canals, pools left behind by receding rivers, and rainwater collecting in natural depressions (Gillies and De Meillon, 1968). The choice of oviposition site for mosquitoes in general seems to be related to chemical cues given off from the breeding sites (McCall and Eaton, 2001, Takken and Knols, 1999). The eggs hatch into larvae, which remain at the surface of the water where they are adapted to feed. The larvae undergo three successive moults during their development and change into pupae after the fourth instar stage. The pupa does not feed and it breathes from the water surface by means of respiratory trumpets. The adults later emerge from the pupae and disperse (Fig.1).

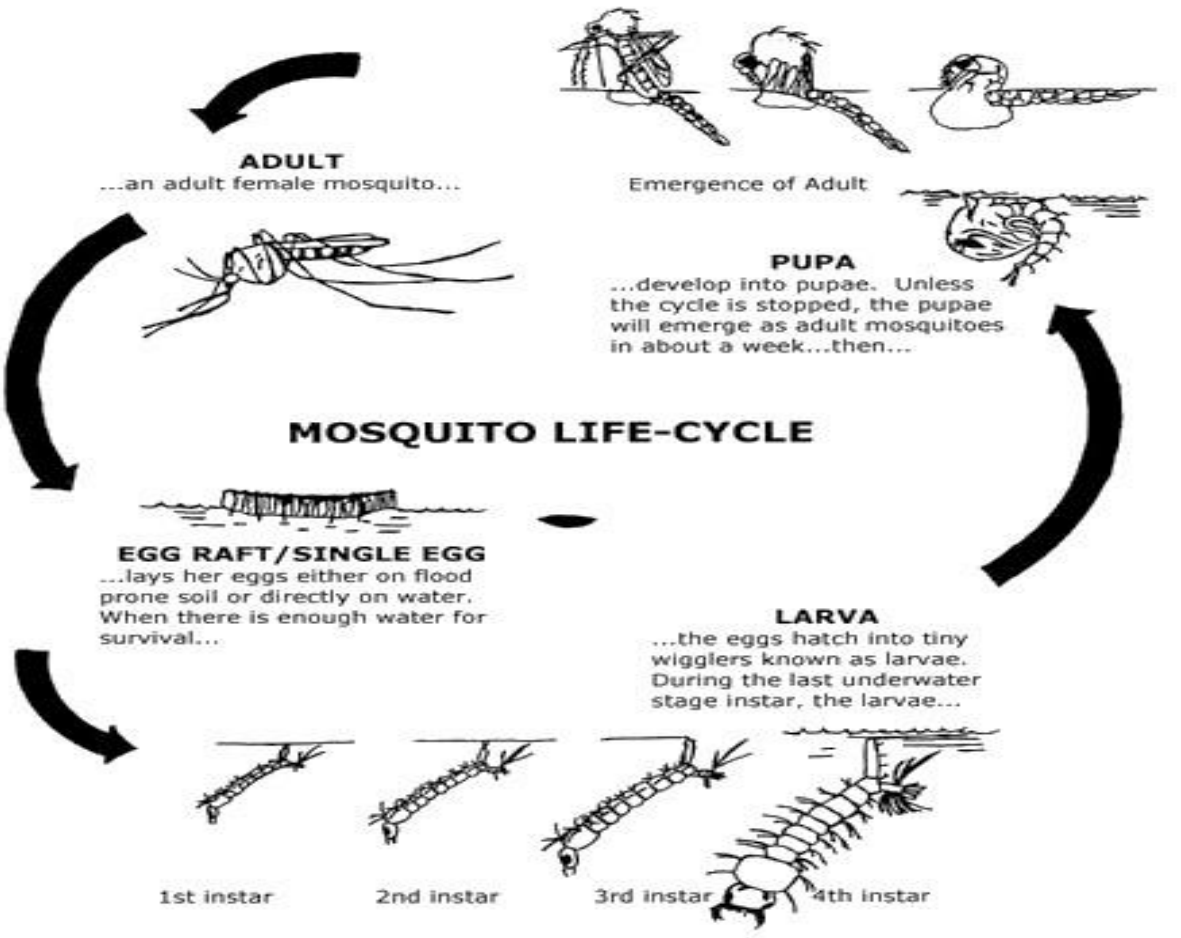

Figure 1: The life cycle of the mosquitoes

Source: https://www.google.com.gh/

2.3. An. gambiae s.l larva feeding behavior

Larvae of *An. gambiae* spend most of their time lying horizontally beneath the surface of the water where they feed and obtain atmospheric oxygen. Hydrophobic organic matter that accumulates at the surface film of water bodies forms microlayers (Maki and Hermansson, 1994) which consist of a wide range of particles including living and dead matter as well as dissolved organic particles. The micro-organisms proliferate in the surface microlayers by feeding on this accumulated organic

matter. Larvae are known to ingest a wide variety of dissolved and particulate organic matter from, within and just below these surface microlayers (Walker and

Merritt, 1993) and the micro-organisms found there (Laird, 1988; Maki and Hermansson, 1994; Merritt *et al*., 1992a). Larval feeding involves a complex pattern of behavior including the generation of water currents by rapid movements of the lateral palatal brushes which deliver water containing particulate food to the pre-oral cavity (Merritt *et al*., 1992b) from where they are ingested.

2.4. An. gambiae s.l adult feeding behavior

Both male and female anopheles mosquitoes feed on plant nectar and honey dew which is principally composed of fructose, sucrose and dextrin (Auclair, 1963; Wykes, 1952). Males have mouth parts that enable them to seep fluids while females can probe flowers and pierce skin. Plant juices thus provide an important source of energy during most of the adult life of both sexes (Clements, 1992). Females and males mate only once but a male can mate with several females. Females in addition take a blood meal mainly to produce eggs. They locate their host and feed mainly at night with biting activity peaking towards midnight (Gillies, 1957, Maxwell *et al*., 1998). Host location and selection is generally mediated by either carbon dioxide and/or other host-specific chemicals (Costantini *et al*., 1998; Gillies, 1980; Takken and Knols, 1999). During the course of blood feeding, an infective mosquito injects malaria parasites from her salivary glands to the feeding site into the host. Moreover, the female can be infectious only from a second blood meal when a sporozoite cycle is completed in her abdomen.

2.5. Mosquitoes and temperature

Many insects, in particular those with easy access to water, produce and retain drops of fluid, such as nectar, honey-dew, water or urine, depending on species. This production and retaining of fluid evaporate in contact with the air, causing heat loss by evaporative cooling and consequently decrease the temperature of the insect body. Evaporative cooling constitutes an adaptive and effective response to risks associated with high temperature and has been observed in different groups of insects (Adams and Heath, 1964; Mittler, 1958). This decrease of temperature helps them to avoid the deleterious physiological consequences of thermal stress. Some insects such as honeybees and bumblebees produce heat with their thoracic muscles while flying (endothermy) and regurgitate a droplet of nectar through their mouthparts to cool down their head, thus keeping the brain safe from overheating (Heinrich. 1975, Heinrich and Casey, 1978). Moths emit fluid, which is retained on the proboscis to refresh their head whereas others, such as aphids, excrete honey-dew through their anus that consequently refreshes their abdomen. The recorded loss of temperature is between 2 and 8° C depending on species (Prange, 1996).

Mosquitoes, being insects, are subject to the same constraints of temperature, and Trpis (1972) has clearly shown that normal functioning can only occur within a limited temperature range. Leeson (1939) reported that the aquatic stages of tropical anophelines failed to develop or breed below 16°C. Insect sex ratio can also be modified by temperature. Anderson and Horsfall (1963) reported that under natural conditions for development of *Aedes stimulans* (5-20°C) 50% of the population is limited to being female since half of the individuals are homozygous for female characters. The other half has the potential to develop into males or females for they are heterozygous for sex. The heterozygous component expresses itself entirely as males when reared below 23°C and wholly as females when reared above 28°C. At temperatures intermediate between 23°C and 28°C various

intersexes are produced, but the higher the temperature, the more severely are the male characters suppressed.

The development and survival of insects is critically dependent on temperature. As temperature increases, growth rates for mosquitoes generally increase and larval duration decreases (Brust, 1967; Hagstrum and Workman, 1971; Lassiter *et al*., 1995; Lyimo and Takken, 1993, Tun-Lin *et al*., 2000) leading to increased adult turnover. The tracheal system of respiration and the generally small size and large surface area of insects make them very susceptible to desiccation.

The amount of moisture in the air affects the activity and longevity of most terrestrial insects. Mosquitoes tend to be more active and survive longer at higher humidity compared to lower humidity (Dow and Gerrish, 1970, Leeson, 1939; Siddons, 1944), although very high humidity, especially at high temperatures, may not always be favorable (Pal, 1943; Piatt *et al*., 1957; Thomson, 1938). Because humidity can depend on temperature (Unwin and Corbet, 1991), the effects of the two are usually inter-related and quite difficult to separate in the natural environment of the insect.

Some mosquitoes respond to adverse conditions by overwintering either as eggs, larvae or adults. Mogi (1996) investigated the overwintering strategies for several mosquito species in Japan and reported that some *Anopheles* and *Culex spp*. diapause as adults while most *Aedes spp*. do so as eggs or larvae. The tree breeding temperate mosquito *Wyeomyia smithii* overwinters as a larval diapause that is initiated and maintained by short days and terminated by long days (Smith and Brust, 1971). The appropriate sites for aestivation of fertilized female mosquitoes are dark and cool places such as dwelling huts, animal sheds, caves, disused water wells, thatched roofs and uninhabited houses. The female becomes immobile and does not blood feed and her oviposition activity is completely suspended during this time and resumed as soon as conditions become

favourable. In the semi-arid parts of sub-Saharan Africa, these methods were reported to be responsible for the disappearance of active adult malaria vectors during the dry season and their reappearance as soon as the rains resumed (Omer and Cloudsley-Thompson, 1970). It has been suggested that *An. arabiensis* probably survives the dry season in the sub-Saharan Sahel and northern savannah regions of West Africa in a physiologically altered state. The few individual survivors or immigrants from other locations recolonize the site in the subsequent rainy season (Taylor *et al*., 1993). The speed with which biting females reappear with the onset of the rains and the almost immediate resurgence of malaria suggest that aestivating fertilized females may be involved. However recent investigations in a dry savannah zone of East Africa failed to show any evidence of aestivation among the three main malaria vectors *An. funestus*, *An. gambiae* and *An. arabiensis* (Charlwood *et al*., 2000). Other works have also pointed the possibility of egg aestivation in the dry season (Benoit, 2010; Yaro *et al*., 2012). Beier *et al*. (1990) recovered viable *An. gambiae* and *An. arabiensis* eggs from dry soil at known breeding sites in Western Kenya and suggested that the resistant eggs may represent a significant short term survival strategy for the species.

However, the authors could not determine whether the eggs recovered were present in the soil before or after drying up, and they did not report the moisture content of the soil samples collected. A similar study recently reported by Minakawa *et al*., (2001) implicated the presence of one viable egg out of a total of 124 dry soil samples investigated as a dry season survival strategy for *An. gambiae*. Much more research is required to substantiate the hypothesis that *An. gambiae* survives dry conditions as eggs. In the meantime, the most plausible explanation lies in the close association of this species with man. This association can ensure year round presence of breeding sites around homes, farms or river systems and the probable sequestering of the adults in shaded homes or

cultivated vegetation. Behavioral mechanisms such as acclimatization or relocation to more favourable microclimatic environments have been projected for the response of mosquitoes to unsuitable conditions (Haufe and Burgess, 1956; Mellanby, 1954). Endophilic species would not have problems with regulating body temperature as they are mostly protected by the stable conditions inside houses. Activity at night would also imply that these insects would be resting in places away from the harsh conditions such as among vegetation or other shade providing materials. It is necessary that in the overall estimation of global climate change and insect vector models, such subtle microclimatic adaptations be given due consideration (Lindsay and Birley, 1996).

2.6. Mosquito survival and mortality

The main causes of mortality in mosquitoes include competition for food and space, adverse climatic conditions, parasitism and predation (Service, 1993). The impact of these may vary with the life stage of the insect. Mortality rates are often described by the use of life tables (Gomez *et al*., 1977; Lansdowne and Hacker, 1975). Life tables provide a summary of either age-specific or time-specific mortality operating within a population. (Service, 1993) suggests that because laboratory conditions cannot perfectly mimic field situations, mortality estimates based on laboratory data are of limited value. However, the probability of survival in the laboratory, where food and space is abundant and climatic conditions are favorable gives an estimate of the potential of the insect in nature and this could be of use in generalizations. Also, field conditions cannot be easily controlled and therefore controlled laboratory studies are the best way possible to estimate the cumulative effect of all the various factors in the field on insect mortality. Field estimates of mortality of the immature stages of mosquitoes often require the estimation of the duration of each instar stage and the construction of an age distribution curve. After larval sampling, the different

instars are sorted and counted and each total is divided by the duration of the specific instar. The values are then plotted against age in days. A smooth line through the points represents the age-specific age distribution, which is equivalent to the time-specific survivorship curve. The assumption made is that the population is in a steady state where the number of additions cancels out the number of deaths.

For age-specific life tables, a series of samples are taken at different times and the numbers in each age class determined. The differences between successive estimates represent the numbers lost from the population, which is only valid where there is no overlap of generations. In the laboratory these factors can all be controlled since you introduce a cohort of 1st instars and observe the daily number of losses till the very last individual (Hassell *et al*., 1976). Adult survival rate is the most important factor in determining the stability of any mosquito population.

It affects total egg output and also the potential of mosquitoes to be vectors of disease organisms. Females that become infected when taking a blood meal must survive through the incubation period of the parasite before they can transmit the disease. The probability of survival of the vector is thus critical in the transmission cycle. It is therefore of great significance that the survival and mortality of adult mosquitoes be thoroughly investigated or described. Direct measurements of mosquito survival and mortality rates in nature are difficult to obtain. Some investigators have made use of data on the proportions of females that have laid one or more batches of eggs or of those that have not yet laid eggs. This ratio and knowledge of the duration of the first gonotrophic cycle can be used to estimate daily survival rate (Davidson, 1954).

This is applicable on the assumption that mortality rates are independent of age and the population is stable. Others have estimated the daily survival rates from the results of markrelease-recapture experiments (Service, 1993). If the ages of the captured mosquitoes are correctly established, an

estimation of survival rates can be made. A direct method for establishing the physiological age of individuals has been based on the observation that irreversible changes occur in the internal reproductive organs of female mosquitoes at every oviposition (Detinova, 1962). During each gonotrophic cycle, there is formation of bead-like dilatations in most ovarioles, resulting from either the distension of certain ovarian membranes by the developing oocyte or from the residue of resorbed follicle. The changes that occur during and after follicular development are used to determine the age of the individual (Tyndale-Biscoe and Hughes, 1968; Wall *et al*., 1991; Hoc and Wilkes, 1995).

It has been shown in many mosquito species including *An. gambiae* (Gillies and Wilkes, 1965) that a relationship exists between the number of gonotrophic cycles completed and the largest number of dilatations in any ovariole. Using mark-recapture experiments, they were able to correlate the reproductive history of recaptured *An. gambiae* females with their chronological age. However, recent investigations have reported significant weaknesses in this approach and there is the need for improved methods. Modern concepts of the epidemiology of malaria are based largely on the model developed by Macdonald (1952).

Using published data on mosquito survival in the laboratory and in the field, the author has pointed out that the intensity of the environmental hazards from which female mosquitoes die in nature is the same for all adult age classes. Thus considering that death rate should not change with age; he assumed an exponential model of mortality, based on the mathematical treatment of survival on the factor *p*, the probability of the mosquito surviving through one day. Estimates of *p*, have been employed in models of mosquito population dynamics (Miller *et al*., 1973), vectorial capacity (Garrett-Jones, 1964) climate change and malaria transmission (Lindsay and Birley, 1996; Martens, 1998) and in the assessment of control measures (Molineaux and

Gramiccia, 1980). However, re-analyzing Gillies and Wilkes' survival data, Clements and Paterson (Clements and Paterson, 1981), pointed out that there was the tendency for female mortality rate to increase with age over the adult life span as a whole, thus finding the simple exponential model less satisfactory. Gillies and Wilkes (1965) observed with three species of *Anopheles*, that female mortality rates increased during the later gonotrophic cycles but stated that in *An. gambiae* and *An. funestus* the mortality rates remained constant up to and including the age at which most malaria transmission occurs. This may be the reason why recent contributors to the model of malaria epidemiology have continued to use the exponential model of survival in calculating longevity factors for the vector species.

2.7. Mapping *An. gambiae s.l* distribution

Mapping the distribution of insects has in recent times taken the spotlight in many disciplines of biology and medicine. Like all maps, these distribution maps provide a clear and instant image of the situation being described. In the past, distribution maps took the form of point or range maps. Point maps consisted of dots indicating sites where the organisms were found and range maps were produced by interpolating between these dots (White, 1989). Point maps give little or no information about adjacent sites and to provide a broader picture, extensive investigations demanding large quantities of limited resources are required. The range maps rely on expert opinion and are based on the use of sparse data usually to interpolate to large areas. Due to these deficiencies new approaches have been developed for mapping insect distribution. Insects usually occur within specific ranges of certain climatic or environmental factors. The beauty of our knowledge of the relationship between climate and insects is that if we can mathematically describe the climate envelope in which an insect survives we can use the derived equations to map its distribution over large areas. There have been two main approaches in describing the

relationship between climate and insect distributions. In the empirical or statistical method, the specific climate factors to which the insect is adapted are determined experimentally and projected on maps without specifying any underlying biological mechanisms, while in the process-based or physiological method the biological responses of the insect to climate are also incorporated. Biologically based responses to climate allow process models to address growth in current conditions and those that may occur in the future.

Both methods have been possible because of the marked improvement in spatial characterization tools and computation. For instance, the availability of the Geographical Information Systems (GIS) software, coupled with use of satellite imagery of potential breeding sites (Hayes *et al.*, 1985) and adult abundance (Thomson *et al*., 1996) has made mapping mosquito distributions easier and more accurate. These tools have also made possible the generation of maps predicting insect distributions with climate change (Beerling *et al*., 1995). (Lindsay *et al*., 1998) used these basic principles to map the distribution of *An. gambiae s.s*. and *An. arabiensis* across Africa with very high precision. It should be pointed out that within the climate space the distribution of a species is likely to be further constrained by such factors as vegetation, soil type, and competition as well effects of biotic factors associated with the insect or on a smaller scale density-independent factor such as microclimate. However, the maps present the situation at the macro level that forms the basis for further investigations or actions.

2.8. Insecticide resistance mechanisms

According to the World Health Organization, resistance is defined as the ability of an insect to withstand the effects of an insecticide by becoming not affected to its toxic effects by natural selection and genetic mutations (Davidson, 1957). This definition is differently interpreted by the Insecticide Resistance Action Committee (IRAC, 2011) [www.irac-online.org] that gathers

independent scientists and experts belonging to agrochemical companies who described operational (field) resistance as a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species. Even though the IRAC‟s definition seems to be pragmatic, it‟s less “sensitive” with the scope to implement early insecticide resistance management strategies in the field. In both cases however, appropriate tools (biological, biochemical and/or molecular) are needed to identify the mechanisms involved and to conduct surveillance at individual and/or population levels (WHO, 2012).

The various mechanisms that enable insects to resist the action of insecticides can be grouped into four distinct categories; metabolic resistance, target-site resistance, reduce penetration and behavioral avoidance. The description of these mechanisms is illustrated in the figure 2 below.

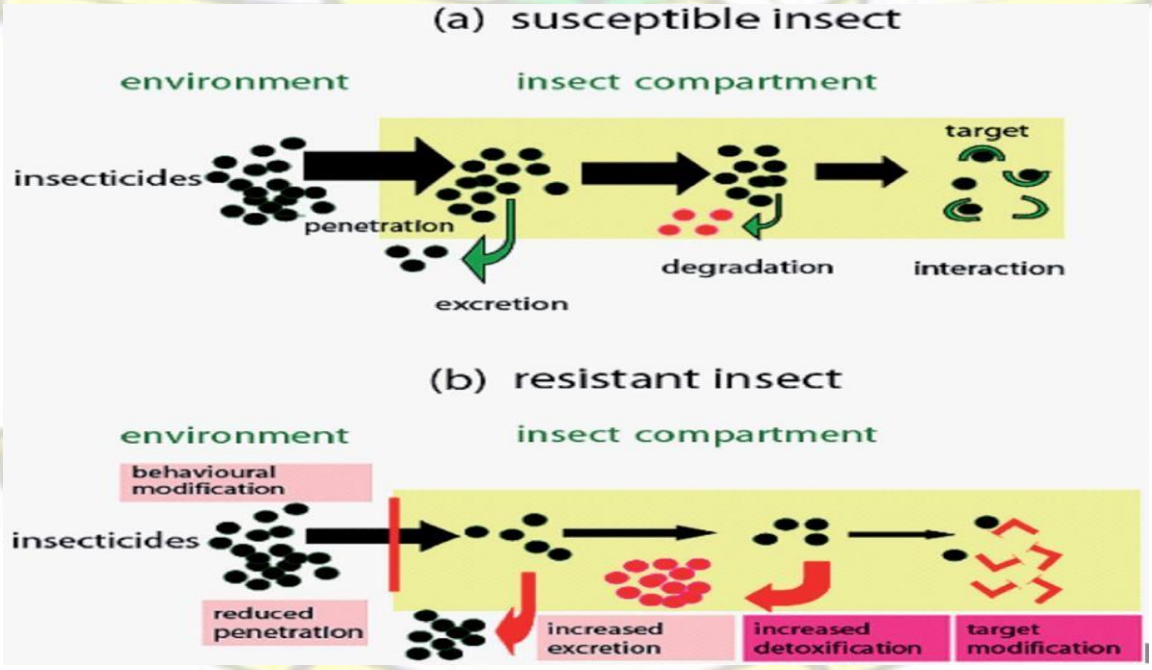

Figure 2: *Scheme of behavioral and physiological changes associated with insecticide resistance in malaria vectors; (a) susceptible insect; (b) resistant insect. Source: (Lapied et al., 2009)*

The metabolic resistance is the most common resistance mechanism that occurs in insects. This mechanism is based on the enzyme systems which all insects possess to help them detoxify naturally occurring xenobiotics/insecticides. It is commonly accepted that insect detoxification systems derived from the plant-insect evolutionary arm race and several insect detoxification enzymes have been associated to the detoxification of plant toxins and all types of chemicals, including insecticides (Despres *et al*., 2007). Over-expression of enzymes capable of detoxifying insecticides or amino acid substitutions within these enzymes, which alter the affinity of the enzyme for the insecticide, can result in high levels of insecticide resistance. Three categories of enzymes, namely esterases, P450s and glutathione-S-transferases are known to confer resistance to insecticides in insect pest such as malaria vectors (Ranson *et al*., 2011).

The other most common resistance mechanism occurring in insects is target-site resistance (Fanello *et al*., 2003). Insecticides generally act at a specific site within the insect, especially within the nervous system (e.g. OP, carbamate, DDT and pyrethroid insecticides). The site of action can be modified in resistant strains of insects such that the insecticide no longer binds effectively. Reduced sensitivity of the target receptors to insecticide results from non-silent point mutations in the gene encoding the protein.

Modifications in the insect cuticle or digestive tract linings that prevent or slow the absorption or penetration of insecticides can be found is some resistant insects. This resistance mechanism is not specific and can affect a broad range of insecticides. Reduced uptake of insecticide, often referred to as cuticular resistance, is frequently described as a minor resistance mechanism (Ranson *et al*., 2011).

2.9. Definition of a species

A species is defined as a group of organisms having many characteristics in common and ranking below a genus. Organisms that reproduce sexually and belong to the same species interbreed and produce fertile offspring. In that sense, a species is a big gene pool possible under natural conditions. However, this definition of a species might have limitation. In nature, there are lots of places where it is difficult to apply this definition. For example, many bacteria reproduce asexually. Therefore, the definition of a species as a group of interbreeding individuals cannot be applied to organism that reproduce only or mainly asexually.

For *Anopheles*, the genus currently includes 465 formally named species that are disproportionately divided between seven subgenera: *Anopheles* (cosmopolitan, 182 species); *Baimaia* (oriental, one species), *Cellia* (Old World, 220 species), *Kerteszia* (Neotropical, 12 species), *Lophopodomyia* (Neotropical, six species), *Nyssorhynchus* (Neotropical, 39 species) and *Stethomyia* (Neotropical, five species) (Harbach Accessed on April 01, 2013). Four of the subgenera, *Anopheles, Cellia, Kerteszia* and *Nyssorhynchus*, include the species that transmit human malarial parasites. Most vector species of *Anopheles* have been found to comprise complexes of sibling species. Historically, the taxon *Anopheles* (*Cellia*) gambiae Giles was known as a major vector of malaria in Africa, exhibiting a wide range of biological attributes (Gillies, 1968).

Many plants, and some animals and insects including *An. gambiae* mosquitoes, form hybrids in nature. *An. gambiae s.s* M molecular form and S molecular form look different, and largely mate within their own groups, but in some areas, they hybridize. Should they be considered the same species or separate species?

2.10. Definition of gene flow and introgression

In population genetics, gene flow (also known as gene migration) is the transfer of alleles or genes from one population to another and introgression is defined to be the movement of a gene from one species into the gene pool of another by the repeated backcrossing of an interspecific hybrid with one of its parent species.

Migration into or out of a population may be responsible for a marked change in allele frequencies (the proportion of members carrying a particular variant of a gene). Immigration may also result in the addition of new genetic variants to the established gene pool of a particular species or population.

There are a number of factors that affect the rate of gene flow between different populations. One of the most significant factors is mobility, as greater mobility of an individual tends to give it greater migratory potential (Rhymer, 1996).

Maintained gene flow between two populations can also lead to a combination of the two gene pools, reducing the genetic differentiation between the two groups. It is for this reason that gene flow strongly acts against speciation, by recombining the gene pools of the groups, and thus, repairing the developing differences in genetic variation that would have led to full speciation and creation of daughter species.

Gene flow, like other forces, may be higher in some parts of the genome and lower in others. For example, favorable genes can still be exchanged successfully even when barriers to gene flow are strong. Such genes could be at loci that confer local adaptations and at any linked loci. The significance of this is that gene flow, even if estimated accurately, may still fail to account for variation among different parts of the genome. This effect may be particularly strong for genes

contained within inversions, both because of potentially strong selection and because of linkage imposed by the reduced recombination associated with inversions (Tripet *et al*.,2005a).

Horizontal gene transfer (HGT) refers to the transfer of genes between organisms in a manner other than traditional reproduction, either through hybridization, antigenic shift, or re-assortment. It is sometimes an important source of genetic variation. Viruses can transfer genes between species. Bacteria can incorporate genes from other dead bacteria, exchange genes with living bacteria, and can exchange plasmids across species boundaries. Sequence comparisons suggest recent horizontal transfer of many genes among diverse species including across the boundaries of phylogenetic "domains". Thus determining the phylogenetic history of a species cannot be done conclusively by determining evolutionary trees for single genes (Nei, 1972).

2.11. Background of *An. gambiae s.s* hybridization

The *An. gambiae* genome is organized on three chromosomes; two sub metacentric autosomes and X/Y sex chromosomes, with males being the heterogametic sex. For descriptive purposes the autosomes are divided into two “arms” at the centromere. The longer arm is referred to as the right arm and the shorter the left arm. A high degree of chromosomal polymorphism, in the form of para centric inversions, has been described in populations of *An. gambiae* (Lanzaro and Lee, 2013, Pombi *et al*., 2008)

An attempt to develop a molecular diagnostic for the chromosomal forms of *An. gambiae* identified 10 nucleotide residues that differ between the “Mopti” and the “Savanna” or “Bamako” chromosomal forms in a 2.3 kb fragment at the 5‟ end of the rDNA IGS region located on the X chromosome (Favia *et al*., 2001). These findings led to the development of a

PCR speciation based diagnostic to differentiate Mopti chromosomal forms from Bamako and Savanna forms based on a single base pair substitution at the 540th nucleotide position in a 28S rDNA amplicon sequence. Mopti form individuals carry a C/C genotype and both Bamako and Savanna individuals a T/T genotype (Genbank accession number AF470112-6). Individuals carrying C/C are referred to as M molecular form and those carrying the T/T genotype are known as S molecular form. There is good correspondence between the M molecular form and the Mopti chromosomal form in Burkina Faso and Mali, however, the Bamako and Savanna chromosomal forms cannot be distinguished (both are of the S molecular form).

The association of M and S molecular forms and chromosomal forms breaks down at other locations in West Africa. For example, in Western Senegal and Gambia the association between the Savanna chromosomal form and S molecular form does not hold (della Torre *et al*., 2005) and the Forest form contains both M and S individuals. The M and S molecular forms, therefore, largely fail as a diagnostic for these chromosomal form. However, the significance of the M and S forms of *An. gambiae* goes well beyond their utility as proxies for identifying chromosomal forms. The molecular form concept has now largely replaced chromosomal form for defining discrete sub-populations of *An. gambiae* that are to some extent reproductively isolated. M and S forms occur in sympatry at many sites in West and Central Africa, and typically there was a high degree of reproductive isolation between the two forms. M/S hybrids (C/T genotype) produced in the laboratory did yield clearly distinguishable hybrid patterns in females. Surprisingly, however, field collected individuals carrying "hybrid" karyotypes (putative hybrids between different chromosomal forms) did not produce results consistent with their being hybrid, but rather produced either M or S patterns (Favia *et al*., 1997). This observation supports the notion that certain karyotypes, thought to be fixed in one chromosomal form or another, are in fact shared, occurring

commonly in one form and rarely in another, due to ancestral polymorphism and/or ongoing gene flow (Costantini *et al*., 2009, Simard *et al*., 2009). This diagnostic now forms the basis of recognizing two distinct subpopulations of *An. gambiae*, known as molecular forms (M and S).

Understanding the relationship between the two molecular forms has been the focus of an intense and ongoing research effort. The S form has the broadest distribution occurring throughout subSaharan Africa, whereas the M form occurs throughout West and parts of Central Africa. With the exception of a single site in northern Zimbabwe (Santolamazza *et al*., 2011), M is absent from Eastern Africa (Fig. 3) (della Torre *et al*., 2005).

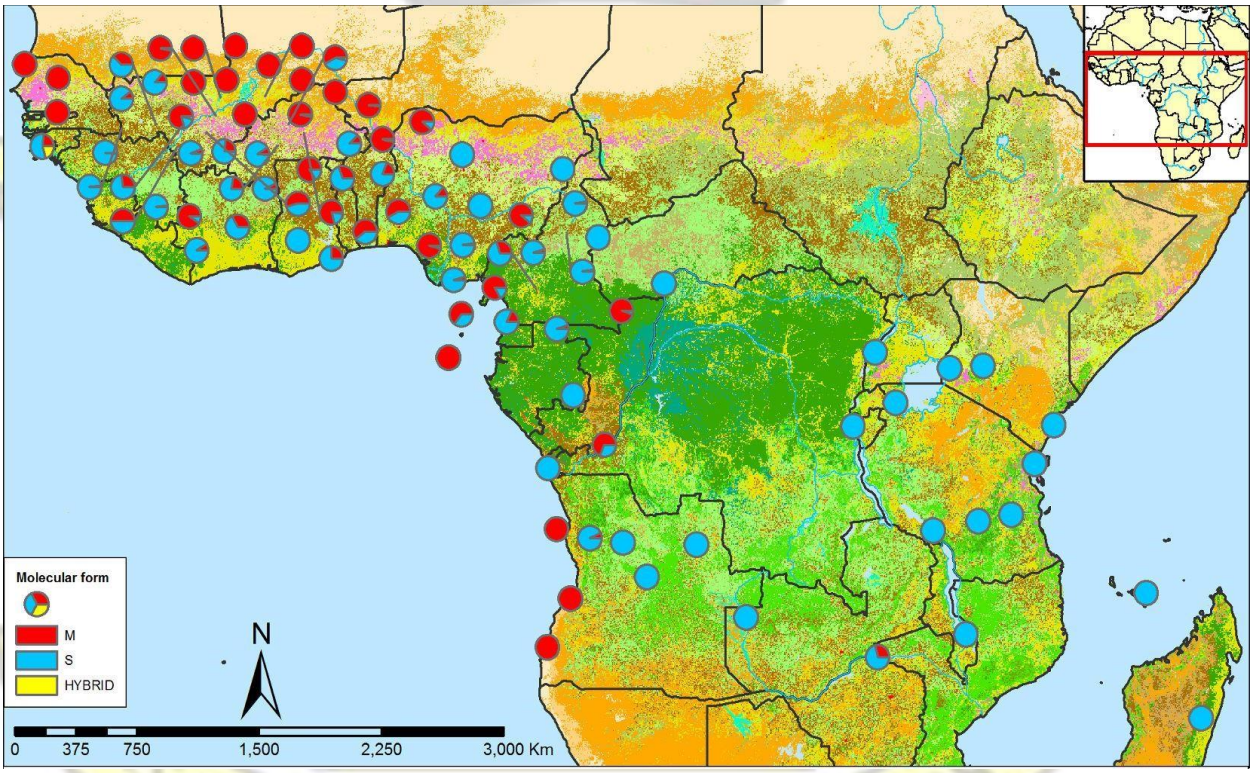

Figure 3: Distribution of *An. gambiae* molecular forms in Sub-Saharan Africa (Lanzaro and Lee, 2013)

2.12. Global vector control approaches

Vector control remains the most preferred strategy for reducing malaria transmission. The two main methods of malarial vector control are indoor residual spraying and long lasting insecticide treated nets (WHO, 2006; 2007). The combination of the two methods using different classes of insecticides have shown efficacy in some *An. gambiae s.l* resistant areas (Djenontin *et al*., 2009).

Currently, synthetic pyrethroids are the only group of insecticides licensed for use in ITNs (Vezenegho *et al*., 2009) while pyrethroids and carbamates (Osse *et al*., 2012) and dichlorodiphenyltrichloroethane (DDT) are used in IRS.

The challenges facing the use of insecticides are the development of resistance which has been reported in the whole sub Saharan Africa countries and exceptionally in the West and Central Africa (Corbel *et al*., 2007; Dabire *et al*., 2008; Dabire *et al*., 2006; N'Guessan *et al*., 2007; Santolamazza *et al*., 2008; Yadouleton *et al*., 2010).

3. Integrated Vector Management

In an effort to combat the spread and impact of malaria the adoption of a combination of control strategies through Integrated Vector Management (IVM) has gained popularity. An IVM strategy is a rational decision-making process for the optimal use of resources for vector control. Integrated vector control or vector management relies on a number of factors, but foremost (as given in the World Health Organization (WHO), strategic framework for integrated vector management (WHO, 2004) is the „selection of proven vector control methods based on knowledge of local vector biology and ecology, disease transmission and morbidity"; essentially, knowing which vector species is present and understanding how it behaves.

It is essentially a management approach to improve the efficiency, effectiveness and ecologically soundness of vector control interventions given the available tools and resources (Chanda *et al*., 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

An. gambiae S form obtained from Tiassalé in Côte d‟Ivoire was established in the insectary since 2010. Mosquito larvae were collected in irrigated rice fields surrounding Tiassalé, Southern Côte d‟Ivoire (5°52′47″N; 4°49′48″W) and transferred to the insectary. The strain was colonized since 2010 and was at the 65th generation since its establishment in the laboratory.

Anopheles gambiae Kisumu originally from Kenya was used as susceptible strain for all the WHO susceptibility tube tests as well as PCR. The colony is also S molecular form. The *An. gambiae* Kisumu eggs were shipped from LSTM (Liverpool School of Tropical Medicine) and maintained in the same insectary since 2011.

3.2.Study site

Larvae of *An. gambiae* M form were collected from the rice irrigation site of Okyereko (Fig.4) in the Central Region in Ghana. Okyereko is a village located in the central region of Ghana, about 50km away from Accra the national capital (5°24‟57.68”” N and 0° 35‟53.99”” W). The climate is coastal savannah vegetation with an annual average rainfall of 750mm. The project consists of an earthen dam with a catchment area of about 1685 km2. The reservoir is fed by the tributary of the Ayensu River. Two canals on the left and right banks of the tributary convey water to the irrigable area below the dam. The irrigation project was started in 1973 and fully completed in 1982 with the aid of Japanese Government. Eighty one out of 125ha available were developed, including 42ha irrigated by the project with an average production of 4000 bags of rice per year.

Mosquito larvae were collected from Okyereko and brought to laboratory insectary and reared till adult emergence. Sub generations were completed by successive blood feedings.

3.3. Methods

3.3.1. Mosquito collections

To establish laboratory *An. gambiae s.l* mosquito colonies, the initial specimens are mostly collected at their larval stage. Due to the fact that the insectary was maintaining only S molecular form specimens, we investigated on getting an M molecular form colony following some works already done in Ghana (Charlwood *et al*., 2012; Okoye *et al*., 2005). *An. gambiae* Okyereko was identified to be composed of at least 95% M form. Therefore, mosquito larvae were collected from Okyereko and brought to the laboratory insectary. The colony was characterized by PCR for confirmation of the molecular form and the resistance status.

All mosquito populations were maintained at Vestergaard- NMIMR Vector Labs at Noguchi Memorial Institute for Medical Research in Legon, University of Ghana. The strains were maintained in the same insectary bay, under controlled conditions of 27°C (±2), 70% (±10) relative humidity, and a 12 L: 12 D hour light-dark cycle. Larvae were reared in plastic trays (27 x 16 x 6.5 cm) containing 2 litres of deionized water at a density of approximately 200 larvae per litre, and fed a daily diet of a mixture of finely ground tropical fish pellet (Fig. 5A). Pupae were transferred to 0.27 m^3 screened cages, where emerged adults were maintained on 10% sugar solution (Fig. 5B).

Figure 4: Mosquito larvae collection

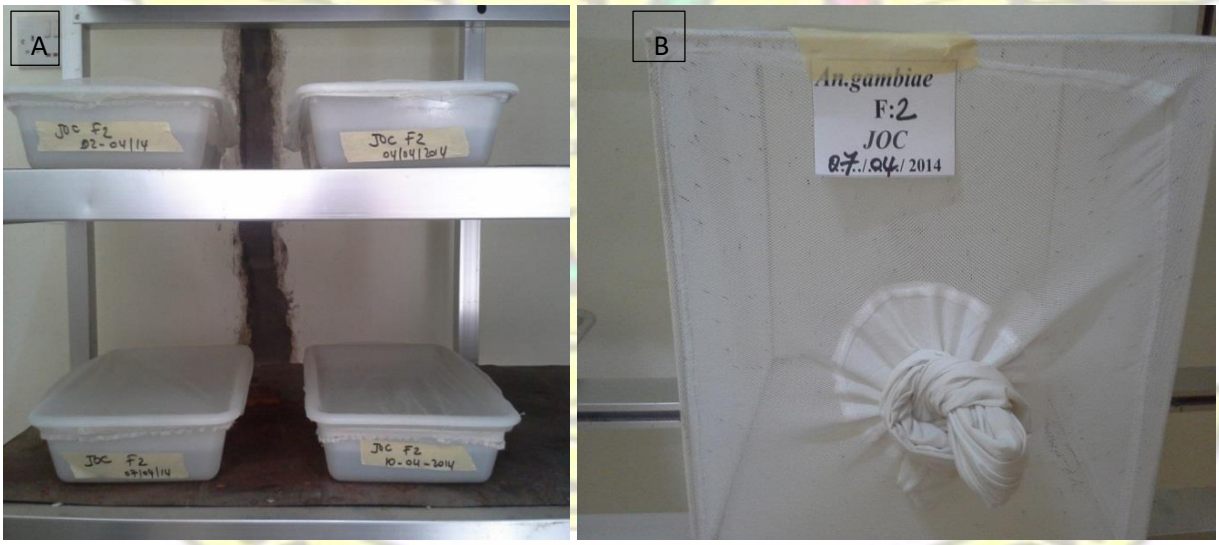

Figure 5: Insectary larval trays (A) and adult holding cage (B)

3.3.2. WHO susceptibility testing

Mortality and knock-down of *An. gambiae* females resulting from tarsal contact with insecticide treated Whatman 1 CH filter paper were measured using WHO test kits (WHO, 1998) (Fig. 6).

Six sheets of clean white paper (12 x 15 cm), rolled into a cylinder shape, were inserted into six holding tubes (one per tube) and fastened into position with a steel spring-wire clip. The tubes were attached to slide units. About 120–150 active female mosquitoes were aspirated (in batches) from the mosquito cage into the six holding tubes through the filling hole in the slide to give six replicate samples of 20–25 mosquitoes per tube. Once the mosquitoes have been transferred, the slide unit was closed and the holding tubes set in an upright position for one hour. At the end of this time, any damaged insects were removed.

Six exposure tubes were prepared in much the same way. Each of the 4 red dotted exposure tubes were lined with a sheet of insecticide-impregnated paper, while the 2 yellow-dotted control exposure tubes were lined with oil-impregnated papers; each was fastened into position with a copper spring-wire clip. The empty exposure tubes were attached to the vacant position on the slides and with the slide unit open the mosquitoes were blown gently into the exposure tubes. Once all the mosquitoes were in the exposure tubes, the slide unit was closed and the holding tubes were detached and set to one side. Mosquitoes were kept in the exposure tubes, which were set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour.

At the end of the 1-hour exposure period, the mosquitoes were transferred back to the holding tubes by reversing the procedure outlined above. The exposure tubes were detached from the slide units. A pad of a cotton-wool soaked in sugar water is placed on the mesh-screen end of the holding tubes. Mosquitoes were maintained in the holding tubes for 24 hours (the recovery period). Temperature and humidity values were recorded during the recovery period. At the end of recovery period (i.e. 24 hours post-exposure), the number of dead mosquitoes was counted and recorded.

An adult mosquito was considered to be alive if it was able to fly, regardless of the number of legs remaining. Any knocked down mosquitoes, whether or not they have lost legs or wings, were considered moribund and were counted as dead.

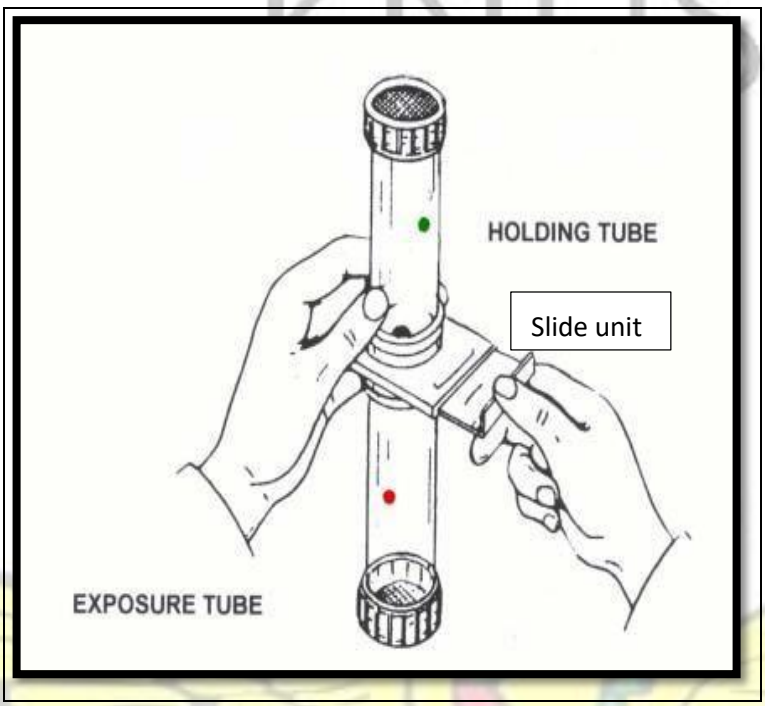

Figure 6: WHO susceptibility tube testing procedure

3.3.3. Profiling of the colonies

The resistance status of each colony was obtained using the WHO susceptibility tube assays as described above. Four classes of insecticides were used for determining the resistance status of each colony following WHO standard doses. Permethrin 0,75% and deltamethrin 0.05% (pyrethroids), DDT 4% (organochlorine), fenitrothion 1% and malathion 5% (organophosphates) and bendiocarb 0.1% and propoxur 0.1% from the class of carbamates.

In addition, 120 specimens were randomly sampled for PCR for the detection of resistance mechanisms such as *kdr* (knock down resistance) and *ace-1* (acetylcholinesterase).

3.3.4. Crossing of the strains

To correct a certain bias which can occur in the M molecular form blood feeding rate due to the fact that the strain was newly colonized, the adults emerging from the larval collection were blood fed for production of new generations in the laboratory. The first two generation blood feedings were completed using human hand feeding, due to the fact that the mosquitoes were wild. Afterwards, the blood feeding process was continued using a rabbit from the third generation (F_3). Thus, the colony was maintained for three months to get the third generation before crossing with the laboratory S form colonies. The mating experiments were performed either by a slight mass crossing or single mating and the percentage of hybrid specimens was checked after each crossing and subsequent generations. Each S molecular form colony as well as resistant and susceptible was crossed with the M molecular form which was also characterized resistant to DDT, pyrethroid and carbamates.

Thus, the pupae of each colony were individually placed in 20ml plastic tube covered with a small untreated net and allowed to emerge (Fig. 7). The following day, 10 virgin females of each colony were transferred into 15x15x15cm cages in two replicates, giving a total of eight cages. Afterwards, a slight mass crossing was done by inversely adding 10 males of each colony into each cage and allowed to mate (Fig. 8). All the cages were maintained in the same conditions as described above for 4 to 5 days before blood feeding. An oviposition tray was put in the cage 48 hours later and all collected eggs were washed in deionized water. The larvae were also maintained till adult emergence following the previously described procedure. A mean of 50 virgin males and females‟ progeny resulting from each crossing were sampled and used for PCR characterization of the molecular forms. The initial parents of each form were similarly characterized.

Figure 7: Separation of mosquito pupae for obtaining of virgin specimen

Figure 8: Mass crossing process. A: Resistant M and resistant S crossing cages; B: Resistant M and susceptible S crossing cages.

For a single crossing, the same procedure was followed as described above. Each single female and male of inverse colony were maintained in a 150 ml disposable cup and allowed to mate (Fig. 9). The females were individually blood fed and the progeny reared in separate trays.

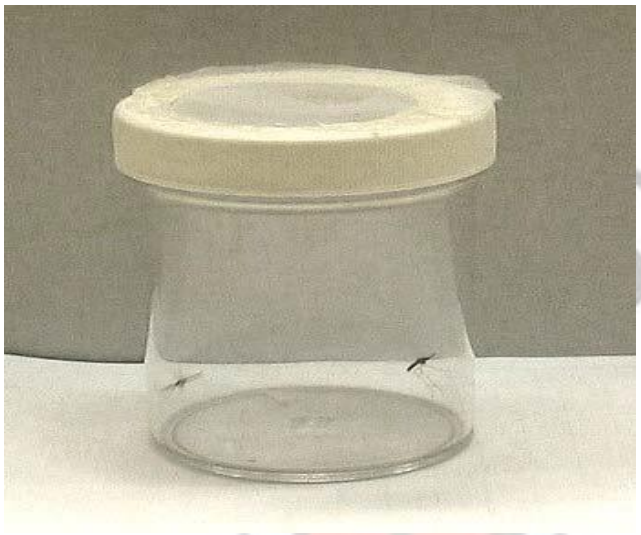

Figure 9: Procedure of *An. gambiae* single pair mating

The subsequent generations were completed by blood feeding of generation to generation following the diagram below (Fig.10) by considering the origin and the status of the initial parents. Also the estimate ratio per generation of each molecular form as well as males and females was described using PCR methods after each progeny.

The five consecutive generations after the parent crossing were followed as described in the diagram.

Crossing way 1 **Crossing way 2**

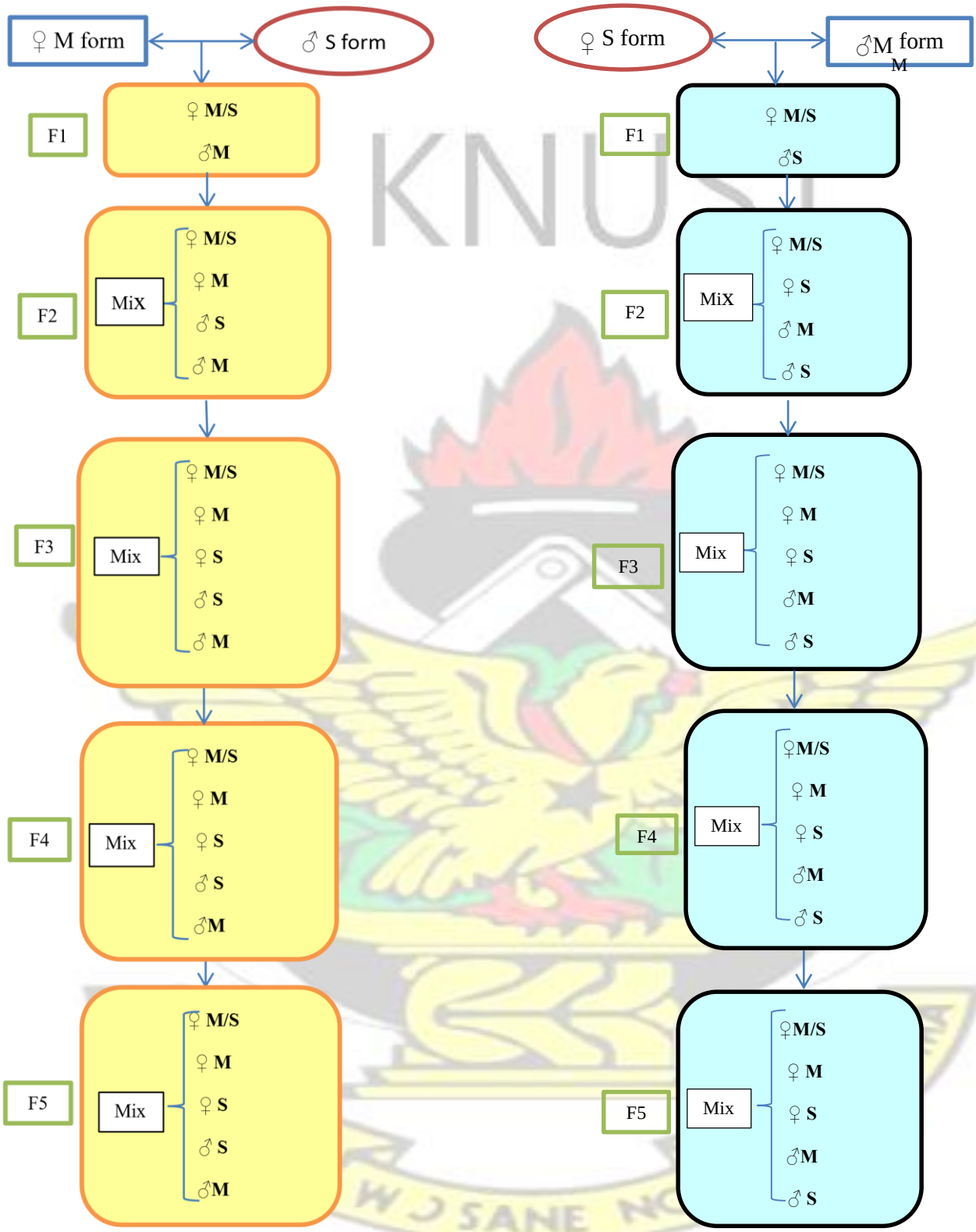

Figure 10: Diagram of *An. gambiae* M and S molecular form crossing experiments

3.3.5.Mosquito DNA extraction

Mosquito DNA were obtained after extraction of the whole mosquito using a slightly modified version of the protocol designed by Collins *et al*. (1987). A single mosquito was homogenized in a 1.5 ml Eppendorf tube containing 200 µl of CTAB buffer (100 mM Tris HCL, pH 8.0, 10 mM EDTA, 1.4 M NaCl, 2% CTAB (cetyl trimethyl ammonium bromide)) and incubated at 65°C for 5 minutes. 200 µl of chloroform was added and mixed by inversion of the tube. After centrifugation at 12000 rpm at room temperature for 5 minutes, the supernatant in the first layer of the solution was pipetted into a new 1.5 ml tube. 200 µl of isopropyl alcohol was added, mixed and centrifuged at 12000 rpm for 15 minutes. Afterwards the supernatant was discarded and the DNA pellet formed at the bottom of tubes was purified with 70% ethanol, dried and reconstituted in 20 µl DNase-free water.

3.3.6. Species identification of *An. gambiae complex*

DNA extracts from each single mosquito was used for a template in a PCR-mix following a modified protocol of Fanello *et al*. (2002) (Appendix I). One fortieth (1/40) dilution of the extracted DNA was prepared and 3 µl of the diluted DNA extract was used as template in the PCR master mix. A 20 µl PCR Master mix consisted of 12.5 µl of 1X Green Go *Taq* buffer (Promega) mixed with 0.6 µl of 0.15 µM universal primer UN (5‟- GTG TGC CCC TTC CTC GAT GT -3‟), 0.6 µl each of species-specific primers such as 0.15 µM primer GA (5‟- CTG GTT TGG TCG GCA CGT TT -3‟) for *An. gambiae*, 0.15 µM primer AR (5‟- AAG TGT CCT TCT CCA TCC TA -3‟) for *An. arabiensis*, 0.15 µM primer ME (5‟- TGA CCA ACC CAC TCC CTT GA -3‟) for *An. melas*, 2.1 µl of DNase-free water and 3 µl the DNA template. The Go *Taq* in the PCR master mix was comprised of PCR buffer (Flexi Buffer), 25 mM MgCl2, 0.2 mM of each dNTP, 1.25 Units of Go *Taq* DNA polymerase and a loading dye. PCR runs were done in a thermocycler (Gene Amp PCR System 9700) using PCR reaction condition consisting of initial

denaturation of 3 minutes at 94 °C to activate the DNA polymerase followed by 33 cycles of 30 seconds each for final denaturation step at 94°C, 30 seconds annealing at 50 °C and 30 seconds extension at 72 °C; and a final extension step of 5 minutes at 72 °C. After amplification, 2% agarose gel with ethidium bromide solution added during its preparation was casted and made to stand for about 20 minutes for it to become semi-solid. The electrophoresis machine (Bio-Rad Power Pac 3000) was filled with 1x TAE Buffer to a level to cover the surface of the gels. The semi-solid gel were removed from the set up and submerged in the TAE Buffer. 5-10µl amplicon as well as a DNA Marker (100bp) was loaded into wells created by the combs of the electrophoresis set-up. Loading of amplicons into the wells of the 2% agarose gel did not require any loading buffer because the Flexi Buffer already contains a loading dye that gives the Go *Taq* its green coloration. The electrophoresis run was done for about 45 minutes. The gel was thereafter observed under a UV illuminator (TOYOBO Trans illuminator Model TM-20 connected to a monitor for printing pictures of electrophoregrams). The expected band size for the different sibling species from the PCR products is shown in the appendix I.

3.3.7. Identification of *An. gambiae s.s* molecular forms

The SINE PCR described by Santolamazza *et al*. (2008) was followed for the identification of M and S molecular forms using Primers F6.1a (TCGCCTTAGACCTTGCGTTA) (Sigma80125654050060) and R6.1b (CGCTTCAAGAATTCGAGATAC) (Sigma80125654050070).

The reaction was carried out with DNA of 1/40th dilution of a single mosquito in a 25 µl volume with a final concentration of 0.4 µM for each primer and Go *Taq* (Promega). Reaction conditions were 94°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 1

minute and a final extension at 72°C for 10 minutes. PCR products were run on 2% agarose gels stained with ethidium bromide and visualized under UV light (Appendix II).

3.3.8. Detection of knock down resistance (*kdr*) genotype of *An. gambiae s.l*

Polymerase chain reaction diagnostic test for detection of *kdr* mutations was carried out on *An.gambiae* mosquitoes as described by Martinez-Torres *et al.* (Martinez-Torres, 1999, Martinez-Torres *et al*., 1998) and confirmed by Real Time PCR following the protocol of Bass *et al*. (2007).

Classical PCR was performed as described by Martinez- Torres *et al.* (Martinez-Torres, 1999, Martinez-Torres *et al*., 1998). A 25 µl reaction volume consisting of 12.5 µl of Go *Taq*, 4.5 µl of Dnase free water, 4 µl of 1/40 dilution of DNA template and 1 µl each of 20 µM of primers AGD1 (ATAGATTCCCCGACCATG); AGD2 (AGACAAGGATGATGAACC); AGD3 (AATTTGCATTACTTACGACA) and AGD4 (CTGTAGTGATAGGAAATTTA) was used. Reaction conditions were 94°C for 3 minutes, 35 cycles of 94°C for 30seconds, 55°C for 30seconds, 72°C for 20 seconds and a final extension at 72°C for 5 minutes. PCR products were run in 2% agarose gels stained with ethidium bromide and visualized under UV light.

The protocol of Bass *et al*. (2007) was used for the Real-Time detection of *Kdr* genotype of *An. gambiae*. The protocol uses two probes, first labeled with VIC which is specific for the wild type and the second labeled with FAM (6-carboxyfluorescein), specific for the mutant allele (*kdr-W*). A 20 µl PCR reaction mixture was prepared including 4 µl of DNase-free water, 1.6 µl of Primer *kdr*-forward (800 nM final concentration) CATTTTTCTTGGCCACTGTAGTGAT (Sigma 80158365110010), 1.6 µl of Primer kdr-reverse (800 nM final concentration) CGATCTTGGTCCATGTTAATTTGCA (Sigma 80158365110020), 0.4 µl of TaqMan MGB probe (200 nM final) WT VIC-CTTACGACTAAATTTC, 0.4 µl of TaqMan

MGB probe (200 nM final) kdrW 6FAM-ACGACAAAATTTC, 10 µl of DyNAmo SNP genotyping master mix (1x final) (Thermo Scientific F-480) and 2 µl of 1/40 dilution of DNA template was prepared.

The PCR process was carried out in a real time machine (Agilent Technologies Stratagene Mx3000P) and the cycling conditions were an initial denaturation step of 1 cycle at 95°C for 10minutes, 40 cycles of final denaturation at 95°C for 10 seconds, annealing at 60°C for 45 seconds and an extension at 72°C for 30 seconds. The results were interpreted following the fluorescence indication of each specimen tested. A substantial increase in VIC fluorescence indicates a homozygous wild type (susceptible), a substantial increase in FAM fluorescence indicates a homozygous mutant (resistant), and an intermediate increase in both signals indicates a heterozygote (Appendix III)

3.3.9. Detection of acetylcholinesterase (*ace-1*) resistance genotype of *An. gambiae s.l*

The PCR-RFLP diagnostic test was used to detect the presence of *G119S* mutation (*ace-1* gene) as described by Weill *et al*. (2003) resulting from the mosquitoes developing resistance towards two classes of insecticides; carbamates and organophosphates.

A 25 µl PCR reaction mixture was prepared and this consisted of 1 µl each of 10 µM Primers EX3AGdir (GATCGTGGACACCGTGTTCG) and EX3AGrev (AGGATGGCCCGCTGGAACAG), 12.5 µl of Go *Taq*, 9µl of DNAse-free water and 1.5 µl of 1/40 dilution of DNA template. Reaction conditions were 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 5 minutes. An enzymatic digestion step was followed after the PCR reaction. This consisted of a 20 µl PCR reaction mixture of 2 µl of enzymatic buffer B 10X (Promega R002A), 0.2 µl of Acetylated

BSA at 10 µg/µl (Promega R396D), 0.5 µl of Restriction Enzyme (Alu1) 10 U/µl (Promega R628A), 12.3 µl of DNase-free water and 5 µl of PCR products. The mixture was incubated at 37°C for 4 hours in a thermocycler and afterwards 5-10 µl of amplicons was electrophoresed in 2% agarose gel stained with the ethidium bromide (Appendix IV).

3.3.10. DNA sequencing and gene flow

Positive DNA bands were purified using ExoSapIT protocol (Affymetrix). Sequencing was performed using amplification PCR primers as described by Santolamazza *et al*. (2008). Cycling sequence was performed with Big Dye terminator version 3.1 kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions.

The sequence loaded plate run on automated machine (3130XL analyzer) (Applied Biosystems, Foster City, CA) (Appendix V).

3.3. Data analysis

The susceptibility status of the colony against each insecticide was determined following WHO criteria (WHO, 2013). The population was considered resistant when the mortality after 24h was in the range of 0-89%; suspicion of resistance between 90 and 97% and susceptible when 98100% of the specimens were killed.

Abbott formula was applied when the mortality of the controls was between 5 and 10% (Abbott, 1925).

The *kdr* and *ace-1* frequencies were calculated using the Hardy Weinberg formula (Rousset and Raymond, 1995) and compared for each population using the exact probability test of XLSTAT software.

The DNA sequence data analysis were done using sequence analysis software (Bioedit version 7.0.0) (Hall, 1999) and compared using DnaSP version 5.10.01.

CHAPTER FOUR

RESULTS

4.1. Colony Susceptibility Status and Resistance Mechanisms

4.1.1. *An. gambiae* Kisumu

An. gambiae Kisumu represents the standard World health organization pesticide evaluation scheme (WHOPES) susceptible strain. The colony was susceptible to all the classes of insecticide. The tests resulted in 100% mortality for all the insecticides tested after 24h post exposure (Fig. 11).

An. gambiae Kisumu being a susceptible strain was characterized as 0% *kdr* and *ace-1* frequencies. The colony was found to be 100% S molecular form following PCR characterization of a subsample of about 100 mosquitoes.

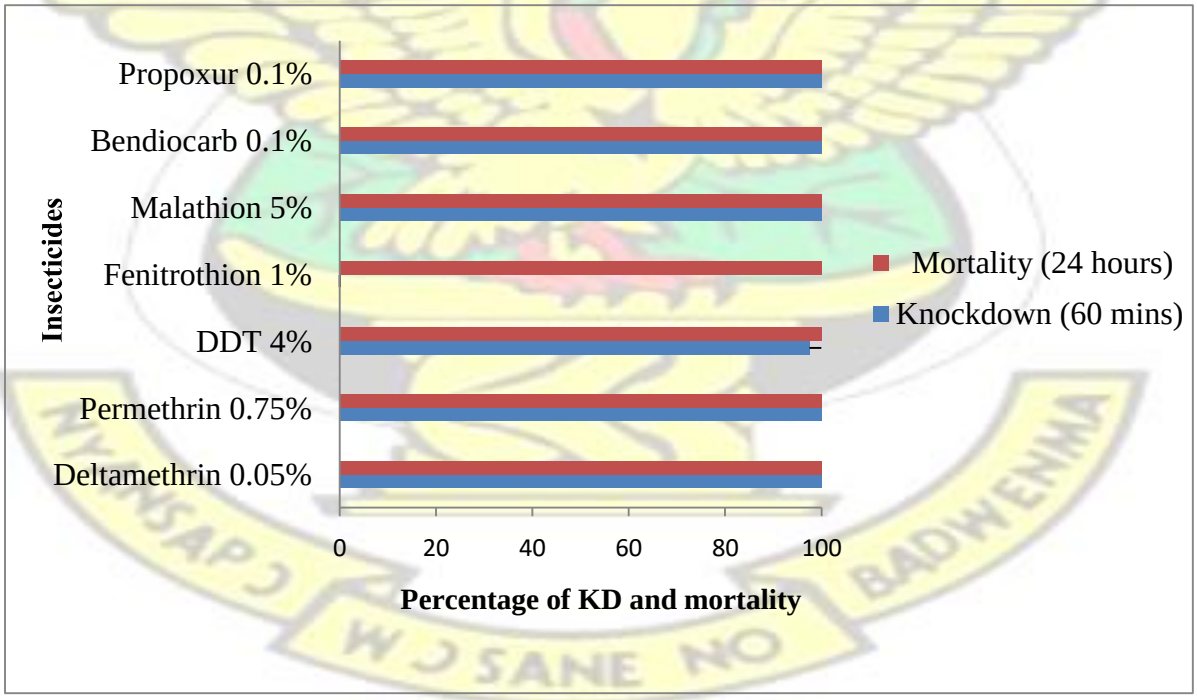

Figure 11: Susceptibility status of *An. gambiae* Kisumu to different insecticides

4.1.2. *An. gambiae* Tiassalé

An. gambiae Tiassalé was described resistant to all the classes of insecticide tested. The colony showed very high resistance to DDT with only about 4% mortality, bendiocarb and propoxur recorded 0% mortality whilst deltamethrin and permethrin recorded 40 and 35% mortality respectively. Malathion yielded the highest mortality of 86% (Fig. 12). The frequencies of the resistance mechanisms showed 0.85 and 0.62 for *kdr* and *ace-1* mutation respectively which confirmed the observations from the tube testing. The characterization of the molecular form showed that the colony was also 100% S molecular form resultant of the 100 specimen analyzed.

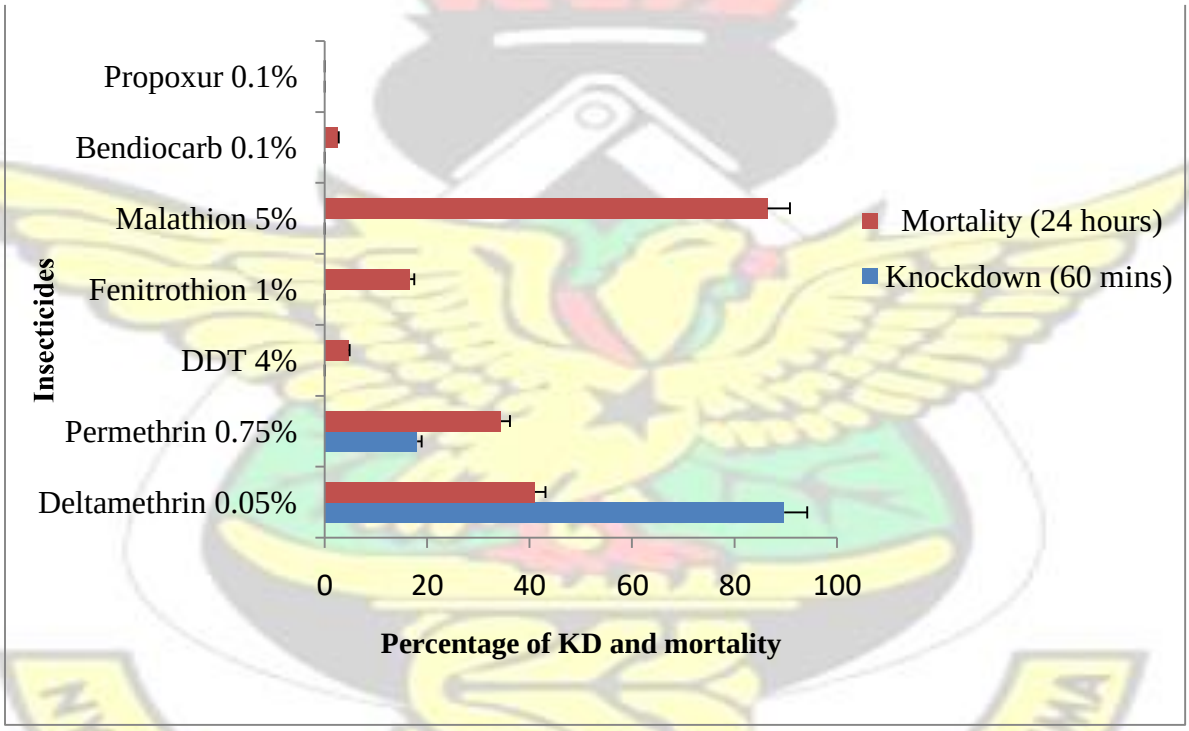

Figure 12: Susceptibility status of *An. gambiae* Tiassalé to different insecticides

4.1.3 An. gambiae Okyereko

An. gambiae Okyereko showed high resistance to DDT and pyrethroid with a range of 0 to 18% mortality. The resistance level was slightly lower with the carbamates and organophosphates (Fig. 13). The knockdown resistance (*kdr*) and acetylcholine esterase (*ace-1*) frequencies observed were 0.9 and 0.15 respectively, showing that the colony is highly resistant to pyrethroid and DDT. The colony was characterized to be 96% M form and few specimens of S form following analysis of a subsample of 218 mosquitoes.

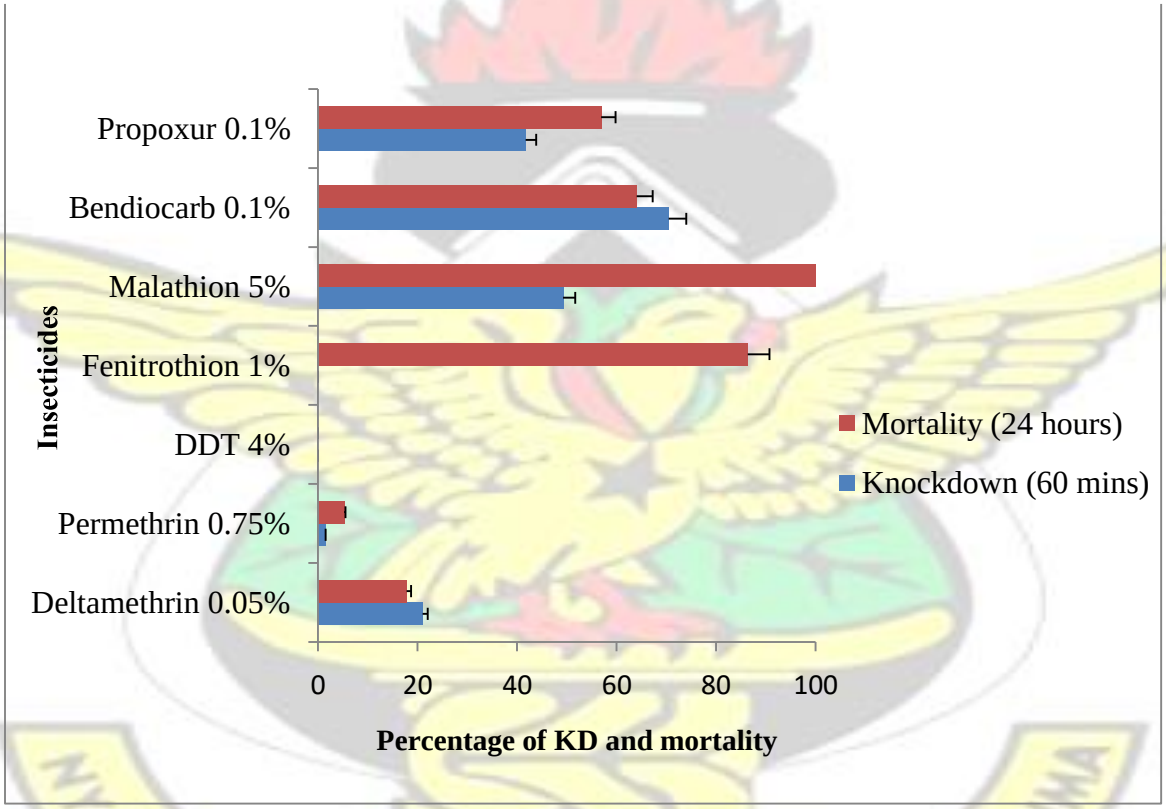

Figure 13: Susceptibility status of *An. gambiae* Okyereko to different insecticides

4.2. Mating experiments

4.2.1. Crossing of the resistant strain of M and S molecular forms (crossing experiment 1 and experiment 2)

The percentage of M/S hybrids, M and S forms obtained among all the mosquitoes according to their sex is described below. Approximately, 1500 PCR reactions were run for the identification of the molecular forms of the different generations (Fig. 14). The proportion of each molecular form and M/S hybrids resultant of the crossing are shown in the figures 15 and 16 according to the origin and the resistance status of the parents.

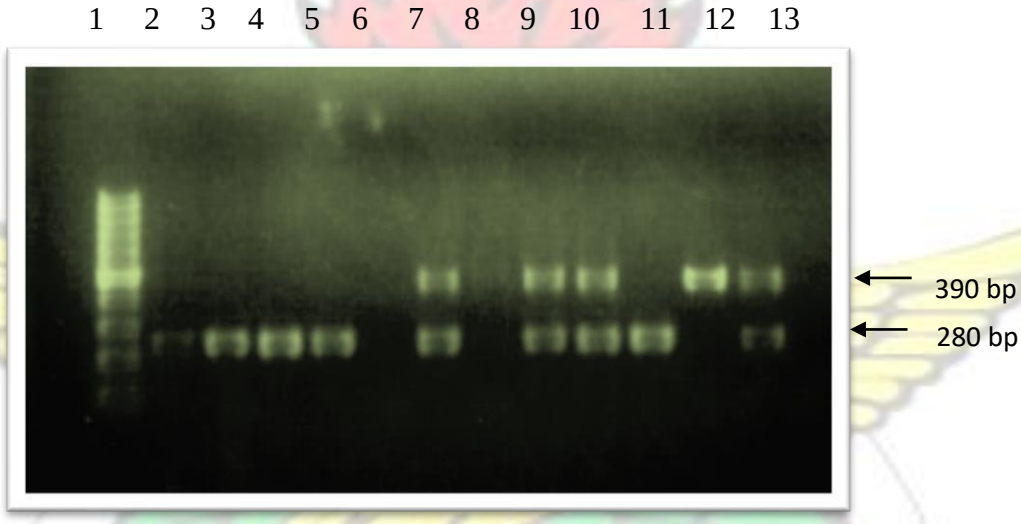

Figure 14: Ethidium bromide-stained 2% agarose gel electrophoregram of Hha1restriction of *An. gambiae s.s* amplified PCR products of F_1 molecular forms from the female M form and male S form parental crossing
L1: 100bp molecular weight marker; L2-5: male S; L7 and L9-10: M/S hybrid; L11: Control S; L12: Control M and L13: Control M/S

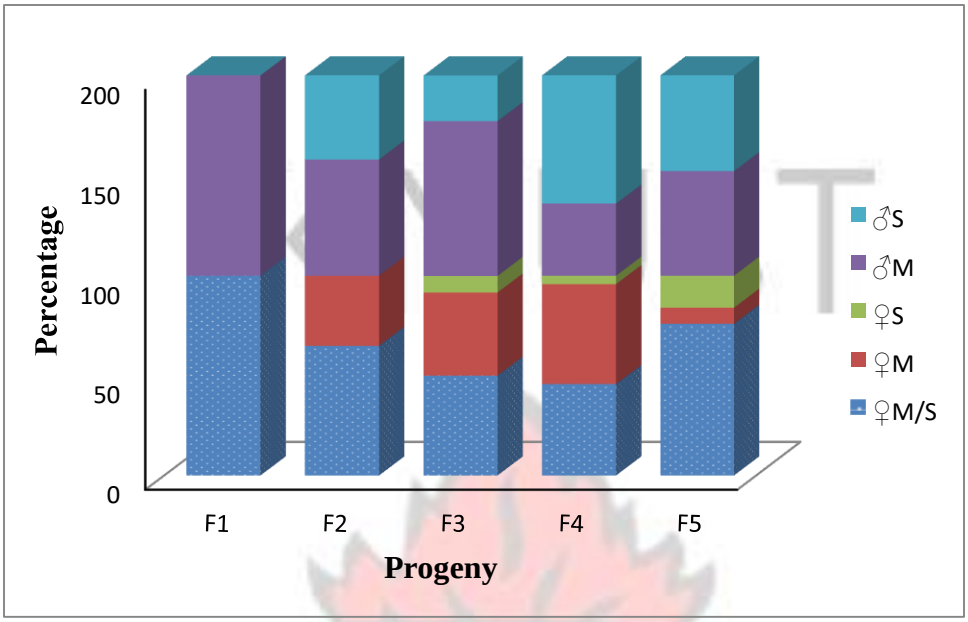

Figure 15: Percentage of M/S hybrid female mosquitoes observed from the different progenies of both resistant female M form and male S form experiment

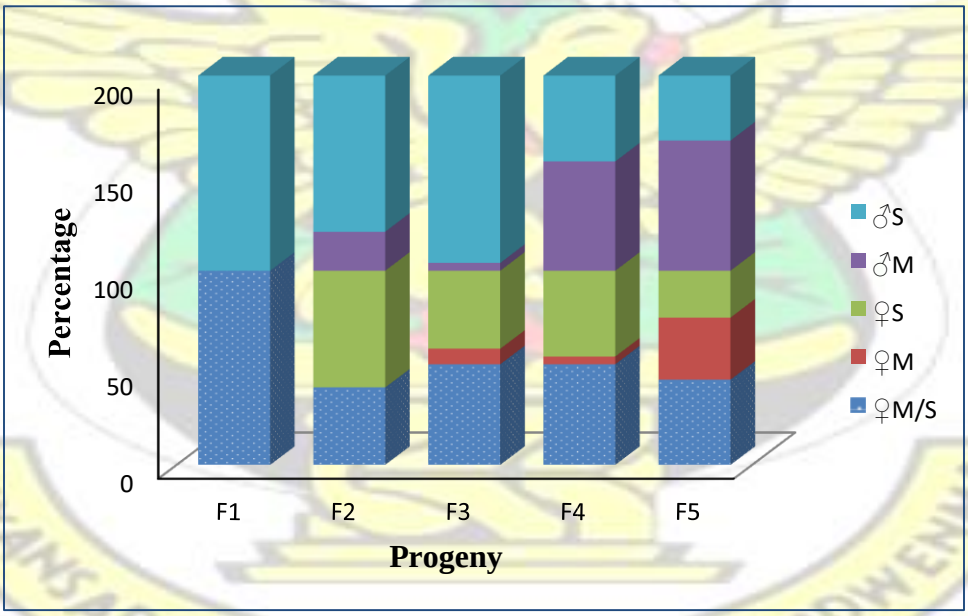

Figure 16: Percentage of M/S hybrid female mosquitoes observed from the different progenies of both resistant female S form and male M form experiment.

4.2.2. Crossing of the resistant M form and susceptible S form strains (crossing experiment 3 and experiment 4)

The protocol used for the crossing of both resistant strains was followed using the susceptible *An. gambiae* Kisumu as S molecular form and resistant *An. gambiae* Okyereko representing the M form. The same diagram as described above was also followed for these crossing experiments. The different proportion per generation of each form and M/S hybrids are described in the figures below (Fig. 17 and 18).

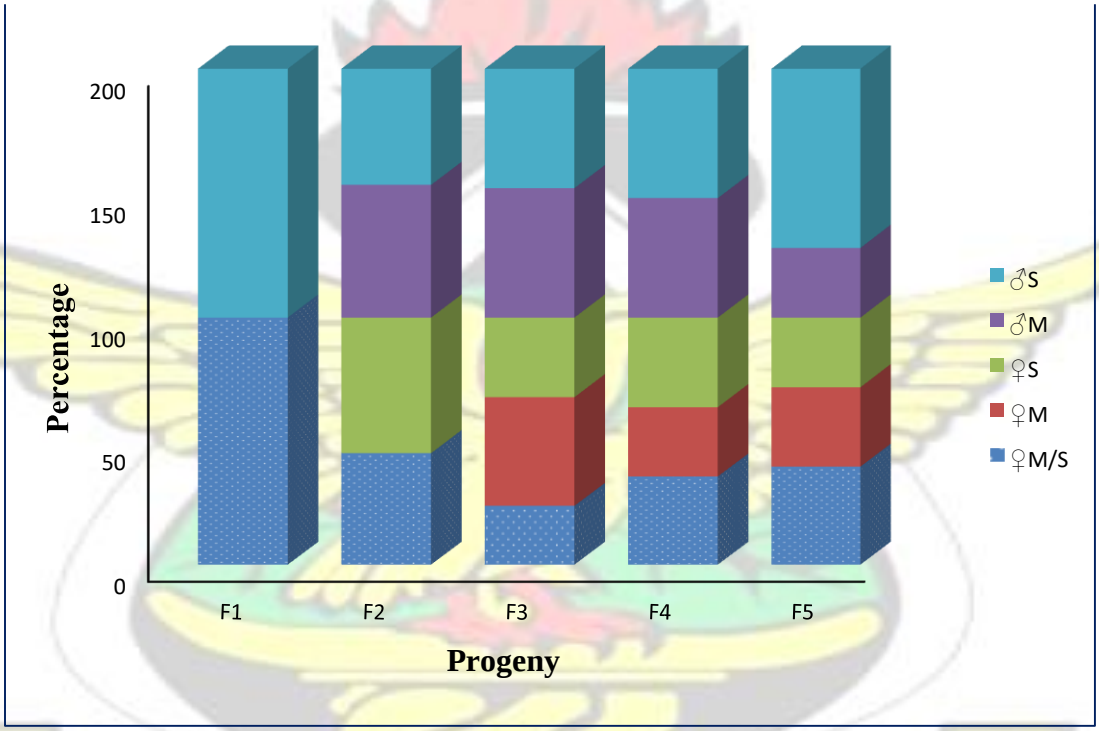

Figure 17: Percentage of M/S hybrid female mosquitoes observed from the different progenies of susceptible female S form and the resistant male M form experiment.

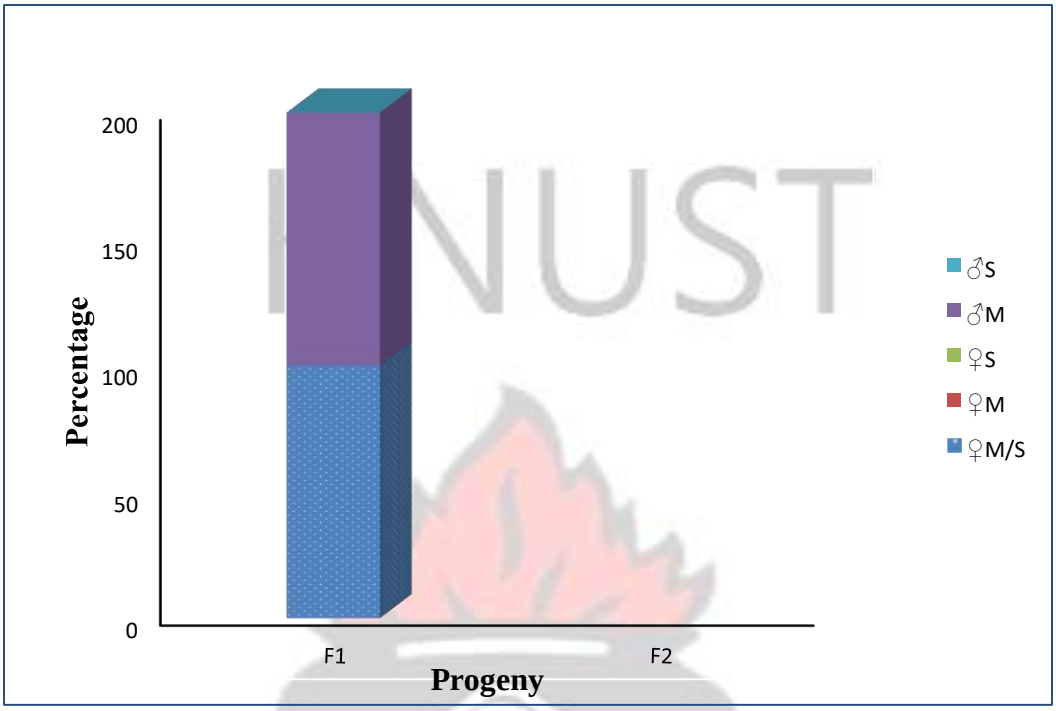

Figure 18: Percentage of M/S hybrid female mosquitoes observed from the different progenies of the resistant female M form and the susceptible male S form experiment

4.3 Resistance status of the M/S hybrid mosquitoes

4.3.1 *Kdr* genotype of the M/S hybrid mosquitoes

The *kdr* L1014F genotype expressed by the first hybrid progeny of each crossing was detected using the PCR methods and described in the figure 19 below. The results of each crossing progeny are shown in the figures 20 to 22 below. All the 50 hybrid specimen resulting from the crossing of the resistant strain and the susceptible colony were 100% heterozygous RS (Fig. 20). In contrast, the progeny of the two resistant parents showed 60% and 40% homozygous RR and heterozygous RS respectively for the female M form parent (Fig. 21). Only a couple of susceptible SS was found among the progeny of the resistant female S and male M in addition to the 47% RR and 37% RS (Fig. 22).

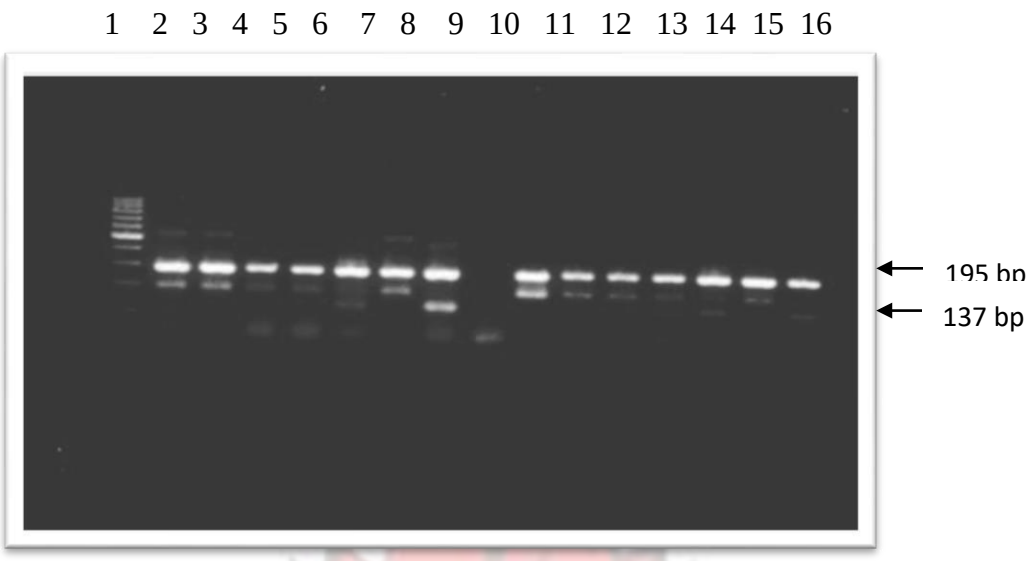

Figure 19: **Ethidium bromide-stained 2% agarose gel electrophoregram of *An. gambiae s.s* amplified PCR products for the detection of the *kdr* mutation.**
L1: 100bp molecular weight marker; L2-7: RR; L8: SS; L10-16: RR

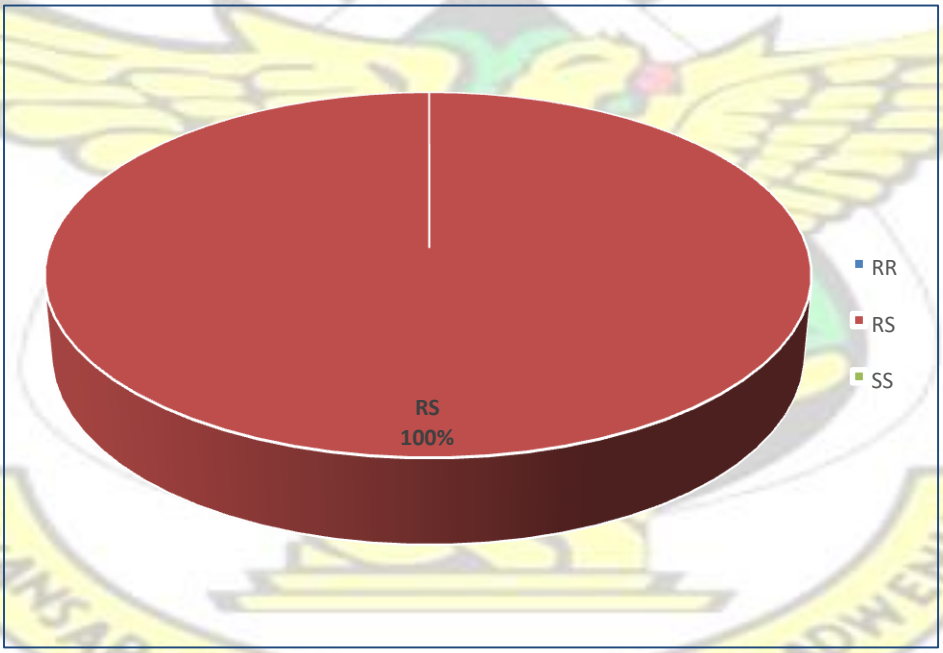

Figure 20: *Kdr* genotypes of the F_1 M/S hybrid mosquitoes resulting from the crossing of the susceptible female S and male M

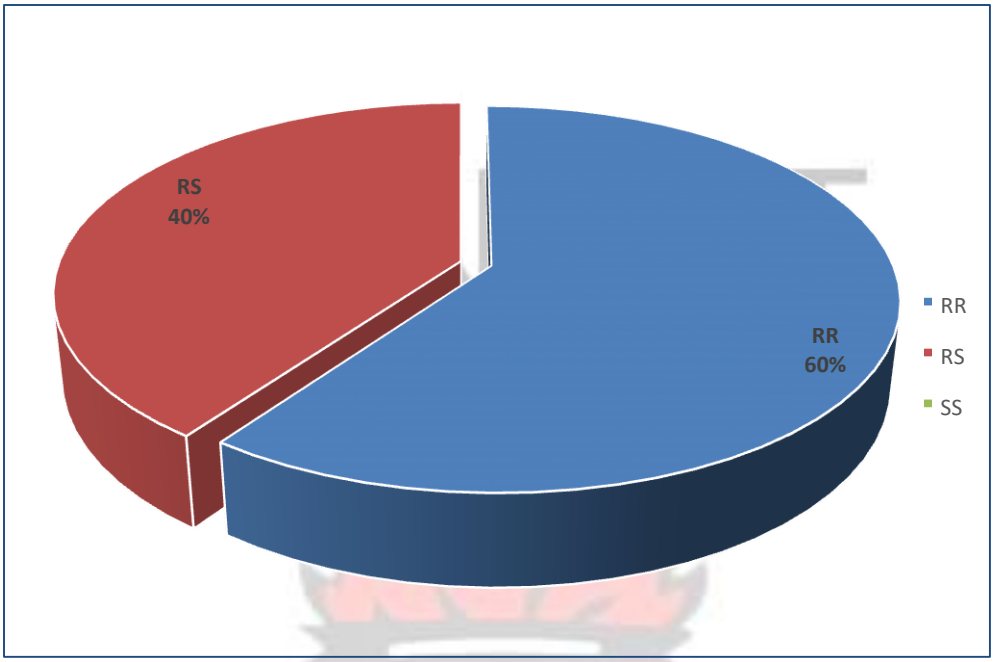

Figure 21: *Kdr* genotypes of the F_1 M/S hybrid mosquitoes resulting from the crossing of the both resistant female M and male S

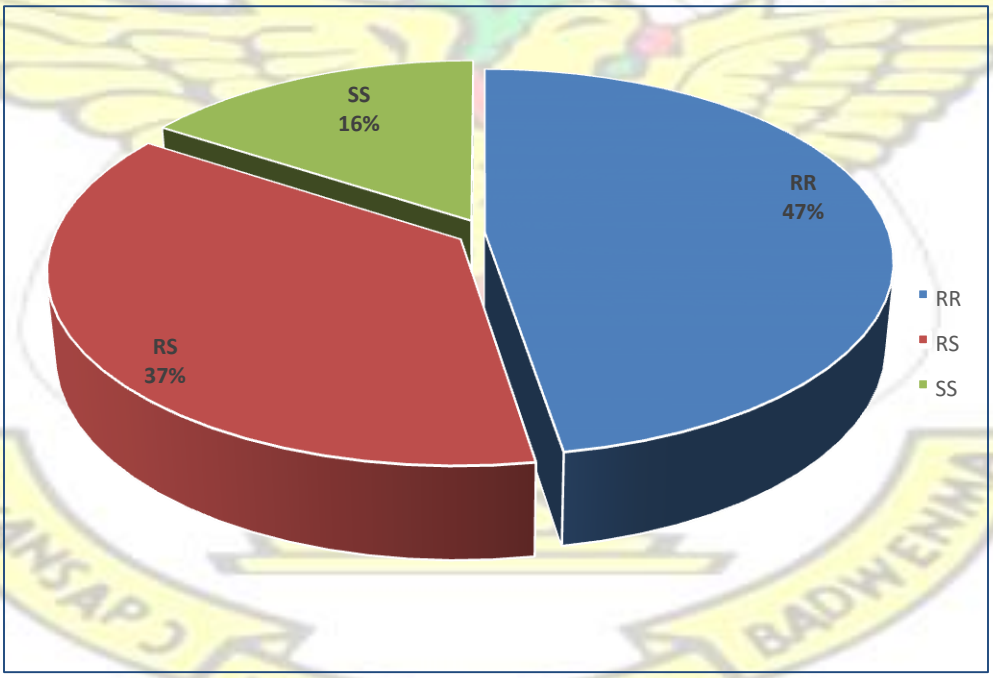

Figure 22: *Kdr* genotypes of the F_1 M/S hybrid mosquitoes resulting from the crossing of the both resistant female S and male M

4.3.2 *Ace-1* genotype of the M/S hybrid mosquitoes

The introgression of the *ace-1* mutation in the hybrid progeny was also detected using the PCR methods as describe above and the gel electrophoregram shown below (Fig. 23). The results of the different progenies are described in the figures 24 to 26 below.

All M/S hybrids, progeny of the crossing with the male M parent showed 100% heterozygous hybrid, irrespective of the resistant status of the female (Fig. 24 & 25). A single susceptible SS and two RR were detected in the progeny of the resistant female M and male S forms, giving 88% of heterozygous out of the 25 specimen tested (Fig. 26). But overall, the frequencies of *ace1* expressed in all the progenies were not significantly different.

Figure 23: **Ethidium bromide-stained 2% agarose gel electrophoregram of *An. gambiae s.s* amplified PCR products for the detection of the *ace-1* mutation.** *L1: 100bp molecular weight marker; L2-3; L5: RS; L7 & 9: SS; L4; L5-8 & L10; 12-13: SS*

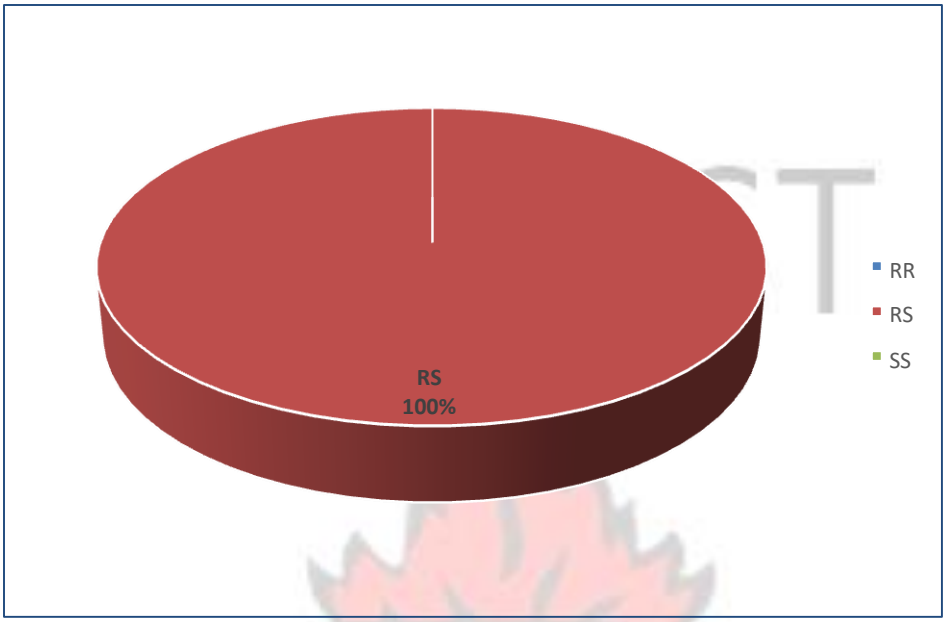

Figure 24: *ace-1* genotypes of the F_1 M/S hybrid mosquitoes resulting from the crossing of the both resistant female S and male M

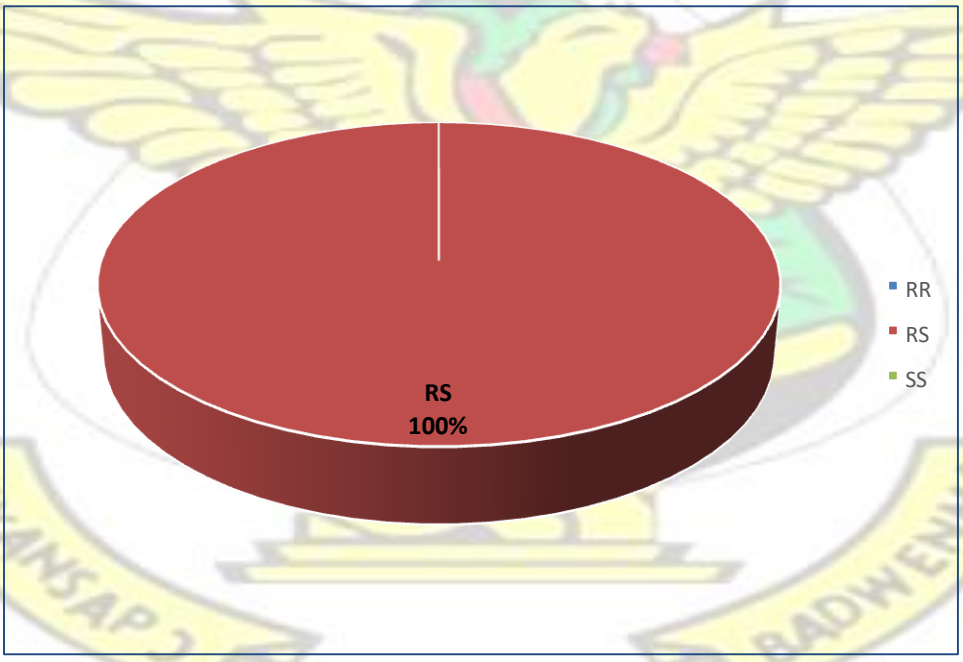

Figure 25: *ace-1* genotypes of the F_1 M/S hybrid mosquitoes resulting from the crossing of the susceptible female S and male M

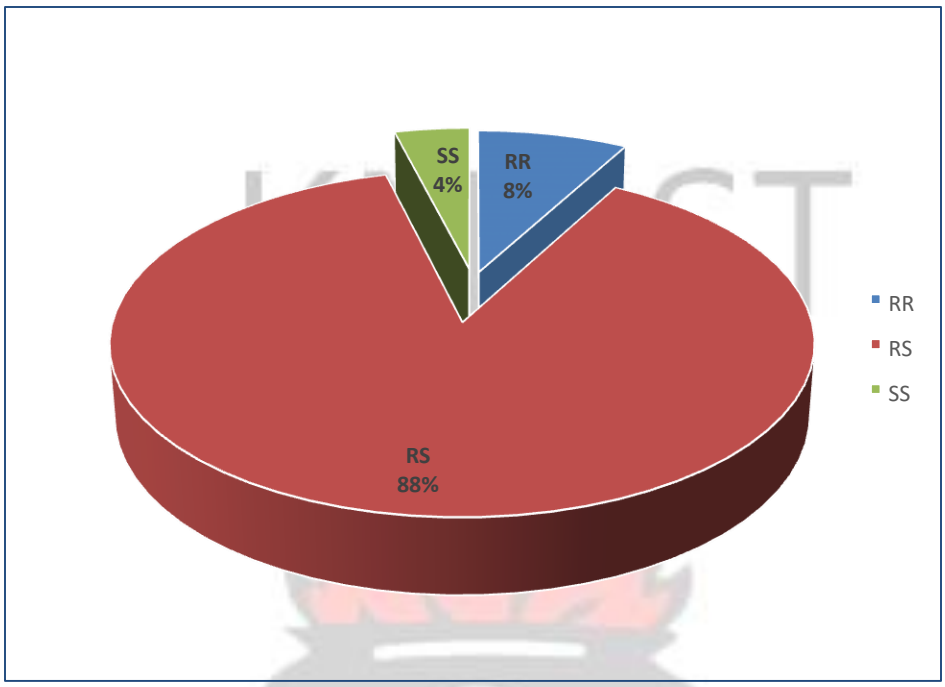

Figure 26: *ace-1* genotypes of the F_1 M/S hybrid mosquitoes resulting from the crossing of the both resistant female M and male S

4.3.3 *Kdr* and *ace-1* frequency of the M/S hybrid mosquitoes

The *kdr* and *ace-1* genotypes of the females M/S hybrid of the F_1 progeny of each crossing experiment were determined. A total of 70 samples of mosquitoes were run to determine the *kdr* and the *ace-1* frequency among the different colonies. It was noted a high *kdr* frequency for the progenies of resistant M and S molecular form parent crosses than the susceptible S and resistant M parents. The resistant parent progenies showed between 0.66 and 0.8 *kdr* frequencies, whilst the resistant M and susceptible S progeny yielded 0.5. In contrast, the *ace-1* frequencies were not significantly different among the progenies, which was about 0.50 for all the experiments (Table 1).

Table 1: *Kdr* and *ace-1* frequency of the M/S hybrid female mosquitoes of each first progeny (N= number of mosquito analyzed).

Progeny of crosses	Frequency	
	Kdr	*Ace-1*
Female resistant M x Male resistant S	0.80[a] *(N=25)*	0.52[a] *(N=25)*
Female resistant S x Male resistant M	0.66[b;a] *(N=19)*	0.50[a] *(N=17)*
Female susceptible S x Male resistant M	0.50[b] *(N=25)*	0.50[a] *(N=25)*

**Two values in the same column sharing the same letter do not significantly differ (P>0.05).*

4.4 Resistance status of M/S hybrid mosquitoes using WHO susceptibility testing The WHO susceptibility was conducted on the progeny of two of crossing experiments. One with both resistant female S and male M parents and the second tested with the susceptible female parent S and the male resistant M. The data observed are shown below.

An increase of the resistance level was observed with the progeny of resistant parents. The progeny was highly resistance to the two pyrethroid insecticides and DDT tested (Fig. 27). In contrast, the progeny of the susceptible female parent showed in lower extent a resistance to the same insecticides (Fig. 28). This confirmed the *kdr* expressed by both progenies with most of the homozygous RR found in the resistant parental progeny.

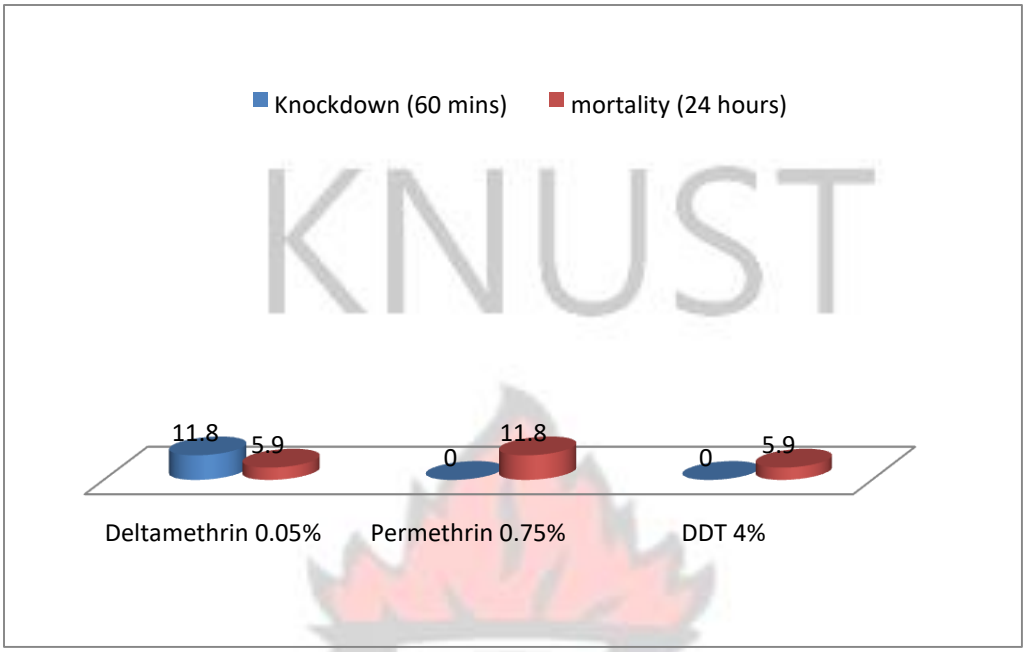

Figure 27: Resistance status of M/S hybrid mosquitoes of both resistant female S form vs male M progeny.

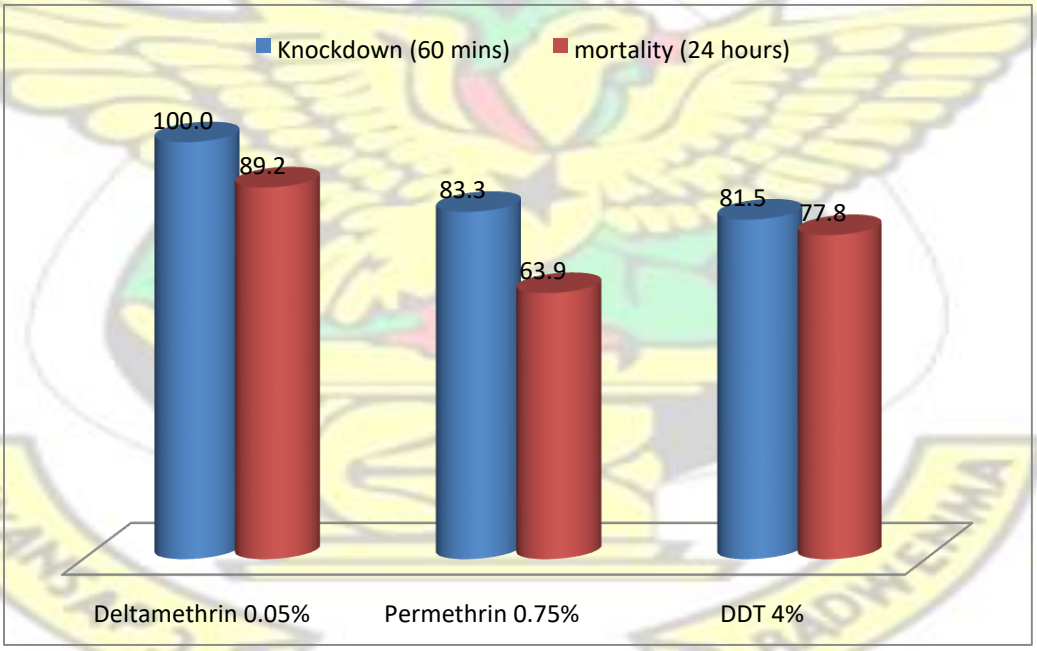

Figure 28: Resistance status of M/S hybrid mosquitoes of the female susceptible S form vs resistant male M progeny

4.5 DNA sequences of M/S hybrid mosquitoes

All M/S hybrid DNA sequenced showed similar sequences whatever the crossing way and generation tested and similar with those in the GenBank. Only a single nucleotide was shown to be different at the 101 sequence position by showing either a G or A nucleotide (Appendix V). However, that status was shown to not impact the similarity of the sequences. The comparison done using DnaSP 5.10.1 did not reveal any significant difference between the different M/S sequences analyzed ($p = 0.4652$).

DISCUSSION

Understanding the hybridization process between M and S forms of *An. gambiae s.s* and how its genetic and geographical distributions impact on malaria vector control has become extremely necessary and increasingly important. It is widely noted that the major malaria vector *An. gambiae s.s.* is undergoing a process of speciation within the M and S molecular forms (della Torre *et al*., 2001). Although the divergence of M and S forms is likely proceeding in many parts of West and Central Africa (Reidenbach *et al*., 2012), the results of this laboratory study conducted, showed similarity with those of other recent surveys of wild populations in the extreme West of Africa (Caputo *et al*., 2011, Marsden *et al*., 2011, Oliveira *et al*., 2008, Weetman *et al*., 2012) which indicated that hybridization is truly occurring and becoming common among the M and S *An. gambiae s.s* populations. Most investigations have shown that evidence and have confirmed the ongoing speciation among the M and S forms occurring in the nature (Caputo *et al*., 2011, Lee *et al*., 2013, Nwakanma *et al*., 2013). Furthermore, some studies have exploited different approaches on the hybridization process and the gene flow between the two molecular forms and also within the whole complex (Pates *et al*., 2014, Weetman *et al*., 2014). Suspicion in the hybrid progeny fertility is still unclear for the M and S molecular forms.

Whilst these two forms were considered to be unique (della Torre *et al*., 2001, della Torre *et al*., 2005). Research has been undertaken to investigate the way in which the occurrence of hybridization has been described to enable correct mapping of *An. gambiae s.s* speciation (Santolamazza *et al*., 2011).

A wide range of proportion of M/S hybrid mosquitoes has been increasingly reported in the western countries of Africa like Senegal, Bissau Guinea and Gambia (Santolamazza *et al.*, 2011) and that bring the need of a good understanding and clear description of the fertility and survival

of hybrid mosquitoes (Lee *et al*., 2013). The observation that the two forms were able to mate confirms the assertion of ongoing hybridization occurring in the field. This result brings into question the issue of describing M and S as separate or sibling species and therefore, further studies to be undertaken to definitively specify the hybridization of the two sibling species. In areas where the two species are living in sympatry, hybrids should be expected, especially hybrid females (Lanzaro and Lee, 2013). This study furthermore demonstrated that the crossing of either an M or S female parental line, with a male S or M form, respectively, yielded 100% M/S hybrid females irrespective of the origin of the parents. Interestingly, the first progeny of males were identified carrying the same form as their female parent. Thus, the progeny of the female M crossed with the male S form yielded 100% males M form at the F_1 generation and inversely for female S and male M. This shift of male form is shown for the first time in this study.

This observation may explain the non-survival of hybrid males expressed by some authors (Tripet *et al*., 2005b). Moreover, the fact that only the females were hybrids indicates that the gene flow between the two molecular forms could probably be sex linked and carried by the female. Since the occurrence of the hybridization trends, all the M/S hybrids found were only females and this study confirmed the observations of other studies (Lanzaro and Lee, 2013, Nwakanma *et al*., 2013). A decrease in hybrid ratio was observed in subsequent generations due to the fact that the males were either M or S form. This indicated that the gene responsible may be recessive. The second generation (F_2) progeny of the different crossing experiments showed a reconstitution of the female parent form at almost 50% whatever the origin of the female in addition to the M/S hybrid female proportion observed. All the forms as well as females and males were fully reconstituted after the third generation (F_3) of each crossing. The shift of male form was shown as an impact to the proportion of the hybrids, M and S forms on the subsequent generations after the F_1. More than

40% of M/S hybrid was noted among the females of each generation (F_2 to F_5) for all the experiments. This stability of the hybrid female among the population after several generations should be considered following the increasing report of the hybrid in some countries (Nwakanma *et al.*, 2013). The shift of the male form at the first generation could also lead to a change in the molecular forms in areas where hybridization occurs. Though the trend gradually changed after the third generation of the same progenies, it should be considered the way the mating can occur in nature during the swarm (Dabire *et al*., 2014, Sawadogo *et al*., 2013). Furthermore, this study has demonstrated the fertility of the hybrid mosquitoes, following the maintenance of the colonies to over five generations post crossing. In contrast with some statements made by Lanzaro G. (Personal communication: University of California - Davis. "Hybrid 'super mosquito' resistant to insecticide-treated bed nets." Science Daily. Science Daily, 12, January 2015) when describing the M/S hybrid mosquitoes as mentioned hereafter: “Monitoring of the two species since they were first recognized had demonstrated that hybrids would periodically appear in natural mosquito populations, but then they would disappear. Presumably nothing favored their survival over the pure species, if they were even fertile. Thus, researchers knew that the two populations tended to remain more or less reproductively isolated, even where they coexisted”. This study demonstrated the reproduction and surviving ability of the M/S hybrids in the laboratory and therefore casts doubts of the M/S hybrid‟s fertility and viability. This trend could be expected in nature even though it was noted the absence of male hybrids. In addition, it can also bring new challenges in vector control considering that the resistant genotype is differently expressed in the two forms and the insecticide resistant hybrids are more successful in breeding with the M form (Norris *et al*., 2015). Several studies have described the S form as more resistant than the M form (Dabire *et al*., 2008, Nwane *et al*., 2013) where both forms are found with a promoted introgression of the *kdr* mutation from the S to the M molecular form.

The resistance mechanisms expressed by the hybrid mosquitoes may be linked to the resistance status of the parents. The frequency of *kdr* expressed by the hybrid progeny resulting from each crossing way was impacted by the fact that either both colonies were resistant or not. The WHO susceptibility test performed on the F_1 generation in this study described the evidence of this assertion, where the progeny of the resistant parents showed higher levels of the resistance than the progeny of the resistant male M and female susceptible S forms. The introgression of the resistance mechanisms from parents to progeny was shown by the increase frequency of either the *kdr* or the *ace-1* mutation expressed by the M/S hybrid mosquitoes which can unexpectedly promote the evolution of the insecticide resistance mechanisms. This trend was already evidence between the two molecular forms and mostly reported in Sub-Saharan African countries. The introgression of the *kdr* from the S form to the M form was well described in countries like Benin, Burkina Faso and Cameroon (Dabire *et al*., 2009, Etang *et al*., 2009, Weill *et al*., 2000, Lehmann and Diabate 2008). Furthermore, the rapid increase of the *kdr* frequency in the M form in the field following introgression must have been outweighed by insecticidal selection on the mutation channel (Clarkson *et al*., 2014). That introgression was described to be coinciding with the increasing to the insecticide exposure of *An. gambiae s.s* which acted as a selective force to drive the introgression of the *kdr* genome across the reproductive barrier separating both M and S forms (Norris *et al*., 2015). But the specifications on the hybrid resistance mechanisms are still unclear and need more investigations in order to foresee control measures. Additional mutations like *ace-1* and the metabolic enzymes activities of the M/S hybrids require attentions even though this study has partially showed the probable introgression with regard to the *ace-1* frequency of the hybrids.

Through this study, certain similarities among the data observed from each crossing experiment and inversely expressed following the form of the female and male parents were noted. Unfortunately, the descendants of the crossing experiment of female resistant M and susceptible male S forms crashed after the F_1. It was observed that the slightly longer period required to enable the male to mate with the female could not maintain the males alive. Therefore, most of the males died before mating with the resistant female M form. Thus, fewer eggs were collected from the F_1 and the colony was crashed a generation after. However, the data from the three other experiments had described the expectations on the hybrid proportion per generation to enable some conclusions to be made.

The mosquito DNA sequences analyzed demonstrated similarities among all the hybrid mosquitoes irrespective of the origin and the generation ($p = 0.4652$). As described by (Weetman *et al*., 2012), the hybridization rates in most sampled areas suggest interform gene flow in excess of that required to prevent extreme divergence in the absence of selection (Slatkin, 1987, Wright, 1931) when using any plausible estimate of population size. However, selection against F_1 hybrids in the wild can be very strong which was indeed, the case in this study with a large number of the hybrids detected at F_1 rather than more advanced crosses or backcrosses. However, this trend was observed as stipulated earlier for the fact that only females were hybrids and the gene flow was then maintained by the female through heredity. This explains why the gene flow between M and S forms was much lower after several generations (White *et al*., 2010).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

This study revealed that hybridization occurring between *An. gambiae s.s* M and S molecular forms is real. The suspicion of the gene flow between them should be reviewed. Moreover, the specification of the gene flow in only females was well described by this study, where only female M/S hybrid specimen were found after five generations post crossing. Therefore, this study showed the ability of the M/S hybrids to survive and fertilize. This study also described the non-existence of hybrid males, showing the limitation of the gene flow which may be sex linked and then impacted the subsequent generations post crosses.

Although the introgression of the *kdr* mutation from the S to the M form where the two forms were found living in sympatry is well described, the hybrid resistance status is still not well delimited. Nevertheless, the results of the WHO susceptibility tube testing could guide on the expectation of the progeny status following the parental resistance profiles. As demonstrated in this study, the hybrids resulting from the crossing of the two resistant strains showed more ability to withstand the insecticide effect than a crossing of the resistant and the susceptible strains which was confirmed by the frequency of the *kdr* allele mutation in the different hybrid colonies.

This study also showed that all the M/S hybrid mosquitoes have similar DNA sequences, irrespective to the crossing way and the progeny.

6.2. Recommendations

This study showed lightly the introgression of the resistance mechanisms and required additional genome sequence analysis. Also, further approaches should be made to design new markers for the identification of male hybrid if possible. In addition, more investigations have to be completed on the characterization of the resistance status of the different generation post crossing to enable the specification of each form and hybrid resistance profiles. Furthermore, studies must be undertaken to assess the susceptibility of M/S hybrids to the different malaria *plasmodium* following the increasing reports of hybridization in the wild.

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APPENDICES

Appendix I: PCR conditions for identification of *An. gambiae s.l* complex.

Ref: Fanello C., Santolamazza F., and della Torre A. 2002 "Simultaneous identification of species and molecular forms of the Anopheles gambiae complex by PCR-RFLP." Med Vet Entomol 16 (4):461-4.

Primers

UN: 5‟- GTGTGCCGCTTCCTCGATGT - 3‟

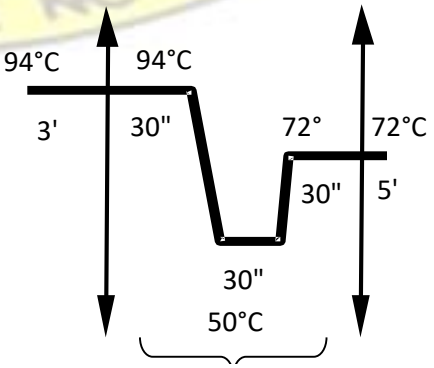
AG: 5‟- CTGGTTTGGTCGGCACGTTT - 3‟

AA: 5‟- AAGTGTCCTTCTCCATCCTA - 3‟

AM: 5‟- GTGACCAACCCACTCCCTTGA - 3‟

PCR Conditions

With Go *Taq* for final volume of 20 µl per reaction



Reagents	Final concentration	For 1 reaction at 20 µl
Go *Taq*	1X	12.5 µl
Primer UN (10 µM)	0.15 µM	0.6 µl
Primer AG (10 µM)	0.15 µM	0.6 µl
Primer AA (10 µM)	0.15 µM	0.6 µl
Primer AM (10 µM)	0.15 µM	0.6 µl
ddH_2O		2.1 µl
DNA template (1/40)		3.0 µl

Amplification: 3‟ (30,,,,; 30,,,,; 30,,,,)$_{33\ cycles}$ 5‟ at 72°C

Lane waited

An. gambiae: 390 bp

An. arabiensis: 315 bp

An. melas: 464 bp

Appendix II: PCR conditions for identification of *An. gambiae s.s* M and S molecular forms.

Ref: Santolamazza F., Mancini E., Simard F., Qi Y., Tu Z. and della Torre A. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of Anopheles gambiae molecular forms Malaria Journal 2008, 7:163

Primers

F6.1a: 5‟- TCGCCTTAGACCTTGCGTTA - 3‟

R6.1b: 5‟- CGCTTCAAGAATTCGAGATAC - 3‟

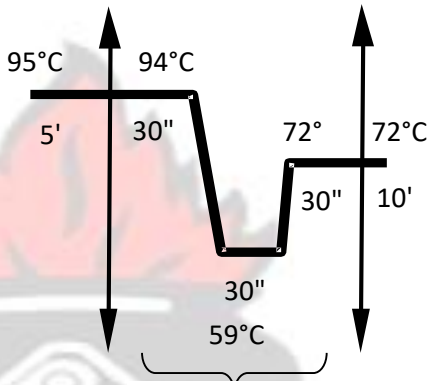
PCR Conditions

With Go *Taq* for final volume of 25 µl per reaction

Reagents (Initial Concentration)	Final Concentration	For 1 reaction at 25 µl
Go *Taq*	1X	12.5 µl
F6.1a(100 µM)	10 µM	1.0 µl

R6.1b (100 µM)	10 µM	1.0 µl
DNase free water		6.5 µl
DNA template (1/40)		4.0 µl
Total		**25.0 µl**

Amplification: 5‟ (30,,,,; 30,,,,; 30,,,,)$_{35\ cycles}$ 10‟ at 72°C



Lane waited

An. gambiae s.s. S form: 249 bp

An. gambiae s.s M form: 479 bp

Appendix III: PCR for characterization of *An. gambiae s.l kdr* mutation genotype

Ref: Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AI,
Guillet P, Pasteur N, Pauron D. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s.* Insect Mol Biol. 1998, 7(2): 179-84

Primers

AGD1: ATAGATTCCCCGACCATG

AGD2: AGACAAGGATGATGAACC

AGD3: AATTTGCATTACTTACGACA

AGD4: CTGTAGTGATAGGAAATTTA

PCR Conditions

With Go *Taq*, for a final volume of 25 µl per reaction

Reagents	**Final concentration**	**For 1 reaction at 25 µl**
Go *Taq*	1X	12.5 µl
Primer D1 (10 µM)	20 µM	1 µl
Primer D2 (10 µM)	20 µM	1 µl
Primer D3 (10 µM)	20 µM	1 µl
Primer D4 (10 µM)	20 µM	1 µl
ddH_2O		4.5 µl

DNA template (1/40)		4 µl

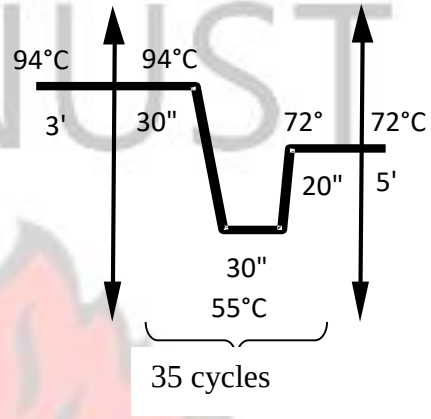
Amplification: 3‟ (30,,,,; 30,,,,; 20,,,,)$_{35\ cycles}$ 5‟ at 72°C

Lane waited

D1/D2: 293 bp

D1/D3: 195 bp **Resistant**

D2/D4: 137 bp **Susceptible**



Appendix IV: PCR condition for characterization of *An. gambiae s.l ace-1* mutation

Ref: Weill, M., G. Lutfalla, K. Mogensen, F. Chandre, A. Berthomieu, C. Berticat, N. Pasteur, A. Philips, P. Fort, and M. Raymond. 2003. "Comparative genomics: Insecticide resistance in mosquito vectors." Nature 423 (6936):136-7.

Primers

Ex3 AGdir: GATCGTGGACACCGTGTTCG

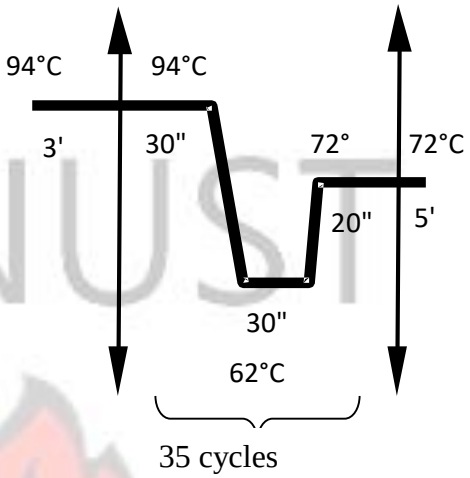
Ex3 AGrev: AGGATGGCCCGCTGGAACAG

PCR Conditions

With Go *Taq*, for a final volume of 25 µl per reaction

Reagents	**Final concentration**	**For 1 reaction at 25 µl**
Go *Taq*	1X	12.5 µl
Primer Ex3AGdir (10 µM)	10 µM	1.0 µl
Primer Ex3AGrev (10 µM)	10 µM	1.0 µl
ddH_2O		9 µl
DNA template (1/40)		1.5 µl

Amplification: 3‟ (30,,,,; 30,,,,; 20,,,,)$_{35\ cycles}$ 5‟ at 72°C



Lane waited

ExAG: 541 bp

Verify 5 µl of PCR product on agarose gel at 2%

Enzymatic digestion

For a final volume of 20 µl per reaction

Reagents	**Final concentration**	**For 1 reaction at 25 µl**
Enzym buffer	10 X	2 µl
Acetylated BSA	10 µg/l	0.2 µl
Alu 1 (5U/µl)	10 U/ µl	0.5 µl
ddH_2O		12.3 µl
PCR product		5.0 µl

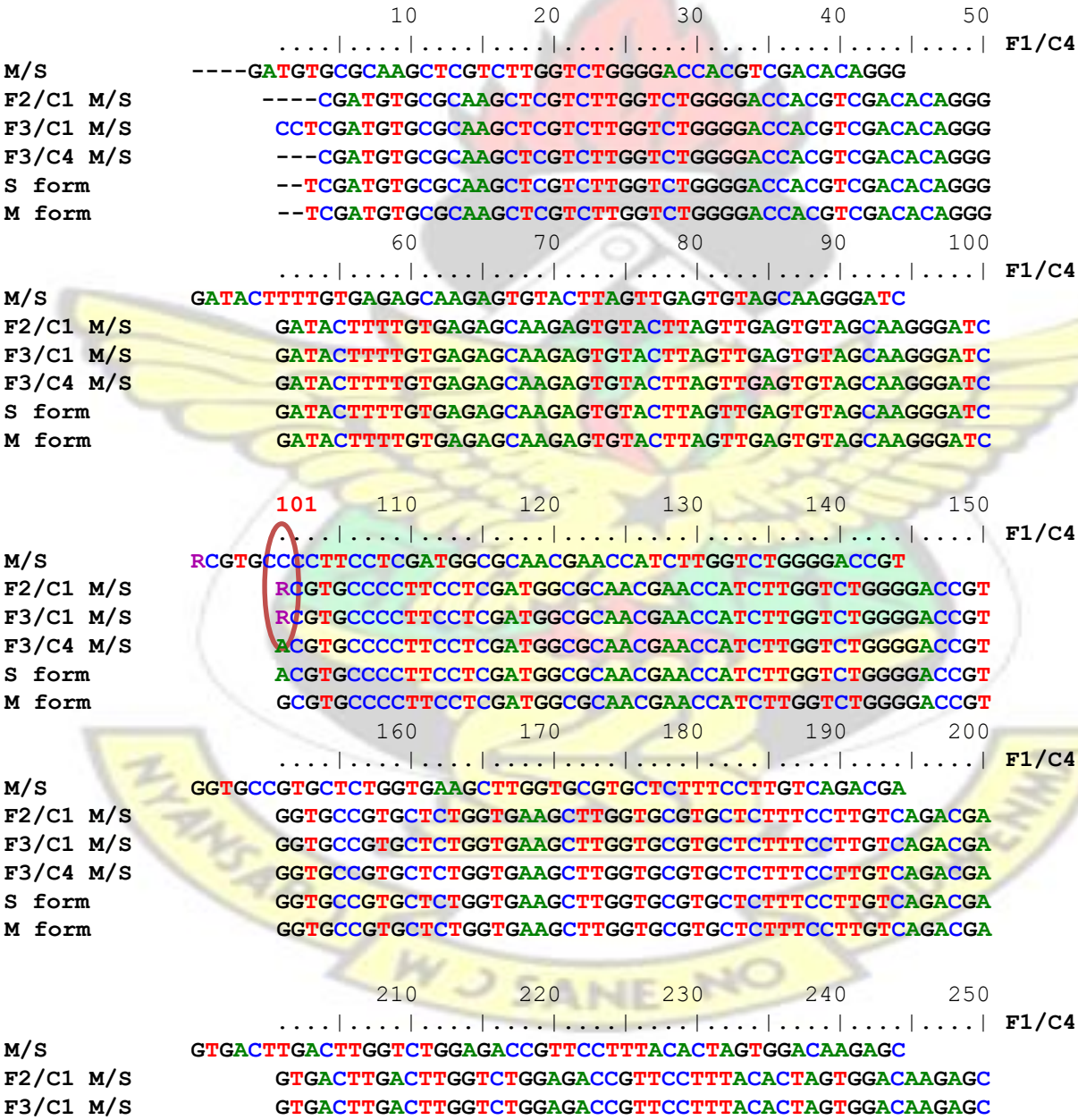
Incubation at 37°C for 4h or the whole night (better)

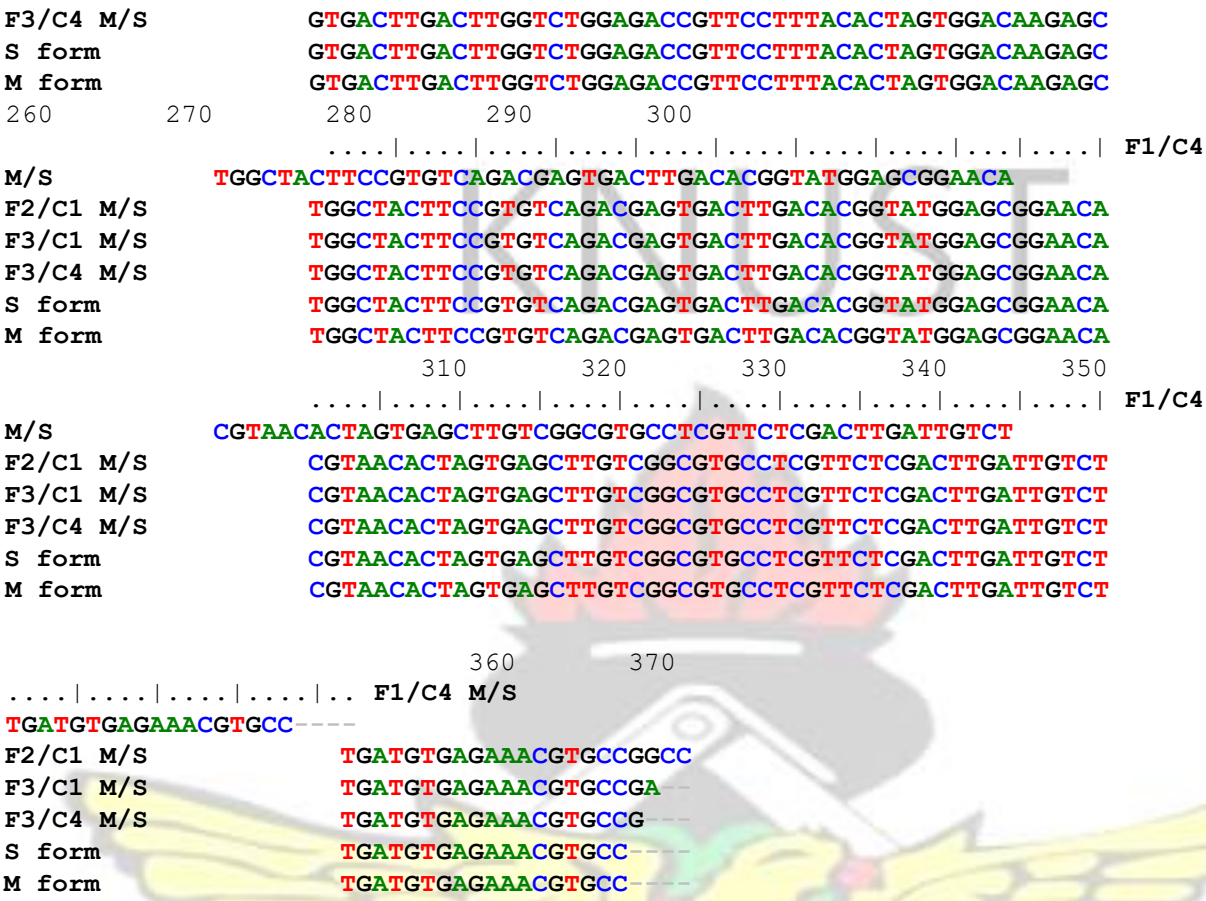
Run on agarose gel at 2%

Lane waited

Genotype	**SS**	**RR**
Lane	403 bp	253 bp
		150 bp
	138 bp	138 bp

Appendix V: DNA sequences of *An. gambiae* M, S forms and M/S hybrids




F3/C4 M/S GTGACTTGACTTGGTCTGGAGACCGTTCCTTTACACTAGTGGACAAGAGC
S form GTGACTTGACTTGGTCTGGAGACCGTTCCTTTACACTAGTGGACAAGAGC
M form GTGACTTGACTTGGTCTGGAGACCGTTCCTTTACACTAGTGGACAAGAGC
260 270 280 290 300
F1/C4
M/S TGGCTACTTCCGTGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACA
F2/C1 M/S TGGCTACTTCCGTGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACA
F3/C1 M/S TGGCTACTTCCGTGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACA
F3/C4 M/S TGGCTACTTCCGTGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACA
S form TGGCTACTTCCGTGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACA
M form TGGCTACTTCCGTGTCAGACGAGTGACTTGACACGGTATGGAGCGGAACA
310 320 330 340 350
F1/C4
M/S CGTAACACTAGTGAGCTTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCT
F2/C1 M/S CGTAACACTAGTGAGCTTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCT
F3/C1 M/S CGTAACACTAGTGAGCTTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCT
F3/C4 M/S CGTAACACTAGTGAGCTTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCT
S form CGTAACACTAGTGAGCTTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCT
M form CGTAACACTAGTGAGCTTGTCGGCGTGCCTCGTTCTCGACTTGATTGTCT

360 370
F1/C4 M/S
TGATGTGAGAAACGTGCC----
F2/C1 M/S TGATGTGAGAAACGTGCCGGCC
F3/C1 M/S TGATGTGAGAAACGTGCCGA--
F3/C4 M/S TGATGTGAGAAACGTGCCG---
S form TGATGTGAGAAACGTGCC----
M form TGATGTGAGAAACGTGCC----

Appendix VI: M/S DNA sequence analysis

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Gene Flow and Genetic Differentiation

Input Data File: C:\...\All MS hybrid alignment.fas
Number of Populations Included: 2
Selected region: 1-372 Number of sites: 372
Sites with alignment gaps are: Excluded
Total sites (excluding alignment gaps): 364

Population 1: MS_hybrid_of_female_M_and_male_S
Number of sequences: 4
Number of segregating sites, S: 1
Number of haplotypes, h: 2
Haplotype diversity, Hd: 0.50000
Average number of differences, K: 0.50000
Nucleotide diversity, Pi: 0.00137
Nucleotide diversity with JC, PiJC: 0.00138

Population 2: MS_hybrid_of_female_S_and_male_M
Number of sequences: 4
Number of segregating sites, S: 1
Number of haplotypes, h: 2
Haplotype diversity, Hd: 0.66667
Average number of differences, K: 0.66667
Nucleotide diversity, Pi: 0.00183
Nucleotide diversity with JC, PiJC: 0.00183
Total Data Estimates
Number of sequences: 8

Number of segregating sites, S: 1
Number of haplotypes, h: 2
Haplotype diversity, Hd: 0.53571
Average number of nucleotide differences, Kt: 0.53571
Nucleotide diversity, PiT: 0.00147

========== Genetic Differentiation Estimates ========== Chi-square (table), Chi2: 0.533 P-value of Chi2: 0.4652 ns; (df = 1) ns, not significant; *, 0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001
HBK 1992, Hs: 0.58333 Hst: -0.08889
HBK 1992, Ks: 0.58333 Kst: -0.08889
HBK 1992, Ks*: 0.40434 Kst*: -0.08889
HBK 1992, Z: 14.16667 Z*: 2.58677
Hudson 2000, Snn: 0.37500

========== Gene Flow Estimates ==========
Genome: Diploid-Autosome
Haplotype Data Information
Nei 1973
Gst: -0.07692 Nm: -3.50
Sequence Data Information
Nei 1982
DeltaSt: 0.00009 GammaSt: 0.06667 Nm: 3.50
Lynch and Crease 1990 (with Jukes and Cantor correction)
Nst: -0.16667 Nm: -1.75
Hudson, Slatkin and Maddison 1992
Fst: -0.16667 Nm: -1.75
