INSECTICIDE RESISTANCE ASSOCIATED WITH 2La INVERSION AND MICROSATELLITE LOCI POLYMORPHISM IN *Anopheles gambiae s.s.* POPULATIONS FROM LAGOS AND OYO STATES, NIGERIA

BY

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ABSTRACT

Dichlorodiphenyltrichloroethane (DDT) and deltamethrin are insecticides frequently used in malaria vector control interventions in Africa. Resistance to these insecticides has emerged in the malaria vector, *Anopheles*. However, the assortment of two genetic mechanisms, 2La inversion and the polymorphism of microsatellite loci, have also been associated with insecticide resistance in *Anopheles* populations in several countries with limited studies on these resistance mechanisms in Nigeria. This study was therefore designed to determine DDT and deltamethrin insecticide resistance, associated with 2La inversion and microsatellite loci polymorphism in *Anopheles gambiae s.s.* populations from Lagos and Oyo States.

Larval samples of *Anopheles* were collected from six localities each in Lagos and Oyo States and were morphologically identified using standard methods. Emerged adult females (Lagos: n = 1,822, Oyo: n = 1,810) were exposed to 4% DDT and 0.05% Deltamethrin insecticides separately for one hour, according to WHO insecticide susceptibility criteria. The mosquitoes were characterised using PCR and restriction enzyme digestion (for M and S forms). Resistant mosquitoes to DDT were further subjected to 2La inversion and microsatellite loci characterisation. Genotyping of DDT resistant mosquitoes to 2La inversion was performed on 30 selected *Anopheles gambiae s.s.* (M molecular form) from each locality using PCR. Ten microsatellite loci, selected close to documented insecticide resistance genes within and outside 2La, were examined for polymorphic alleles using standard methods. Lagos and Oyo resistant *Anopheles* populations were compared using descriptive statistics. The 2La inversion data for the two states were analysed using Wright F-statistic, Chi-square and Hardy-Weinberg equation. Microsatellite data were subjected to linkage disequilibrium and one-way ANOVA at $\alpha_{=0.05}$.

Mosquitoes from Lagos were more resistant to DDT and deltamethrin with 0.0-34.5 and 50.0-92.7% mortalities, respectively compared to those from Oyo with 13.3–84.0 and 80.0–100% mortalities, respectively. Significant difference in resistance profile between Lagos and Oyo *Anopheles* population was recorded only for DDT with deltamethrin showing insignificant values between populations. *Anopheles gambiae*

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s.s. was the only species found in all the localities surveyed in Lagos and all belonged to the M molecular form. Oyo State populations contained more *Anopheles arabiensis* (58.0%) than the *Anopheles gambiae s.s.* (42.0%) with sympatric occurrence of the M and S molecular forms. The DDT resistance profile patterned the 2La inversion karyotype (Lagos:F_{ST}=0.104; Oyo:F_{ST}=0.043) with the Chi square values falling within Hardy-Weinberg estimates (χ^2=0.001-3.81, p=0.096 - 0.999) in all populations. Microsatellite genotypic linkage disequilibrium occurred in 24.0% of the loci (χ^2=10.6 - 25.0, p=0.00005-0.032) between Lagos and Oyo populations. Six out of ten polymorphic alleles had significantly high genetic differentiation values, AG2H26 (F_{ST}=0.2938), AG2H175 (F_{ST}=0.0595), AG2H590 (F_{ST}=0.0519), for Lagos and Oyo populations; three of which AG2H637 (F_{ST}=0.1134), AG2H772 (F_{ST}=0.3246), AG2H143 (F_{ST}=0.0817), were located within inversion 2La. Resistance to dichlorodiphenyltrichloroethane and deltamethrin in *Anopheles* population was established in Lagos and Oyo States. The resistance profile associated with 2La inversion karyotypes, and polymorphism of six microsatellite loci may be used as genetic markers in malaria vector control interventions in Lagos and Oyo States, Nigeria.

Keywords: *Anopheles gambiae s.s.*, Insecticide resistance, 2La inversion, Microsatellite loci Polymorphism

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DEDICATION

This project is dedicated to GOD almighty, who has always been my strength and shield. To my supervisors, family, colleagues and friends for their support during this program.

CERTIFICATION

I certify that this work was carried out by Mr. Adedapo Olufemi ADEOGUN in the Department of Zoology, University of Ibadan.

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CHAPTER ONE
INTRODUCTION

Some of the world's most devastating vector-borne diseases (VBDs) are transmitted to people by blood-sucking arthropods, particularly mosquitoes. In most tropical and subtropical disease endemic countries, these vector-borne diseases which are of serious public health concern, affect billions of people globally. Population growth, poorly managed urbanization, the greater incursion of human activities into natural ecosystems, and the transition and expansion of the geographical distribution of vectors due to climatic changes have contributed to an unpresented growth in several vector-borne diseases, particularly malaria. This situation has been aggravated by the accidental spread of vectors and pathogens through increased global travel, and the collapse of vector control in public health programmes (WHO, 2015).

The conventional methods of controlling disease vectors, for example mosquito populations, which involve insecticide fogging, aerosol space spraying, larviciding, indoor residual insecticide spray have proved largely effective in reducing vector density (Awolola *et al.,* 2005b). However, the indiscriminate use of insecticides for malaria vector control activities and the lack of evidence based interventions has led to the selection of malaria vectors that survived the insecticides interventions, often referred to as insecticide resistance. Resistance to insecticides in these major malaria vector, *Anopheles gambiae*, has largely contributed to the failure of malaria control interventions. This resistance confer an adaptive potential on the vectors to explore former inhospitable environments (Hougard *et al.,* 2002).

In the last two decades, the ineffectiveness of malaria control interventions, due to insecticide resistance has led to the development of the application of molecular biology and genetic engineering in vector control (Ayala *et al.,* 2014). This is largely

dependent on the concept of evolutionary forces such as selection and migration, which shape adaptive processes and the species genome reflect this evolutionary process through modifications in its sequence and architecture (Lenormand, 2002). Among the most prominent adaptation mechanisms are chromosomal inversions (Krimbas and Powell, 1992; Hoffman *et al.*, 2004). Inversions join two evolutionary characteristics making them one of the most effective instruments for local adaptation: they involve several or even hundreds of genes, and recombination is drastically reduced in heterozygote state (Stump *et al.*, 2007; Kulathinal *et al.*, 2009). Inversions can also affect fitness by influencing the expression and/or structure of the genes located near there break points (Calvete *et al.*, 2012).

Inversions in *Anopheles*, however, have been associated with several phenotypic traits including insecticide resistance (Brooke *et al.*, 2002). Of great importance is the frequency of alternate arrangement on chromosome 2 (inversions 2La and 2Rb) in *Anopheles gambiae s.s.*. These were shown to correlate with ecological/climatic factors and resistance to insecticides, suggesting an adaptive potential for inversions and different combinations favoring survival under a variety of environmental and anthropogenic conditions (Coluzzi *et al.*, 1979; Coluzzi *et al.*, 1985; Toure *et al.*, 1994; 1998; Brooke *et al.*, 2002; Wondji *et al.*, 2002). An inversion on the left arm (2La) is a critical component to the ecological differentiation in this medically important species (Coluzzi *et al.*, 1979; Simard *et al.*, 2009). Specifically, 2La inversion frequency has been associated with resting and feeding behavior (Coluzzi *et al.*, 1979), susceptibility to *Plasmodium* (Petrarca and Beier, 1992; Sharakhov *et al.*, 2006), thermal tolerance (Rocca *et al.*, 2009) and insecticide resistance (Brooke *et al.*, 2000) in *Anopheles gambiae*. Understanding the role of inversions in insecticide resistance could have direct implication on the success of malaria control programmes by helping us understand the spread and introgression of resistance alleles within and between natural populations.

Furthermore, the examination of Variable Number of Tandem Repeats (VNTR) on the second chromosome may also shed more light on the study of population genetics of *An. gambiae*. They are characterized by microsatellite loci, which are about 2-6bp

repeats (Lanzaro *et al.,* 1998). They have been proven useful in the analysis of paternity and kinship (Queller *et al.,* 1993) and in the probability of sample identity at both the individual (Edwards *et al.,* 1992) and population levels (Paetkau *et al.,* 1995). Microsatellite variation has been used to study the amount of hybridization between closely related species and comparison of levels of variation between species and populations (Gotelli *et al.,* 1994; Roy *et al.,* 1994). It has also proven useful in the assessment of overall genetic variation (Taylor *et al.,* 1994). Hence, they can be used to gain insight into the degree of population substructure and genetic relationships among various subpopulations of *Anopheles gambiae* (Lade *et al.,* 1996).

In Nigeria, DDT and Deltamethrin insecticides are extensively used in malaria vector control programs. DDT has been extensively used in Indoor Residual Spray (IRS) while deltamethrin insecticides are used in both impregnating Long Lasting Insecticide Treated Nets (LLIN) and Indoor Residual Spray (Oduola *et al.,* 2012). Until recently, resistance to Deltamethrin has been patchy with low amount of resistance in most *Anopheles* populations (Awolola *et al.,* 2005a). Earlier studies recorded very high efficacy of the insecticide DDT in malaria vector control programs and the insecticide was regarded as one of the leading insecticides adopted for use (Armstrong *et al.,* 1957; Ramakrishna and Elliot, 1959). Resistance to this insecticide in *Anopheles* populations became widespread in the 70's and 80's and in some mosquitoes, there was evidence that DDT resistance causes cross-resistance to pyrethroids (Prasittisuk and Busvine, 1977; Prasittisuk and Curtis, 1982). In recent studies, DDT resistance in the major malaria vector, *Anopheles gambiae* has been alarming with 100% survival in most populations exposed to diagnostic concentrations (Oduola *et al.,* 2010). The use of DDT has been suspended in malaria vector control interventions in Nigeria due to issues bordering around resistance. Genetic analysis had revealed that the suspension of DDT in malaria control interventions may not produce a significant effect on the pressure of natural selection against DDT resistance genes. A state's policy of simply alternating DDT with other insecticides in space or time could in the long run prevent the frequency of DDT resistance genes rising to unacceptable levels (Curtis *et al.,* 1978; Prasittisuk and Curtis, 1982). There is need for extensive research on certain genetic mechanisms that aid the survival of the major malaria vectors in Nigeria. The

unacceptable level of insecticide resistance in *Anopheles gambiae* populations in Nigeria and lack of information on genetic resistance mechanisms prompted this study to examine the relationship, if any, between insecticide resistance and two resistance mechanisms in *Anopheles* populations in Nigeria.

1.1 Aim of this Study

The aim of this study is to determine the DDT and Deltamethrin insecticide resistance status of *Anopheles gambiae s.s.* populations in Lagos and Oyo States, Nigeria and evaluate the association between the insecticide resistance phenotypes and genetic resistance mechanisms, 2La inversion and microsatellite loci polymorphism.

1.2 Problem Statement

The paucity of insecticide resistance data on the frequently used insecticides in public health interventions in Nigeria, and the incipient speciation in the efficient malaria vector, *Anoheles gambiae s.s.*, capable of transmitting malaria and exhibiting different adaptation mechanisms could pose a major threat to the success of malaria control interventions. There is then need to provide information on the resistance status of the malaria vectors in Nigeria and determine, if any, an association between these resistance profile and certain genetic mechanisms that are crucial to the survival of *Anopheles* in Nigeria.

1.3 Objectives of this study

1. To carry out morphological and molecular identification of the major malaria vectors in selected study sites in Lagos and Oyo state.
2. To determine the spatial distribution of *Anopheles gambiae s.s.* molecular forms in the selected sites.

3. To determine the susceptibility status of *Anopheles gambiae s.s.* to DDT and Deltamethrin insecticides in Lagos and Oyo state.

4. To assess the association between 2La inversion karyotype frequencies and insecticide resistance in *Anopheles gambiae s.s.* populations breeding in the selected areas.

5. To assess the plausible role of insecticide resistance on the polymorphism of microsatellites in *Anopheles gambiae* populations from the selected localities.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria burden and the biology of its vector

2.1.1 The global burden of malaria

Malaria remains a global disease burden which demands a collective global attention. In 2006, 3.2 billion people from 109 countries were reported to be under the risk of malaria transmission; 45 of these countries fall within the WHO African region (World Malaria Report, 2009). The annual estimates of reported clinical malaria cases were 243million. In 2006, estimated malaria deaths was 863,000, of which 89% were in Africa and 85% were of children under 5 years of age. Eighty-six percent, or 212 million cases were in the African region. Eighty percent of the cases in Africa were in 13 countries, and over half were in Nigeria, Democratic Republic of Congo, Ethiopia, United Republic of Tanzania and Kenya (World Malaria Report, 2008; 2009).

In 2013, there were an estimated 198 million cases of malaria worldwide, and an estimated 584 000 deaths. About 90% of all malaria deaths occur in Africa (World Malaria Report, 2013). An estimated 437 000 African children died before their fifth birthday due to malaria. Globally, the disease caused an estimated 453 000 under-five deaths. Between 2000 and 2013, an expansion of malaria interventions helped to reduce malaria incidence by 30% globally, and by 34% in Africa. During the same period, malaria mortality rates decreased by an estimated 47% worldwide and by 54% in Africa. In the under-five age group, mortality rates have declined by 53% globally, and by 58% in Africa (World Malaria Report, 2013). New analysis reveals that the prevalence of malaria parasite infection (including both symptomatic and asymptomatic infections) has decreased significantly in Africa

since 2000. The number of people infected decline from 173 million in 2000 to 128 million in 2013 – a reduction of 26%. This has occurred despite a 43% increase in the African population living in malaria transmission areas.

According to the World Malaria Report, 2014, 97 countries and territories had ongoing malaria transmission. An estimated 3.3 billion people are at risk of malaria, of which 1.2 billion are at high risk. In high-risk areas, more than one malaria case occurs per 1000 population. In Nigeria, malaria accounts for much of the disease burden with about 97% of the approximately 150 million people at risk. It accounts for 25% of all infant related mortality, 30% of child related mortality and 11% of maternal mortality (World Malaria Report, 2009). A large percentage of the population affected with this disease live in extreme poverty in rural areas with few having access to good healthcare facilities (Otubanjo and Mafe, 2002; Amexo *et al.*, 2004; Obrist *et al.*, 2007).

The human malaria parasite in Nigeria include: *Plasmodium falciparum, Plasmodium ovale and Plasmodium malariae*. However, *P. falciparum* is responsible for more than 95% of all malaria cases transmitted. Malaria parasites are usually transmitted through bites of an infected female mosquito of the genus *Anopheles.* This species is widely distributed across the different ecological zones in Nigeria where suitable sub Saharan climatic conditions exist (Molineaux and Gramiccia, 1980; Kiszewski, 2004).

Studies on malaria transmission conducted in Nigeria have identified eleven species of *Anopheles* mosquitoes: *A. gambiae sensu stricto, A. arabiensis, A. funestus, A. rufipes, A. pharoensis, A. wellcomei, A. squamosus, A. coustani, A. maculipalpis, A. nilli,* and *A. pretoriensis* of which two species; *A. gambiae* and *A. funestus* are regarded as main vectors (Bruce-Chwatt, 1951; Hanney, 1960; Service, 1965; Boreham *et al.*, 1979; Molineaux and Grammicia, 1980; Rishikesh *et al.*, 1985; Oyewole and Awolola, 2006; Oduola *et al.*, 2010; 2012).

Reviewed studies on malaria vector characterisation in Nigeria relied mainly on the use of morphological keys of identification (Okorie, 1973; Mafiana *et al.*, 1998). However, the advent of molecular and immune diagnostic tools have alleviated the difficulties associated with identifying morphological indistinguishable members belonging to

species complexes and the incrimination of *Anopheles* species that are involved in malaria transmission (Service, 1993). Despite the development of molecular techniques, only few studies have utilized them in malaria vector research in Nigeria (Awolola *et al.*, 2003; Onyabe and Conn, 2003; Okwa *et al.*, 2007; Oyewole *et al.*, 2007; Rousseau *et al.*, 2007; Oyewole and Awolola, 2006; Oduola *et al.*, 2010; 2012).

2.1.2. Biology of malaria vector *Anopheles*

Malaria is caused by the *Plasmodium* parasite, which spends its life cycle both in humans and certain species of mosquitoes belonging to the genus *Anopheles*. In humans, the cycle involves trophozoites and meroziotes production followed by differentiation into gametocytes. In the mosquito host, the parasite could be digested at the gametocyte stage during blood feeding. The remaining gametocytes exflagellate and mate to produce zygotes. From the zygotes follows the ookinetes, the oocytes and finally the sporozoite stage. The sporozoite then migrate to the salivary gland ready for inoculation during another blood feeding of the mosquito (Fig. 2.1).

2.1.2.1 Mating and blood feeding of adult Anopheles

Adult *Anopheles* usually mate within a few days (2-3 days) after emergence from the pupal stage. In most species, the males form large swarms, usually around dusk, and the females fly into the swarms to mate. Males live for about a week, feeding on nectar and other sources of sugar. Females will also feed on sugar sources for energy but usually require a blood meal for the development of eggs. After obtaining a full blood meal, the female will rest for a few days while the blood is digested and eggs are developed. This process depends on temperature but usually takes 2-3 days in tropical conditions (WHO, 2002). During blood digestion, females abdomen undergo series of changes from unfed (tiny abdomen) to blood-fed (red abdomen), then semi-gravid (half red and half whitish) to gravid (whitish); then the female lays eggs and resumes host seeking. This marks the end of a gonotrophic cycle and the beginning of a new one. The cycle repeats itself until the female dies.

2.1.2.2. Ecology of breeding sites

Gravid *Anopheles* lay their eggs in different types of breeding sites depending on the species (Savage, 1990). Most *Anopheles* species prefer clean water and edges of streams, while others thrive in irrigation areas, rice fields, grassy ditches and reservoirs. Some species require extensive vegetative cover for oviposition while others would prefer water bodies with dark or light bottom pools. Others will prefer swamps and other permanent water bodies laden with dissolved organic matter (Mc Crae 1983; 1984; Huang *et al.*, 2005). Many of these sites develop into zones of transmission due to the concomitant increase of human populations moving to these areas. Ecological disturbance as a direct result of human activity may also increase the number of breeding sites. Road construction and maintenance projects often impede drainage of runoff from rainfall. Clogged drainage ditches along roads left by logging and construction activities are ideal places for floodwater mosquitoes. Around the house, objects such as empty cans, discarded tires, potted plants, and similar objects used as a result of human activities are often responsible for the collection of rainwater which allows mosquitoes to breed (Kitron, 1989; Tadei, 1998).

2.1.2.3. Preferred sources of blood meal

One important behavioral factor is the degree to which an *Anopheles* species prefers to feed on humans (anthropophily) or animals such as cattle or pigs (zoophily). Anthropohilic *Anopheles* are more likely to transmit the malaria parasite from one person to another. Most *Anopheles* mosquitoes are not exclusively anthropophilic or zoophilic. The primary malaria vectors in Africa, *A. gambiae* and *A. funestus*, are strongly anthopophilic and consequently, are two of the most efficient malaria vectors in the world (Macdonald, 1957).

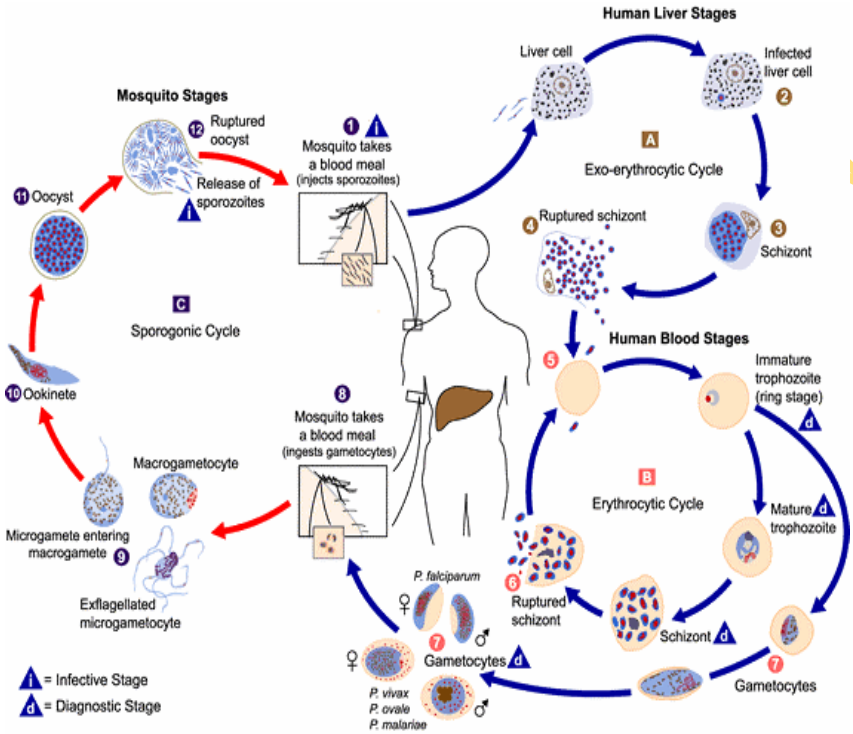

Figure 2.1: **The Life cycle of malaria parasite**

Source: **WHO, 2002**

2.1.2.4. Patterns of feeding and resting

Anopheles mosquitoes are crepuscular (active at dusk or dawn) or nocturnal (active at night). Some *Anopheles* mosquitoes feed indoors (endophagic) while others feed outdoors (exophagic). After blood feeding, some *Anopheles* mosquitoes prefer to rest indoors (endophilic) while others prefer to rest outdoors (exophilic). Biting by nocturnal, endophagic *Anopheles* can be markedly reduced through the use of insecticide treated bed nets (ITNs) or through improved housing construction (e.g. window and door screens) whereas, exophagic vectors are best controlled through breeding sites destruction. Endophagic *Anopheles* have an increase contact with humans and consequently are likely to be able to transmit more cases of malaria (Macdonald, 1957).

2.2 Major vectors of malaria in Sub-saharan Africa

There are several species of malaria vectors in Africa. Two members of these species have been reported to be widely distributed and being able to efficiently transmit the malaria parasite: the *Anopheles gambiae* complex and the *Anopheles funestus* group. Both species belong to a complex comprising of morphologically indistinguishable species (Service, 1993).

2.2.1 The *Anopheles gambiae s.l.* complex

A. gambiae is the principal vector of malaria in tropical Africa. It has the capacity to colonise sunlit, emporary small water bodies that are scattered, around human dwellings (Minakawa, 1999; Gimnig, 2001). The complex was initially considered to be of a single species until much later when it was confirmed using molecular tools to be made up of seven named species: *A. gambiae s.s.* (*sensu stricto*), *A.arabiensis, A. merus, A. melas, A. bwambe,* and *A. quadriannulatus* A and B (Hunt *et al.*, 1998;

Brooke *et al.,* 2002). All members of *A. gambiae* complex are morphologically identical but have few molecular differences. *A. gambiae s.s.* and *A. arabiensis* are most widespread of these groups with *A. arabiensis* broadly distributed in arid regions. Both species occur in sympatry and are can breed in temporary stagnant water often associated with human activities (Coetzee and Fontenille, 2004). Another member of the group, *A. quadriannulatus species* A and *A. quadriannulatus species* B, are known to have a restricted distribution which is limited to South-East Africa and Ethiopia (Fettene and Temu, 2003). *A. melas* and *A. merus* are the salt water species, and their breeding is confined respectively to coastal regions of Africa (Moreno *et al.*, 2004).

2.2.2 The *Anopheles funestus* group

Members of the group are widespread throughout sub-Saharan Africa and Madagascar (Mouchet *et al.,* 1998). Species of this group include *A. funestus s.s.*, *A. parensis*, *A. aruni*, *A. vaneedeni*, and *A. rivulorum*. Of these species, *A. rivulorum* has few morphological features which can be used for identification at the adult stage (Gillies and Coetzee, 1987).

2.3 Identification of *Anopheles*

2.3.1 Morphological identification

The identification of the exact *Anopheles species* responsible for transmission is pertinent to vector control programs. Most mosquitoes belonging to the genus *Anopheles* are identified through dichotomous taxonomic keys with morphological characteristics that are species specific (Gillies and Coetzee, 1987). However, there is a limitation to the use of morphological characteristics in distinguishing related organisms sharing similar morphological features. There are a number of biological species sharing similar morphological features but completely reproductively isolated. These are known as cryptic species, sibling species or isomorphic species such as the members of the *A. gambiae* and *A. funestus* complex (Hunt, 1998). Vector identification has helped to quantify the role of several cryptic species belonging to

major groups in disease transmission (Coetzee and Fontenille, 2004). The occurrence of species complexes is often accompanied by genetic variations. As a result, proper species identification allows appropriate decision making for better control strategies (Weeto, 2004).

2.3.2 Cytogenetic and Molecular techniques of identification of *Anopheles*

2.3.2.1 Cytogenetic technique for *An. gambiae* complex

As *A. gambiae* has tracked humans across temporally and spacial diverse habitats. It appear to have been force to undergo extensive ecological adaptation, which in turn drives population divergence (Bradley, 2010). The first evidence for ecological adaptation of *A. gambiae* came via the examination of chromosomal inversions, which occur when a segment of a chromosome breaks off, flips 180 degrees, and becomes inserted into same position (Hoffmann and Rieseberg, 2008). This event causes gene order within the inversion to be reversed relative to that of an ancestral chromosome. By viewing the characteristic banding pattern and/or loops on these chromosomes under a phase contrast microscope, researchers have not only determined speciation in *Anopheles* but have named five non-Linneaean chromosomal forms: Forest, Savanna, Mopti, Bamako, Bissau (Coluzzi *et al.*, 1985; Toure *et al.*, 1998; Powell *et al.*, 1999; Brooke *et al.*, 2002).

2.3.2.2 Polymerase Chain Reaction (PCR)

The use of PCR created a revolution in diagnostic research by providing new ways of studying parasites, vectors and their hosts (Greenwood, 2002). The technique involves repeated amplification of small fragments of DNA present in a test sample. This involves the use of specific primers designed for specific and conserved regions of the DNA of the different members of species complexes. Therefore, in a single PCR run, the seven members of the *A. gambiae* complex can be distinctly differentiated based on the sizes of each fragment (Scott *et al.*, 1993; Fanello *et al.*, 2002). PCR has a major

advantage because it utilizes DNA which is relatively robust and can be easily transported from field and stored in the laboratory for long periods (Li *et al.*, 1997).

2.3.2.3 Restriction fragment length polymorphism for *An. gambiae s.s.* molecular form

Subsequent analysis of PCR products of members belonging to the *Anopheles gambiae s.s.* by restriction endonucleases (Hha I) has revealed that the species can further be divided into two molecular forms: M and S. This is as a result of the variation in the sequences on the intergenic spacers of ribosomal DNA located on the X chromosome (Favia *et al.*, 1997; Gentile *et al.*, 2002). Recently, the M molecular form has been named *Anopheles colluzzi* while the S molecular form called *Anopheles gambiae s.s.* (Coetzee *et al.*, 2013). Based on the rationale that reproductive isolation would lead to genome-wide heterogeneity within species, there is considerable evidence that these two molecular forms are reproductively isolated (Chanre *et al.*, 1999; della Torre *et al.*, 2001; Wondji *et al.*, 2002). Hybrids between these molecular forms are rare (Tripet *et al.*,2001; Edillo *et al.*, 2002; Onyabe *et al.*, 2003; Awolola *et al.*, 2005a). Gentile *et al.*, (2002) proposed that M and S molecular forms may have mosaic genome consisting of parts completely differentiated between which gene flow is barred, whereas other parts of the genome are free to pass between forms. Although interbreeding between M and S forms yield fertile projeny, M-S hybrids are rarely observed in nature. If correct, these suggest that the genetic response rate to environmental factors in M and S forms would differ considerably as suggested by the presence of *kdr* gene in S form and its absence in M (Awolola *et al.*, 2003), and the circulation of such traits as insecticide resistance may be hindered between the two forms owing to incipient speciation.

2.4 Vector control strategies

Vector control strategies aim at limiting the spread of malaria by reducing the population density of the vector *Anopheles*. Control of mosquitoes may prevent

malaria as well as several other mosquito borne diseases. House screening to prevent the entry of mosquitoes, combined with larval habitat destruction and treatments have led to the elimination of malaria in several North American countries. Most vector control strategies are insecticide driven and focused mainly on: indoor residual spraying, insecticide treated bed-nets and larviciding (WHO, 2012). Other methods including the release of genetically modified *Anopheles* have been proposed with little field application.

2.4.1 Indoor Residual Spray (IRS)

Residual house spray of insecticides is usually termed the most efficient approach to the control of malaria transmission, because the chance of killing an *Anopheline* mosquito is repeated every time the mosquito enters a house to bite and before it reaches the age of transmitting mature sporozoites (Curtis *et al*., 2000). This method involves spraying of the walls and other surfaces of the house with residual insecticide (WHO, 2006). Historically, the best control results have been achieved by IRS (Brooke *et al.*, 2000). IRS with DDT and dieldrin was the primary malaria control method used in South Africa during the global malaria eradication campaign from 1955 to 1969. The campaign did not achieve its stated objective but it did eliminate malaria from several areas and sharply reduced the burden of malaria disease in others (MacDonald, 1957). The negative publicity due to the failure of the malaria eradication campaign, and environmental concerns about residual insecticides accounted negatively for the up scaling of IRS. However, the more recent success of IRS in reducing malaria cases in South Africa by more than 80% has revived interest in this malaria prevention tool and has also reignited the debate over whether or not, DDT should have a place in malaria control (WHO, 2006). More recently, Insecticide treated plastic sheets (ITPS) and Zero Vector Durable Lining (ZVDL) have been proposed to cater for the drawbacks of IRS. Insecticide treated materials are placed directly on walls. Used as a wall covering, ITPS or ZVDL may be likened to long -lasting indoor residual spray (IRS) treatment in which the substrate requires only a single treatment instead of annually and can last for longer periods on surfaces. It can also provide aesthetic than mud or cement plaster.

Reports have shown the potentials of ITPS in providing up to 84.7 percent in the entry rate of total mosquitoes and 56.2 percent immediate mortality and this confirms the feasibility of this intervention in high transmission in difficult areas. There has also been convincing evidence of durable lining in providing protection in insecticide resistance situation (Chandre *et al.*, 2010).

2.4.2 Insecticide – Treated Bednets (ITN)

The concept of ITN is based on impregnating net materials (bednets or curtains) with insecticide solutions mostly pyrethroids. The efficacy of ITNs has been clearly established in malaria control (Dariet *et al.*, 1984). In West Africa, three main factors stimulate the purchase of bednets in communities: the noise made by mosquitoes, their bites and the disease they cause (Akogbeto *et al.*, 2004). In Cote d'Ivore and Cameroon, the evidence that ITN.s function effectively despite the presence of vector populations with high frequency of the knock down resistance gene (*kdr*) that confers resistance to pyrethroids have been demonstrated (Etang *et al.*, 2003).

Nets are made of polyester (Permanet®) but they are also available in cotton, or polyethelene (Olyset®). Currently, only pyrethroid insecticides are approved for use on ITNs (WHO, 2006). These insecticides have very low mammalian toxicity but are highly toxic to insects and have rapid knock-down effect, even at very low doses. Pyrethroids have a relatively high residual effect: they do not rapidly break down unless washed or exposed to sunlight. Previously, nets had to be retreated at intervals of 6-12 months and more frequently if the nets are washed. The need for retreatment, the lack of understanding of the importance of bednets, and the additional cost for insecticides resulted in very low retreatment rates in most African countries and constituted the major barrier to full implementation of ITNs in endemic countries (Binka *et al.*, 1998). This condition has led to the development of long lasting insecticide treated nets (LLINs) (WHO, 2006). More recently, several companies have developed long-lasting insecticide treated nets (LLINs) that retain lethal concentrations of insecticide for at least 3 years (WHO, 2006). LLINs has also recently been modified

specifically for insecticide resistant mosquitoes with proven efficacy in high insecticide resistant populations (Adeogun *et al.*, 2012).

2.4.3 Larval control

The use of larvicides in mosquito control require several prerequisites: the knowledge of laying behaviors of anopheles in the locality, mapping and constant monitoring of breeding sites, and the composition and activity of the larvicide to be used. Larvicides are mostly biological (*Bacillus thuringiensis, Bacillus sphaericus*, larvivoros fish e.t.c). These bacteria release toxins which are ingested by larvae and have cytotoxic activities in the midgut cells of the insect larva. Several trials to combat mosquito larvae with Bacillus have been successful at low scale in Cote d'ivore and India (Becker *et al.*, 1994; Yapabandara *et al.*, 2002) until the development of resistance in *Culex species* which necessitated that larviciding should be part of an integrated control strategy (Nielsen-Leroux *et al.*, 2001).

2.4.3.1 The potential advantages of larviciding

In most settings insecticide treated nets (ITNs) - which include long-lasting insecticidal nets (LLINs) - and indoor residual spraying (IRS) are the most powerful, reliable and practicable tools for malaria vector control; however these two interventions are not perfect, and they cannot serve all vector control purposes in all settings. For example, it has often been observed in Africa that indoor transmission can be greatly reduced by careful indoor residual spraying (IRS) (Kouznetsov, 1977), but outdoor transmission may persist and prevent the complete interruption of transmission. However, it is important to note that major African malaria vectors prefer to rest indoors, where they are exposed to insecticides, even if they sometimes bite outdoors. Larviciding has the potential to overcome this problem, because it is expected to affect indoor and outdoor biting vectors equally. Similarly, larviciding may sometimes have the potential to play a role in insecticide resistance management, although as of yet, there is no direct evidence that such a strategy will work (WHO, 2012). Of the larvicides that are recommended by the WHO Pesticide Evaluation Scheme (WHOPES), the majority

have never been used to kill adult mosquitos and are unaffected by the resistance mechanisms currently spreading through malaria vector populations in Africa. Consequently, larviciding can only potentially play important role in those settings where the procedure is feasible and cost-effective (WHO, 2012).

2.4.4 Genetically Modified *Anopheles* (GMAs)

The *Anopheles* genome sequence provides an architectural scaffold for mapping, identifying, selecting and exploiting desirable insect vector genes. It also promotes understanding of mosquito biochemistry, physiology, and behavior as well as of malaria epidemiology, and spurs development of new public health interventions (Hemingway *et al.*, 2002). Two orientations are given in the development of GMAs: the first consists of developing refractory mosquitoes and the second is to generate and release sterile males with the low outcome of available vector control tools, the TDR and the MacArthur Foundation conveyed a meeting in Tucson, Arizona, in 1991. Here, a small group of scientists proposed the GMAs so that it could no longer harbour or transmit the *Plasmodium* parasite. This revolutionary idea, accepted by the Joint Coordinating Board of TDR, launched the field of molecular entomology of GMAs. The 20 year plan had three principal goals: (i) to develop basic tools for the stable transformation of *Anopheline* mosquitoes by the year 2000; (ii) to engineer a mosquito incapable of carrying the malaria parasite by 2005; and (iii) to run controlled experiments to test how to drive the engineered genotype into wild mosquito populations by 2010. The first goal have already been achieved in *Anopheles*. A strain of *An. stephensis* that is unable to transmit malaria parasite in mice has already been engineered (Tu Zhijian, 2001).

The current big challenges are: driving of refractory genotypes in wild strains, studying the bio-ecology of engineered mosquitoes (Scott *et al.*, 2002) and getting information on the stability of engineered genes (Tu Zhijian, 2001). A full understanding of the oxidative stress of the mosquitoes which appear to be important in refractory strains to resist parasite infections and to drive refractory gene into wild populations of

Anopheles (Hemingway *et al.,* 2002) and getting communities involved in the process. The use of genetically modified insect vectors in the field will require careful consideration of bio-safety, ecological, ethical, legal, and social issues to ensure public acceptance.

2.4.5 Other less Implemented Vector Control Strategies

Other vector control strategies with less implementation in community programs include: (i) Fogging or outdoor spraying which is primarily reserved for emergency situations such as halting epidemics or rapidly reducing adult mosquito populations when they have become sever pests; (ii) the use of repellents such as DEET (Fradlin and Day, 2002), wearing light colored clothes, long pants and long sleeved shirts (NIH-USA, 2009).

2.5 Public Health insecticides and there mode of action

Insecticides are primarily employed in vector control. They act by mainly disturbing the transfer of impulses in the nervous system by either maintaining opened sodium ion channels (leading to tetanization) or inhibiting activities of acetylcholinesterase (leading to paralysis). These insecticides can be grouped under four main families: Organochlorine, Organophosphates, Carbamates and Pyrethroids. Currently a total of 12 insecticides from these families are used in public health against mosquitoes at adult stage: 7 pyrethroids, 3 organophosphate, 1 carbamate and 1 DDT (dichloro diphenyl trichloroethane) (WHOAfro, 2003).

2.5.1 Organochlorine Insecticides

This family of insecticides is divided into three subgroups based on their chemical structure and there mode of action. The main members of the family are: DDT and its analogues, lindane and cyclodiene. DDT was discovered in 1939 by Paul Muler in Switzerland and tested in 1942 as an antimosquito spray in army camps in the United

States and the United Kingdom. In 1944, DDT was tested for the first time in civilian areas at Voluntoro, Italy. The first trial with DDT, as a residual spray against adult mosquitoes in the field, was highly successful (Singh, 1962). In 1950, DDT water dispersible powder containing 50-75% technical grade DDT was made available and its remarkable convenience in application prompted it to be an ultimate choice in anti-malaria campaigns. Its efficacy in agriculture and public health generated a great interest of WHO and led to the launching of malaria eradication program in the 50's (Mouchet, 1994). DDT has a complex chemical structure. Its activity is focused on peripheral and central nervous system of insects (Hassal, 1990). It has a rapid knock down effect on mosquito populations. Despite these high performances, its bioaccumulation in the environment and the appearance of cases of resistance in some regions brought WHO to stop using and even ban it in many countries.

Lindane and cyclodiene are subgroups in the family of organochlorine. A known member of this family is dieldrine. Their activities are focused on the central nervous system where they inhibit chlorine channels, the main receptors of gamma-aminobutyrique acid (GABA). This set of insecticides was also banned because of their bioaccumulation, their toxicity and the emergence of resistance in vectors.

2.5.2 Organophosphate Insecticides

These are derived from phosphoric acid and replaced organochlorine because they are less toxic. The members of this family of insecticides are malathion, fenitrothion. When coupled with oxygen molecules, organophosphates are good inhibitors of acetylcholinesterase. This enzyme degrades activities of acetylcholine which neuromediates cholinergic synapses, located in the central nervous system of insects. The fixation of organophosphates on acetylcholinesterase leads to the accumulation of acetylcholine at the synaptic junction. When the levels of acetylcholine becomes too high, the acetylcholine receptors are blocked. It is this blockage that leads to paralysis and eventual death of insects (Keith, 2005).

2.5.3 Carbamates Insecticides

These compounds are synthetically derived from serine. They act like organophosphates by inhibiting activities of acetycholecterase. The family is made up of carbamate and bendiocarb. These insecticides are derived from carbonic acids. They are less used because of their cost and their toxicity to mammals (Keith, 2005).

2.5.4 Pyrethroids Insecticides

They are synthesized from pyrethrins which are natural extracts from *Chrysanthemum cinerariaefolium* flowers. First generations of pyrethroids were very volatile and therefore less persistent. With advanced works, this instability was overcome and more stable molecules developed (Elliot and James, 1978). Pyrethroids are divided into two groups based on their alpha radicals (group I: permethrin and group II: deltamethrin, lambda-cyhalothrin, cypermethrin). Pyrethroids act on sodium channels in the nervous system by keeping it open which in turn accelerate the speed of nervous impulses. The insect ends up dying by tetanization (Keith, 2005). Pyrethroids have a rapid knock down effect coupled with high excite-repulsive action and are less toxic to mammals at operational doses. These features explain why pyrethroids were quickly welcomed and are the only insecticides currently used in the impregnation of net materials.

2.6 Resistance of *Anopheles* to insecticides

It is conventional in writing about malaria to list insecticide resistance of vectors as one of the important factors interfering with efforts to control the disease. As defined by the W.H.O. as the occurrence in a population of a set of individuals capable of tolerating doses of chemicals which under normal condition would kill the majority of the population (Hamon and Mouchet, 1961). In *Anopheles gambiae* various factors in the environment has been directly linked to the development of insecticide resistance. The spillage of oil products in certain areas of Nigeria and Benin republic have been reported to constitute greatly to the development of resistance to pyrethroids (Rousseau

et al., 2007). Urbanization as an entity has also been linked with insecticide resistance in *Anopheles gambiae* (Oyewole and Awolola, 2006). The toxicity of an insecticide result from interaction between the insecticide and the biological set-up of the mosquito. Various steps are necessary for this to take place: the insecticide must get in contact with the insect, enter the insect, be transformed into a metabolite and carried to the target site for expression. All these steps are governed by either one or several genes of which any structural or functional modification could lead to resistance (Soderlund and Bloomquist, 1990). Modifications can lead to a change in the behavior of the insect by either escaping the contact with the insecticide or reducing its absorption process (behavioral resistance). The second set of mechanism developed by mosquitoes is to elevate excretion and detoxification process (metabolic resistance) and the third method is the modification of the target site of insecticides:

2.6.1 Behavioral mechanism of resistance

The irritant property of some insecticides can cause a proportion of mosquitoes to leave sprayed surfaces before acquiring a lethal dose so that repeated contact is required before mortality occurs. Refractory types of behavior or the evasive habits due to the presence of insecticides are often referred to as "Bavioristic resistance", which means development of the ability to avoid dose which would prove lethal (W.H.O., 1957). Behavioristic resistance is often reserved for populations that have been changed by selection and therefore genetically inherited to produce increase in frequency of avoidance; it is not always applied to populations which show pronounced irritability to evasive habits as their normal reaction to certain insecticides in which case is termed "protective avoidance" (Muirhead-Thomson, 1960). With the publication of mosquito genome, investigations are currently focused on genes responsible for neurosensory perception and chemical detection by the mosquito (Ranson *et al.*, 2002).

2.6.2 Metabolic mechanism of resistance

In metabolic resistance, the pathways of the insect become modified in ways that detoxify the insecticide, or disallow metabolism of the applied compound into its toxic forms. Metabolic resistance to insecticides is mediated by qualitative and quantitative changes in proteins that can often be difficult to define precisely at the biochemical level. Three families of proteins are largely responsible for metabolizing insecticides: the cytochrome-P450s (oxidases), carboxylesterases (esterases) and the glutathione-S-transferases (GST). A recent analysis of the *A. gambiae* genome identified 111 genes putatively encoding P450s, 51 genes encoding esterases and 31 genes for GST (Ranson *et al.*, 2002).

Cytochrome P450s exist in insects in very diverse family. Certain subfamilies of P450s have been widely implicated in the metabolism of insecticides (Feyereisen, 1995). Elevated P450 activities have been widely implicated in resistance to pyrethroids in many species, but the lack of sensitivity of biochemical assays designed to detect increases in P450s in individual insects and the paucity of knowledge on the role of individual P450 enzymes in insecticide metabolism have presented an accurate assessment of this mechanism (Ranson *et al.*, 2002). However, elevated expression of a particular P450 gene has been associated with resistance to pyrethroids in *A. gambiae* from East Africa (Nikou *et al.*, 2003) but preliminary findings need further verification.

The family of carboxylesterases are extensive in insects. This include enzymes like acetycholinesterases which is found at the synaptic junctions and are responsible for degrading acetylcholine. Carboxylesterase proteins do not hydrolyse organophosphates but act by sequestration because of their high affinity with this family of insecticides (Cuany *et al.*, 1993). Insensitive acetylcholine (*Acer-1*) has been reported in malaria vectors from Sri Lanka (Karunaratine, 1999). In West Africa, Djogbenou *et al.* (2008) identified and mapped the distribution of *Acer-1* in *A. gambiae* samples from Benin and Burkina Faso. Elevated frequencies of *Acer-1* mutation are associated with resistance to organophosphate and carbamate (Djogbenou *et al.*, 2008). Depending on the esterases involved, resistance can be specific to a particular insecticide or can

confer broad spectrum resistance to a number of different insecticides (Oakeshott *et al.*, 1999).

Glutathione-S-transferase on the other hand, binds on insecticides and produces less toxic products. The most significant one is DDT-ase which degrades DDT in several *Anopheles* populations (Prapanthadara *et al.*, 1993; 2000). Recently, a glutathione transferase responsible for resistance to DDT in *A. gambiae* have been elucidated (Ranson *et al.*, 2001). In other insects such as *Drosophilia*, glutathione transferase has been implicated in resistance to pyrethroids (Vontas *et al.*, 2001) and to organophosphates (Huang, 1998).

2.6.3 Target site modification (Knock-down mutation)

The term "knock-down" as applied in entomology denotes paralysis in insects whether reversible or not. Target site of insecticides are either receptors or enzymes of the nervous system like acetylcholinesterase, sodium channel and the gamma acetyl-butyric-acid (GABA) receptors. Structural modifications of these targets either reduce binding affinity or change the synthesis of enzymes leading to resistance. Target modifications are powerful mechanisms of resistance in the sense that they lead to cross resistance of all families of insecticides targeting the same pathway. It is associated to point or multiple mutations on nucleotide sequences. Mutations affecting sodium channels and GABA receptors have been identified in various species of mosquitoes (Coustau and French-Constant, 1995; Martinez-Torres *et al.*, 1998). Once insecticide resistance is developed, the genes can persist in the insect population for 30 years or more but at low levels.

The knock down resistance (*kdr*) is a target site modification generated by a mutation in the voltage-gated sodium channel of the insect's nervous system. This target is similar for both DDT and pyrethroid insecticides. This resistance mechanism has evolved at least twice in *A. gambiae* (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000) and is now present at very high levels in some regions of Africa (Akogbeto and Yakoubou, 1999; Chandre *et al.*, 1999). With the *kdr bearing A.gambiae* collected

from West Africa, the point mutation on the sodium channel leads to a different amino acid synthesis: leucine is replaced by phenyl alanine (Leu-Phe). In East Africa, the same *kdr* mutation leads to the replacement of leucine by serine (Leu-Ser). The *kdr* gene has also been detected in *A. sacharovi* (Luleyap *et al.*, 2002) and *A. stephensi* (Enayati *et al.*, 2003). Once identified, the mutation can be detected using Polymerase Chain Reaction (PCR) technique (Martinez-Torres *et al.*, 1998). The knock down resistance mechanism has evolved at least twice in *A. gambiae* (Martinez-Torres *et al.*, 1998); Ranson *et al.*, 2000) and is now present at very high levels in some regions of Africa (Akogbeto and Yakoubou, 1999; Chandre *et al.*, 1999; Adasi and Hemingway, 2008).

2.7 Determination of Insecticide Resistance in *Anopheles* (Susceptibility test)

The detection of insecticide resistance in Anopheles populations is highly important for health policies and decision making in the type of vector control strategy to be implemented in a given locality. This detection provides information on the susceptibility to insecticides of mosquito populations and the potential mechanisms of insecticide resistance involved. Four tools are routinely used for basic detection of resistance in field Anopheles populations: the "WHO susceptibility kits" in tubes with adult mosquitoes, the "bottle tests" with synergists, biochemical assays to determine elevated enzyme activities related to resistance, and polymerase chain reaction (PCR) for detection of target sites modification in the mosquito.

2.7.1 Bioassays for determining insecticide resistance in *Anopheles*

The WHO test kits for insecticide susceptibility tests are used. This is generally composed of papers impregnated with technical grade insecticides at discriminating concentrations (Fig. 2). Females of *Anopheles* are exposed to different impregnated papers for one hour and the mortality recorded after 24hours monitoring in the

insectary (WHO, 1986; WHO, 1998; WHO, 2013). This assay segregates resistant and susceptible phenotypes and allows the characterization of *Anopheles* populations as resistant and susceptible. The validation of results from this bioassay depends immensely on the total number of exposed mosquitoes which should be about 100 (WHO, 1986). The main difficulty in this diagnostic technique is getting enough Anopheles from the same locality all aged between 2-5days.

2.7.1.1 Procedure for measuring susceptibility to insecticides in adult mosquitoes: the WHO bioassay test

Six sheets of clean white paper (12 x 15 cm), rolled into a cylinder shape, are inserted into six holding tubes (one per tube) and fastened into position with a steel spring-wire clip. The tubes are attached to slides. At least 120–150 active female mosquitoes are aspirated (in batches) from a 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 is closed and the holding tubes set in an upright position for one hour. At the end of this time, any damaged insects are removed.

Six exposure tubes are prepared in much the same way. Each of the 4 reddotted exposure tubes are lined with a sheet of insecticide-impregnated paper, while the 2 yellow-dotted control exposure tubes are lined with oil-impregnated papers; each is fastened into position with a copper spring-wire clip. The empty exposure tubes are attached to the vacant position on the slides and with the slide unit open the mosquitoes are blown gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, the slide unit is closed and the holding tubes can be detached and set to one side.

Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (60 minutes). At the end of the 1-hour exposure period, the mosquitoes are transferred back to the holding tubes. The exposure tubes are 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 are maintained in the holding tubes for 24 hours (the recovery period). During this time, it

is important to keep the holding tubes in a shady, sheltered place free from extremes of temperature (an insectary is ideal). Temperature and humidity should be recorded during the recovery period.

At the end of recovery period (i.e. 24 hours post-exposure), the number of dead mosquitoes is counted and recorded. An adult mosquito is considered to be alive if it is able to fly, regardless of the number of legs remaining. Any knocked down mosquitoes, whether or not they have lost legs or wings, are considered moribund and are counted as dead. On completion of the susceptibility test, mosquitoes may be transferred to individual, clearly labelled Eppendorf tubes (separating dead and live mosquitoes into separate tubes) for storage until such time that they can be transferred to suitable facilities for species identification and supplementary testing if necessary.

2.7.1.2 Discriminating concentrations

The concept of discriminating or diagnostic concentrations (or dosages) is now well established and has been widely adopted for the purposes of monitoring insecticide resistance in mosquitoes and other disease vectors (WHO, 1998; Oduola *et al.*, 2010). Discriminating concentrations have been established under standardized laboratory conditions for all insecticides currently used in malaria control programmes (Fig. 2.2). Discriminating concentrations for a range of pyrethroid insecticides were included for the first time in the 1998 guidelines, having been the subject of a multi-centre study involving nine institutes (WHO, 1998). The anopheline species used in this study were *An. aconites*, *An. albimanus*, *An. arabiensis*, *An. dirus*, *An. freeborni*, *An. gambiae s.s*, *An. maculatus*, *An. minimus* and *An. stephensi*. Since then, discriminating concentrations have been established for a further four insecticides, although as yet these are tentative pending confirmation by WHO's Pesticide Evaluation Scheme (WHOPES).

Papers already impregnated with insecticide at the appropriate diagnostic concentrations are provided as part of the test kits supplied (Table 2.1). In order to be certain that all susceptible mosquitoes are killed, WHO has traditionally defined its discriminating concentrations in one of two ways, that is, as either: twice the lowest concentration that gave systematically 100% mortality after 60 minutes exposure and a

holding period of 24 hours on a susceptible strain or a susceptible population; or twice the $LC_{99.9}$ value as determined by baseline susceptibility testing of a susceptible strain or a susceptible population.

2.7.1.3 Recording and reporting susceptibility test results

The mortality of test sample is calculated by summing the number of dead mosquitoes across all four exposure replicates and expressing this as a percentage of the total number of exposed mosquitoes according to WHO 2013 criteria:

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

A similar calculation should be made in order to obtain a value for the control mortality. If the control mortality is above 20%, the tests must be discarded. When control mortality is greater than 5% but less than 20%, then the observed mortality has to be corrected using Abbots formula, as follows:

$$\frac{(\%\ \text{observed mortality} - \%\ \text{control mortality})}{(100 - \%\ \text{control mortality})} \times 100$$

If the control mortality is below 5%, it can be ignored and no correction is necessary.

Pyrethroids and DDT are fast-acting insecticides which have a knock-down effect. When knock-down resistance (*kdr*) is involved, the rate of knock down (KD) has been shown to be a sensitive indicator for early detection of resistance. Observations of the number of knocked-down mosquitoes are made during the hour-long exposure period. A mosquito is considered knocked down if it is unable to stand or fly in a coordinated way; it will usually fall to the bottom of the exposure tube. It is recommended that observations are made at regular intervals, usually after 10, 15, 20, 30, 40, 50 and 60

minutes into the exposure period, with the last observation just before transfer to the observation tube. If, after 60 minutes, the observed KD rate is less than 80%, another count at 80 minutes should be made of the mosquitoes in the observation tube. The holding container may be tapped a few times before this final determination is made. In very susceptible populations, the recording of knock down should be done more frequently, every 3 minutes.

From the observed KD counts, it is possible to calculate knock-down rates for 50%, as well as 95%, of mosquitoes (KD50 and KD95, respectively), either graphically using log-probit paper or by computer using a log time-probit statistical model. Although the calculation of KD50 and KD95 values is a relatively simple procedure, these measures are not widely used for routine monitoring of susceptibility for operational purposes.

2.7.1.4 Interpretation of susceptibility test results

In light of new knowledge and the need for prompt action to counter the spread of resistance among vector populations, guidance on interpreting the results of the WHO bioassay has been revised. In the current recommendations (WHO, 2013), a mortality in the range 98–100% indicates susceptibility, a mortality of less than 98% is suggestive of the existence of resistance and further investigation is needed. If the observed mortality (corrected if necessary) is between 90% and 97%, the presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests with the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions) and/ or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98%, then resistance is confirmed. If mortality is less than 90%, confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary, as long as a minimum of 100 mosquitoes of EACH species was tested. However, further investigation of the mechanisms and distribution of resistance should be undertaken.

When resistance is confirmed, pre-emptive action must be taken to manage insecticide resistance and to ensure that the effectiveness of insecticides used for malaria vector control is preserved.

Insecticide class	Insecticide	Discriminating concentration (1-hour exposure period)
Organochlorines	DDT	4%
	Dieldrin[a]	4%0.4%
		4%
Organophosphates	Malathion	5%
	Fenitrothion[b]	1%
	Pirimiphos methyl[c,d]	0.25%
Carbamates	Propoxur	0.1%
	Bendiocarb	0.1%
	Carbosulfan[c,e]	0.4%
Pyrethroids	Permethrin	0.75%
	Deltamethrin	0.05%
	Lambda-cyhalothrin	0.05%
	Cyfluthrin	0.15%
	Etofenprox	0.5%
Pyrroles	Chlorfenapyr[c,f]	5%
Phenyl pyrazoles	Fipronil[c,g]	2%

Table 2.1: Discriminating concentrations of insecticides for adult Anopheline mosquitoes

Source: WHO, 2013

2.7.2 The bottle tests with synergists

The bottle bioassay described by Allister and Brogdon (1999) can be used to assess the biochemical mechanisms of resistance development for mosquito populations collected in the field. The technique is based on coating of bottles. Once resistance is detected, another set of coated bottle prepared using 2 synergists: Piperonyl butoxide (PBO) and S.S.S-tributylphosphorotrithioate (DEF). PBO is used for detecting the presence of elevated oxidases activities in the mosquitoes whereas DEF is for esterases (Allister and Brogdon, 1999).

2.7.3 Polymerase Chain Reaction (PCR) for target site modification

PCR analysis provides insight information on the sequence arrangements, the presence or absence of specific nucleotides in the DNA of the field collected mosquitoes. This sequences arrangement profile is used for molecular characterization of resistant genes in sampled *Anopheles* populations. The most common PCR for target modification is the PCR *kdr* used in knock down resistance. The technique is based on detection of single nucleotide polymorphism following DNA extractions and using appropriate primers (Martinez-Torres *et al.*, 1998). This PCR allows determination of various resistant alleles (RR, RS, SS) and their respective frequencies in mosquito populations could be inferred. The acetylcholinesterase target site mutation (*Ace-1*) known to confer carbamate and organophosphate resistance could also be screened in the field populations of *A. gambiae* using PCR protocols described by Weill *et al.*, (2004).

2.8 Reported Cases of Resistance in *Anopheles gambiae* to insecticides in Nigeria

Few studies have been conducted on vector resistance to insecticides in Nigeria. Awolola *et al.*, (2003) studied the resistance of *A. gambiae* to insecticides in Lagos, Nigeria. The study identified the presence of resistance in some *Anopheles* populations and established the presence of M and S molecular forms of existing as single or in sympatry in some localities in Nigeria. Mojca *et al.*, (2003) also reported on the low

presence of *kdr* mutation in *Anopheles* populations from Ogun State in the South-western Nigeria. Awolola *et al.*, (2005a) investigated the distribution of the molecular forms M and S of *A. gambiae* and the *kdr* gene associated with pyrethroid and DDT resistance in *A. gambiae s.s.* at 13 localities across Nigeria. The report showed that the overall collection was a mix of the molecular M and S forms across the mangrove (63:37%), forest (56: 44%), and transitional (36: 64%) ecotypes, but almost a pure collection of the S form in the Guinea and Sudan-savanna. Results of insecticide resistance tests showed that mosquitoes sampled at seven localities were susceptible to permethrin, deltamethrin, and DDT, but populations of *A. gambiae* resistant to these insecticides were recorded at six other localities mainly in the transitional and Guinea-savannah ecotypes. The *kdr* gene was found only in the molecular S forms, including areas where both forms were sympatric. The overall *kdr* frequency was low: <47% in forest, 37-48% in the translational, and 45-53% in the Guinea-savanna. More recently, resistance to pyrethroid has also been reported by Rousseau *et al.*, (2007) with strong link to the impact of spilled petroleum products from South- western Nigeria. Oduola *et al*., (2010), also detected high resistance to DDT from rural, semi urban and urban communities in Nigeria.

2.9 The spread of insecticide resistance genes in populations

Resistance of operational importance will eventually emerge to any insecticide that continues to be widely used. Insecticide resistance genes have clearly been spreading and will spread further, particularly in the face of continuing selection pressure (Brooke *et al.*, 2000). Most cases of resistance in the field are attributable to a few genes of major effect. Therefore the spread of resistance throughout mosquito populations requires understanding of the evolution of those genes. A resistance gene starts as a rare gene, but, with further exposure to the same insecticide, the frequency of the gene increases until it becomes common in a population (Fig. 2.2) (WHO, 2012). Other factors being equal, resistance is likely to evolve more quickly if it is functionally dominant in the field exposures. It is also likely to evolve more quickly in

isolated (e.g. on islands) and uniformly exposed vector populations because there is less dilution from susceptible inward migrating vector populations (WHO, 2012).

2.10 Mapping of Insecticide resistance genes and genetic studies of *A. gambiae*

A genetic map of *An. gambiae* is currently available for genetic studies (Zheng *et al.*, 1996) (Fig. 2.3). While efforts are ongoing to study the population genetics of *A. gambiae*, the genes conferring resistance to Permethrin and DDT have been mapped and presented (Ranson *et al.*, 2000, 2004). Genes conferring resistance to DDT has been mapped to chromosome 2 and 3, and tagged *rtd2* and *rtd1* respectively (Fig. 2.4) (Ranson *et al.*, 2000) while those conferring resistance to pyrethroid insecticides were named *rtp1* mapped to chromosome 2, and *rtp2* and *rtp3* mapped to chromosome 3 respectively (Ranson *et al.*, 2004) (Fig. 2.5). Further work still needs to be conducted on the population genetics of *A. gambiae* and resistance mechanisms especially in Nigeria where there are a handful of reports on insecticide resistance and their associated mechanisms.

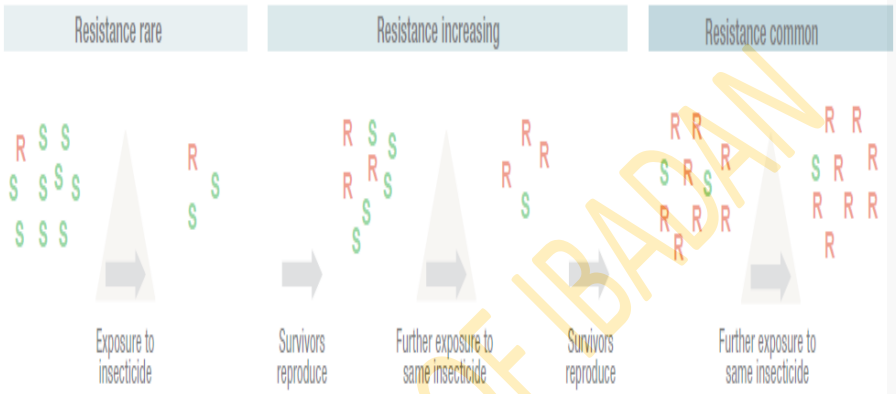

Figure 2.2: **Genetic heritability drives increased resistance in the face of continued pressure on mosquito populations**

Source: **WHO, 2012**

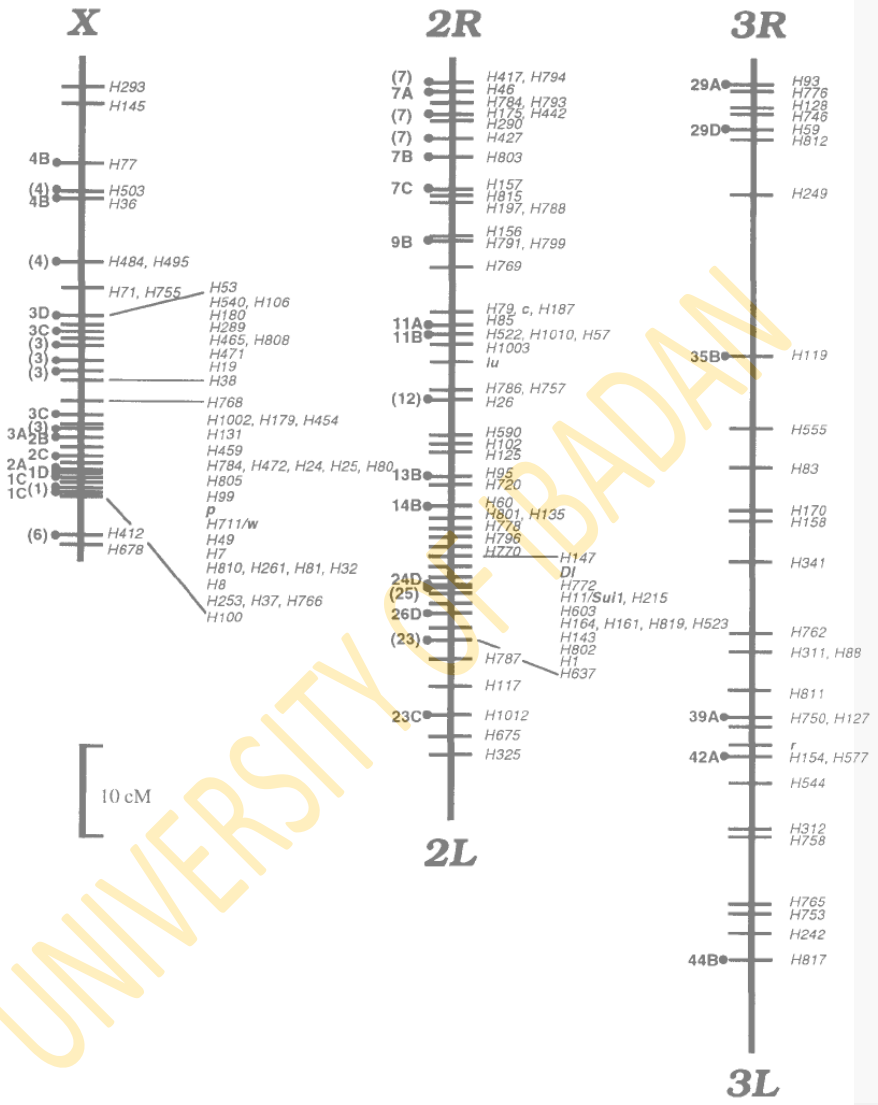

Figure 2.3: **Genetic map of *Anopheles gambiae***

Source: **Zheng *et al.*, 1996**

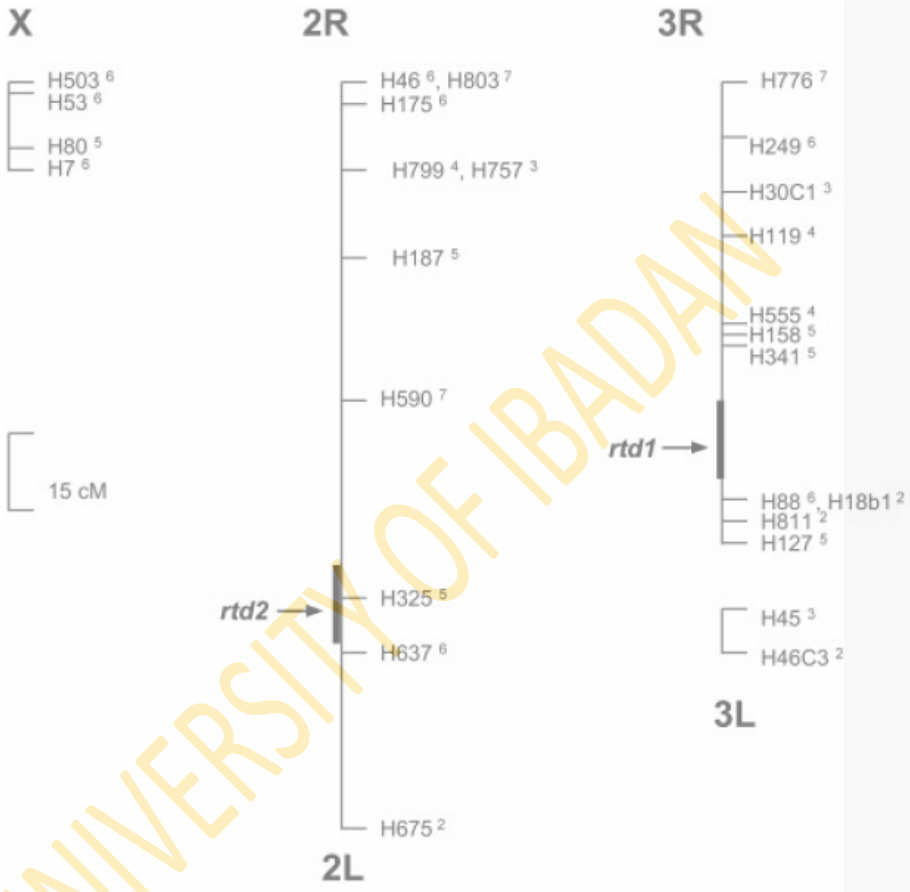

Figure 2.4: **Genes conferring resistance to DDT in *Anopheles***

Source: **Ranson *et. al.*, 2000**

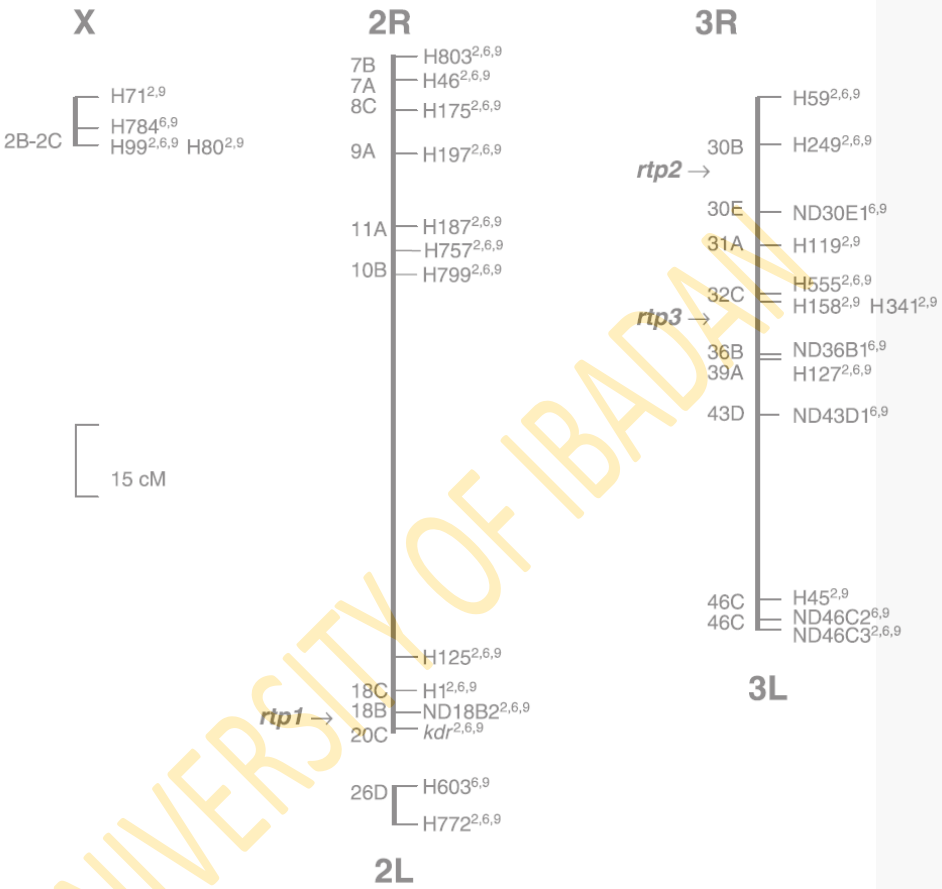

Figure 2.5: **Genes conferring resistance to Pyrethroids in *Anopheles***

Source: **Ranson *et. al.*, 2004**

2.10.1 Importance of inversion 2La in *A. gambiae* population genetics

The impressive geographic and seasonal distribution of *A. gambiae* is hypothesized to originate in local adaptations facilitated by inversion polymorphisms (della Torre *et al.*, 2002). Frequencies of alternate arrangements, especially involving inversions on chromosome 2, were shown to correlate with ecological/ climatic factors such as the degree of aridity of the environment, suggesting an adaptive potential of inversions, different combinations favoring survival under a variety of environmental conditions (Coluzzi *et al.*, 1979; Coluzzi, 1992; Toure *et al.*, 1994; Wondji *et al.*, 2002). *Anopheles gambiae* also presents clines in inversion frequencies, as has been repeatedly observed along transects ranging from equatorial forests in southern Nigeria and Cameroon to arid savannahs in the north (Coluzzi *et al.*, 1979; Simard *et al.*, 2009). It is hypothesized that these inversions are also associated with specific phenotypes that are under differential selection, maintaining the inversion clines and ultimately permitting range expansion of the vector mosquito (Rocca *et al.*, 2009).

On the left arm of chromosome 2 and subsuming roughly one half of its length, inversion 2La is a critical component to the ongoing speciation and ecological differentiation in this medically important species. Recent cytologic and molecular studies of 2La, long considered the derived arrangement relative to an arbitrary standard, leave little doubt that 2La is the ancestral arrangement from which $2La^{+}$ arose (Sharakhov *et al.*, 2006). However, *A. gambiae* remains highly polymorphic for the two arrangements, although they are non-randomly distributed temporally and spatially with respect to degree of humidity East and West Africa (Powell *et al.*, 1999). The 2La arrangement is reported to be absent in southern Nigeria and southern Cameroon and increases progressively to reach fixation in the north if these countries (Coluzzi *et al.*, 1979; Simard *et al.*, 2009).

Apart from studies of 2La with degree of aridity, the inversion has been recently linked to insecticide resistance and adaptation in *A. gambiae* (Brooke *et al.*, 2002; White *et al.*, 2007) making this inversion central to population genetic studies. Moreover, progress at understanding this phenomenon more deeply at the genetic and molecular level has been stalled for lack of key tools (White *et al.*, 2007). A significant remaining

barrier to studying inversions in *A. gambiae* is the requirement for karyotyped specimens: those whose chromosomal banding pattern has been read from polytene chromosomes by a skilled cytogeneticist with the aid of a microscope. Polytene chromosomes favorable for interpretation of the banding pattern are limited to one tissue and developmental stage of one sex: the large nurse cells within the ovaries of half-gravid females. Such a constraint increases the time, effort, and expense needed for fresh sample collection while precluding the use of any previous collections that were inadequately preserved for cytogenetics, of the wrong sex or the incorrect developmental stage. This makes karyotype analysis labour intensive and requires uncommon expertise.

The recent molecular cloning and sequence characterization of the 2La breakpoints delimited this rearrangement with a high degree of precision relative to previous cytogenetic estimates (Sharakhov *et al.,* 2006). Importantly, these data also provide the basis for a DNA- based strategy to determine the 2La karyotype of both sexes and all developmental stages, overcoming the major limitations to traditional karyotype analysis. A major molecular karyotyping of this inversion 2La has been reported (White *et al.,* 2007) and is currently used in molecular studies of 2La inversion polymorphism. In this report, expected product sizes for the 2La and $2La^{+}$ arrangements were 492 and 207bp respectively. If one of the 492bp or 207bp band appear entirely in the gel electrophoresis process, the species is homozygous for the allele. If the two bands appear during electrophoresis, the species is heterozygous (2La/2La+) for the allele. However, anomalies has been reported in this technique, a 687bp fragment has been reported (Ng'habi *et al.,* 2008) to appear alongside the reported sequences.

2.10.2 Microsatellite analysis of *Anopheles* population

Genetic analysis of natural populations has allowed biologists to ask a wide variety of questions which previously could only be answered by extensive observation of the group in question. Understanding mutational processes is essential before relationships

between observed variation and genetic distance or population substructure can be inferred. A number of genetic markers have proven to be useful. These include mitochondrial DNA, Major Histocompatibility Complex loci, allozyme loci, and Variable number of Tandem Repeats (VNTR) markers. VNTR are characterized by core sequences which consist of a number of identical repeated sequences. They can be divided into categories based on the repeat length. These are minisatellites, 15-70 base pairs (bp), and microsatellites, 2-6bp. Recently, microsatellites have been increasingly used as the marker of choice (Edwards *et al.,* 1992; Lanzaro *et al.,* 1998; Balloux and Lugon-Moulin, 2002; Onyabe and Conn, 2001; Norris *et al.,* 2001). They are originally utilized for genetic mapping (Weissenbach *et al.,* 1992) and have been useful in the analysis of both individual and population structure (Edwards *et al.,* 1992; Norris *et al.,* 2001).

Microsatellites have been estimated to mutate at rate between 10^3 and 10^5 mutations per gamete (Edwards *et al.,* 1992; Bowcock *et al.,* 1994; Forbes *et al.,* 1995). However, the mechanisms by which microsatellites mutate are poorly understood. Two main mechanisms have been proposed, which may act in concert; 1) unequal crossing over in meiosis and 2) strand – slippage replication (Levinson and Gutman, 1987). Of these, strand – slippage replication appears to be the predominant mode at microsatellites (Wolf *et al.,* 1989) which is speculated to occur primarily during lagging strand synthesis (Schlotterer and Tautz, 1992).

Whatever the mutation process, there does appear to be some biases in the mutation rate. An *in votro* study has found evidence that repeat length and base composition affect the mutation rate, i.e. dinucleotide repeats mutate faster than tri's, and sequences with high AT content mutate faster than those with a GC content (Schlotterer and Tautz, 1992). Most polymorphism is ascribed to allelic length variation, which is a difference in the number of repeat units between alleles, and proves most informative in studies of population structure in *An. gambiae* (Lanzaro *et al.,* 1995; Lehmann *et al.,* 1996; Donnelly and Townson 2000). Even though there may be bias towards an increase in repeat length, it is clear from empirical data that there is a size limitation on the number of repeats (Bowcock *et al.,* 1994). For instance, of the 383 CA

microsatellite repeats found in humans, only 45 had over 20 repeats (Valdes *et al.*, 1993). However, the mechanism for limiting the number of repeats is still not known (Fu *et al.*, 1991).

A large number of microsatellite loci from *An. gambiae* have been identified and used to develop an integrated genetic map of *An. gambiae* (Zheng *et al*., 1993; Zheng *et al*., 1996) which are mainly found in the non-coding regions. Recent examination of genetic structuring within *A. gambiae* populations in West Africa using microsatellite DNA analysis have revealed that gene flow varies among regions of the mosquito genome (Lanzaro *et al.*, 1998; Walton *et al.*, 1998). A study using microsatellite loci throughout the genome showed low levels of gene flow between *An. gambiae* and *An. arabiensis* (Besansky *et al.*, 2003). These results were similar to those based on observed frequencies of hybrid karyotypes in natural populations (Lanzaro *et al.*, 1998). Gene flow has been strongly correlated with distances ranging from 62 to 536km using microsatellite loci in *An. gambiae* from Mali, with no major differences among chromosomes. The genetic differentiated microsatellite loci corresponded with traditional models of isolation by distance (Carnahan *et al.*, 2002). Lehmann *et al.*, (1996, 1997) found no differences in microsatellite frequencies between populations of the Savanna chromosomal form in *An. gambiae* on the east and west coasts of Africa, thousands of kilometers apart. However, Wang *et al.*, (2001) have measured the genetic differentiation between *An. gambiae* and *An. arabiensis*, as well as between the M and S form of *An. gambiae* using 25 microsatellite loci. They found significant differences between *An. gambiae* and *An. arabiensis* from X-linked chromosomal loci within the *Xag* inversion, as well as between M and S forms at two loci from the proximal region of the X chromosome, outside the *Xag* inversion but not at most autosomal loci. Lehmann *et al.*, (2003) also found significant divergence at one locus located on the X chromosome near the centromere between allopatric populations of M from Ghana and S from Gabon, as well as between sympatric M and S populations from Mali and the Democratic Republic of Congo. These data support the proposals that the two molecular forms of *An. gambiae* represent genetically different entities.

In Nigeria, the magnitude of gene flow across ecological zones is unclear from Coluzzi *et al.* (1979). However, chromosome inversions may be poor indicators of gene flow because they are not selectively neutral. The distribution of inversions across Nigeria suggests that gene flow is restricted by geographical distance, that is, isolation by distance as the largest disparities in inversion frequencies were between the extremes of the country (Coluzzi *et al.*, 1979). Thus, parts of the genome that are located within inversions, especially on chromosome II, might be expected to measure higher levels of differentiation than those that are located outside inversions (Lanzaro *et al.*, 1998; Black and Lanzaro, 2001). Few studies using microsatellite as a tool to analyze population structure have been conducted in Nigeria. Onyabe and Conn, (2001) investigated gene flow from eight localities across Nigeria using 10 microsatellite loci. They reported extensive gene flow across the country but three loci located within inversions on chromosome II counters the homogenizing effect of gene flow. Onyabe and Conn, (2003) also reported selection as a major factor shaping genetic differentiation of *A. gambiae* across Nigeria. This indicates that microsatellite markers on chromosome II, may provide information on population structure of *A. gambiae* in Nigeria.

2.10.2.1 Analysis of genetic differentiation in microsatellite loci examinations

Three common mutation models are used to describe the nature of mutation at microsatellite loci:

1. Infinite Allele Model (IAM): Kimura and Crow (1964) developed this model to describe mutation at microsatellite loci. Under the IAM, a mutation involves any number of tandem repeats and always results in an allele state not previously encountered in the population.

2. Stepwise Mutation Model (SMM): This model developed by Ohta and Kimura (1973) describes mutation of microsatellite alleles by the loss or gain of a single tandem repeat, and hence alleles may possibly mutate towards alleles states already present in the population.

3. Two Phase Model (TPM): DiRienzo *et al.,* (1994) introduced this model, where mutations introduce a gain or loss of X repeats. It assumed that whilst most mutations involve a single repeat unit, mutations of two or more repeats units also occur.

It is worth noting that it seems rather difficult to reconcile empirical data to any of the existing models. Neither of the mutation models proposed by population geneticists (IAM, SMM, TPM) appeared to perfectly account for the observed patterns of microsatellite mutations. There mutation pattern probably lies somewhere between two extreme models (IAM or SMM) (Balloux and Lugon-Moulin, 2002). Most statistics that describe genetic differentiation from genetic markers rely solely on allele identity information. Hence, the difference in size between two alleles might be informative: the larger the difference, the higher the number of mutation events (thus time lapse) is expected to have occurred since common ancestry. There is thus a "memory" of past mutation events (Hardy *et al.,* 2003). F_{ST} and R_{ST} are often used in interpreting microsatellite data.

Several definitions can be given for F_{ST}. Originally, a fixation index was developed by Wright (1921) to account for the effect of inbreeding within samples. He defined this quantity in terms of correlation coefficient. Later Wright (1951) expanded this concept to a population subdivided to a set of sub-populations, leading to traditional hierarchical F-statistics, F_{IS}, F_{ST}, F_{IT} (where I stands for individuals, S for subpopulations and T for the total population). He defined F_{ST} as the correlation between two alleles chosen at random within subpopulations and relative to alleles sampled at random from the total population (Wright 1951, 1965). For the interpretation of F_{ST}, it has been suggested that a value lying in the range 0 - 0.05 indicates little genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation; and values above 0.25, very great genetic differentiation (Wright, 1978; Hart and Clark, 1997). The main problem affecting F-statistics when working with microsatellites is their sensitivity to mutation rates. It should also be noted that F_{ST} values could be deflated in the presence of high mutation rates (Hardy *et al.,* 2003).

Alternative solutions to the danger of using F_{ST} in statistical analysis with high mutation rates have been proposed using statistics accounting for allele size information, such as R-statistics (Slatkin, 1995; Rousset, 1996). Conversely, under a strict SMM, R_{ST} is independent of mutation rate. Indeed, R_{ST} is an analog of F_{ST} based on allele size differences; it is a parameter defined as the correlation of allele sizes (rather than allele state) between genes sampled within populations or equivalent, $R_{ST} \equiv (S_b - S_w)/S_b$, where S_w (S_b) is the mean square difference in allele size for two genes from same population (different populations; Excoffier, 2001, a definition slightly different from Slatkin, 1995). R_{ST} will be deflated when the mutation pattern includes mutations involving more than one repeat when the number of possible allelic states is finite (Slatkin, 1995). R_{ST} is nevertheless expected to give, on average, more accurate differentiation estimates than F_{ST} as long as there if some memory in the population. If the value of $R_{ST} > F_{ST}$ in a population, then there is a contribution of SMM to genetic differentiation but if $R_{ST} = F_{ST}$, then there is no contribution of SMM to genetic differentiation (Hardy *et al.,* 2003). Hence, R_{ST} appears to be a better predictor of interspecific divergence, that is, it better detects longer historical separations than F_{ST}. On the other hand, F_{ST}, appeared to be more sensitive to detect intraspecific differentiation (Forbes *et al.*, 1995; Lugon-Moulin *et al.*,1999; Balloux and Lugon-Moulin, 2002.

2.11 Operational impact of insecticide resistance

Experts agree that if nothing is done to reduce selection pressure, insecticide resistance will ultimately have an operational impact that will lead to widespread control failure (WHO, 2012). While the high frequency of *kdr* resistance, notably in West Africa, has not been accompanied by an obvious attributable increase in the number of malaria cases, several reports indicate that resistance could have an operational impact and lead to control failure (Ranson *et al.*, 2011; WHO, 2012). For instance, a national decision was made in South Africa in 1996, to change from DDT to pyrethroid for IRS. By 2000, however, the number of reported malaria cases had multiplied by approximately four. *An. funestus*, a vector that had been eliminated by DDT spraying in the 1950s,

reappeared, and bioassays showed that the species was susceptible to DDT but resistant to pyrethroids and furthermore has a sporozoite rate of 5.4% (Hargreaves *et al.*, 2000) which is remarkable high by South African standards.

Another example is the case of Benin, where several small trials were conducted to test the efficacy of IRS and LLINs against resistant vectors (N'Guessan *et al.*, 2007; 2010). In one trial, IRS and LLINs were tested at two sites, one with *kdr* resistance to pyrethroids (Ladji) and one with susceptibility (Melanville). Holes were made in the nets to mimic worn nets. In the huts at the site with resistance (Ladji), the efficacy of the insecticide appeared to be significantly reduced: vector mortality was lower and the level of blood feeding was the same as in huts with untreated nets. However, it is suspected that metabolic resistance was also present at Ladji as results from a similar experimental hut trial in northern Benin with *kdr*-resistant mosquitoes did not show a significant effect (WHO, 2012).

CHAPTER THREE

MATERIALS AND METHOD

3.1. Description of sampling sites

Mosquito samples were collected from six localities each in Lagos (Lekki, Ajah, Badagry, Yaba, Ikorodu, and Magodo) (Fig. 3.1) and Oyo State (Oluyole, Eruwa, Oyo, Ojoo, Bodija and Ogbomoso) (Fig. 3.2). The localities within Lagos and Oyo states were selected based on information available on the presence of insecticide resistant species of *Anopheles* (Rousseau *et al.*, 2007; Oduola *et al.*, 2010) and a geographical distance of about 50km between localities within Lagos and Oyo State, within which substantive genetic differentiation has not been reported between *Anopheles* populations. However, Lagos and Oyo States are about 120km apart, a geographical distance in which genetic differentiation has been reported between *Anopheles* populations (Carnahan *et al.*, 2002). Therefore the data obtained from Lekki, Ajah, Badagry, Yaba, Ikorodu, and Magodo were pooled to represent Lagos State while data from Oluyole, Eruwa, Oyo, Bodija and Ogbomoso were also pooled to represent Oyo State.

3.2 Collection of mosquitoes and determination of larval preference of *Anopheles* in the study localities

Standing water points found in each of the selected localities were systematically scrutinized for mosquito larvae (Plates 3.1 and 3.2). Sites with breeding water containing *Anopheles* and/ or Culicines were considered as breeding habitats of mosquitoes. Following a standard protocol (Service, 1971), larval samples were collected by lowering white dippers gently into identified breeding sites at an angle of 45^{o} to the surface until one side is just below the surface. These were moved along the breeding site, skimming the surface of the water with the dipper and raised out of the

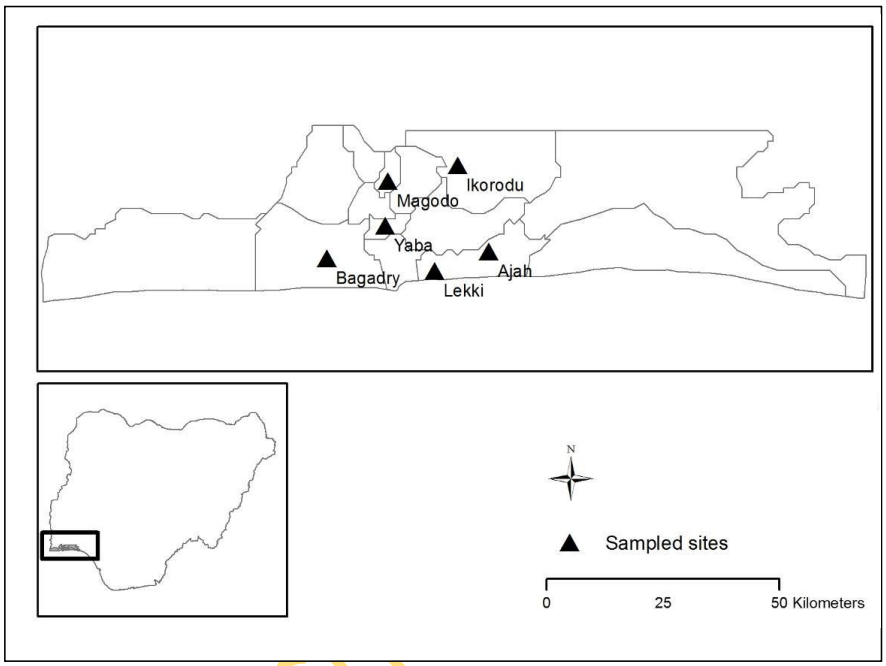

Figure 3.1: Mosquito sampling sites in Lagos State, Nigeria

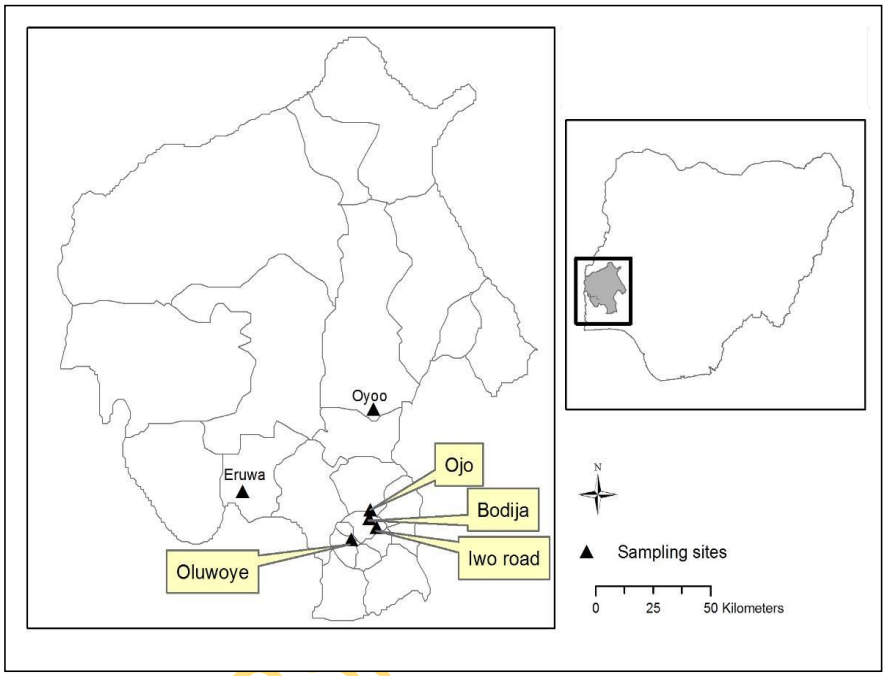

Figure 3.2: Mosquito sampling sites in Oyo State, Nigeria

Plate 3.1: Collection of *Anopheles* mosquito larva at Lekki, Lagos State

Plate 3.2: Collection of *Anopheles* mosquito larva at Eruwa, Oyo State

water, ensuring that the water containing the larvae and pupa did not spill. In each breeding site screened per locality, larvae of all available instars, or pupae, or both were collected from footprints, ponds, pool, puddle, tire track and tanks within a radius of 1km. Samples were collected from at least 8 habitats per locality but sample size per habitat was not determined and collections from all habitats within each locality was pooled. Each locality sampled was mapped with Garmin GPS eTrexLegend personal navigator. The larval and pupal samples were transferred into collection bottles, properly labelled per locality and taken to the Molecular Entomology and Vector Control Research laboratory at the Nigerian Institute of Medical Research, Yaba, Lagos. Larval samples were maintained at a temperature between 27-29^{o}C and humidity 70-80%, with a 12hr day/night cycle (Das *et al.*, 2007). Emerged adults were fed with 10% glucose solution.

3.3 Identification of *Anopheles* mosquitoes from the sampled localities

3.3.1 Morphological Identification of *Anopheles* mosquitoes collected

A total of 3,632 mosquitoes (containing 1,822 from Lagos and 1,810 mosquitoes from Oyo state respectively) were morphologically identified across all localities. Morphological identification was carried out with the aid of standard identification guides (Gillet 1972; Gilles and Coetzee, 1987). Culicines and Anopheles were separated using the Gillet, 1972 identification guide while *Anopheles* mosquitoes present were further identified with the aid of the Gilles and Coetzee 1987 keys. Using these morphological keys, mosquitoes with speckled legs, hind tarsus 4 and 5 not entirely pale, abdominal segments without laterally projecting tufts of scales, 3 pale bands on antenna and third main dark area of vein 1 on wing with pale interruption sometimes fused with preceding pale spots were identified as members of the *Anopheles gambiae s.l.* . These were separated for molecular identification.

3.3.2 Molecular identification of *Anopheles* mosquitoes morphologically identified

3.3.2.1 DNA extraction

A total of 100 *Anopheles gambiae s.l.* were selected from each locality and analyzed with PCR. DNA extraction was conducted with the aid of a genomic DNA extraction kit *prep*GEM^{TM} insect produced by ZyGEM Corporation Limited, New Zealand. As specified by the manufacturer, master mix (Enzyme and 10x extraction buffer) were prepared and legs of mosquitoes removed and crushed in the master mix. The extraction solution with the legs were incubated at 75^0C for 15minutes (to activate proteinase, lyse cells, destroy nucleases and remove nucleoproteins), and 95^0C for 5 minutes (to inactivate proteinase) using a Primus® 96 well thermo cycler. The extracted DNA's were kept in the -20^0C freezer inside 0.2ml eppendorf tubes. DNA was also extracted from the positive controls (*Anopheles gambiae s.s.*, "BOA" and "NAG", *Anopheles merus/melas* "ZAM" and *Anopheles arabiensis* "KGB", *Anopheles quadriannulatus* "SANGWE" from the National Institute for Communicable Diseases, NICD in South Africa and *A. gambiae s.s.* "KISUMU" strains from the Nigerian Institute of Medical Research, NIMR, in Lagos, Nigeria). These have been maintained in the insectary at both the Vector Control Research Unit (VCRU) at NICD, South Africa (BOA and NAG) and at the Molecular Entomology and Vector Control Unit, Public Health Division of the Nigerian Institute of Medical Research (NIMR) for a minimum of 10 years.

3.3.2.2 Molecular identification of mosquitoes using PCR and Enzyme digest

Molecular identification was conducted using 1µl of the DNA extract from each mosquito samples as template for the Polymerase Chain Reaction (PCR) process using standard methods (Scott *et al.*, 1993). A master mix solution containing 2.5µl of 10x PCR reaction buffer, 2.5µl of dNTP, 1µl of MgCL solution, 1µl of *An. quadriannulatus* primer, 2µl each of the other primers (Universal, *Anopheles gambiae*

s.s., *Anopheles arabiensis*, *Anopheles merus/melas*), 4.9µl of sterile distilled water and 0.2µl Taq polymerase enzyme was made in a 0.2ml eppendorf tube for each sample and transferred to a Primus® 96 well thermo cycler that was used for the PCR process. The thermo cycler was programmed thus: 94^0C for 2min (denaturation phase), the 30 cycles of 94^0C for 30 seconds, 50^0C for 30seconds, and 72^0C for 30seconds (Hybridization and Extension phase), and a final extension phase of 72^0C for 5min. The discrimination of the members of the *Anopheles gambiae* complex was done using the following primers *Anopheles gambiae s.s.* (CTGGTTTGGTCGGCACGTTT), *Anopheles arabiensis* (AAGTGTCCTTCTCCATCCTA), *Anopheles merus/melas* (TGACCAACCCACTCCCTTGA), *Anopheles quadriannulatus* (CAGACCAAGATGGTTAGTAT) and a Universal primer (GTGTGCCCCTTCCTCGATGT) which anneals to the same position of the rDNA of all the five subspecies that could not be differentiated using morphological means. A 12.5µl reaction volume of the product was electrophoresed through 2.5% SEAKEM® agarose gel containing ethidium bromide and photographed under ultraviolet light illuminator.

The *Anopheles gambiae s.s.* present in the population were selected for further M/S molecular form identification. The remaining 12.5µl of the product was further digested by adding in a reaction mixture, 2µl of *Heamophilus haemolyticus* (HhaI) restriction enzyme, 18µl of distilled water, and 2µl of 10x Buffer Tango™. Reaction mixture was incubated for 4hours at 37^0C according to standards (Favia *et al.*, 1997). The BOA and NAG which were used as control for S and M form respectively, were also digested using the same process. The reaction was stopped by adding 2µl of 0.5M EDTA. The entire product was also electrophoresed through 2.5% SEAKEM® agarose gel containing ethidium bromide and photographed under ultraviolet light illuminator. In the respective localities, where adequate (30) number of M form mossquitoes were not detected, more mosquitoes were screened to make up the number for subsequent tests.

3.4 Determination of the susceptibility status of *Anopheles* populations to DDT and Deltamethrin insecticides

3.4.1 Insecticide susceptibility tests

Insecticide susceptibility tests were carried out on the identified *Anopheles gambiae s.l.* mosquitoes by exposing 2-5 day old adults to 4% Dichlorodiphenyltrichloroethane (DDT) and 0.05% Deltamethrin insecticides according to standard protocol (WHO, 1998; 2013) (Plate 3.3). A total of 1,822 (Lagos State = 900; Oyo State= 922) and 1,810 (Lagos State = 900; Oyo State = 910) adult female Anopheles mosquitoes were exposed to DDT and deltamethrin insecticides respectively across all localities. For each locality, the number of adult female mosquitoes used for the test varied between 140 - 170 (Lagos = 300 female mosquitoes from Ikorodu, Lekki, Ajah, Magodo, Yaba, and Badagry respectively; Oyo State = 280 female Anopheles from Oluyole, 300 from Iwo road, Bodija, and Oyo respectively, and 310 and 342 for Ojoo and Eruwa respectively) depending on the availability of mosquitoes. According to WHO criteria (WHO, 1998; WHO, 2013), 25 mosquitoes were transferred into each holding tube in four replicates except for Ojoo and Eruwa mosquito populations where more mosquitoes were used while each exposure had a minimum of 40 mosquitoes (20 mosquitoes in two replicates containing silicon oil) as control. The exposure period lasted for one hour for each insecticide after which mosquitoes were transferred to holding tubes and provided with cotton pads soaked in 10% sucrose solution. Knockdown was taken after one hour while final mortality values were recorded after 24hours according to WHO standards (WHO, 1998 and modified in WHO, 2013). A mortality value between 97% and 100% indicate that the population is susceptible to the insecticide; if the mortality value is between 95% and 97%, the population is said to have reduced susceptibility to the insecticide used. However, if the mortality value is less than 95%, the population is resistant to the diagnostic concentration of insecticide used (WHO, 2013). The survivors and dead mosquitoes were kept in silica gel separately and properly labeled for each locality.

Plate 3.3 **Exposure of the *Anopheles* mosquitoes collected to insecticide impregnated papers using WHO criteria**

3.5 Assessment of 2La inversion frequency and Microsatellite loci polymorphism in the identified *Anopheles gambiae s.s.*, M molecular form mosquitoes

The frequency of inversion 2La and the polymorphism of microsatellite loci were assessed in 30 DDT resistant *Anopheles gambiae s.s.*, M molecular form mosquitoes previously identified by PCR. However, for consistency, the polymorphism of microsatellites was assessed on the same samples used for 2La inversion analysis.

3.5.1 Determination of 2La inversion frequency in the mosquito populations

Inversion 2La was assessed in the selected mosquitoes previously identified from each locality with the aid of White *et al.,* (2007) protocol. Thirty samples were selected from the mosquitoes that survived exposure to DDT from each site. PCR reaction was carried out in a 12 µl reaction that included 1.25µl 10x PCR buffer, 1.25µl $MgCl_2$, 0.5µl dNTP, 1µl primers (2La, $2La^+$ and Universal primers), 5.4µl of distilled water and 0.1µl of Dream Taq DNA polymerase and 0.5µl of the extracted DNA was used in the reaction mixture. Thermocycler conditions were 94^0C for 2 minutes; 35 cycles of 94^0C for 30 seconds, 60^0C for 30 seconds and 72^0C for 45seconds; a final elongation at 72^0C for 10 minutes and a 4^0C hold. Stock PCR primers for molecular karyotyping of 2La and $2La^+$ chromosomes were acquired from Inqaba biotec^{TM}, South Africa with the respective sequences ACACATGCTCCTTGTGAACG for 2La (27A2), GGTATTTCTGGTCACTCTGTTGG FOR $2La^+$ (DPCross5) and CTCGAAGGGACAGCGAATTA for the Universal target (23A2). The resulting products were analyzed on 1.5% agarose gel, stained with ethidium bromide, for 2hours.

3.5.1.1 Extraction and sequencing of anomalous 2La band detected within the Lagos populations

The anomalous 2La band, which has not been reported in previous studies, discovered in this study was excised from SEAKEM® low melting agarose gel for purification (Fig 3.3). Extraction procedure was carried out using a QIAquick Gel Extraction Kit Protocol from QIAquick® Spin handbook, 2006 by QIAGEN (at www.qiagen.com).

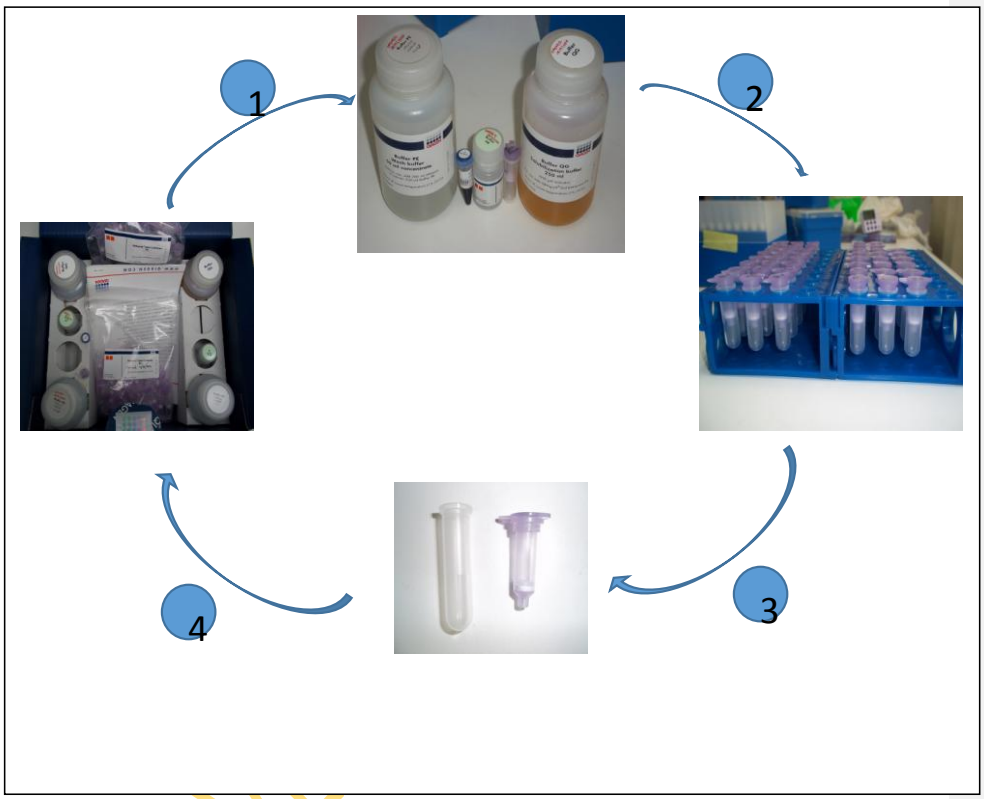

Figure 3.3 Extraction of anomalous 2La band using gel extraction kit

1. DNA extraction kit: instructions and steps in the manufacturer protocol (www.qiaquick.com) were followed
2. Transfer of Gel excised into QIAquick column for separation of fragment
3. Elution of DNA fragment trapped in the QIAquick membrane column
4. Preparation of eluted DNA for sequencing

Gel slice were weighed in a colorless tube and Buffer QG was added to the gel at 3:1 volume. The reaction was incubated at 50^0C for 10 min for gel slice to dissolve completely until reaction turns yellow. A total of 1ml gel volume of isopropanol was added to reaction volume. Samples were then transferred into the QIAquick column and centrifuged for 1min. Flow-through was discarded and QIAquick column placed back into the same collection tube. To wash the trapped fragment, 0.75ml of Buffer PE was added to QIAquick column and centrifuged for 1 minute. DNA fragment was then eluted by adding 50µl of buffer EB (10mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and column centrifuged for 1 min. The extracted DNA fragment was sent to Macrogen for sequencing (at http//dna.macrogen.com/eng). Sequences were aligned on PUBMED.

3.5.2 Assessment of the association between the insecticide resistance profile and 2La inversion frequency

The insecticide resistance profile of the *Anopheles* mosquito populations were recorded for each locality and the percentage mortality values were converted to % Survival. The %Survival values were plotted against the 2La inversion frequency data.

3.5.3 Determination of polymorphic alleles using Microsatellite PCR sequencing

3.5.3.1 Selection of Microsatellite loci

Microsatellite PCR was conducted on the same samples used for 2La inversion karyotyping. Microsatellite data were obtained from published genomic map of *Anopheles gambiae s.s.* (Zheng *et al.*, 1996). The selection of loci with respect to the location of insecticide genes (Table 3.1) was determined from genomic (Zheng *et al.*, 1996) and genetic maps conferring DDT, and Pyrethroid resistance (Ranson *et al.*, 2000). Ten microsatellite loci (AG2H637, AG2H143, AG2H523, AG2H603, AG2H772, AG2H590, AG2H26, AG2H79, AG2H197, AG2H175) were selected, five of these loci (AG2H637, AG2H143, AG2H523, AG2H603, AG2H772) are located within 2La inversion, while the rest are located outside inversion 2La (Table 3.1). Loci were selected based on their level of polymorphisms, distance to DDT/ pyrethroid

resistance genes (Ranson *et al.,* 2000; 2004) and position along the chromosome (Zheng *et al,* 1996).

3.5.3.2 Microsatellite PCR amplification and sequencing

Microsatellite primers used for this study were labelled with FAM and HEX fluorescent dye. The forward primers of AG2H637, AG2H143, AG2H772, AG2H79, and AG2H175 were labeled with 5'FAM while AG2H523, AG2H603, AG2H590, AG2H26 and AG2H197 were labeled with 5'HEX. Primer sequence 5'-TCGAAATGTATGCGAAATGCAG-3' and 5'-CCTTCTTTCCTCGATGCATTCC-3' was designed for the forward and reverse sequences of microsatellite loci AG2H637; 5'-CGTACGAGTGAGTGAGTTGG-3' and 5'-CAAAAATAGCATCACGGCCG-3' for microsatellite loci AG2H143; 5'-CTCGTTAGGCGCTTGTGAAC-3' and 5'-CACTTCACGACTGTGAGCAC-3' for microsatellite loci AG2H523; 5'-TGCACCGTTGATGCACATGC-3' and 5'-GTGGACGATGTGAAAGATAAGG-3' for loci AG2H603; 5'-TACAGCTGTTTGGGAGTTGG-3' and 5'-GGGTCGGCTTTTATTTCCTCG-3' for loci AG2H772; 5'-CGGGAAAGCGAAGTGTACGA-3' and 5'-TGCGGCTGGTGAACATTTTC-3' for microsatellite loci AG2H590; 5'-GGTTCCTGTTACTTCCTGCC-3' and 5'-CCGGCAACACAAACAATCGG-3' for microsatellite loci AG2H26; 5'-CGGGTAGCGCTAGAAGTATG-3' and 5'-AGAGAAATGTGCCGAAGGGG-3' for microsatellite loci AG2H79; 5'-TACCTCTGTGTTCGGTTTCC-3' and 5'-GGTGGTATGGCGATGGAAGG-3' for microsatellite AG2H197; and 5'-AGGAGCTGCATAATTCACGC-3' and 5'-AGAAGCATTGCCCGCATTCC-3' for the forward and reverse primers of microsatellite loci AG2H175.

The reaction mixture for PCR analysis contained 0.5µl of the extracted DNA, 1.5µl 10x PCR buffer, 0.9µl of 25mM $MgCl_2$, 1.2µl dNTP, 0.6µl of the forward and reverse primers, 10.1µl of nuclease free water and 0.1µl of Taq polymerase, to make a total volume of 15µl. PCR amplification and electrophoresis was carried out as described by Onyabe and Conn, 2001. However, PCR optimization temperature of each primer used differ as a result of the variations in the melting temperatures among the primers.

Thermocycler condition was programmed at 94^0C for 5min (denaturation phase), the 35 cycles of 94^0C for 30 seconds, 57^0C for 30seconds (for microsatellite loci AG2H590, AG2H772, AG2H175, AG2H143, AG2H79, and AG2H197. For loci AG2H637 and AG2H523 hybridization temperature was 53^0C. For loci AG2H26, the hybridization temperature was 55^0C while the hybridization optimization temperature for AG2H603 was 51^0C), and 72^0C for 40seconds (Hybridization and Extension phase), and a final extension phase of 72^0C for 10min. PCR products were packed and sent for analysis at Macrogen (Plate 3.4).

3.6 Data Analysis:

Data on the longitude and latitude of the surveyed localities were analyzed with Arc-view software and projected on the map of Nigeria. The map also included the susceptibility status of the mosquitoes in each locality to DDT and Deltamethrin as developed from the GPS coordinates obtained. This was also used to determine the spatial distribution of the mosquito populations from each locality. Resistance data between Lagos and Oyo populations were compared with descriptive statistics using SPSS v. 2010. Resistance status of populations in each locality was determined and compared with inversion 2La data. The 2La inversion frequency data was analysed using Wright F-statistics (Brown, 1970), where $F = (4ac\text{-}b^2)/[(2a + b)(2c + b)]$, with a and c being the absolute frequency of the two homozygous classes and b the frequency of the heterozygote. Absolute frequencies of F value karyotypes were calculated by applying the following formulas (where p and q were the frequencies of the standard and inverted arrangements) (Petrarca and Beier, 1992):

Standard homokaryotypes = $N[pF + p^2 (1 – F)]$

Heterokaryotypes = $N[2pq(1 – F)]$

Inverted homokaryotypes = $N[qF + q^2(1 – F)]$

Table 3.1: Microsatellite loci studied among *Anopheles gambiae s.s.** populations from Lagos and Oyo States Nigeria

Locus	Cytological location ŧ	Inversion Φ	Allele size	Repeat motif	QTL close to locus
AG2H637	2L	2La	107	(CA) 5+ 6	rtd 2 (resistance to DDT)
AG2H143	2L	2La	160	(TC) 9	rtd 2 (resistance to DDT)
AG2H523	2L	2La	188	(GT) 19	rtd 2 (resistance to DDT)
AG2H603	2L	2La	109	(GT) 8	rtd 2 (resistance to DDT)
AG2H772	2L	2La	116	(GT) 8	*Dl* (dieldrin resistance)
AG2H590	2R	OI 2La	125	(GT) 11 + 8	Cyp 4 (Pyrethroid resistance)
AH2H26	2R:12	OI 2La	154	(GT) 8 + 29 + 4	Cyp 4 (Pyrethroid resistance)
AG2H79	2R	OI 2La	201	(GT) 20	Cyp 4 (Pyrethroid resistance)
AG2H197	2R	OI 2La	85	(GT) 8	Unknown
AG2H175	2R	OI 2La	97	(CA) 8	Unknown

* Data from Zheng *et al.* (1996)

ŧ R refers to right arm of chromosome and L to the left arm

Φ OI refers to outside inversion

QTL refers to Quantitative Trait Loci

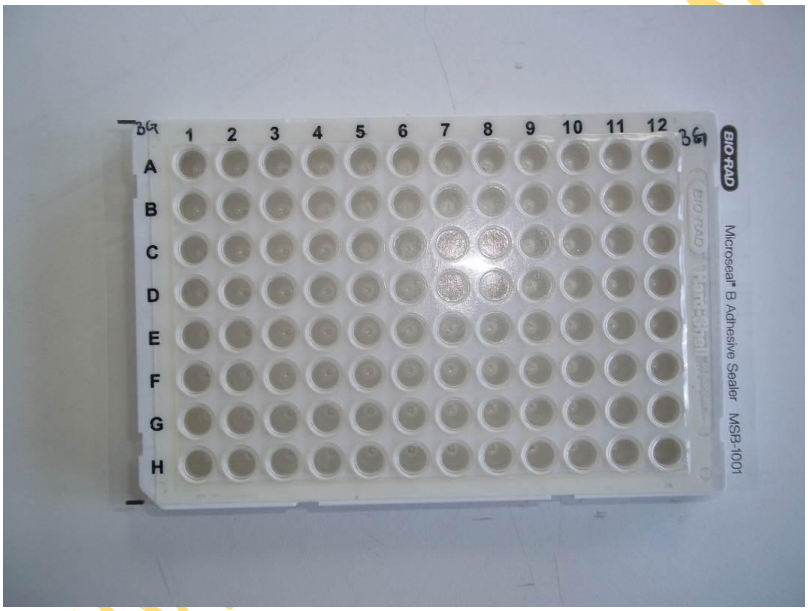

Plate 3.4: Preservation of PCR products in Bio-rad Sequencing plate for microsatellite sequencing

Hardy-Weinberg chi squares estimates for 2La were also calculated for each locality. The trend of association between the insecticide resistance profile and the 2La genetic differentiation index (FST) using descriptive statistics (correlation) was also calculated using SPSS v. 2010. Microsatellite data were first interpreted using Peak Scanner™ software version 1.0. Peak Scanner results were then converted and analyzed online using GENEPOP software version 4.0.10 (http://genepop.curtin.edu.au). Alele frequency based correlations (F_{ST} and R_{ST}) were compared between Lagos and Oyo State populations. Number of migrants within and between the two states were also determined. Linkage disequilibrium was conducted using Fisher's method between Lagos and Oyo populations all on the GENEPOP software (http://genepop.curtin.edu.au/) version 4 at 1000 number of iterations per batch, 1000 dememorization number and 100 number of batches. All analysis were at α=0.05.

CHAPTER FOUR

RESULT

4.1 *Anopheles* mosquitoes collected and Identified from study localities

4.1.1 Larval preference of mosquitoes in the study localities

A total of 120 mosquito larval habitats were sampled from the twelve localities surveyed (Table 4.1). Anopheline larvae were found in a total of 96 habitats out of which 43 (44.8%) had only anophelines. Culicine larvae were found in 77 sites and 24 (31.2%) of these habitats had only culicines. Anophelines and Culicnes cohabit in 28 (46.8%) and 25 sites (43.1%) from Lagos and Oyo States respectively, suggesting that anopheleines and culicines coexist in majority of the habitats. The habitat type distribution for the habitats with anopheline only or culicine only larvae was not different in the two states (Table 4.1; χ^2 = 9.73, degree of freedom [df] = 5, *P*> 0.01 and χ^2 = 5.25, degree of freedom [df] = 5, *P*> 0.01 for Lagos and Oyo states respectively) and not significant even between states (Table 4.1; χ^2 = 3.67, degree of freedom [df] = 5, *P*> 0.01).

4.2 Spatial distribution of *Anopheles* mosquitoes identified from the study localities in Lagos and Oyo State

All the 3,632 mosquitoes identified morphologically across all localities were members of the *Anopheles gambiae s.l.* Polymerase chain reaction (PCR) identification was conducted on a total of 1,200 female *Anopheles* (Table 4.2, Appendix 1 and Appendix 2). The remaining specimens could not be identified due to lack of PCR products. *Anopheles gambiae s.s.* was the only species found in all the localities surveyed in Lagos state and all belonged to the M molecular form (100% M form) (Table 4.2, Table 4.3, Fig. 4.1, Fig. 4.2). Oyo state populations contained more *An. arabiensis* (58%) than the *An. gambiae s.s.* (42%). Samples from Iwo road and Bodija had higher proportions of *An. arabiensis* (77% and 83% respectively) than *An. gambiae s.s.*.

Table 4.1: Distribution of Anopheline and Culicine mosquito larvae in a total of 120 aquatic habitats sampled for mosquito larvae from the selected localities.

State	No of habitats examined (%)	Larval habitat type					
		Footprints	Ponds	Pool	Puddle	Tire tracks	Tanks
Lagos							
Anopheline larvae only	31 (50%)	7	0	0	20	4	0
Culicine larvae only	3 (4.8%)	0	0	0	3	0	0
Anopheline and culicine	28 (46.8%)	1	0	3	21	3	0
Total	62 (100%)	8	0	3	44	7	0
Oyo							
Anopheline larvae only	12 (20.7%)	0	0	0	3	9	0
Culicine larvae only	21 (36.2%)	17	2	0	2	0	0
Anopheline and culicine	25 (43.1%)	9	0	11	1	4	0
Total	58 (100%)	26	2	11	6	13	0

All the samples analysed from Ojoo population were *An. arabiensis* (100%). However, *An. gambiae s.s.* predominated at Oluyole, Oyo town and Eruwa (Table 4.2, Fig. 4.1). In all the *An. gambiae s.s.* further identified from Oyo populations, the M molecular form had high percentage occurrence (Oluyole, Iwo road, Bodija with 100% M form and 95% in Oyo town population) except for the samples from Eruwa where the M and S form occur in sympatry (50% M and 50% S form respectively) (Table 4.3, Fig. 4.2).

4.3 Susceptibility of *Anopheles* populations to DDT and Deltamethrin insecticides in Lagos State and Oyo State

A total of 3,632 adult *Anopheles* mosquitoes were exposed to diagnostic concentrations of DDT and Deltamethrin insecticides, according to WHO standards (WHO, 1998; WHO, 2013). 1,822 *Anopheles* mosquitoes were exposed to the DDT insecticide (Lagos = 900; Oyo = 910) (Table 4.4) while a total of 1,810 *Anopheles* mosquitoes were exposed to deltamethrin insecticide (Lagos = 900; Oyo = 922) (Table 4.5) across all localities.

4.3.1 Susceptibility of *Anopheles* populations to DDT in Lagos and Oyo State

There was no mortality in five out of six populations examined in Lagos (Ikorodu= 0%; Lekki= 0%; Ajah= 0%; Magodo= 0%; Badagry= 0% and Yaba= 34.5% mortalities respectively) except in Yaba where 34.5% of the mosquito populations were killed (Table 4.4, Fig. 4.3), which gave a mean mortality of 5.75%. The populations from all the six localities from Oyo State died more when exposed to the diagnostic concentrations of DDT with a mean mortality of 53.55% but according to WHO criteria, the populations were resistant to DDT. However, the populations from Oluyole (13.3% mortality) had the lowest mortality value compared with all other localities from Oyo State while populations from Iwo road had the highest mortality value of 84% (Table 4.4).

Table 4.2: Distribution of members of *An. gambiae* complex in the sampled localities

State	Localities	No. identified	PCR – Species Identification		
			% *An. gambiae s.s.*	% *An. arabiensis*	% *An. melas*
Lagos	Ikorodu	100	100%	-	-
	Lekki	100	100%	-	-
	Ajah	100	100%	-	-
	Magodo	100	100%	-	-
	Yaba	100	100%	-	-
	Badagry	100	100%	-	-
Oyo	Oluyole	100	86.7%	13.3%	-
	Iwo road	100	23.3%	76.7%	-
	Bodija	100	16.7%	83.3%	-
	Ojoo	100	-	100%	-
	Oyo	100	73.3%	26.7%	-
	Eruwa	100	53.3%	46.7%	-

***** Number of mosquitoes that did not amplify using PCR even after 2-3 runs are not included in the table

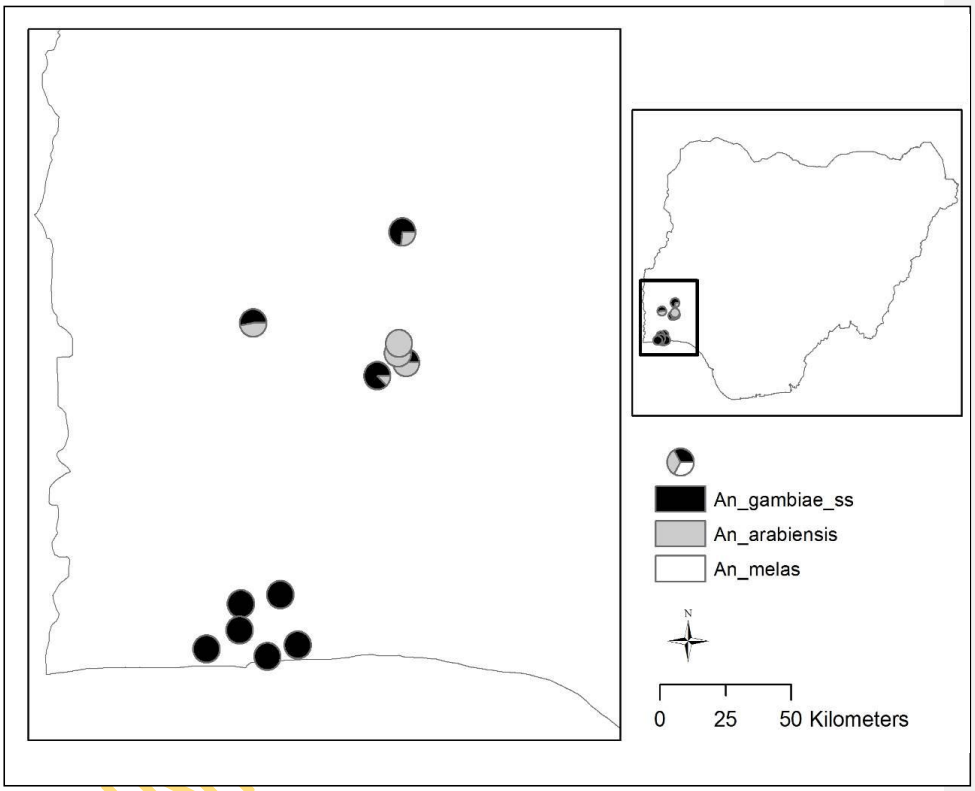

Figure 4.1: Species composition of *Anopheles* in the sampled localities

Table 4.3: Distribution of the molecular forms M/S in the sampled localities

State	Localities	No. of *An. gambiae s.s.* identified	PCR - Form	
			% "S"	% "M"
Lagos	Ikorodu	100	-	100%
	Lekki	100	-	100%
	Ajah	100	-	100%
	Magodo	100	-	100%
	Yaba	100	-	100%
	Badagry	100	-	100%
Oyo	Oluyole	85		100%
	Iwo road	20	-	100%
	Bodija	15	-	100%
	Ojoo	-	-	-
	Oyo	70	4.8%	95.2%
	Eruwa	52	50%	50%

***** Number of mosquitoes that did not amplify using PCR even after 2-3 runs are not included in the table

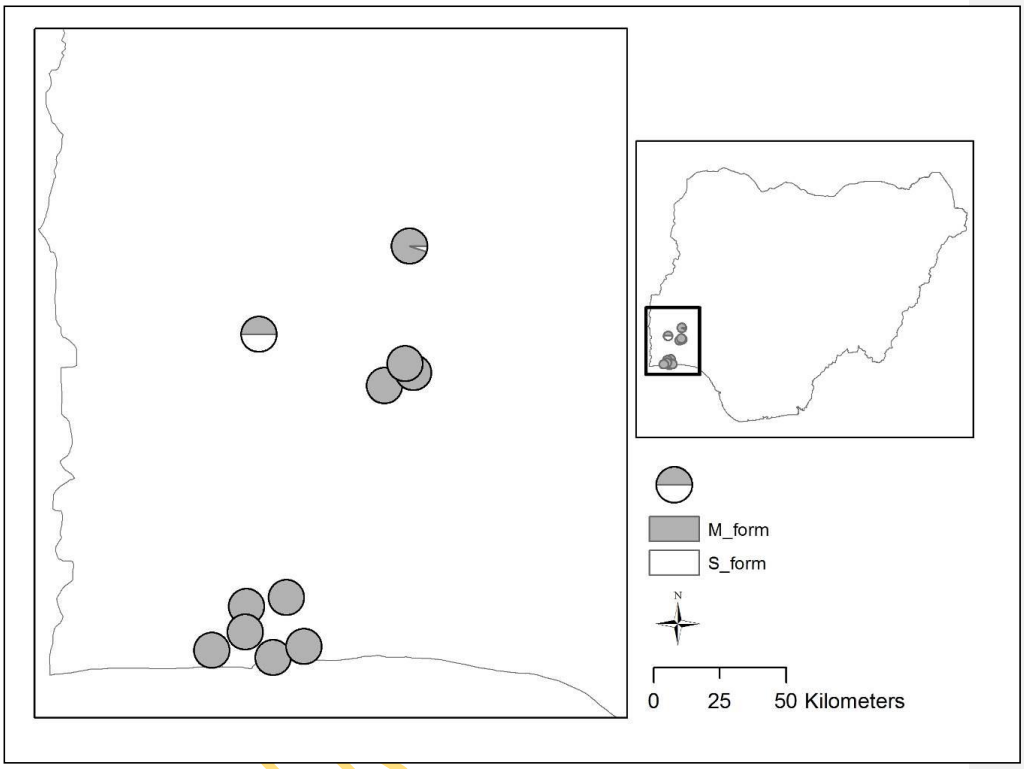

Figure 4.2: Molecular forms of *Anopheles gambiae s.s.* in the sampled localities

Table 4.4: Susceptibility status of *Anopheles* populations to DDT in Lagos and Oyo State

States	Localities	Latitude	Longitude	No. tested	Mortality (%)	Susceptibility status
Lagos	Ikorodu	6°38.013’	3°30.644’	150	0%	Resistant
	Lekki	6°25.746’	3°27.983’	150	0%	Resistant
	Ajah	6°28.018’	3°34.238’	150	0%	Resistant
	Magodo	6°36.176’	3°22.558’	150	0%	Resistant
	Yaba	6°30.987’	3°22.275’	150	34.5%	Resistant
	Badagry	6°27.228’	3°15.470’	150	0%	Resistant
Oyo	Oluyole	7°21.404’	3°50.598’	140	13.3%	Resistant
	Iwo road	7°24.042’	3°56.496’	150	84%	Resistant
	Bodija	7°25.901’	3°54.815’	150	52%	Resistant
	Ojoo	7°27.812’	3°55.017’	160	82%	Resistant
	Oyo	7°49.923’	3°55.727’	150	30%	Resistant
	Eruwa	7°31.894’	3°25.077’	172	60%	Resistant

Susceptibility criteria: 100 – 97% Mortality= Susceptibility; 97 - 95% Mortality = Reduced susceptibility; below 95% = Resistance (WHO, 2013).

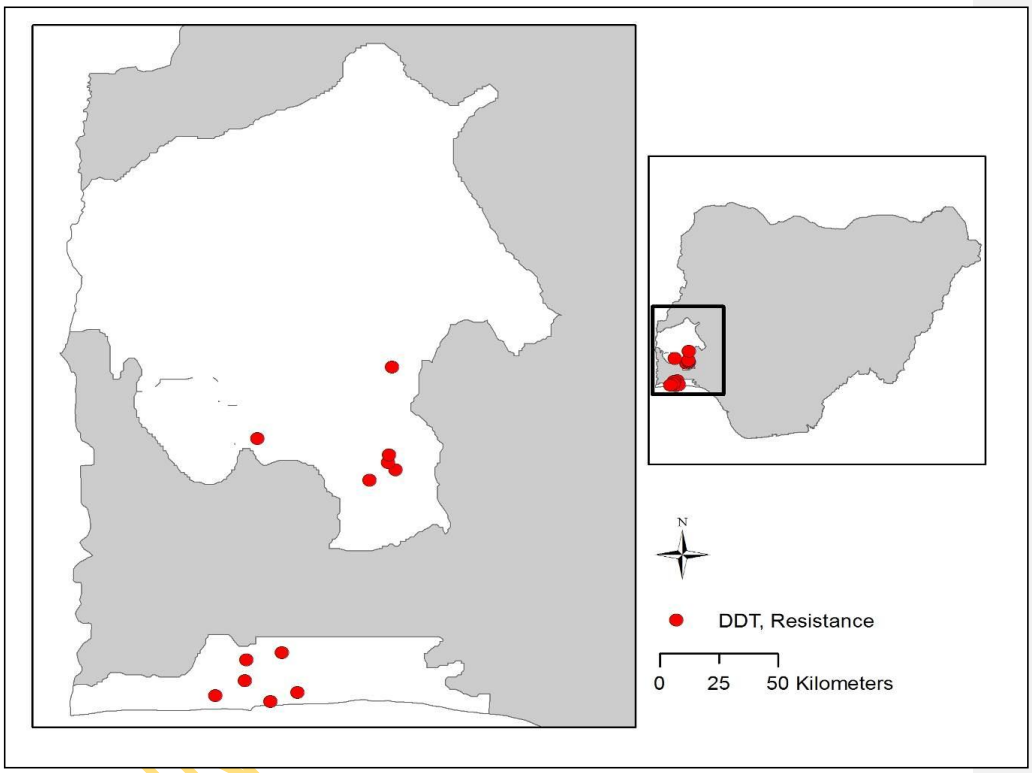

Figure 4.3: Susceptibility status of *Anopheles* populations to DDT in Lagos and Oyo States

4.3.2 Susceptibility of *Anopheles* populations to Deltamethrin in Lagos and Oyo State

Anopheles mosquitoes from all the six localities from Lagos were resistant to deltamethrin while varying level of susceptibility was recorded in samples collected from Oyo State (Table 4.5, Fig. 4.4). In Lagos, all the mosquito populations exposed were resistant to deltamethrin insecticide (92.7%, 87.5%, 86.8%, 70%, 65%, and 50% for Yaba, Ikorodu, Magodo, Lekki, Ajah, and Badagry respectively) according to WHO criteria. Though, Yaba population had the highest observed mortality value while Badagry populations had the lowest. Susceptibility status data of the *Anopheles* mosquitoes to deltamethrin in Oyo state vary considerably. Resistance was recorded in *Anopheles* populations from 3 localities (Ojoo, Bodija and Oluyole with 90%, 88% and 80% mortalities respectively) while Iwo road and Oyo town populations were susceptible to deltamethrin with 100% and 98% mortalities respectively. Observed mortality value recorded from Eruwa populations indicate reduced susceptibility to deltamethrin at 95% mortality according to WHO, 2013 criteria.

Data analysis revealed that, only the DDT resistance profile showed significant deviations from the mean between Lagos and Oyo state (0.023) susceptibility data. However, deltamethrin resistance showed non-significant value of 0.094 (Appendix 3).

4.4 Frequency of Inversion 2La karyotypes in *Anopheles gambiae s.s.* populations from Lagos State and Oyo State

A total of 333 "M" molecular form of the resistant *An. gambiae s.s.* mosquitoes were positively analyzed from Lagos and Oyo State with an average of 30 samples per locality (Table 4.9, Appendix 4). All the 180 *An. gambiae s.s.* (M molecular form) samples analyzed from Lagos populations gave good bands while a total of 153 *An. gambiae s.s.* M molecular form produced good bands from Oyo state populations (Table 4.6).

Table 4.5: Susceptibility status of *Anopheles* populations to Deltamethrin in Lagos and Oyo State

States	Localities	Latitude	Longitude	No. tested	Mortality rate (%)	Susceptibility status
Lagos	Ikorodu	6°37.606'	3°30.383'	150	87.5%	Resistant
	Lekki	6°25.746'	3°27.983'	150	70%	Resistant
	Ajah	6°28.018'	3°34.238'	150	65%	Resistant
	Magodo	6°38.315'	3°23.420'	150	86.8%	Resistant
	Yaba	6°30.987'	3°22.275'	150	92.7%	Resistant
	Badagry	6°27.228'	3°15.470'	150	50%	Resistant
Oyo	Oluyole	7°21.404'	3°50.598'	140	80%	Resistant
	Iwo road	7°24.042'	3°56.496'	150	100%	Susceptible
	Bodija	7°25.901'	3°54.815'	150	88%	Resistant
	Ojoo	7°27.812'	3°55.017'	150	90%	Resistant
	Oyo	7°49.923'	3°55.727'	150	98%	Susceptible
	Eruwa	7°31.894'	3°25.077'	170	95%	Reduced Susceptibility

Susceptibility criteria: 100 – 97% Mortality= Susceptibility; 97 - 95% Mortality = Reduced susceptibility; below 95% = Resistance (WHO, 2013).

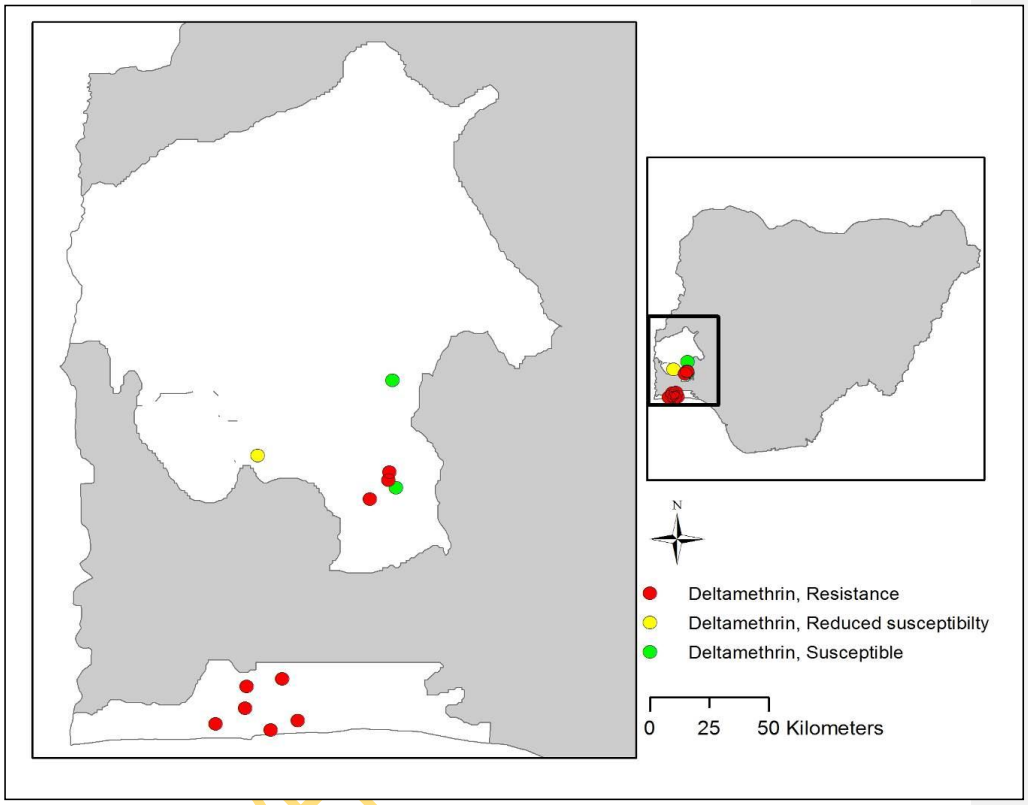

Figure 4.4: Susceptibility status of *Anopheles* populations to Deltamethrin in Lagos and Oyo States

Significant genetic differentiation values were observed in Ikorodu, Lekki, Ajah, Magodo and Badagry samples which follows the same trend with their susceptibility status (0% mortality from all the sites). However, the only site with the highest mortality data in Lagos, Yaba, had a non-significant $F_{ST} < 0.05$ (Table 4.6). This also followed the same trend with susceptibility data. In Oyo state populations, significant genetic differentiation values were observed in four out of the five localities analyzed (Oluyole, Bodija, Oyo, Eruwa) while an insignificant value was observed in Iwo road population $F_{ST} < 0.05$ (Table 4.6). The susceptibility status of the populations from Oyo state also followed the same trend as the genetic differentiation indices (Table 4.6). Iwo road populations had the highest mortality value of 84% with the lowest/ non-significant F_{ST} value of 0.029 while Oluyole populations with the lowest mortality value of 13.3% had the highest genetic differentiation value F_{ST} of 0.3 (Table 4.6).

Chi-square results were less than tabulated value of 3.8 across all localities from Lagos with P values higher than 0.05 (χ^2 = 0.001-0.711 and P=0.999-0.702) except Magodo samples (χ^2 = 4.689, P= 0.0096) (Table 4.7). In Oyo populations however, Chi-square values were less than tabulated value of 3.8 across all localities with P values higher than 0.05 (χ^2 = 0.0025-2.749 and P=0.981-0.253) except Eruwa samples (χ^2 = 3.86, P= 0.140) (Table 4.8).

A summary of the genetic analysis of the Lagos and Oyo populations indicate that genetic differentiation is higher in Lagos state (F_{ST}= 0.104) as compared with Oyo state (F_{ST}= 0.043) which correlates with their insecticide susceptibility values (mean mortality= 5.75% and 53.55% for Lagos and Oyo States respectively) (Table 4.9). The allele frequency data showed that the heterozygous form 2La/2La+ had the highest observed frequency (46.8%) followed by the 2La+/2La+ (37.8%) and then the 2La/2La (15.3%) (Table 4.9).

4.4.1 Trend of Association between DDT Insecticide resistance profile (% Survival rate) and 2La genetic differentiation index (F_{ST}) in Lagos and Oyo state populations

The population's 2La genetic differentiation followed the same trend as the insecticide resistance status of the mosquitoes in both state. Lagos State F_{ST} gave a correlation coefficient of +0.500 while Oyo State coefficient was +0.520 (Fig. 4.5, Fig. 4.6). This indicate a strong association between insecticide resistance and genetic differentiation in Anopheles populations in Lagos and Oyo states.

4.4.2 Detection, Isolation and sequencing of anomalous band from Lagos State populations

Anomalous 400bp bands were consistently detected in the 2La inversion PCR of the Lagos populations which was completely absent in the samples from Oyo State. A total of 37 (20.8%) out of the 180 samples analyzed from Lagos populations produced the anomalous band, which were isolated from the gel and sequenced (Appendix 4). Base sequence of the band produced a 361bp product (Appendix 5), which was subjected to the Basic Local Alignment Sequence Tool (BLAST) that produced the respective protein sequence. Information on this protein and the alignment on the NCBI page gave the AGAP001652-PA [*Anopheles gambiae str.* PEST] (Apendix 6) with ascension number AAAB01008987.1 .

Table 4.6 **Observed 2La allele frequencies of *An. gambiae s.s.* (M form) across all localities sampled in Lagos and Oyo State**

States	Localities	% Mortality	No of mosquito analyzed	Allele frequency per locality			F_{ST}
				2La/2La	2La/2La^{+}	2La^{+}/2La^{+}	
Lagos	**Ikorodu**	**0%**	**30**	3	16	11	0.148
	Lekki	**0%**	**30**	3	14	13	0.050
	Ajah	**0%**	**30**	2	14	14	0.111
	Magodo	**0%**	**30**	0	17	13	0.395
	Yaba	**34.5%**	**30**	4	14	12	0.005
	Badagry	**0%**	**30**	0	8	22	0.154
	Total	**x= 5.75%**	**180**	**12**	**83**	**85**	**0.104**
Oyo	**Oluyole**	**13.3%**	**30**	8	19	3	0.303
	Iwo road	**84%**	**30**	5	15	10	0.029
	Bodija	**52%**	**29**	5	12	12	0.121
	***Ojoo**	**82%**	**0**	-	-	-	-
	Oyo	**30%**	**30**	7	16	7	0.067
	Eruwa	**60%**	**34**	14	11	9	0.339
	Total/mean	**x= 53.55%**	**153**	**39**	**73**	**41**	**0.043**

F_{ST} = 0.0 – 0.05 (little genetic differentiation), 0.05 – 0.15 (Moderate genetic differentiation), 0.15 – 0.25 (great genetic differentiation), >0.25 (very great genetic differentiation)

***** Inversion 2La is fixed in *An. arabiensis*, therefore the population was not analyzed for 2La

Tables 4.7: Observed and expected karyotype frequencies of chromosome inversion 2La in *An. gambiae s.s.* populations sampled in Lagos

Localities		Karyotype frequencies			χ^2	P value
		2La/2La	2La/2La+	2La+/2La+		
Ikorodu	Observed frequencies	3	16	11	**0.655**	**0.721**
	Expected frequencies	3.99	13.92	12.09		
Lekki	Observed frequencies	3	14	13	**0.073**	**0.964**
	Expected frequencies	3.36	13.38	13.28		
Ajah	Observed frequencies	2	14	14	**0.365**	**0.833**
	Expected frequencies	2.82	12.78	14.40		
Magodo	Observed frequencies	0	13	13	**4.689**	**0.096**
	Expected frequencies	2.43	12.21	15.42		
Yaba	Observed frequencies	4	14	12	**0.001**	**0.999**
	Expected frequencies	4.05	13.95	12.06		
Badagry	Observed frequencies	0	8	22	**0.711**	**0.702**
	Expected frequencies	0.54	6.96	22.56		

Table 4.8: Observed and expected karyotype frequencies of chromosome inversion 2La in *An. gambiae s.s.* populations sampled in Oyo

Localities		Karyotype frequencies			χ^2	P value
		2La/2La	2La/2La+	2La+/2La+		
Oluyole	Observed frequencies	8	19	3	**2.749**	**0.253**
	Expected frequencies	10.23	14.61	5.22		
Iwo road	Observed frequencies	5	15	10	**0.025**	**0.987**
	Expected frequencies	5.22	14.58	10.2		
Bodija	Observed frequencies	5	12	12	**0.477**	**0.788**
	Expected frequencies	3.92	13.59	11.63		
Ojoo	Observed frequencies	-	-	-	-	-
	Expected frequencies	-	-	-		
Oyo town	Observed frequencies	7	16	7	**0.133**	**0.936**
	Expected frequencies	7.5	15	7.5		
Eruwa	Observed frequencies	14	11	9	**3.86**	**0.140**
	Expected frequencies	11.19	16.63	6.15		

Table 4.9: Summary of the polymorphic inversion 2La frequencies in Lagos and Oyo States

State	Observed Allelic frequencies			Mean Genetic differentiation F_{ST}	Mean Mortality
	2La/2La	2La/2La^{+}	2La^{+}/2La^{+}		
Lagos	12 (6.7 %)	83 (46.1%)	85 (47.2%)	**0.104**	**5.75%**
Oyo	39 (25.5%)	73 (47.7 %)	41 (26.8%)	**0.043**	**53.55%**
Total	**51 (15.3%)**	**156 (46.8 %)**	**126 (37.8 %)**		

F_{ST} = 0.0 – 0.05 (little genetic differentiation), 0.05 – 0.15 (Moderate genetic differentiation), 0.15 – 0.25 (great genetic differentiation), >0.25 (very great genetic differentiation)

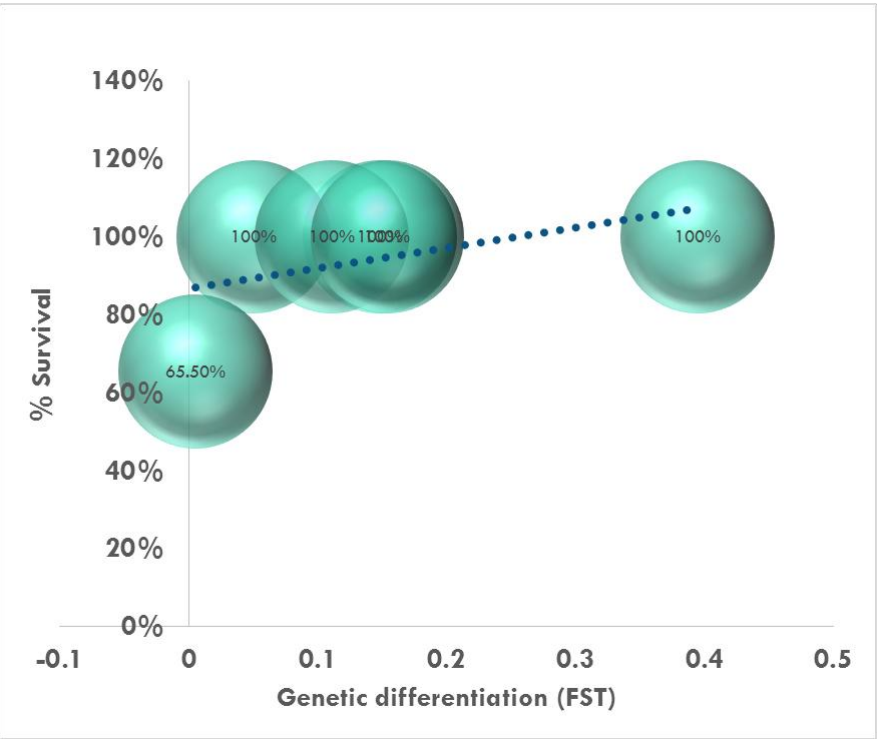

Figure 4.5: Correlation between percentage survival and genetic differentiation F_{ST} among Lagos populations

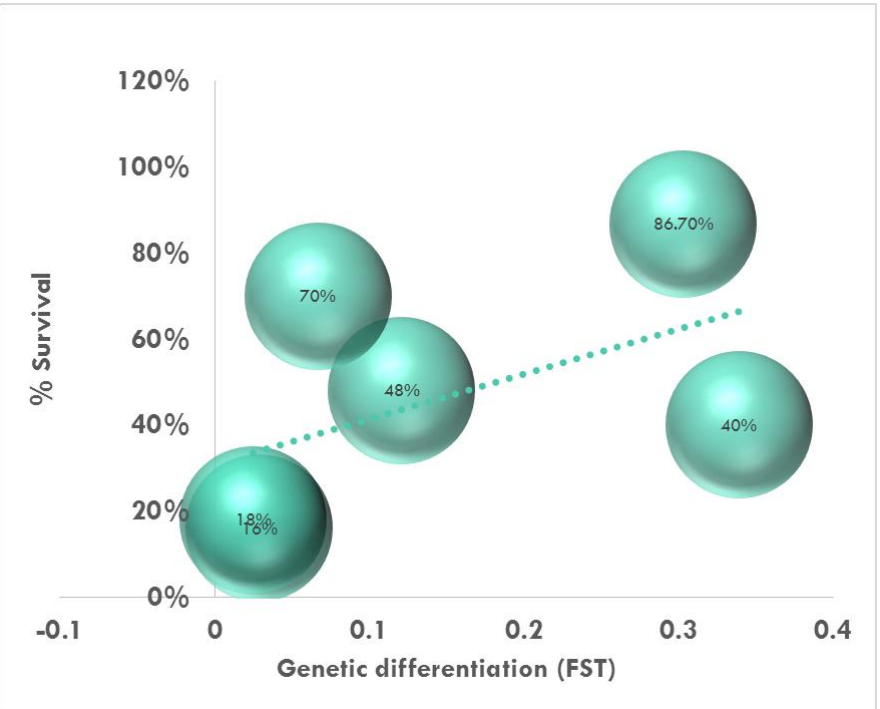

Figure 4.6: **Correlation between percentage survival and genetic differentiation F_{ST} among Oyo populations**

4.5 Microsatellite analysis of *Anopheles gambiae s.s.* populations from Lagos and Oyo State

Results of the microsatellite sequenced data are presented in Appendix 7 and Appendix 8. Translation of the results into GENEPOP format is presented (Appendix 9). The results of population analysis is presented in Table 4.10, Table 4.11 and Table 4.12.

4.5.1 Allele frequency based correlation between Lagos and Oyo State populations

Significant genetic differentiation values were recorded on six microsatellite loci (AG2H26, AG2H175, AG2H590, AG2H637, AG2H772 and AG2H143 (Table 4.13). However, comparison of F_{ST} and R_{ST} values shows that loci AG2H79, AG2H590 and AG2H772 had higher R_{ST} data as compared to F_{ST} (Table 4.10).

4.5.2 Number of migrant (Nm) within and between Lagos and Oyo populations

Samples from Lagos state had higher number of migrants of 5.94813 while the samples from Oyo state gave a lover value of 2.07774 after correction for size (Table 4.11). However, the migration index Nm gave a much lower value (Nm= 1.41934) when the migration index between Lagos and Oyo state populations were computed.

4.5.3 Linkage disequilibrium across all loci between Lagos and Oyo State population

Linkage distribution data showed that 24% of the locus pair had significant chi square values and corresponding P values that were less than 0.05 (Table 4.12).

Table 4.10: Allele frequency based correlation of the 10 microsatellites between Lagos and Oyo State populations

Allele frequency-based correlation (Fis, Fst, Fit/Ris, Rst, Rit)

Multilocus estimates for diploid data (Lagos State and Oyo State)

Locus	Fwc(is)	Fwc(st)	Fwc(it)
AG2H175	0.1325	0.0595	0.1841
Ag2H143	0.1802	0.0817	0.2471
Ag2H26	0.0508	0.2938	0.3297
AG2H637	0.3892	0.1134	0.4585
AG2H79	0.0330	0.0120	0.0446
AG2H590	0.3105	0.0519	0.3463
AG2H772	0.1462	0.3246	0.4233
AG2H603	0.4408	0.0409	0.4637
AG2H523	0.5317	0.0222	0.5420
AG2H197	0.2226	0.0183	0.2368
All:	**0.2514**	**0.1095**	**0.3334**

Locus	Rho(is)	Rho(st)	Rho(it)
AG2H175	0.0003	-0.0017	-0.0014
AG2H143	0.1030	0.0078	0.1100
AG2H26	0.3795	0.0115	0.3867
AG2H637	0.3691	0.0354	0.3915
AG2H79	0.0421	0.0324	0.0732
AG2H590	0.6224	0.2726	0.7254
AG2H772	0.4284	0.4151	0.6656
AG2H603	0.4283	0.0242	0.4421
AG2H523	0.4691	-0.0077	0.4650
AG2H197	0.1210	0.0311	0.1483
All:	0.3318	0.0758	0.3824

File: 013302, One locus estimates following standard ANOVA as in Weir and Cockerham (1984) using GENEPOP version 4.0.10 (http://genepop.curtin.edu.au)

Table 4.11: Number of migrants (Nm) within and between Lagos and Oyo State populations

Number of migrants using private alleles
Lagos State populations

Mean sample size: 24.7667
Mean frequency of private alleles p(1)= 0.0279449
Number of migrants for mean N=10: 17.0809
Number of migrants for mean N=25: 5.89262
Number of migrants for mean N=50: 3.64416
Number of migrants after correction for size= 5.94813
Oyo State populations

Mean sample size: 24.78
Mean frequency of private alleles p(1)= 0.0512011
Number of migrants for mean N=10: 4.95138
Number of migrants for mean N=25: 2.05945
Number of migrants for mean N=50: 1.35487
Number of migrants after correction for size= 2.07774
Lagos and Oyo State populations

Mean sample size: 136.25
Mean frequency of private alleles p(1)= 0.026945
Number of migrants for mean N=10: 18.4022
Number of migrants for mean N=25: 6.27741
Number of migrants for mean N=50: 3.86771
Number of migrants after correction for size= 1.41934

File: 021954 and 022412 Number of migrants (see Barton & Slatkin, Heredity (1986),56:409-415)using GENEPOP version 4.0.10 (http://genepop.curtin.edu.au)

Table 4.12: Linkage distribution of allele pairs across all loci

Locus pair		Chi2	df	P-Value
AG2H175	& Ag2H143	10.773553*	4	0.029230
AG2H175	& Ag2H26	5.790883	4	0.215319
Ag2H143	& Ag2H26	15.702335*	4	0.003446
AG2H175	& AG2H637	5.567701	4	0.233842
Ag2H143	& AG2H637	5.629691	4	0.228563
Ag2H26	& AG2H637	8.634192	4	0.070922
AG2H175	& AG2H79	3.853115	4	0.426249
Ag2H143	& AG2H79	12.365228*	4	0.014832
Ag2H26	& AG2H79	11.244069*	4	0.023954
AG2H637	& AG2H79	Infinity	4	Highly sign.
AG2H175	& AG2H590	Infinity	4	Highly sign.
Ag2H143	& AG2H590	9.463585	4	0.050501
Ag2H26	& AG2H590	7.043294	4	0.133618
AG2H637	& AG2H590	3.155895	4	0.532084
AG2H79	& AG2H590	Infinity	4	Highly sign.
AG2H175	& AG2H772	3.738836	4	0.442503
Ag2H143	& AG2H772	14.981685*	4	0.004739
Ag2H26	& AG2H772	Infinity	4	Highly sign.
AG2H637	& AG2H772	7.789292	4	0.099609
AG2H79	& AG2H772	4.498036	4	0.342781
AG2H590	& AG2H772	Infinity	4	Highly sign.

AG2H175	& AG2H603	7.350171	4	0.118500
Ag2H143	& AG2H603	6.983882	4	0.136742
Ag2H26	& AG2H603	8.380467	4	0.078594
AG2H637	& AG2H603	Infinity	4	Highly sign.
AG2H79	& AG2H603	4.771245	4	0.311585
AG2H590	& AG2H603	Infinity	4	Highly sign.
AG2H772	& AG2H603	10.596468*	4	0.031494
AG2H175	& AG2H523	7.221807	4	0.124621
Ag2H143	& AG2H523	6.127013	4	0.189862
Ag2H26	& AG2H523	21.754550*	4	0.000224
AG2H637	& AG2H523	Infinity	4	Highly sign.
AG2H79	& AG2H523	Infinity	4	Highly sign.
AG2H590	& AG2H523	8.514824	4	0.074439
AG2H772	& AG2H523	Infinity	4	Highly sign.
AG2H603	& AG2H523	Infinity	4	Highly sign.
AG2H175	& AG2H197	25.025630*	4	0.000050
Ag2H143	& AG2H197	15.058584*	4	0.004581
Ag2H26	& AG2H197	4.920039	4	0.295601
AG2H637	& AG2H197	Infinity	4	Highly sign.
AG2H79	& AG2H197	14.880138*	4	0.004956
AG2H590	& AG2H197	13.854643*	4	0.007774
AG2H772	& AG2H197	4.057286	4	0.398309
AG2H603	& AG2H197	Infinity	4	Highly sign.
AG2H523	& AG2H197	Infinity	4	Highly sign.

*Calculated values were higher than tabulated values with p values less than 0.05 (significant allele pair combinations)

Null hypothesis = no linkage disequilibrium (linkage equilibrium)

Alternate hypothesis = Linkage disequilibrium

CHAPTER FIVE

DISCUSSION

This study demonstrates the spatial clustering of *Anopheles* mosquitoes from Lagos and Oyo states. Spatial homogeneity was observed in the distribution of *Anopheles* larval habitats from Lagos but heterogeneity was found in the distribution of the populations from Oyo state. However, this study did not identify the possible environmental variables that determine anopheline occurrence and abundance in relation to larval habitats. This is because the spatial heterogeneity in *An. gambiae* species composition has been reported to be affected either by many variables, each of which has a small effect, or by other important variables that have not yet been measured under field conditions (Minakawa *et al.*, 1999). This is also consistent with the results of Robert *et al.* (1998) who found that the occurrence and abundance of one of the major malaria vectors, *An. arabiensis* larvae in permanent habitats in Dakar, Senegal, are determined by many physicochemical and biological variables.

To examine the association between larval preference and mosquito occurrence/abundance, multiple linear or multiple logistic regression analysis is more appropriate than simple linear or logistic regression (Robert *et al*, 1998). Although, this study did not examine the influence of environmental variables on larval site preference, it seem Anophelines and Cilucines primarily breed and coexist freely in most of the habitats examined as this study did not detect any statistically significant associations between breeding site preference and mosquito occurrence and abundance.

Malaria vector control either by Indoor Residual Spray (IRS), Long Lasting Insecticide Treated Nets (LLINs) or genetic control strategies require accurate mosquito identification and information on the behaviour of vector species which informs the choice of control strategies to deploy. Although the distribution of members of the

Anopheles gambiae is well documented in Africa (Gillies and Coetzee, 1987), much of the work is still not well documented in Nigeria. Most of the work conducted in Nigeria focused on the dynamics and insecticide resistance status of the malaria vectors (Awolola *et al.*, 2002, Oduola *et al.*, 2010; 2012). However, there has been less emphasis on the spatial distribution of these important vectors especially in the studied localities (Onyabe *et al.*, 2003, Awolola *et al.*, 2005a). Our study shows that other species of *Anopheles* are completely absent in Lagos state except the molecular M form recently named as *Anopheles coluzzii* (Coetzee *et al.*, 2013). This agrees with the findings of Oduola *et al.* (2010) who reported the same in samples exposed to diagnostic concentrations of insecticides. In contrast, Onyabe *et al.* (2003) and Awolola *et al.* (2005a) had earlier reported the presence of the molecular S form in Lagos State though at relatively low frequencies. It is believed that there has been a gradual range expansion of the molecular M form and subsequent replacement of the S molecular form in Lagos state.

There has been no report on the spatial distribution of these malaria vectors in Oyo State. Data available are that of spot checks involving samples exposed to insecticides (Rousseau *et al.*, 2007). The map in this study shows the sympatric occurrence of *Anopheles gambiae s.s.* and *Anopheles arabiensis* across all sites examined in Oyo State, with the absence of other species of *Anopheles.* The paucity of data from these areas affirms the presence of both species of *Anopheles* as detected in this study. This would serve as a guide for vector control activities in the localities. The dominance of the M molecular form in the *Anopheles gambiae s.s.* populations reported from Oluyole, Iwo road, Bodija and Oyo town is not surprising and may be due to the factors earlier discussed (Urbanization/ Industrialization). Both the M and the S molecular form occur in sympatry at Oyo town and Eruwa. The presence of these molecular forms in certain localities in this study confirms the earlier reports by Awolola *et al.*, (2005b) on the presence of the two molecular forms in certain parts of South west Nigeria. However, this report is the first to present data on the presence of the M and S molecular form of *Anopheles gambiae s.s.* occurring in Sympatry in Oyo state. This study did not detect *Anopheles gambiae s.s.* in Ojoo. However, the data is in contrast to an earlier report (Rousseau *et al.*, 2007) which may be attributed to the

method of collection and the low number of *Anopheles* mosquitoes tested by PCR in earlier studies from this locality.

Series of insecticide bioassays conducted in this study revealed a good spread of insecticide resistant phenotypes in the malaria vector, *Anopheles*, in Lagos and Oyo State. The presence of *Anopheles* populations capable of withstanding diagnostic doses of insecticide was first reported in Sokoto, Nigeria by Elliot and Ramakrishna (1956) and subsequently reported by Armstrong *et al.* (1957) and Ramakrishna and Elliot (1957). Although these studies were conducted in the northern parts of Nigeria, the spread of *Anopheles* resistance seem to go beyond the northern parts of the country. However, most of the studies earlier conducted in the north involved detections of resistance to the insecticide dieldrin.

In the southern part of Nigeria, resistance of adult *Anopheles* populations to insecticides was initially reported by Awolola *et al.* (2003) and Mojca *et al.* (2003). Although there studies were confined to Lagos and Ogun states respectively, the spread of *Anopheles* resistance seem to go beyond those two localities. *Anopheles* populations collected from all the 12 localities in this study were resistant to DDT. Likewise, *Anopheles* populations from 10 out of the 12 localities surveyed were resistant to Deltamethrin insecticide. In West Africa, several works have been published on the presence of resistant populations of *Anopheles* (Akogbeto and Yakoubou, 1999; Chandre *et al.*, 1999; Diabate *et al.*, 2002; N'guessan *et al.*, 2003; Rousseau *et al.*, 2007). With the increasing flow of human populations and probably mosquito populations in the western coast of Africa, insecticide resistance observed in these South western states in Nigeria could be from either migration of resistant strains of *Anopheles* from Benin Republic where high levels of resistance were documented as early as 1999 (Akogbeto and Yakoubou, 1999) or could be locally selected by specific factors. With the phenomenon of resistance being dynamic, it is certain that the remaining 2 populations that were susceptible to deltamethrin in this study may soon be colonized by resistant strains of mosquitoes unless the source of selection is removed.

A recent study indicate very high resistance of *Anopheles* populations to the insecticides DDT and deltamethrin in South-west Nigeria (Oduola *et al.*, 2010). The study was conducted in urban, semi- urban and rural communities in Lagos state and observed that resistance profiles of *Anopheles* mosquitoes are higher in urban localities as compared with semi- urban and rural communities. According to the World Bank Report in 2009, there are more urban settlements in Lagos as compared with Oyo state. This could partly explain the results of this study. A mean higher resistance value to both DDT and Deltamethrin insecticides in Lagos state as compared with Oyo state in this study, suggests that urbanization/ industrialization remain a key factor in the selection of physiologically resistant phenotypes in Lagos and Oyo states. A factor that is also confirmed in the resistance profile of *Anopheles* from Oyo state. Oluyole populations, an industrialized area in Oyo state which had the highest insecticide resistance profile as compared with the other localities in the state. This is consistent with past studies conducted in South West Nigeria (Oduola *et al.*, 2010).

Student t-test indicate that only the DDT resistance profile differ significantly between Lagos and Oyo state populations. An insignificant value with deltamethrin insecticide resistance profile indicate that there were no phenotypic resistance to detamethrin between Lagos and Oyo populations which made it difficult to proceed to resistant mechanisms using deltamethrin exposed samples. However, the 100% survival rate recorded in the DDT resistance profile in most of the populations especially from Lagos made it impossible to evaluate the dead mosquito populations. Analysis of the dead mosquito populations would have helped in understanding the genetic mechanisms that made certain individuals within the populations to survive insecticide exposure. Hence, the only option left was to evaluate the degree of resistance and the genetic mechanisms that made certain populations to survive insecticide exposure more than others in the resistant mosquito populations. It was expected that the Lagos populations with higher DDT resistance profile and lower mortality values should have a higher frequency of the genes conferring resistance than the Oyo State populations with lower insecticide resistance profile and higher mortality data.

Previous reports have shown the near absence of the 2La homokaryotype in Southern Nigeria and Southern Cameroon which then increases in frequency progressively to reach fixation in the north of these countries (Coluzzii *et al.,* 1979). In this study, the 2La homokaryotype detected in Lagos state, though at low frequencies, is suspected to have occurred as a result of the sensitivity of the technique used (Microscopy vs PCR). The results shown here shows a higher percentage of the heterokaryotype in both populations; indicating positive selection on heterokaryotypes i.e. positive heterosis. The absolute F_{ST} values for the populations of *An. gambiae s.s.* (M form) found in Lagos State was higher than the populations from Oyo State (M form). This indicates higher genetic differentiation on the *Anopheles gambiae s.s.* mosquitoes in Lagos state which follows the same trend as the DDT resistance data recorded and as confirmed by the correlation coefficient (a positive value of 0.5 shows strong association in trend between genetic differentiation and resistance to DDT). The fact that inversion 2La is associated with insecticide resistance in *Anopheles* mosquitoes suggest that our data on the association between this inversion and DDT insecticide resistance is valid as the rtd2 gene responsible for DDT resistance (Ranson *et al.,* 2000) is located close to this inversion and may assort with 2La.

In the past six years, there has been massive vector control activities including Indoor Residual Spray in Lagos State as compared with other states in Nigeria (National Malaria Elimination Programme, 2014) with IRS pilot studies in three local governments in Lagos which has recently been scaled up. In Oyo state, however, extensive programmatic malaria vector control activities has not been implemented. This might have had a profound effect on the selection of *Anopheles* populations capable of withstanding doses of the insecticides as reflected in the insecticide susceptibility data in this study. The resistance data follows the same trend as the genetic differentiation index (F_{ST}) and the corresponding chi square values. Chi square has been used as an index of determinant for insecticide selection pressure in Nigerian laboratory colony exposed to insecticides (Brooke *et al.,* 2002) but this data has not yet been verified in field collected samples. The genetic differentiation values in this study and there corresponding chi square index, indicate that insecticide resistance is maintained in most of these populations as a result of insecticide selection pressure

possibly due to extensive malaria control activities in Lagos as compared with Oyo state, Nigeria. This suggests a strong association between DDT resistance and 2La inversion polymorphism in *Anopheles gambiae s.s.* in Lagos and Oyo States. Also, the low/ non-significant value of chi square across all the populations indicate that so long as the factor that is responsible for the selection of resistance is present, genetic population differentiation will increase to a stage where it becomes significant with chi square. According to chi square, if populations are within chi square estimates, the factor that is selecting population differentiation will disappear after one generation. This means that if the factor selecting resistance in the populations in this study are tackled on time, the genetic differentiation occurring within the populations will disappear after a single generation.

Inversion 2La shows strong association with climate (Coluzzi *et al.*, 1979), resistance to dieldrin/fipronil (Brooke *et al.*, 2000, 2002) and thermal tolerance (Rocca *et al.*, 2009) with resistance to drought (Gray *et. al.*, 2009). This has helped *Anopheles gambiae* to invade and adapt to most ecosystems (Coluzzi *et al.*, 2002), hence transmission. The introduction of PCR into the detection of 2La inversion opened up a new era into the karyotyping of this inversion (White *et al.*, 2007). Ng'habi *et al.*, (2008) published an article on the clarification of anomalies in the application of 2La inversion and discovered certain bands that were sequenced and aligned to the region. This study identified a PCR fragment that was not consistent with previous studies. These fragments were detected in the industrialised/urbanised state (Lagos State). The sequencing of these fragment and subsequent BLAST on NCBI gave the "triacylglycerol lipase (TAG)" in which there has been no previous information on its involvement in resistance in *Anopheles gambiae s.s.*

Triacylglycerol lipase is found to play a major role in organisms found in industrialised areas causing the condition obesity which results from an abnormal increase in white adipose tissue mass (in form of triacylglycerides), and in humans is thought to be caused by a complex array of genetic, environmental, and hormonal factors (Jenkins *et. al.*, 2004). Triacylglycerol/fatty acid recycling is an important mechanism by which adipocytes modulate fatty acyl flux in response to changing metabolic conditions. The

TAG metabolic cycle encompasses both *de novo* triacylglycerol synthesis, which is thought to me mediated primarily through the concerted activities of glycolytic/ glyceroneogenic enzymes, acyl CoA dependent acyltransferases, and phosphatidic acid phosphatases, and TAG hydrolysis catalyzed by triacylglycerol lipases (Jenkins *et al.*, 2004).

In *Anopheles* mosquitoes however, the synthesis and hydrolysis of lipids has been linked to the presence of alternate 2La inversions. By weighing the dry carcass of mosquitoes, studies have revealed that 2La+ females boosted their lipid stores while 2La females elevated there glycogen content during drought resistance (Gray *et. al.*, 2009). This study identified one of the enzymes involved in lipid hydrolysis in *Anopheles gambiae s.s.* However, this data still needs further verification with genotypic association studies.

There is only one published report on microsatellite polymorphism in *Anopheles gambiae s.s.* populations in Nigeria (Onyabe and Conn, 2001). The work examined microsatellites present on the three chromosomes of mosquitoes and report that microsatellites on chromosome 2 are the ones mainly responsible for most of the genetic differentiations among *Anopheles gambiae* populations in Nigeria. This informed the selection of microsatellites mainly on chromosome 2 for this study.

Six loci, AG2H26, AG2H175, AG2H590, AG2H637, AG2H772 and AG2H143 were responsible for all the genetic differentiation in this study. AG2H26 is located within inversion 2Rb while three of the microsatellites (AG2H637, AG2H772 and AG2H143) are located within inversion 2La. Like the microsatellite loci, the frequencies of these inversions varies clinally from North to South in Nigeria (Coluzzi *et al.*, 1979, 1985). Removal of these six loci from the data- set resulted in low or insignificant estimate of differentiation even between localities. The preceding observation is that gene flow is extensive across the *Anopheles* populations in Lagos and Oyo State but that selection on genes located within some inversions on chromosome II counters the homogenizing effect of gene flow. It is likely that the six microsatellite loci above merely hitch- hike on nearby genes that are under insecticide selection pressure and can capture certain genes that are important to the survival or adaptation of the mosquitoes to there ever

changing environment. This is a major factor that was considered during the selection of these microsatellites. Also, the three significantly polymorphic microsatellites that are located within inversion 2La have been mapped to a locus that is close to the *kdr* gene (also called "knock down resistant gene) (Ranson *et al.,* 2000; 2004). This is a very important gene in the development of resistance to pyrethroids and cross resistance to DDT. This study did not screen for *kdr* gene mutations in the populations as the microsatellite loci close to this gene has provided the information and this suggests that the *kdr* frequency will be high in the populations studied.

Moreover, local selection which probably results in adaptation to ecological zones (Coluzzi *et al.,* 1979) can result in differentiation by reducing survival and fecundity of immigrants. If, for example, an immigrant does not carry a particular inversion, it may experience reduced survival and reproduction (Onyabe and Conn, 2001). The extensive genetic exchange measured by parts of the genome that are located outside inversion suggests that migrants survive and reproduce. Hence, there is probably recombination among regions outside inversions such that inversion heterozygous offspring give rise to a mixture of gametes but only zygotes that possess the inversion survive and reproduce. Though, microsatellites were not selected on other chromosomes in this study, but reports have indicated the importance of polymorphic microsatellites on chromosome II in the genetic differentiation of *Anopheles* populations in Nigeria (Onyabe and Conn, 2001). This study is hence, in agreement with Lanzaro *et al.* (1998) and Onyabe and Conn (2001) who both concluded that selection on genes located on chromosome II, but not on other chromosomes, is responsible for genetic differentiation between Bamako and Mopti form in Mali and between North to South clines of *Anopheles gambiae s.s.* in Nigeria respectively.

On the basis of simulation results, Gaggiotti *et al.*,(1999) suggested that for most typical sample sizes and genetic parameters encountered in experimental studies, F_{ST} should be preferred over R_{ST} to estimate gene flow parameters with microsatellites because it generally gave a lower mean square error of Nm estimates. A similar study (Balloux and Goudet, 2002) showed that F_{ST} is more efficient in the case of high levels of gene flow whereas R_{ST} better reflects population differentiation under low gene

flow. Comparing F_{ST} and R_{ST} values computed on the same data can provide valuable insights into the main causes of population differentiation, i.e., drift *vs* mutation because these statistics share equal expectations when differentiation is caused solely by drift, whereas R_{ST} is expected to be larger than F_{ST} under contribution of Stepwise-like mutations (Balloux and Lugon-moulin, 2002).

In this study, F_{ST} and R_{ST} values indicate higher R_{ST} values as compared with F_{ST} data on three microsatellite loci AG2H79, AG2H590 and AG2H772. Earlier studies have identified locus AG2H79 as one of the locus responsible for differentiation in *Anopheles gambiae* across Nigeria (Onyabe and Conn, 2001). However, locus AG2H772 and AG2H590 are both located within inversion 2La and responsible for most of the differentiation in this study. Furthermore, AG2H590 locus is close to the rtd2 gene (resistance to DDT gene) that is responsible for resistance to DDT (Ranson *et al.*, 2000). These microsatellites can have profound effects on genetic differentiation with degree of resistance as an $R_{ST} > F_{ST}$ follows the stepwise mutation model which can explain the association between increase in genetic differentiations of the 2La heterokaryotypes and physiological increase in resistance profile in this study.

The migration rate Nm, examined for the Lagos and Oyo populations ranged from 5.94813 to 2.07774. However, both states are located in the Forest region of Nigeria and this level of gene flow exceeds the threshold ($Nm < 1$) at which substantial differentiation by genetic drift may accure (Slatkin, 1987). Hence, this finding is consistent with chromosome inversion data from Nigeria (Coluzzi *et al.*, 1979, 1985; Onyabe and Conn, 2001) which states that *An. gambiae* samples from the forest zone are virtually uniform for the standard arrangement on chromosome 2.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

This study investigate the association between insecticide resistance and the genetic mechanisms involved in the development of these resistance in *Anopheles* populations from Lagos and Oyo States, Nigeria. Resistance to the Insecticide Dichrlorodimethrytrichloroethane and Deltamethrin was established in *Anopheles* populations present in Lagos and Oyo States Nigeria with *Anopheles* populations from Lagos state having a higher resistance profile as compared with the populations of mosquitoes from Oyo state.

There is yet, no spatially- continuous map of *Anopheles* mosquitoes from the selected localities. The introduced maps in this study has however, yielded more finely resolved *Anopheles gambiae s.l.* distribution in Lagos and Oyo state. These maps provide valuable information for selective and targeted malaria vector control in Lagos and Oyo State.

This study also confirmed an association between inversion 2La and the polymorphism of six microsatellite loci with the development of resistance to DDT in *Anopheles gambiae s.s.* (M molecular form) populations from Lagos and Oyo State. It is not clear what strategy will be employed for releasing transgenic mosquitoes. Assuming a transposable element is found that is capable of germ line transformation, this study hence, reveal that the spread of the transposable element will be rapid provided the insertion is not biased towards 2La and the six microsatellites detected in this study.

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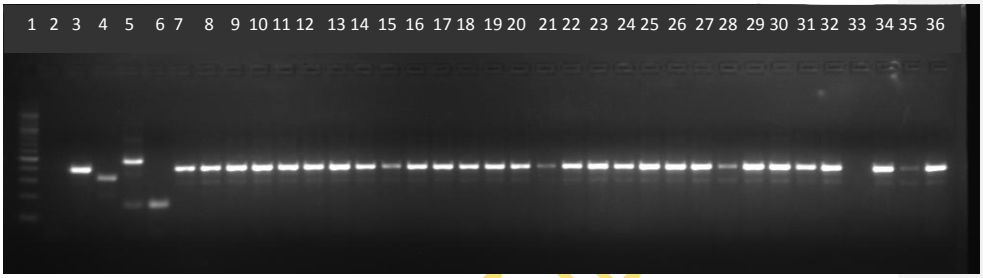
APPENDIX

APPENDIX 1: **Identification (PCR species) of the members of *Anopheles gambiae* complex in the population of mosquitoes used for the study (a few members displayed)**

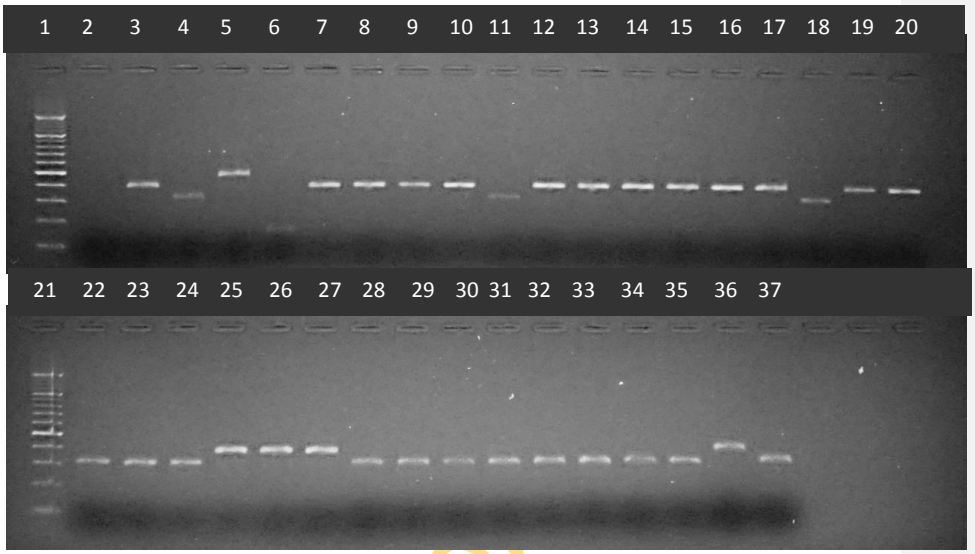
Samples from Lagos, Nigeria



Lane	Code	Identification	Lane	Code	Identification
1	Ladder	Ladder	**19**	IK 13	*An. gambiae s.s.*
2	Neg	-ve control	**20**	IK 14	*An. gambiae s.s.*
3	BOA	*An. gambiae s.s.*	**21**	IK 15	*An. gambiae s.s.*
4	KGB	*An. arabiensis*	**22**	IK 16	*An. gambiae s.s.*
5	ZAM	*An .merus*	**23**	IK 17	*An. gambiae s.s.*
6	SANGWE	*An. quadriannulatus*	**24**	IK 18	*An. gambiae s.s.*
7	IK 01	*An. gambiae s.s.*	**25**	IK 19	*An. gambiae s.s.*
8	IK 02	*An. gambiae s.s.*	**26**	IK 20	*An. gambiae s.s.*
9	IK 03	*An. gambiae s.s.*	**27**	IK 21	*An. gambiae s.s.*
10	IK 04	*An. gambiae s.s.*	**28**	IK 22	*An. gambiae s.s.*

11	IK 05	*An. gambiae s.s.*	**29**	IK 23	*An. gambiae s.s.*
12	IK 06	*An. gambiae s.s.*	**30**	IK 24	*An. gambiae s.s.*
13	IK 07	*An. gambiae s.s.*	**31**	IK 25	*An. gambiae s.s.*
14	IK 08	*An. gambiae s.s.*	**32**	IK 26	*An. gambiae s.s.*
15	IK 09	*An. gambiae s.s.*	**33**	IK 27	*No amplification*
16	IK 10	*An. gambiae s.s.*	**34**	IK 28	*An. gambiae s.s.*
17	IK 11	*An. gambiae s.s.*	**35**	IK 29	*An. gambiae s.s.*
18	IK 12	*An. gambiae s.s.*	**36**	IK 30	*An. gambiae s.s.*

Samples from Oyo State, Nigeria



Lane	Code	Identification	Lane	Code	Identification
1	Ladder	Ladder	**20**	ERW 14	*An. gambiae s.s.*
2	Neg	-ve control	**21**	Ladder	Ladder
3	BOA	*An. gambiae s.s.*	**22**	ERW 15	*An. arabiensis*
4	KGB	*An. arabiensis*	**23**	ERW 16	*An. arabiensis*
5	ZAM	*An .merus*	**24**	ERW 17	*An. arabiensis*
6	SANGWE	*An. quadriannulatus*	**25**	ERW 18	*An. gambiae s.s.*
7	ERW 01	*An. gambiae s.s.*	**26**	ERW 19	*An. gambiae s.s.*
8	ERW 02	*An. gambiae s.s.*	**27**	ERW 20	*An. gambiae s.s.*
9	ERW 03	*An. gambiae s.s.*	**28**	ERW 21	*An. arabiensis*
10	ERW 04	*An. gambiae s.s.*	**29**	ERW 22	*An. arabiensis*
11	ERW 05	*An. arabiensis*	**30**	ERW 23	*An. arabiensis*
12	ERW 06	*An. gambiae s.s.*	**31**	ERW 24	*An. arabiensis*
13	ERW 07	*An. gambiae s.s.*	**32**	ERW 25	*An. arabiensis*
14	ERW 08	*An. gambiae s.s.*	**33**	ERW 26	*An. arabiensis*

15	ERW 09	*An. gambiae s.s.*	**34**	ERW 27	*An. arabiensis*
16	ERW 10	*An. gambiae s.s.*	**35**	ERW 28	*An. arabiensis*
17	ERW 11	*An. gambiae s.s.*	**36**	ERW 29	*An. gambiae s.s.*
18	ERW 12	*An. arabiensis*	**37**	ERW 30	*An. arabiensis*
19	ERW 13	*An. gambiae s.s.*			

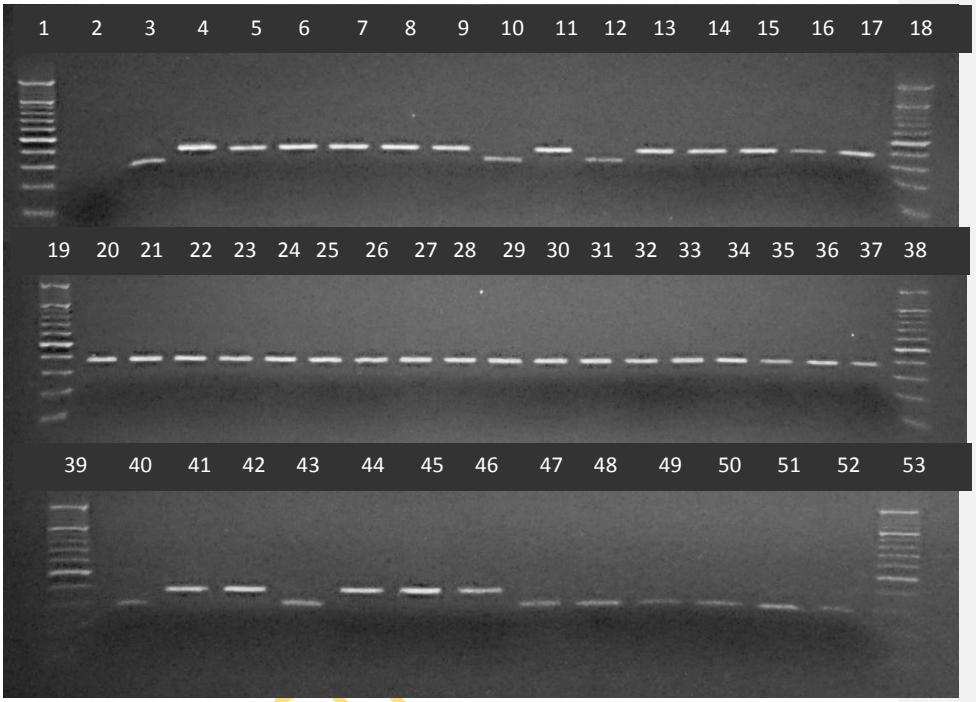
APPENDIX 2: **Documentation of the Molecular M/S form of *Anopheles gambiae s.s.* identified in the study (only a few samples presented)**

Lagos State samples

Lane	Code	Identification	Lane	Code	Identification
1	Ladder	Ladder	**19**	IK 15	M
2	Neg	-ve control	**20**	IK 16	M
3	BOA	S	**21**	IK 17	M
4	NAG	M	**22**	IK 18	M
5	IK 01	M	**23**	IK 19	M
6	IK 02	M	**24**	IK 20	M
7	IK 03	M	**25**	IK 21	M
8	IK 04	M	**26**	IK 22	M
9	IK 05	M	**27**	IK 23	M
10	IK 06	M	**28**	IK 24	M

11	IK 07	M	**29**	IK 25	M
12	IK 08	M	**30**	IK 26	M
13	IK 09	M	**31**	IK 27	No amplification
14	IK 10	M	**32**	IK 28	M
15	IK 11	M	**33**	IK 29	M
16	IK 12	M	**34**	IK 30	M
17	IK 13	M			
18	IK 14	M			

Oyo State samples



Lane	Sample Code	Molecular form	Lane	Sample Code	Molecular form	Lane	Sample Code	Molecular form
1	Ladder		**20**	OYO 19	*M*	**39**	Ladder	
2	-ve		**21**	OYO 21	*M*	**40**	ERW 04	*S*
3	BOA	*S*	**22**	OYO 22	*M*	**41**	ERW 06	*M*
4	NAG	*M*	**23**	OYO 23	*M*	**42**	ERW 07	*M*
5	OYO 01	*M*	**24**	OYO 24	*M*	**43**	ERW 08	*S*
6	OYO	*M*	**25**	OYO	*M*	**44**	ERW	*M*

	02			26			09	
7	OYO 03	*M*	**26**	OYO 28	*M*	**45**	ERW 10	*M*
8	OYO 04	*M*	**27**	OYO 30	*M*	**46**	ERW 11	*M*
9	OYO 05	*M*	**28**	IWO 02	*M*	**47**	ERW 13	*S*
10	OYO 06	*S*	**29**	IWO 08	*M*	**48**	ERW 14	*S*
11	OYO 07	*M*	**30**	IWO 09	*M*	**49**	ERW 18	*S*
12	OYO 08	*S*	**31**	IWO 12	*M*	**50**	ERW 19	*S*
13	OYO 10	*M*	**32**	IWO 21	*M*	**51**	ERW 20	*S*
14	OYO 11	*M*	**33**	IWO 28	*M*	**52**	ERW 29	*S*
15	OYO 13	*M*	**34**	IWO 30	*M*	**53**	Ladder	
16	OYO 16	*M*	**35**	ERW 01	*M*			
17	OYO 17	*M*	**36**	ERW 02	*M*			
18	Ladder		**37**	ERW 03	*M*			
19	Ladder		**38**	Ladder				

APPENDIX 3: **Statistical comparison of DDT and Deltamethrin susceptibility data between Lagos and Oyo State populations using student t-test**

Paired Samples Test

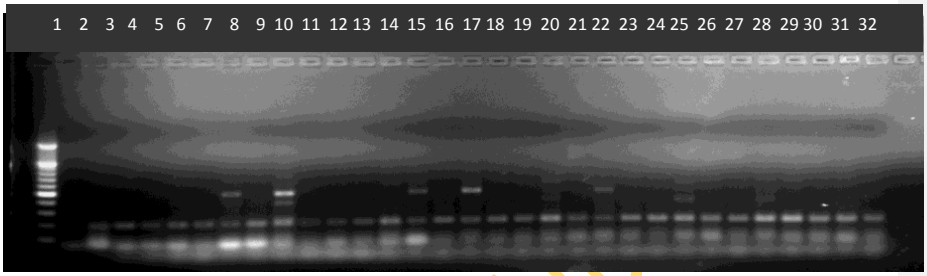
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	DDTresistanceLagos - DDTresistanceOyo	-47.80000	36.25493	14.80101	-85.84722	-9.75278	-3.230	5	.023

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	DelthresistanceLagos - DelthresistanceOyo	-16.50000	19.57079	7.98974	-37.03829	4.03829	-2.065	5	.094

APPENDIX 4: **Polymerase chain reaction (PCR) of inversion 2La karyotypes of the Lagos and Oyo State samples (not all samples presented)**

Lagos State samples

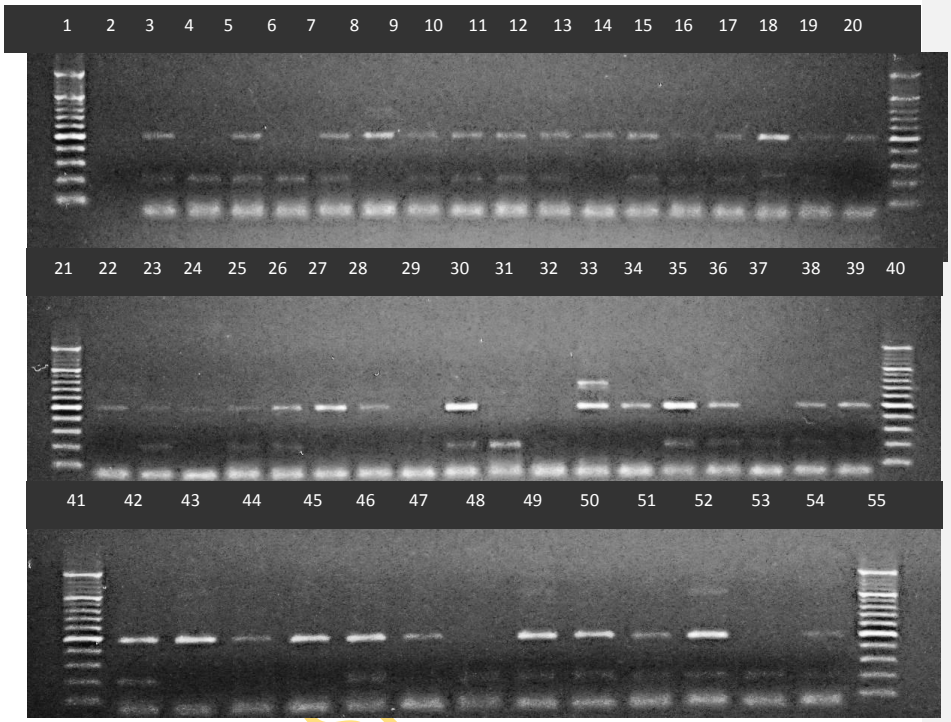


Lane	Code	Identification	Lane	Code	Identification
1	Ladder	Ladder	**19**	BG 17	$2La^{+}/2La^{+}$
2	Neg	-ve control	**20**	BG 18	$2La/2La^{+}$
3	BG 01	$2La^{+}/2La^{+}$	**21**	BG 19	$2La^{+}/2La^{+}$
4	BG 02	$2La^{+}/2La^{+}$	**22**	BG 20	$2La/2La^{+}$
5	BG 03	$2La^{+}/2La^{+}$	**23**	BG 21	$2La^{+}/2La^{+}$
6	BG 04	$2La^{+}/2La^{+}$	**24**	BG 22	$2La^{+}/2La^{+}$
7	BG 05	$2La^{+}/2La^{+}$	**25**	BG 23	$*2La/2La^{+}$
8	BG 06	$2La/2La^{+}$	**26**	BG 24	$2La^{+}/2La^{+}$
9	BG 07	$2La^{+}/2La^{+}$	**27**	BG 25	$2La^{+}/2La^{+}$
10	BG 08	$*2La/2La^{+}$	**28**	BG26	$*2La/2La^{+}$
11	BG 09	$2La^{+}/2La^{+}$	**29**	BG 27	$2La^{+}/2La^{+}$

12	BG 10	$2La^{+}/2La^{+}$	**30**	BG 28	$2La^{+}/2La^{+}$
13	BG 11	$2La^{+}/2La^{+}$	**31**	BG 29	$2La^{+}/2La^{+}$
14	BG 12	$2La^{+}/2La^{+}$	**32**	BG 30	$2La^{+}/2La^{+}$
15	BG 13	$2La/2La^{+}$	**33**		
16	BG 14	$2La^{+}/2La^{+}$	**34**		
17	BG 15	$2La/2La^{+}$			
18	BG 16	$2La^{+}/2La^{+}$			

* Anomalous band detected within Lagos Populations (In well 10, the additional 361 base pair band indicate the unusual band)

Oyo State Samples



Lane	Code	Identification	Lane	Code	Identification	Lane	Code	Identification
1	Ladder	Ladder	**20**	Ladder	Ladder	**39**	OYO 04	$2La/2La^{+}$
2	Neg	-ve control	**21**	Ladder	Ladder	**40**	Ladder	Ladder
3	OL 02	$2La/2La^{+}$	**22**	OL 22	2La/2La	**41**	Ladder	Ladder
4	OL 03	$2La^{+}/2La^{+}$	**23**	OL 23	$2La/2La^{+}$	**42**	OYO 05	$2La/2La^{+}$
5	OL 04	$2La/2La^{+}$	**24**	OL 24	2La/2La	**43**	OYO	2La/2La

							06	
6	OL 05	$2La^{+}/2La^{+}$	**25**	OL 25	$2La/2La^{+}$	**44**	OYO 07	2La/2La
7	OL 06	$2La/2La^{+}$	**26**	OL 26	$2La/2La^{+}$	**45**	OYO 08	2La/2La
8	OL 07	2La/2La	**27**	OL 27	2La/2La	**46**	OYO 10	$2La/2La^{+}$
9	OL 08	$2La/2La^{+}$	**28**	OL 28	2La/2La	**47**	OYO 11	2La/2La
10	OL 09	$2La/2La^{+}$	**29**	OL 29	$2La^{+}/2La^{+}$	**48**	OYO 13	$2La^{+}/2La^{+}$
11	OL 10	$2La/2La^{+}$	**30**	OL 30	$2La/2La^{+}$	**49**	OYO 16	$2La/2La^{+}$
12	OL 11	$2La/2La^{+}$	**31**	BJ 05	$2La^{+}/2La^{+}$	**50**	OYO 17	$2La/2La^{+}$
13	OL 13	2La/2La	**32**	BJ 12	$2La^{+}/2La^{+}$	**51**	OYO 19	$2La/2La^{+}$
14	OL 14	$2La/2La^{+}$	**33**	BJ 16	$2La/2La^{+}$	**52**	OYO 21	$2La/2La^{+}$
15	OL 15	$2La/2La^{+}$	**34**	BJ 23	2La/2La	**53**	OYO 22	$2La^{+}/2La^{+}$
16	OL 16	$2La/2La^{+}$	**35**	BJ 26	$2La/2La^{+}$	**54**	OYO 23	$2La/2La^{+}$
17	OL 18	$2La/2La^{+}$	**36**	OYO	$2La/2La^{+}$	**55**	Ladder	Ladder

				01				
18	OL 19	$2La/2La^{+}$	**37**	OYO 02	$2La^{+}/2La^{+}$			
19	OL 20	$2La/2La^{+}$	**38**	OYO 03	$2La/2La^{+}$			

APPENDIX 5: Base sequence and the corresponding protein alignment of the gel extracted anomalous 2La band

	Sequence size: 361bp
Base sequence	**ATGGAGCAGGTGCACAAACTGACTCAACCGAACCGACTTTCTTAAG TAAAGTGAGATAGAGCGAGAGAGAGCTACAAAAGTTAAGTTGTATA TTTATTTCGAGAAAGAAAATATGGCTTACAAAACAATACTTGGCACT TGAGGGATGTTTGTGAGATAAGAATGTTCTGACGGCTTAACAATAG GTGTATAAAAGCTGCGTGCCCTATGCATTGCAAGGCACTGGAGGTT CGTACGATAGAGAGAGCATGTAGAGTGTAATAATCTCGCTAGAGAG GCACGTTGTTGCTAAAAGTCTTCTTTTGTGTGCGCTTTCCTTCGTTT CGGGTTTCGTTCACAAGAGCCATGGTGTAAAAAAAAC**
Protein alignment	1 mlircgklfr prsalvvitv llltlrpasa dgglfdnfis qlmttaataq nfledaydqr 61 qgrgtepppl aevpsvsaep lspvlipvgs idlsdhqpai psappttfat gtttststtt 121 ttttttstth gtraplpfwn pfvwlrpkep sipynpdtdl stpeiavrhg yqaeshtlkt 181 adgylltlhr lpcgrigcta qggkgtgqpv flqhgllsss adwllsgpek alafiladag 241 ydvwlgnarg ntysrkhvsf ssdetafwdf swhemamydi paeidylynm rerndttrnl 301 lyvghsmgtt mifallasrp eynerleavf alapvafmgh vkspirllap fshdiefmpq 361 nkiirylaky gcelteaeky icentvfvlc gfdkeqynat lmpvifghtp agtstktvvh 421 yaqeihnegn fqlfdygese nqrrygrasp pgynlenist pialfyannd wlagpkdvan 481 lfnqlhrtsi gmfkipndnf nhvdflwgnd apevvykqll mlmqryk

Note: Results as obtained from NCBI through BLAST (http://www.ncbi.nlm.nih.gov/protein/333470113)

APENDIX 6: Aligned 2La unusual band base sequence information as generated from NCBI

AGAP001652-PA [Anopheles gambiae str. PEST]

GenBank: EAA00922.5

LOCUS EAA00922 527 aa linear INV 20-MAY-2011
DEFINITION AGAP001652-PA [Anopheles gambiae str. PEST].
ACCESSION EAA00922
VERSION EAA00922.5 GI:333470113
DBSOURCE accession AAAB01008987.1
KEYWORDS .
SOURCE Anopheles gambiae str. PEST
ORGANISM Anopheles gambiae str. PEST
Eukaryota; Metazoa; Ecdysozoa; Arthropoda; Hexapoda; Insecta;
Pterygota; Neoptera; Endopterygota; Diptera; Nematocera;
Culicoidea; Culicidae; Anophelinae; Anopheles.
COMMENT On May 19, 2011 this sequence version replaced gi:157012647.
Method: conceptual translation.
FEATURES Location/Qualifiers
source 1..527
/organism="Anopheles gambiae str. PEST"
/strain="PEST"
/db_xref="taxon:180454"
/chromosome="2R"
/note="component of assembly AgamP3"
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Region 162..227
/region_name="Abhydro_lipase"

/note="Partial alpha/beta-hydrolase lipase region;
pfam04083"
/db_xref="CDD:202881"

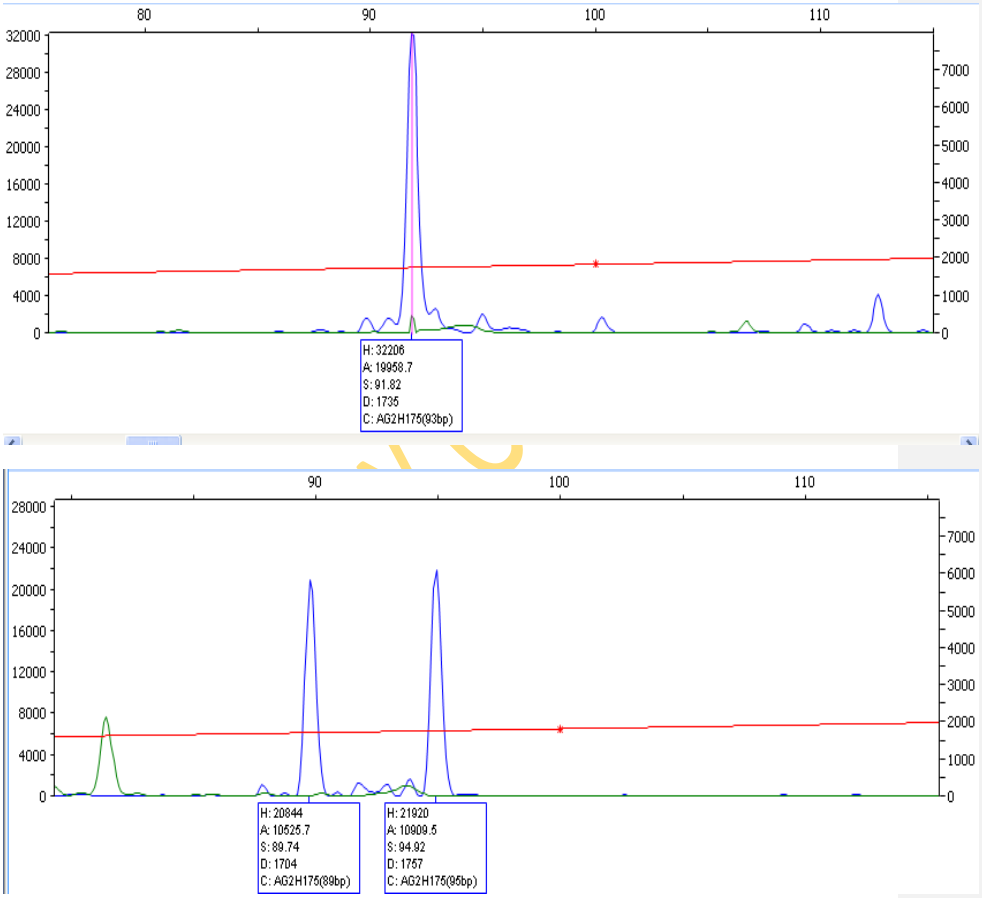
Region 170..520
/region_name="PLN02872"
/note="triacylglycerol lipase"
/db_xref="CDD:166513"

CDS 1..527
/locus_tag="AgaP_AGAP001652"
/old_locus_tag="AgaP_ENSANGG00000019664"
/old_locus_tag="ENSANGG00000019664"
/coded_by="join(AAAB01008987.1:8905343..8905828,
AAAB01008987.1:8909198..8909585,
AAAB01008987.1:8909679..8909872,
AAAB01008987.1:8910889..8911404)"
/note="AGAP001652-PA encoded by AGAP001652-RA"
/db_xref="VectorBase:AGAP001652-PA"
/db_xref="VectorBase:AGAP001652"

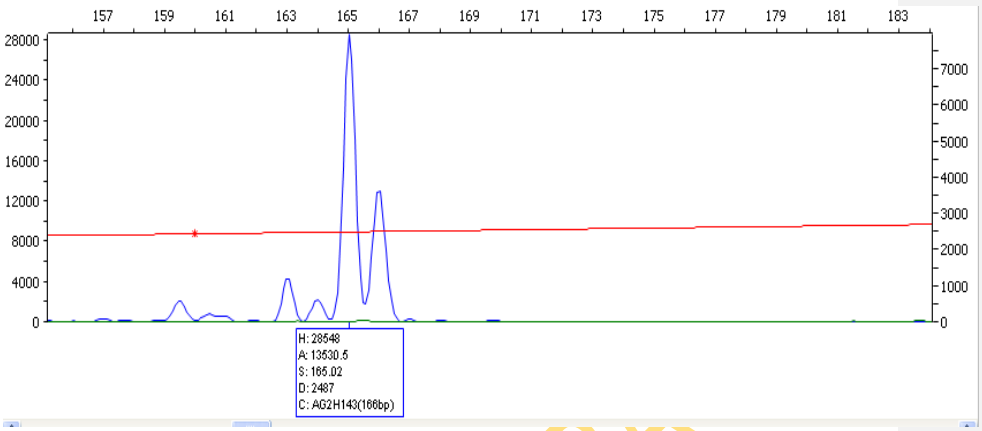
Results as obtained from NCBI through BLAST
(http://www.ncbi.nlm.nih.gov/protein/333470113)

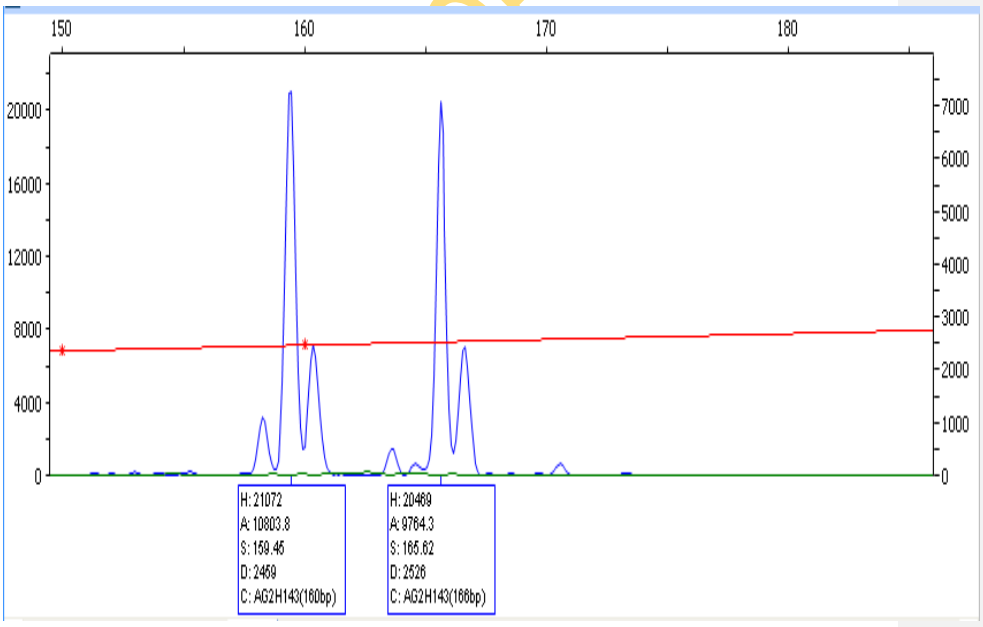
APPENDIX 7: **Idiogram of the ten microsatellite loci examined (Homozygous and Heterozygous sequence graphs) as presented from Peak Scanner v1.0 software**

AG2H175

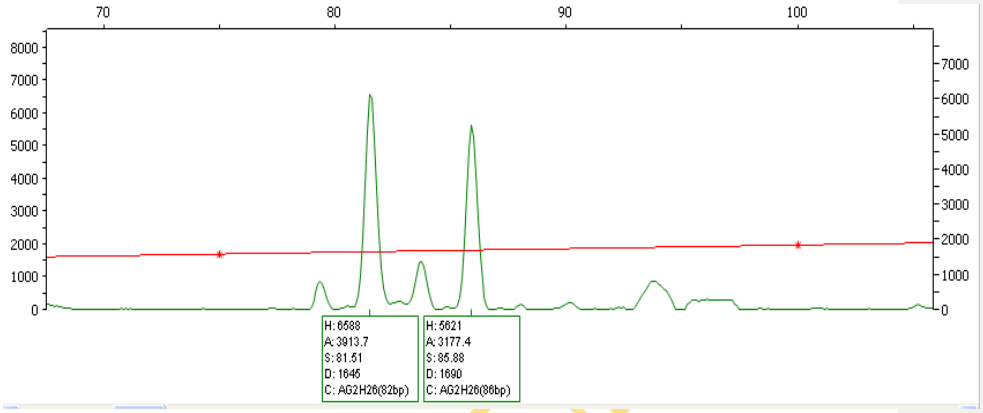


AG2H143


157 159 161 163 165 167 169 171 173 175 177 179 181 183
28000 24000 20000 16000 12000 8000 4000 0
7000 6000 5000 4000 3000 2000 1000 0
H: 28548
A: 13530.5
S: 165.02
D: 2487
C: AG2H143(166bp)


150 160 170 180
20000 16000 12000 8000 4000 0
7000 6000 5000 4000 3000 2000 1000 0
H: 21072
A: 10803.8
S: 159.45
D: 2469
C: AG2H143(160bp)
H: 20469
A: 9764.3
S: 165.62
D: 2526
C: AG2H143(166bp)

AG2H26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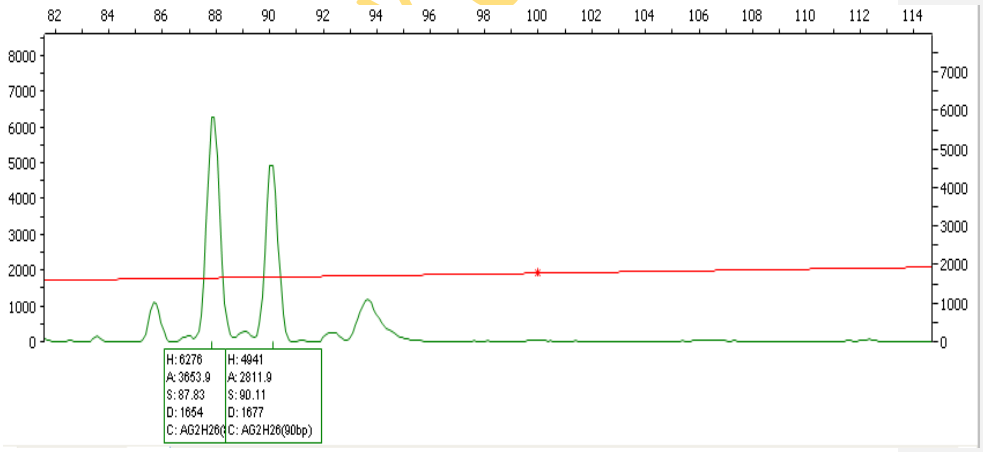
8000
7000
6000
5000
4000
3000
2000
1000
0
H: 6588
A: 3913.7
S: 81.51
D: 1645
C: AG2H26(82bp)
H: 5621
A: 3177.4
S: 85.88
D: 1690
C: AG2H26(86bp)


82
84
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88
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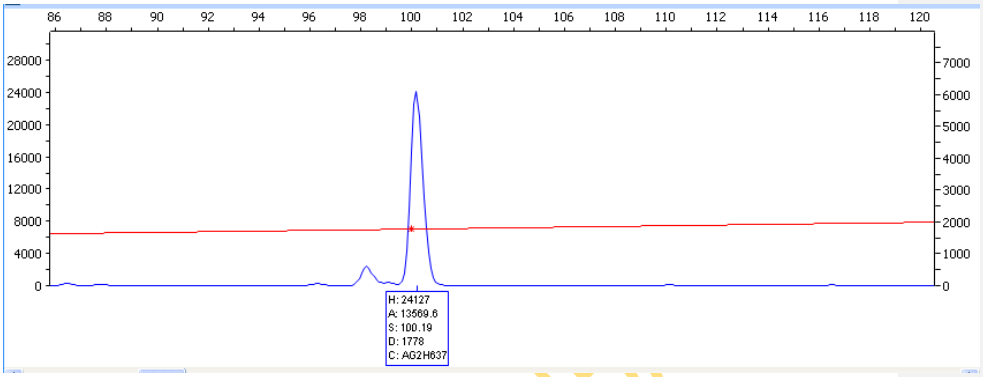
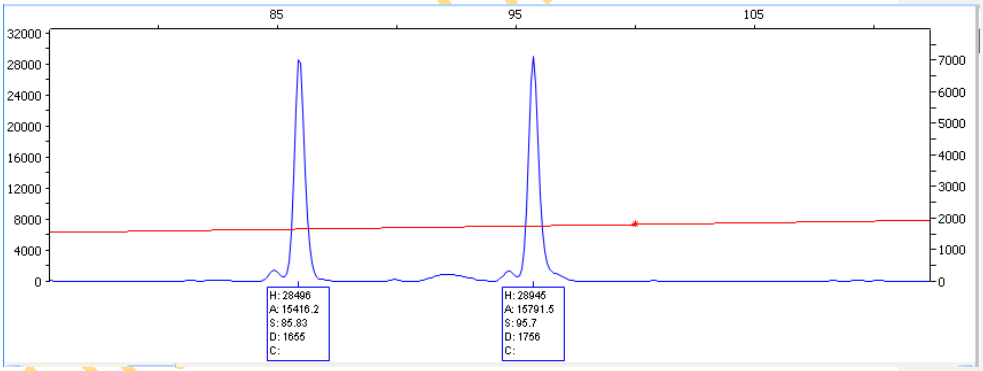
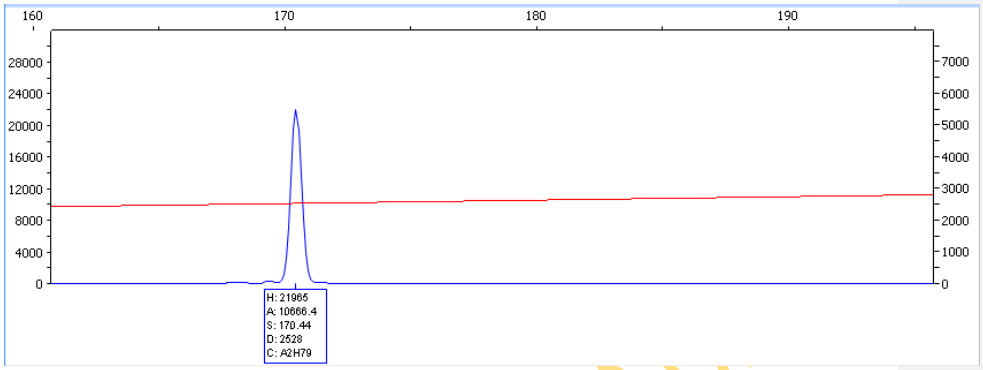
92
94
96
98
100
102
104
106
108
110
112
114
8000
7000
6000
5000
4000
3000
2000
1000
0
H: 6276
A: 3653.9
S: 87.83
D: 1654
H: 4941
A: 2811.9
S: 90.11
D: 1677
C: AG2H26(90bp)

AG2H637


86 88 90 92 94 96 98 100 102 104 106 108 110 112 114 116 118 120
28000 24000 20000 16000 12000 8000 4000 0
7000 6000 5000 4000 3000 2000 1000 0
H: 24127
A: 13569.6
S: 100.19
D: 1778
C: AG2H637


85 95 105
32000 28000 24000 20000 16000 12000 8000 4000 0
7000 6000 5000 4000 3000 2000 1000 0
H: 28496
A: 15416.2
S: 85.83
D: 1656
C:
H: 28945
A: 15791.5
S: 95.7
D: 1756
C:

AG2H79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180
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3000
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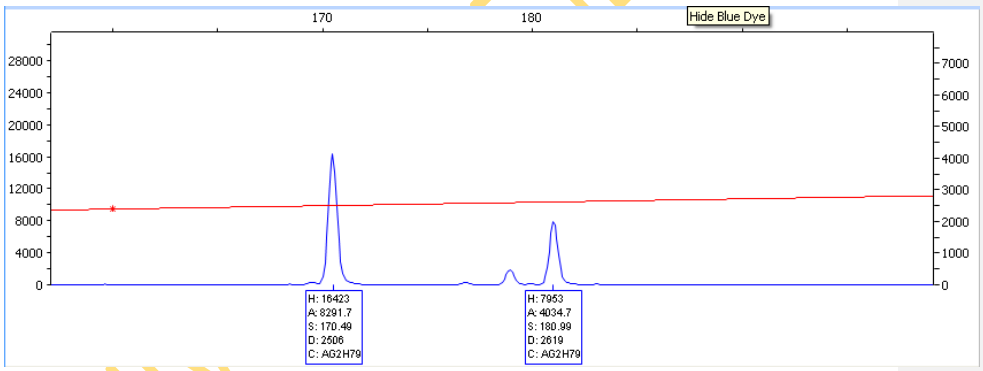
A: 10666.4
S: 170.44
D: 2528
C: A2H79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Hide Blue Dye
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24000
20000
16000
12000
8000
4000
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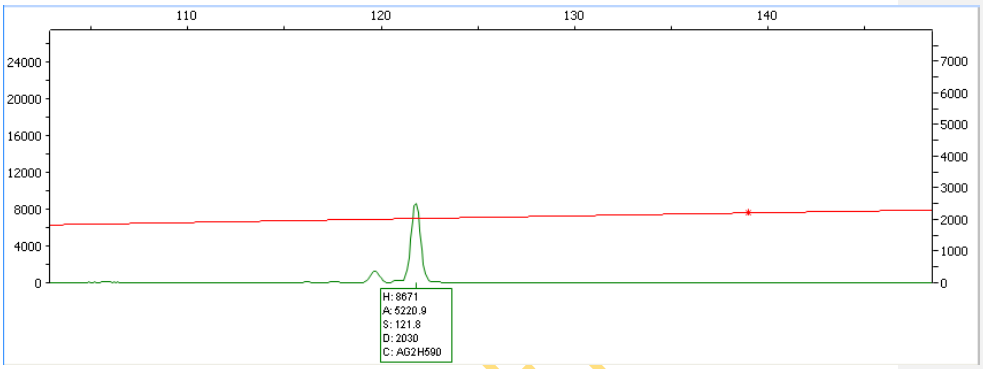
7000
6000
5000
4000
3000
2000
1000
0
H: 16423
A: 8291.7
S: 170.49
D: 2506
C: AG2H79
H: 7953
A: 4034.7
S: 180.99
D: 2619
C: AG2H79

AG2H590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24000
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12000
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2000
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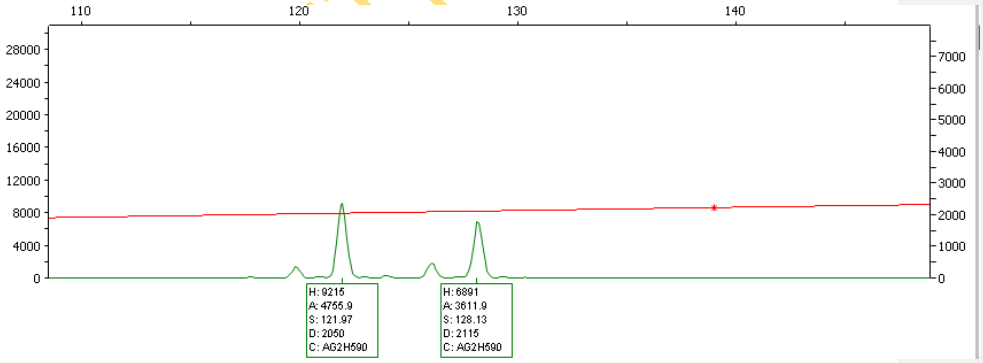
H: 8671
A: 5220.9
S: 121.8
D: 2030
C: AG2H590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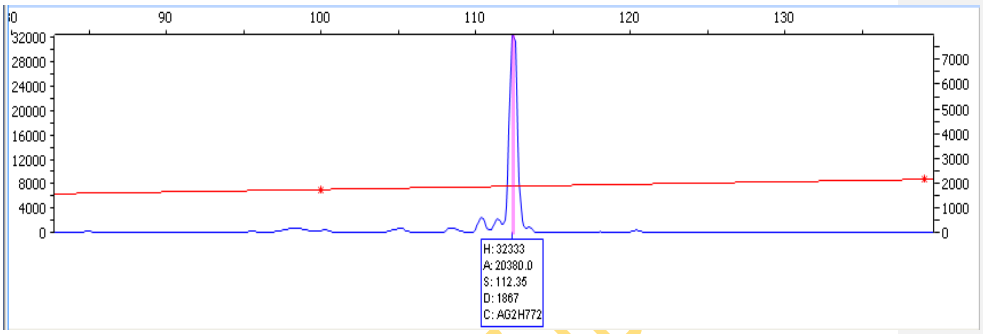
H: 9215
A: 4755.9
S: 121.97
D: 2050
C: AG2H590
H: 6891
A: 3611.9
S: 128.13
D: 2115
C: AG2H590

AG2H772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4000
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2000
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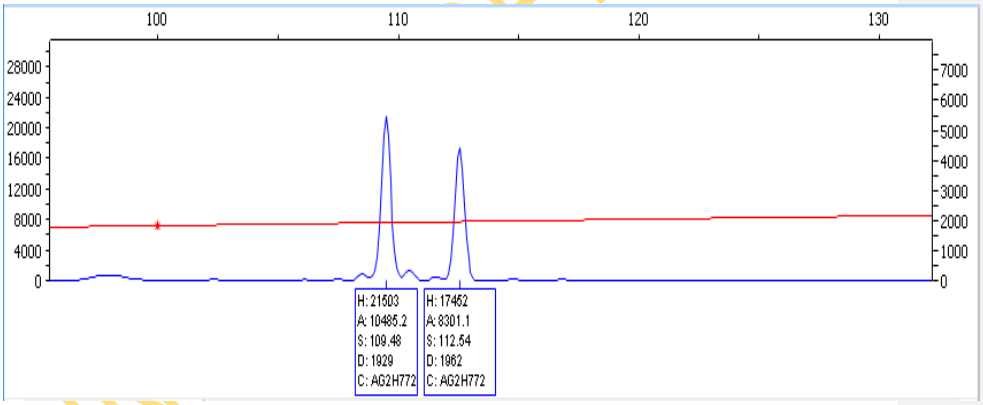
H: 32333
A: 20380.0
S: 112.35
D: 1867
C: AG2H772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3000
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1000
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H: 21503
A: 10485.2
S: 109.48
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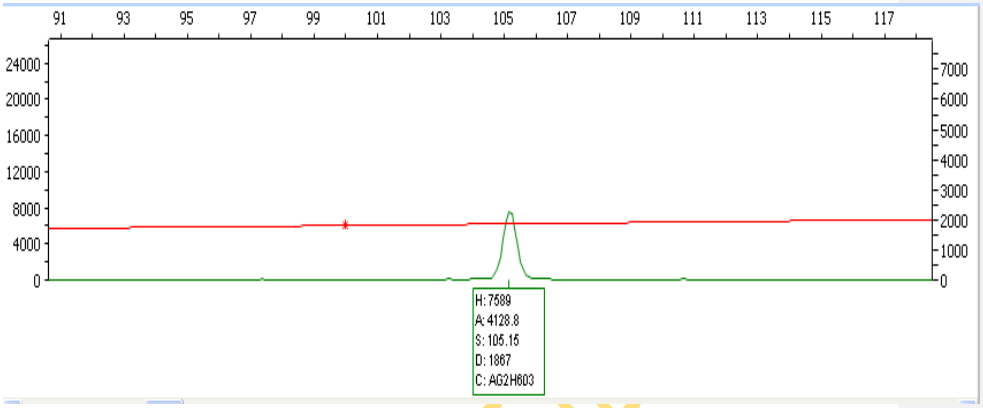
C: AG2H772
H: 17462
A: 8301.1
S: 112.54
D: 1962
C: AG2H772

AG2H603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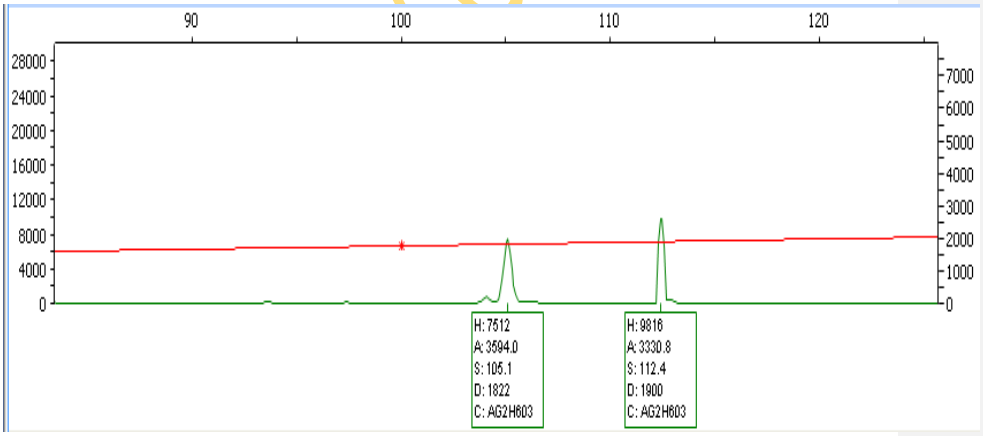
7000 6000 5000 4000 3000 2000 1000 0
H: 7589
A: 4128.8
S: 105.15
D: 1867
C: AG2H603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7000 6000 5000 4000 3000 2000 1000 0
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A: 3694.0
S: 105.1
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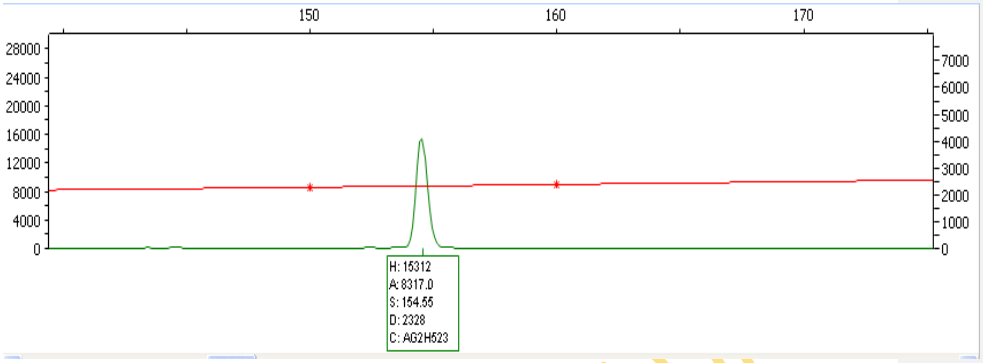
C: AG2H603
H: 9816
A: 3330.8
S: 112.4
D: 1900
C: AG2H603

AG2H523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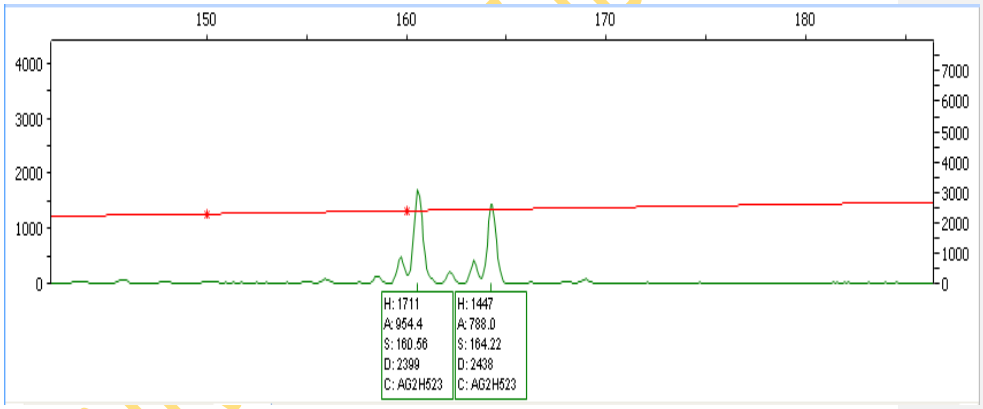
D: 2328
C: AG2H523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A: 954.4
S: 160.56
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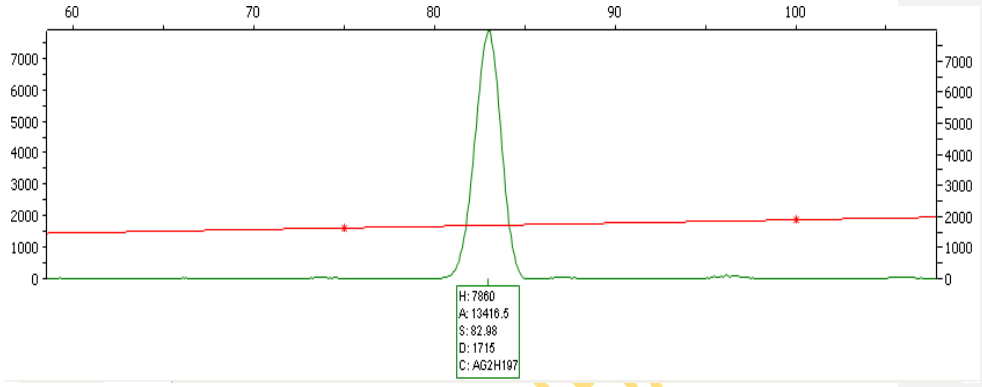
C: AG2H523
H: 1447
A: 788.0
S: 164.22
D: 2438
C: AG2H523

AG2H197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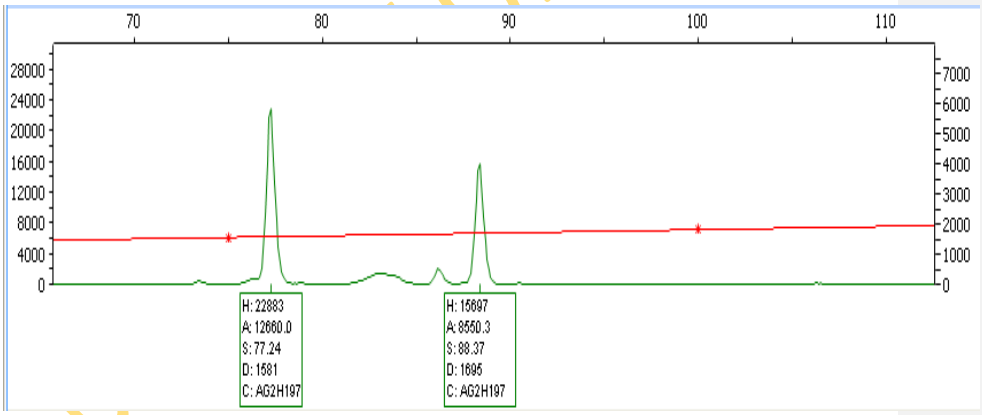
C: AG2H197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1000
0
H: 22883
A: 12660.0
S: 77.24
D: 1581
C: AG2H197
H: 15697
A: 8550.3
S: 88.37
D: 1695
C: AG2H197

APPENDIX 8: **Microsatellite data generated after analysis with Peak Scanner version 1.0 software**

Lagos State Samples

Colum 1 = **Sample names**

Columns 2 to 11 = **Microsatellite loci (AG2H175, AG2H143, AG2H26, AG2H637, AG2H79, AG2H590, AG2H772, AG2H603, AG2H523 and AG2H197)**

Sample codes: **Ajah (AJ), Lekki (LK), Ikorodu (IK), Badagry (BG), Magodo (MG), and Yaba (YB)**

AJ01	93	166	92	95	171	125	112	105	154	87
	93	166	166	105	173	127	114	109	162	89
AJ02	91	162	86	97	173	123	112	105	154	91
	93	166	162	101	173	131	116	117	154	93
AJ03	95	158	80	103	171	129	110	109	162	83
	97	166	82	103	197	131	120	109	178	97
AJ04	95	158	86	109	171	131	112	109	154	87
	99	160	90	109	171	131	120	109	156	103
AJ05	87	158	82	103	173	123	110	105	162	87
	93	164	90	105	173	129	110	111	166	89
AJ06	93	158	82	101	199	121	110	107	154	89
	93	160	86	109	205	121	110	109	156	93

AJ07	87	158	82	101	173	121	112	105	188	81
	99	160	88	101	205	131	114	107	202	83
AJ08	93	158	82	109	159	113	98	105	0	81
	95	160	88	109	171	133	112	109	0	93
AJ09	91	158	86	109	173	121	114	105	162	89
	97	160	88	109	173	121	114	109	174	89
AJ10	93	160	86	103	173	129	110	105	164	81
	95	162	118	103	211	131	112	105	190	93
AJ11	89	158	102	101	171	131	110	105	160	87
	91	160	104	101	199	133	112	109	164	91
AJ12	91	158	82	93	173	129	112	105	158	87
	95	166	86	101	211	131	114	105	166	99
AJ13	93	160	92	112	171	105	110	105	162	81
	93	160	160	114	197	127	112	117	162	91
AJ14	91	160	82	99	171	127	112	105	170	81
	93	166	108	101	173	131	112	113	170	91
AJ15	89	160	82	97	171	121	110	109	160	87
	95	160	82	101	207	123	110	109	162	87
AJ16	91	160	86	101	175	123	112	105	162	81
	95	168	106	101	209	127	124	107	164	89
AJ17	91	158	86	103	205	127	110	107	154	81

	91	160	88	109	209	131	110	109	154	89
AJ18	91	158	88	101	173	105	112	105	162	77
	95	162	90	105	199	105	114	109	162	85
AJ19	93	158	86	95	171	125	112	105	154	81
	93	160	92	109	211	129	114	105	156	89
AJ20	91	158	82	101	171	125	112	109	160	83
	93	166	88	101	209	131	112	113	190	89
AJ21	91	160	84	95	173	129	112	131	154	83
	93	160	94	101	199	131	114	131	160	89
AJ22	85	158	82	101	197	113	112	105	154	89
	93	160	118	101	199	127	114	105	162	91
AJ23	91	160	82	105	171	113	110	105	158	87
	93	160	86	109	171	121	114	109	162	87
AJ24	85	160	128	111	171	127	112	105	158	81
	93	172	130	113	219	157	114	105	166	85
AJ25	93	172	86	97	203	121	110	105	162	91
	97	174	94	101	207	127	112	105	162	93
BG01	89	158	82	95	161	129	112	105	156	81
	95	162	86	95	161	131	112	105	188	97
BG02	85	158	88	101	171	121	112	105	154	85
	95	162	156	101	201	129	122	107	154	87

BG03	85	158	82	101	171	129	112	109	158	85
	95	162	84	105	201	129	114	109	148	85
BG04	89	158	82	101	171	129	112	105	154	85
	95	162	86	101	171	129	114	105	194	91
BG05	93	160	82	101	171	127	112	105	158	85
	95	162	86	109	179	129	114	109	158	91
BG06	93	160	96	101	171	129	112	105	160	85
	97	162	158	101	173	131	112	109	194	91
BG07	93	162	82	101	203	121	112	105	160	81
	93	162	86	101	205	129	112	109	160	87
BG08	95	158	82	95	171	121	112	105	154	85
	95	162	86	111	173	131	114	105	154	87
BG09	85	162	82	101	171	121	112	105	158	77
	95	162	86	101	203	125	120	105	158	91
BG10	93	160	82	95	171	123	112	105	158	89
	95	162	161	101	201	129	114	105	158	91
BG11	85	158	84	97	171	121	112	105	158	89
	95	160	160	111	173	125	120	105	162	91
BG12	85	160	84	95	171	121	110	105	164	81
	95	162	162	101	201	127	114	105	164	87
BG13	93	158	84	93	161	121	110	105	160	85

	95	160	160	95	171	127	112	107	188	93
BG14	93	160	84	101	171	123	112	105	160	85
	93	162	154	101	201	127	112	107	160	93
BG15	89	160	86	97	171	129	112	105	160	85
	89	162	86	101	173	131	112	107	160	87
BG16	89	162	84	101	171	121	112	105	154	85
	91	166	88	101	171	127	112	105	154	87
BG17	91	160	88	101	173	131	112	105	160	85
	91	162	154	101	205	133	112	105	160	91
BG18	89	162	86	101	171	121	110	105	158	81
	91	166	166	107	199	129	122	105	158	91
BG19	93	162	158	101	171	121	112	105	158	81
	95	162	162	101	227	129	114	105	158	91
BG20	95	160	84	101	171	127	110	105	164	81
	95	162	86	109	199	129	112	105	164	87
BG21	93	158	88	101	171	121	112	105	158	81
	95	160	156	101	201	121	112	107	158	85
BG22	93	158	156	95	171	127	110	105	154	87
	95	160	158	111	199	127	112	107	160	101
BG23	89	158	94	101	171	123	112	105	158	81
	95	160	158	101	199	127	112	113	160	97

BG24	95	158	126	111	171	121	110	109	158	81
	95	162	154	115	201	127	110	109	158	91
BG25	93	160	94	97	171	121	112	105	154	81
	97	160	156	97	173	127	112	105	154	91
IK01	91	166	86	109	171	107	110	105	154	79
	95	166	94	115	173	131	112	107	160	87
IK02	91	158	84	103	171	129	114	105	154	89
	93	160	86	109	173	131	118	121	154	91
IK03	91	160	84	97	171	121	98	107	158	87
	93	166	86	97	197	127	114	107	158	97
IK04	91	160	84	101	173	129	98	87	158	93
	93	162	86	105	197	129	98	87	158	97
IK05	89	166	82	101	171	129	114	109	154	87
	95	170	82	101	227	129	114	109	158	91
IK06	89	158	82	101	171	129	110	105	154	87
	95	160	112	105	173	133	118	105	154	91
IK07	93	158	94	101	171	129	110	105	154	81
	95	166	94	109	173	129	112	105	160	91
IK08	89	160	94	101	173	131	112	105	162	87
	93	160	94	109	177	131	132	107	162	97
IK09	93	158	88	101	171	131	112	105	162	87

	95	160	158	101	171	135	114	109	170	87
IK10	93	166	82	101	171	123	112	105	158	83
	95	168	172	103	173	129	112	107	162	87
IK11	93	160	86	103	171	131	118	105	154	81
	95	162	94	109	171	133	132	109	154	89
IK12	93	160	88	93	173	131	112	105	162	83
	93	160	92	109	221	131	114	105	162	89
IK13	91	156	84	111	177	107	110	105	154	87
	95	166	86	113	211	131	112	131	160	91
IK14	93	160	84	95	171	127	110	105	154	85
	93	164	86	101	171	127	118	109	154	91
IK15	95	160	84	101	173	129	112	105	164	87
	95	160	84	107	207	153	112	109	168	87
IK16	91	160	86	101	173	129	110	105	142	87
	93	160	102	105	207	129	112	105	156	87
IK17	89	160	86	101	173	121	110	109	154	87
	93	166	86	113	197	121	114	109	154	89
IK18	91	160	84	101	171	125	112	105	154	87
	91	162	84	103	171	131	114	107	154	97
IK19	93	160	84	101	171	123	112	105	154	87
	93	168	84	101	197	135	132	109	154	87

IK20	93	160	84	101	171	125	114	105	156	97
	93	166	86	101	203	131	132	105	156	97
IK21	93	160	86	107	171	125	112	109	154	81
	95	166	86	109	171	131	114	109	164	89
IK22	91	160	84	101	171	123	110	105	160	87
	93	160	86	101	171	129	118	105	160	91
IK23	93	160	86	103	173	121	112	105	162	81
	93	160	160	103	191	131	114	107	162	89
IK24	93	160	86	97	171	87	110	99	160	81
	95	166	86	103	197	121	112	107	160	89
IK25	93	166	84	103	171	125	110	109	154	85
	97	166	86	109	197	129	118	109	154	97
LK01	93	160	86	95	161	121	92	85	154	81
	95	160	154	95	161	121	112	109	154	85
LK02	91	160	86	103	171	119	92	85	154	81
	95	166	86	105	171	131	110	109	154	89
LK03	91	160	84	97	171	87	110	105	138	83
	93	160	86	97	171	87	120	105	154	83
LK04	95	160	86	101	173	119	108	108	154	85
	95	166	88	109	209	133	108	108	162	85
LK05	89	160	84	95	171	125	110	105	144	83

	91	160	90	101	173	125	114	105	196	85
LK06	93	160	86	97	167	87	112	109	138	81
	95	160	86	97	167	87	114	109	164	83
LK07	85	156	86	97	171	113	112	105	154	89
	95	160	86	97	173	121	114	105	154	93
LK08	85	160	86	101	171	131	112	116	154	87
	95	166	94	109	171	171	116	116	160	89
LK09	91	160	94	101	205	121	112	105	154	77
	95	160	94	101	205	121	132	109	154	91
LK10	93	160	82	101	201	125	98	105	160	81
	93	160	82	105	207	131	112	105	160	93
LK11	95	160	94	101	177	129	112	105	142	87
	95	160	94	109	177	133	120	105	156	93
LK12	95	160	90	101	171	121	112	109	154	87
	95	160	94	101	171	127	112	109	154	89
LK13	93	160	85	91	159	113	112	109	154	81
	93	160	85	95	173	127	116	109	154	85
LK14	93	158	158	97	171	121	92	109	154	85
	95	166	166	109	195	127	112	109	154	89
LK15	85	160	86	101	171	121	112	105	156	83
	91	160	160	101	171	121	112	107	156	87

LK16	95	160	116	101	173	129	112	105	0	81
	95	166	116	107	195	129	124	105	0	87
LK17	95	160	84	97	201	113	112	105	156	81
	95	164	88	107	203	133	112	107	162	89
LK18	91	160	82	97	171	121	112	105	170	87
	95	164	90	105	173	121	118	109	170	93
LK19	91	160	88	101	173	121	110	109	0	81
	93	160	90	101	199	127	112	109	0	91
LK20	93	160	94	103	177	135	114	105	154	87
	93	160	94	103	221	135	114	105	154	97
LK21	93	160	82	103	177	123	112	109	162	81
	93	166	94	105	201	127	132	109	162	87
LK22	93	166	86	103	205	123	112	109	154	83
	93	166	104	105	207	123	120	109	160	87
LK23	91	166	86	95	171	127	116	117	196	81
	93	166	86	95	173	127	124	117	196	83
LK24	85	160	126	103	171	127	112	107	154	81
	95	172	126	105	207	131	112	107	154	89
LK25	85	160	94	99	171	125	110	109	170	81
	93	160	104	107	171	131	112	109	172	81
MG01	93	160	88	101	199	127	110	105	170	87

	95	166	94	101	201	127	112	119	174	89
MG02	95	160	86	105	171	121	112	105	154	83
	95	160	108	105	207	127	114	109	162	93
MG03	93	160	86	101	171	0	110	105	158	81
	95	166	116	109	203	0	112	105	158	91
MG04	93	160	86	101	159	119	112	105	154	79
	93	160	92	103	171	121	120	105	160	81
MG05	93	160	84	101	203	133	112	105	154	0
	93	160	88	101	227	133	132	105	154	0
MG06	93	160	84	101	171	121	0	107	156	87
	93	160	88	101	201	127	0	109	170	93
MG07	91	158	156	101	191	123	112	105	160	77
	93	166	158	101	201	129	112	113	164	97
MG08	93	158	86	101	171	107	110	107	0	81
	95	166	92	107	195	129	112	107	0	83
MG09	93	158	86	101	171	125	114	105	156	85
	95	166	92	101	209	127	124	105	156	85
MG10	93	160	158	95	171	125	114	109	154	81
	95	160	160	101	209	133	114	115	156	91
MG11	93	160	84	0	171	0	112	105	154	81
	95	160	92	0	197	0	116	105	190	83

MG12	93	160	86	93	173	105	112	105	162	81
	93	160	86	113	203	161	114	109	162	83
MG13	93	158	82	109	171	105	112	105	162	87
	93	160	94	113	171	119	114	109	174	89
MG14	91	160	86	101	171	127	110	105	160	87
	93	160	92	101	171	129	112	107	194	87
MG15	91	158	80	0	171	129	112	105	156	77
	93	160	86	0	201	131	114	107	162	87
MG16	93	160	158	95	171	123	112	105	188	81
	93	160	160	101	171	135	114	105	202	89
MG17	85	158	84	97	171	131	0	105	154	87
	93	158	118	101	199	133	0	105	154	97
MG18	89	160	82	101	171	131	112	105	162	87
	91	160	84	103	203	133	114	105	164	97
MG19	89	158	86	101	171	121	116	105	162	83
	93	160	94	101	173	127	116	105	164	87
MG20	89	160	90	101	173	119	116	105	0	87
	95	160	160	101	179	129	116	107	0	95
MG21	93	158	102	101	171	127	112	105	158	87
	95	164	106	109	173	131	116	117	164	89
MG22	93	160	86	99	227	101	110	105	154	87

	95	160	86	101	229	129	112	109	158	89
MG23	95	158	116	109	201	111	110	105	162	81
	95	164	118	111	203	135	112	107	162	87
MG24	93	160	106	111	173	105	110	105	158	81
	113	170	162	113	203	161	112	105	162	93
MG25	93	160	82	105	171	121	112	105	166	81
	113	160	88	105	173	131	114	109	166	89
YB01	93	166	156	109	173	125	112	105	164	85
	93	172	156	113	207	133	114	105	170	91
YB02	93	166	156	101	173	121	110	105	160	81
	95	172	156	101	201	131	112	105	162	89
YB03	85	160	86	101	173	121	98	83	162	91
	93	166	100	101	205	131	112	105	162	97
YB04	89	160	160	101	171	121	110	105	160	83
	89	160	160	109	201	125	112	105	162	87
YB05	93	160	82	101	173	125	112	105	154	83
	95	160	94	101	197	135	116	109	162	87
YB06	93	160	86	97	171	121	112	112	162	99
	93	160	86	101	173	127	112	112	162	111
YB07	91	160	84	101	171	125	112	0	162	77
	93	164	84	101	171	131	114	0	162	81

YB08	89	166	82	97	201	121	112	105	156	81
	95	166	82	101	201	131	114	105	156	85
YB09	93	160	88	101	171	129	112	105	156	81
	93	160	90	101	181	131	114	105	156	83
YB10	95	160	82	105	171	121	112	105	154	77
	97	166	86	107	171	121	112	105	158	95
YB11	85	160	92	101	171	127	110	105	154	91
	89	160	160	101	207	133	114	105	160	97
YB12	93	158	82	101	171	127	112	105	154	83
	111	166	122	105	207	131	112	107	162	83
YB13	93	160	86	113	173	121	112	105	164	91
	97	160	86	119	207	127	114	107	170	93
YB14	93	160	160	101	173	121	112	105	164	91
	93	160	160	101	173	127	114	107	164	99
YB15	85	160	160	103	173	125	110	107	162	81
	95	160	160	103	207	131	114	107	162	85
YB16	91	160	84	101	171	125	112	105	154	89
	95	160	94	101	209	131	112	112	154	97
YB17	91	160	92	101	171	123	112	107	156	93
	97	160	92	101	197	133	114	107	156	97
YB18	95	160	82	101	207	121	112	109	160	81

	97	164	82	103	211	131	114	109	162	87
YB19	85	160	82	101	171	121	112	83	160	81
	95	166	82	101	195	127	114	109	164	85
YB20	85	160	86	103	171	127	112	105	154	81
	95	162	100	103	205	127	114	109	156	91
YB21	85	160	82	101	171	121	112	105	158	83
	93	166	82	105	171	127	112	109	160	111
YB22	93	160	84	111	171	127	112	105	0	77
	95	162	86	111	203	131	116	109	0	87
YB23	93	160	86	101	171	131	112	105	154	83
	95	166	88	101	201	131	122	105	154	95
YB24	93	160	86	111	171	121	112	105	154	85
	95	166	94	111	171	121	112	105	154	97
YB25	93	160	82	101	173	121	98	105	160	81
	93	166	94	101	207	121	112	107	162	89

Oyo State samples

Colum 1 = **Sample names**

Columns 2 to 11 = **Microsatellite loci (AG2H175, AG2H143, AG2H26, AG2H637, AG2H79, AG2H590, AG2H772, AG2H603, AG2H523 and AG2H197)**

Sample codes: **Oluyole (OLO), Iwo Road (IWO), Bodija (BJ), Oyo town (OYO) and Eruwa (ERW)**

OLO2	91	0	94	0	171	87	98	109	162	83
	91	0	98	0	173	87	98	109	162	83
OLO3	0	0	94	101	171	0	98	109	162	83
	0	0	98	101	171	0	98	109	162	83
OLO4	93	160	94	99	171	131	98	109	154	83
	93	160	98	105	171	131	98	109	162	83
OLO5	81	160	94	101	171	93	98	109	160	83
	81	160	98	101	171	93	98	109	160	83
OLO6	85	160	94	101	171	121	98	107	156	83
	93	160	98	107	201	127	98	109	156	83
OLO7	85	160	94	99	171	123	98	109	154	83
	93	168	98	99	201	125	98	115	154	83
OLO8	93	160	94	99	171	127	98	109	154	83
	93	168	98	99	171	127	98	107	154	83
OLO9	93	160	94	0	179	81	98	0	0	83

	93	160	98	0	209	81	98	0	0	83
OLO10	93	160	94	99	171	81	98	109	160	81
	93	160	98	99	171	81	98	109	160	81
OLO11	93	160	94	99	171	81	98	105	154	81
	93	160	94	99	171	81	98	107	154	81
OLO13	93	160	94	99	171	127	112	107	154	87
	93	168	94	99	171	127	120	109	154	87
OLO14	87	160	96	99	167	121	98	107	154	83
	95	160	100	99	167	133	98	111	154	83
OLO15	93	0	94	107	171	87	114	0	154	83
	93	0	94	107	201	87	114	0	154	87
OLO16	91	162	94	99	195	121	98	109	154	83
	95	166	98	99	205	127	98	109	154	89
OLO18	93	158	94	99	197	123	112	107	154	83
	93	160	94	99	197	131	120	107	154	83
OLO19	93	160	94	99	171	121	98	107	156	83
	93	160	98	99	173	121	98	107	156	83
OLO20	95	160	94	99	171	121	98	107	154	83
	95	160	98	99	171	127	98	107	154	83
OLO22	85	160	94	99	171	121	98	109	154	83
	93	168	98	109	171	127	98	115	154	83

OLO23	93	160	94	99	171	131	98	105	164	83
	93	164	94	99	177	131	98	109	188	83
OLO24	91	158	94	103	171	81	98	109	162	81
	91	162	98	107	195	81	98	109	162	85
OLO25	93	160	94	101	171	127	98	115	154	87
	93	160	94	107	195	127	98	115	154	87
OLO26	93	160	94	105	173	81	110	105	0	83
	93	160	94	105	173	81	124	107	0	83
OLO27	85	160	94	99	171	87	0	109	154	81
	93	160	94	99	171	87	0	121	154	91
OLO28	93	160	94	99	171	81	98	107	154	89
	93	160	98	99	199	81	98	109	154	93
OLO29	93	160	94	101	171	87	98	107	154	87
	93	160	98	101	201	87	98	107	160	87
OLO30	93	160	94	99	171	125	98	83	154	83
	93	166	98	99	171	125	98	83	154	83
BJ05	87	160	94	101	171	81	98	105	158	85
	93	160	94	101	171	81	98	105	158	85
BJ12	93	160	94	101	173	131	98	105	158	85
	95	160	98	101	197	131	98	105	158	89
BJ16	93	160	94	107	173	127	98	0	154	87

	93	160	98	107	205	131	98	0	154	95
BJ23	0	160	94	107	171	0	98	109	170	87
	0	160	98	109	173	0	98	115	184	91
BJ26	93	160	94	101	171	81	98	83	194	87
	93	160	94	105	173	81	98	83	194	99
OYO1	0	160	94	107	173	81	98	83	154	89
	0	160	98	107	201	81	98	83	162	93
OYO2	93	160	94	94	171	81	98	113	162	93
	93	160	98	101	171	81	98	113	162	93
OYO3	93	160	94	99	177	81	98	107	184	91
	93	160	98	109	199	81	114	109	186	91
OYO4	93	160	94	101	171	121	112	107	166	91
	93	160	94	107	171	133	120	107	166	91
OYO5	93	160	94	99	171	81	98	107	0	87
	93	160	98	99	203	81	98	107	0	87
OYO6	93	164	94	99	171	113	98	111	166	85
	93	164	98	99	173	121	98	119	166	85
OYO7	85	160	94	105	173	127	98	107	154	81
	95	160	94	107	201	135	114	107	166	91
OYO8	93	164	94	99	173	113	98	111	154	81
	97	164	98	107	173	121	98	119	154	83

OYO10	93	160	94	101	177	121	98	107	160	81
	93	164	98	101	205	127	98	109	166	81
OYO11	85	160	94	101	171	127	98	109	154	81
	95	160	98	101	173	135	98	109	154	85
OYO13	91	160	94	99	171	81	98	105	154	93
	93	160	98	99	171	93	98	105	154	93
OYO16	93	160	94	99	171	81	98	105	160	81
	93	160	98	99	173	87	98	115	160	81
OYO17	93	160	94	101	173	81	98	83	154	81
	93	160	98	101	201	81	98	83	154	81
OYO19	93	160	94	105	171	119	98	105	154	91
	93	160	98	105	175	119	98	105	154	91
OYO21	85	166	94	99	173	123	98	109	154	81
	95	166	98	99	201	135	98	109	154	91
OYO22	93	160	94	99	171	81	98	105	162	91
	95	160	98	99	203	129	98	105	186	99
OYO23	97	162	94	99	171	81	98	107	154	83
	97	162	98	99	171	81	98	107	154	83
OYO24	93	158	94	101	171	127	98	105	162	89
	95	158	94	101	173	127	98	105	186	89
OYO26	93	160	94	99	171	121	98	117	154	81

	95	160	98	99	201	127	98	117	154	81
OYO28	93	160	94	101	171	127	98	105	162	87
	95	160	94	101	171	127	98	105	186	89
OYO30	93	160	94	99	171	127	98	105	154	83
	95	160	98	99	199	135	98	105	156	83
IWO2	91	160	94	103	171	81	98	105	154	87
	91	160	98	103	179	121	98	105	186	
		101								
IWO8	93	160	0	101	203	127	114	107	154	87
	93	162	0	105	203	127	124	109	154	87
IWO9	91	160	94	99	171	131	98	107	154	93
	93	160	94	99	171	131	98	107	154	93
IWO12	91	160	94	101	171	125	112	105	154	83
	93	160	94	101	201	133	132	105	154	83
IWO21	93	160	94	99	171	121	114	105	168	81
	93	160	94	99	171	131	124	109	168	87
IWO28	97	160	94	101	171	127	112	0	154	83
	97	160	94	109	171	127	114	0	154	83
IWO30	91	160	94	101	171	125	110	109	162	87
	93	160	94	109	201	131	132	109	162	99
ERW01	85	160	94	99	177	121	98	109	154	89
	91	164	98	99	209	121	98	109	162	89

ERW02	93	160	98	99	171	133	114	101	154	81
	93	162	98	109	173	133	124	107	156	81
ERW03	93	160	94	101	171	123	98	105	172	81
	99	166	98	109	197	127	112	109	190	97
ERW04	91	160	98	101	171	133	112	105	148	83
	93	166	98	101	175	115	118	109	148	89
ERW06	93	160	94	89	171	131	112	109	166	87
	93	166	94	109	173	131	120	125	168	97
ERW07	93	144	98	99	203	131	110	105	152	87
	95	166	98	109	203	131	116	105	156	89
ERW08	93	160	94	101	171	113	116	105	168	81
	93	160	98	101	173	123	122	109	168	81
ERW09	91	164	94	99	171	125	114	105	154	81
	91	166	98	103	203	127	122	109	172	87
ERW10	91	160	94	101	171	123	112	109	156	83
	91	164	98	107	173	123	112	109	156	83
ERW11	93	160	94	101	171	123	112	109	164	87
	93	160	94	101	173	131	112	109	164	87
ERW13	93	160	94	99	215	113	98	105	172	83
	93	160	94	99	215	113	112	105	172	83
ERW14	93	160	94	99	171	119	114	109	168	81

	95	166	94	99	175	119	118	111	168	83
ERW18	93	164	94	99	171	119	98	105	154	81
	97	164	166	99	175	121	98	109	172	87
ERW19	91	160	94	99	171	113	112	105	156	85
	93	160	94	99	173	119	118	105	156	89
ERW20	93	162	94	99	171	119	114	107	168	79
	95	162	94	99	173	125	124	107	168	81
ERW29	97	160	94	101	173	125	114	107	158	89
	97	160	94	101	175	125	116	107	172	89

APPENDIX 9: **Microsatellite data coded to GENEPOP software format for further analysis**

Colun 1: **Sample names**

Column 2 – 11: **Microsat data AG2H175, AG2H143, AG2H26, AG2H637, AG2H79, AG2H590, AG2H772, AG2H603, AG2H523, AG2H197**

Lagos samples

POP

AJ01, 0606 1212 1047 0510 0506 1920 0607 0608 3741 0910

AJ02, 0506 1012 0745 0608 0606 1822 0608 0612 3737 1112

AJ03, 0708 0812 0405 0909 0518 2122 0510 0808 4149 0714

AJ04, 0709 0809 0709 1212 0505 2222 0610 0808 3738 0917

AJ05, 0306 0811 0509 0910 0606 1821 0505 0609 4143 0910

AJ06, 0606 0809 0507 0812 1922 1717 0505 0708 3738 1012

AJ07, 0309 0809 0508 0808 0622 1722 0607 0607 5461 0607

AJ08, 0607 0809 0508 1212 0105 1323 0406 0608 0000 0612

AJ09, 0508 0809 0708 1212 0606 1717 0707 0608 4147 1010

AJ10, 0607 0910 0723 0909 0625 2122 0506 0606 4255 0612

AJ11, 0405 0809 1516 0808 0519 2223 0506 0608 4042 0911

AJ12, 0507 0812 0507 0408 0625 2122 0607 0606 3943 0915

AJ13, 0606 0909 1044 1415 0518 0920 0506 0612 4141 0611

AJ14, 0506 0912 0518 0708 0506 2022 0606 0610 4545 0611

AJ15, 0407 0909 0505 0608 0523 1718 0505 0808 4041 0909

AJ16, 0507 0913 0717 0808 0724 1820 0612 0607 4142 0610

AJ17, 0505 0809 0708 0912 2224 2022 0505 0708 3737 0610

AJ18, 0507 0810 0809 0810 0619 0909 0607 0608 4141 0408

AJ19, 0606 0809 0710 0512 0525 1921 0607 0606 3738 0610

AJ20, 0506 0812 0508 0808 0524 1922 0606 0810 4055 0710

AJ21, 0506 0909 0611 0508 0619 2122 0607 1919 3740 0710

AJ22, 0206 0809 0523 0808 1819 1320 0607 0606 3741 1011

AJ23, 0506 0909 0507 1012 0505 1317 0507 0608 3941 0909

AJ24, 0206 0915 2829 1314 0529 2035 0607 0606 3943 0608

AJ25, 0608 1516 0711 0608 2123 1720 0506 0606 4141 1112

BG01, 0407 0810 0507 0505 0101 2122 0606 0606 3854 0614

BG02, 0207 0810 0842 0808 0520 1721 0611 0607 3737 0809

BG03, 0207 0810 0506 0810 0520 2121 0607 0808 3934 0808

BG04, 0407 0810 0507 0808 0505 2121 0607 0606 3757 0811

BG05, 0607 0910 0507 0812 0509 2021 0607 0608 3939 0811

BG06, 0608 0910 1243 0808 0506 2122 0606 0608 4057 0811

BG07, 0606 1010 0507 0808 2122 1721 0606 0608 4040 0609

BG08, 0707 0810 0507 0513 0506 1722 0607 0606 3737 0809

BG09, 0207 1010 0507 0808 0521 1719 0610 0606 3939 0411

BG10, 0607 0910 0545 0508 0520 1821 0607 0606 3939 1011

BG11, 0207 0809 0644 0613 0506 1719 0610 0606 3941 1011

BG12, 0207 0910 0645 0508 0520 1720 0507 0606 4242 0609

BG13, 0607 0809 0644 0405 0105 1720 0506 0607 4054 0812

BG14, 0606 0910 0641 0808 0520 1820 0606 0607 4040 0812

BG15, 0404 0910 0707 0608 0506 2122 0606 0607 4040 0809

BG16, 0405 1012 0608 0808 0505 1720 0606 0606 3737 0809

BG17, 0505 0910 0841 0808 0622 2223 0606 0606 4040 0811

BG18, 0405 1012 0747 0811 0519 1721 0511 0606 3939 0611

BG19, 0607 1010 4345 0808 0533 1721 0607 0606 3939 0611

BG20, 0707 0910 0607 0812 0519 2021 0506 0606 4242 0609

BG21, 0607 0809 0842 0808 0520 1717 0606 0607 3939 0608

BG22, 0607 0809 4243 0513 0519 2020 0506 0607 3740 0916

BG23, 0407 0809 1143 0808 0519 1820 0606 0610 3940 0614

BG24, 0707 0810 2741 1315 0520 1720 0505 0808 3939 0611

BG25, 0608 0909 1142 0606 0506 1720 0606 0606 3737 0611

IK01, 0507 1212 0711 1215 0506 1022 0506 0607 3740 0509

IK02, 0506 0809 0607 0912 0506 2122 0709 0614 3737 1011

IK03, 0506 0912 0607 0606 0518 1720 0407 0707 3939 0914

IK04, 0506 0910 0607 0810 0618 2121 0404 0303 3939 1214

IK05, 0407 1214 0505 0808 0533 2121 0707 0808 3739 0911

IK06, 0407 0809 0520 0810 0506 2123 0509 0606 3737 0911

IK07, 0607 0812 1111 0812 0506 2121 0506 0606 3740 0611

IK08, 0406 0909 1111 0812 0608 2222 0616 0607 4141 0914

IK09, 0607 0809 0843 0808 0505 2224 0607 0608 4145 0909

IK10, 0607 1213 0550 0809 0506 1821 0606 0607 3941 0709

IK11, 0607 0910 0711 0912 0505 2223 0916 0608 3737 0610

IK12, 0606 0909 0810 0412 0630 2222 0607 0606 4141 0710

IK13, 0507 0712 0607 1314 0825 1022 0506 0619 3740 0911

IK14, 0606 0911 0607 0508 0505 2020 0509 0608 3737 0811

IK15, 0707 0909 0606 0811 0623 2133 0606 0608 4244 0909

IK16, 0506 0909 0715 0810 0623 2121 0506 0606 3138 0909

IK17, 0406 0912 0707 0814 0618 1717 0507 0808 3737 0910

IK18, 0505 0910 0606 0809 0505 1922 0607 0607 3737 0914

IK19, 0606 0913 0606 0808 0518 1824 0616 0608 3737 0909

IK20, 0606 0912 0607 0808 0521 1922 0716 0606 3838 1414

IK21, 0607 0912 0707 1112 0505 1922 0607 0808 3742 0610

IK22, 0506 0909 0607 0808 0505 1821 0509 0606 4040 0911

IK23, 0606 0909 0744 0909 0615 1722 0607 0607 4141 0610

IK24, 0607 0912 0707 0609 0518 0117 0506 0307 4040 0610

IK25, 0608 1212 0607 0912 0518 1921 0509 0808 3737 0814

LK01, 0607 0909 0741 0505 0101 1717 0106 0408 3737 0608

LK02, 0507 0912 0707 0910 0505 1622 0105 0408 3737 0610

LK03, 0506 0909 0607 0606 0505 0101 0510 0606 2937 0707

LK04, 0707 0912 0708 0812 0624 1623 0404 0808 3741 0808

LK05, 0405 0909 0609 0508 0506 1919 0507 0606 3258 0708

LK06, 0607 0909 0707 0606 0303 0101 0607 0808 2942 0607

LK07, 0207 0709 0707 0606 0506 1317 0607 0606 3737 1012

LK08, 0207 0912 0711 0812 0505 2242 0608 1212 3740 0910

LK09, 0507 0909 1111 0808 2222 1717 0616 0608 3737 0411

LK10, 0606 0909 0505 0810 2023 1922 0406 0606 4040 0612

LK11, 0707 0909 1111 0812 0808 2123 0610 0606 3138 0912

LK12, 0707 0909 0911 0808 0505 1720 0606 0808 3737 0910

LK13, 0606 0909 0707 0305 0106 1320 0608 0808 3737 0608

LK14, 0607 0812 4347 0612 0517 1720 0106 0808 3737 0810

LK15, 0205 0909 0744 0808 0505 1717 0606 0607 3838 0709

LK16, 0707 0912 2222 0811 0617 2121 0612 0606 0000 0609

LK17, 0707 0911 0608 0611 2021 1323 0606 0607 3841 0610

LK18, 0507 0911 0509 0610 0506 1717 0609 0608 4545 0912

LK19, 0506 0909 0809 0808 0619 1720 0506 0808 0000 0611

LK20, 0606 0909 1111 0909 0830 2424 0707 0606 3737 0914

LK21, 0606 0912 0511 0910 0820 1820 0616 0808 4141 0609

LK22, 0606 1212 0716 0910 2223 1818 0610 0808 3740 0709

LK23, 0506 1212 0707 0505 0506 2020 0812 1212 5858 0607

LK24, 0207 0915 2727 0910 0523 2022 0606 0707 3737 0610

LK25, 0206 0909 1116 0711 0505 1922 0506 0808 4546 0606

MG01, 0607 0912 0811 0808 1920 2020 0506 0613 4547 0910

MG02, 0707 0909 0718 1010 0523 1720 0607 0608 3741 0712

MG03, 0607 0912 0722 0812 0521 0000 0506 0606 3939 0611

MG04, 0606 0909 0710 0809 0105 1617 0610 0606 3740 0506

MG05, 0606 0909 0608 0808 2133 2323 0616 0606 3737 0000

MG06, 0606 0909 0608 0808 0520 1720 0000 0708 3845 0912

MG07, 0506 0812 4243 0808 1520 1821 0606 0610 4042 0414

MG08, 0607 0812 0710 0811 0517 1021 0506 0707 0000 0607

MG09, 0607 0812 0710 0808 0524 1920 0712 0606 3838 0808

MG10, 0607 0909 4344 0508 0524 1923 0707 0811 3738 0611

MG11, 0607 0909 0610 0000 0518 0000 0608 0606 3755 0607

MG12, 0606 0909 0707 0414 0621 0937 0607 0608 4141 0607

MG13, 0606 0809 0511 1214 0505 0916 0607 0608 4147 0910

MG14, 0506 0909 0710 0808 0505 2021 0506 0607 4057 0909

MG15, 0506 0809 0407 0000 0520 2122 0607 0607 3841 0409

MG16, 0606 0909 4344 0508 0505 1824 0607 0606 5461 0610

MG17, 0206 0808 0623 0608 0519 2223 0000 0606 3737 0914

MG18, 0405 0909 0506 0809 0521 2223 0607 0606 4142 0914

MG19, 0406 0809 0711 0808 0506 1720 0808 0606 4142 0709

MG20,	0407	0909	0944	0808	0609	1621	0808	0607	0000	0913
MG21,	0607	0811	1517	0812	0506	2022	0608	0612	3942	0910
MG22,	0607	0909	0707	0708	3334	0721	0506	0608	3739	0910
MG23,	0707	0811	2223	1213	2021	1224	0506	0607	4141	0609
MG24,	0616	0914	1745	1314	0621	0937	0506	0606	3941	0612
MG25,	0616	0909	0508	1010	0506	1722	0607	0608	4343	0610
YB01,	0606	1215	4242	1214	0623	1923	0607	0606	4245	0811
YB02,	0607	1215	4242	0808	0620	1722	0506	0606	4041	0610
YB03,	0206	0912	0714	0808	0622	1722	0406	0506	4141	1114
YB04,	0404	0909	4444	0812	0520	1719	0506	0606	4041	0709
YB05,	0607	0909	0511	0808	0618	1924	0608	0608	3741	0709
YB06,	0606	0909	0707	0608	0506	1720	0606	1010	4141	1521
YB07,	0506	0911	0606	0808	0505	1922	0607	0000	4141	0406
YB08,	0407	1212	0505	0608	2020	1722	0607	0606	3838	0608
YB09,	0606	0909	0809	0808	0510	2122	0607	0606	3838	0607
YB10,	0708	0912	0507	1011	0505	1717	0606	0606	3739	0413
YB11,	0204	0909	1044	0808	0523	2023	0507	0606	3740	1114
YB12,	0615	0812	0525	0810	0523	2022	0606	0607	3741	0707
YB13,	0608	0909	0707	1417	0623	1720	0607	0607	4245	1112
YB14,	0606	0909	4444	0808	0606	1720	0607	0607	4242	1115
YB15,	0207	0909	4444	0909	0623	1922	0507	0707	4141	0608

YB16, 0507 0909 0611 0808 0524 1922 0606 0610 3737 1014

YB17, 0508 0909 1010 0808 0518 1823 0607 0707 3838 1214

YB18, 0708 0911 0505 0809 2325 1722 0607 0808 4041 0609

YB19, 0207 0912 0505 0808 0517 1720 0607 0508 4042 0608

YB20, 0207 0910 0714 0909 0522 2020 0607 0608 3738 0611

YB21, 0206 0912 0505 0810 0505 1720 0606 0608 3940 0721

YB22, 0607 0910 0607 1313 0521 2022 0608 0608 0000 0409

YB23, 0607 0912 0708 0808 0520 2222 0611 0606 3737 0713

YB24, 0607 0912 0711 1313 0505 1717 0606 0606 3737 0814

YB25, 0606 0912 0511 0808 0623 1717 0406 0607 4041 0610

POP

Oyo State samples

Column 1: **Sample name**

Column 2-11: **Microsatellite loci AG2H175, AG2H143, AG2H26, AG2H637, AG2H79, AG2H590, AG2H772, AG2H603, AG2H523, AG2H197**

pop

OLO2,	0505	0000	1113	0000	0506	0101	0101	0808	4141	0707
OLO3,	0000	0000	1113	0808	0505	0000	0101	0808	4141	0707
OLO4,	0606	0909	1113	0710	0505	2222	0101	0808	3741	0707
OLO5,	0101	0909	1113	0808	0505	0303	0101	0808	4040	0707
OLO6,	0206	0909	1113	0811	0520	1720	0101	0708	3838	0707
OLO7,	0206	0913	1113	0707	0520	1819	0101	0811	3737	0707
OLO8,	0606	0913	1113	0707	0505	2020	0101	0807	3737	0707
OLO9,	0606	0909	1113	0000	0924	0303	0101	0000	0000	0707
OLO10,	0606	0909	1113	0707	0505	0303	0101	0808	4040	0606
OLO11,	0606	0909	1111	0707	0505	0303	0101	0607	3737	0606
OLO13,	0606	0913	1111	0707	0505	2020	0610	0708	3737	0909
OLO14,	0307	0909	1214	0707	0303	1723	0101	0709	3737	0707
OLO15,	0606	0000	1111	1111	0520	0101	0707	0000	3737	0709
OLO16,	0507	1012	1113	0707	1722	1720	0101	0808	3737	0710
OLO18,	0606	0809	1111	0707	1818	1822	0610	0707	3737	0707
OLO19,	0606	0909	1113	0707	0506	1717	0101	0707	3838	0707

OLO20,	0707	0909	1113	0707	0505	1720	0101	0707	3737	0707
OLO22,	0206	0913	1113	0712	0505	1720	0101	0811	3737	0707
OLO23,	0606	0911	1111	0707	0508	2222	0101	0608	4254	0707
OLO24,	0505	0810	1113	0911	0517	0303	0101	0808	4141	0608
OLO25,	0606	0909	1111	0811	0517	2020	0101	1111	3737	0909
OLO26,	0606	0909	1111	1010	0606	0303	0512	0607	0000	0707
OLO27,	0206	0909	1111	0707	0505	0101	0000	0814	3737	0611
OLO28,	0606	0909	1113	0707	0519	0303	0101	0708	3737	1012
OLO29,	0606	0909	1113	0808	0520	0101	0101	0707	3740	0909
OLO30,	0606	0912	1113	0707	0505	1919	0101	0505	3737	0707
BJ05,	0306	0909	1111	0808	0505	0303	0101	0606	3939	0808
BJ12,	0607	0909	1113	0808	0618	2222	0101	0606	3939	0810
BJ16,	0606	0909	1113	1111	0622	2022	0101	0000	3737	0913
BJ23,	0000	0909	1113	1112	0506	0000	0101	0811	4552	0911
BJ26,	0606	0909	1111	0810	0506	0303	0101	0505	5757	0915
OYO1,	0000	0909	1113	1111	0620	0303	0101	0505	3741	1012
OYO2,	0606	0909	1113	0508	0505	0303	0101	1010	4141	1212
OYO3,	0606	0909	1113	0712	0819	0303	0107	0708	5253	1111
OYO4,	0606	0909	1111	0811	0505	1723	0610	0707	4343	1111
OYO5,	0606	0909	1113	0707	0521	0303	0101	0707	0000	0909
OYO6,	0606	1111	1113	0707	0506	1317	0101	0913	4343	0808

OYO7,	0207	0909	1111	1011	0620	2024	0107	0707	3743	0611
OYO8,	0608	1111	1113	0711	0606	1317	0101	0913	3737	0607
OYO10,	0606	0911	1113	0808	0822	1720	0101	0708	4043	0606
OYO11,	0207	0909	1113	0808	0506	2024	0101	0808	3737	0608
OYO13,	0506	0909	1113	0707	0505	0303	0101	0606	3737	1212
OYO16,	0606	0909	1113	0707	0506	0301	0101	0611	4040	0606
OYO17,	0606	0909	1113	0808	0620	0303	0101	0505	3737	0606
OYO19,	0606	0909	1113	1010	0507	1616	0101	0606	3737	1111
OYO21,	0207	1212	1113	0707	0620	1824	0101	0808	3737	0611
OYO22,	0607	0909	1113	0707	0521	0321	0101	0606	4153	1115
OYO23,	0808	1010	1113	0707	0505	0303	0101	0707	3737	0707
OYO24,	0607	0808	1111	0808	0506	2020	0101	0606	4153	1010
OYO26,	0607	0909	1113	0707	0520	1720	0101	1212	3737	0606
OYO28,	0607	0909	1111	0808	0505	2020	0101	0606	4153	0910
OYO30,	0607	0909	1113	0707	0519	2024	0101	0606	3738	0707
IWO2,	0505	0909	1113	0909	0509	0317	0101	0606	3753	0916
IWO8,	0606	0910	0000	0810	2121	2020	0712	0708	3737	0909
IWO9,	0506	0909	1111	0707	0505	2222	0101	0707	3737	1212
IWO12,	0506	0909	1111	0808	0520	1923	0616	0606	3737	0707
IWO21,	0606	0909	1111	0707	0505	1722	0712	0608	4444	0609
IWO28,	0808	0909	1111	0812	0505	2020	0607	0000	3737	0707

IWO30, 0506 0909 1111 0812 0520 1922 0516 0808 4141 0915

ERW01, 0205 0911 1113 0707 0824 1717 0101 0808 3741 1010

ERW02, 0606 0910 1313 0712 0506 2323 0712 0407 3738 0606

ERW03, 0609 0912 1113 0812 0518 1820 0106 0608 4655 0614

ERW04, 0506 0912 1313 0808 0507 2314 0609 0608 3434 0710

ERW06, 0606 0912 1111 0212 0506 2222 0610 0816 4344 0914

ERW07, 0607 0112 1313 0712 2121 2222 0508 0606 3638 0910

ERW08, 0606 0909 1113 0808 0506 1318 0811 0608 4444 0606

ERW09, 0505 1112 1113 0709 0521 1920 0711 0608 3746 0609

ERW10, 0505 0911 1113 0811 0506 1818 0606 0808 3838 0707

ERW11, 0606 0909 1111 0808 0506 1822 0606 0808 4242 0909

ERW13, 0606 0909 1111 0707 2727 1313 0106 0606 4646 0707

ERW14, 0607 0912 1111 0707 0507 1616 0709 0809 4444 0607

ERW18, 0608 1111 1147 0707 0507 1617 0101 0608 3746 0609

ERW19, 0506 0909 1111 0707 0506 1316 0609 0606 3838 0810

ERW20, 0607 1010 1111 0707 0506 1619 0712 0707 4444 0506

ERW29, 0808 0909 1111 0808 0607 1919 0708 0707 3946 1010

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