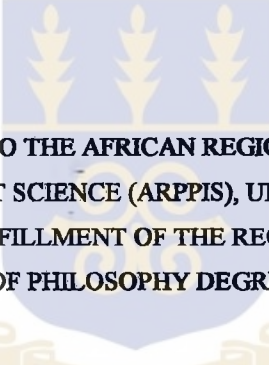


**CYTOLOGICAL AND MOLECULAR CHARACTERIZATION OF
POPULATIONS OF THE *ANOPHELES GAMBIAE* GILES
COMPLEX IN A COASTAL SAVANNA ZONE OF GHANA**

BY

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**A THESIS SUBMITTED TO THE AFRICAN REGIONAL POSTGRADUATE
PROGRAMME IN INSECT SCIENCE (ARPPIS), UNIVERSITY OF GHANA,
LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
AWARD OF MASTER OF PHILOSOPHY DEGREE IN ENTOMOLOGY**

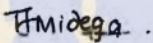
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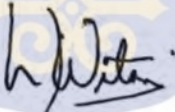
***JOINT INTERFACULTY INTERNATIONAL PROGRAMME FOR THE TRAINING
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COLLABORATING FACULTIES: AGRICULTURE AND SCIENCE**

DECLARATION

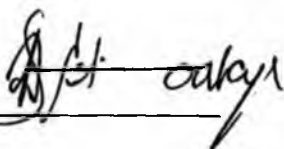
I hereby declare that with the exception of references to other peoples work which I have duly acknowledged all the experimental work described in this thesis was carried out by me and this thesis, either in whole or in part, has not been presented elsewhere for another degree.



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DEDICATION

**To My Parents, Ben and Julie,
and To Professor K.E Senanu.**



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LIST OF ABBREVIATIONS

AgNO ₃	Silver Nitrate
bp	base pair
dATP	deoxyadenosine triphosphate
ddw	distilled de-ionised water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	Disodium ethylene diamine tetraacetate. 2H ₂ O
EthBr	Ethidium Bromide
EtOH	Ethanol
GPS	Global positioning system.
H ₂ O	Water
KAc	Potassium acetate
kb	Kilobase
KOH	Potassium hydroxide
M	Molar
Mw	Molecular weight
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
pH	Hydrogen-ion exponent

pH	Hydrogen-ion exponent
RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal DNA
rpm	revolution per minute
sddH₂O	sterile double distilled water
s.l	Sensu lato
s.s	Sensu stricto
TAE	Tris - Acetate EDTA
TEMED	N,N,N,'N'- tetramethyl ethylene diamine
T_m	Melting temperature
Tris	2 -amino-2-(hydroxymethyl)-1,3 propanediol
μl	microlitre
μM	micromolar
UPGMA	Unweighted Pairgroup Method with Arithmetic Averaging

ABSTRACT

The effective planning of vector control strategies, genetic manipulation of vector species and the management of insecticide resistance require information on the population structure and the assessment of gene flow between vector populations. Although the appetitive flight of mosquitoes is known to be less than 5 km, most studies assessing gene flow between *An. gambiae* populations have been conducted over geographical distances as far apart as 6000km. For vector control, operational purposes such as the use of insecticide treated materials within a particular country setting, the gene flow between populations that are some 10-100 Km is more important. To assess the level of gene flow between populations over short distances, five populations of *Anopheles* mosquitoes from sites covering an area of about 25 km in diameter and located in the Coastal zone of Ghana were studied. One hundred and twenty-nine mosquitoes were identified by cytotaxonomy and PCR. A subset of these were fully karyotyped, but all were analyzed for three microsatellite DNA loci: AGXH7, AG2H26 and AG2H46.

Karyotype analysis revealed the presence of the 'Forest' and 'Savanna' forms of *An. gambiae* s.s. characterized by the karyotypes 2R⁺, 2L⁺ and 2R⁺, 2L^a respectively. The frequency of inversion *a* on the chromosomal arm 2L varied from 0% - 80% between populations that were furthest apart and differing in ecology from coastal forest to coastal savanna.

A total of 30 polymorphic microsatellite alleles were observed. Number of alleles per locus ranged from nine to twelve (Mean = 10 per locus). Inter-population differentiation was highest between populations that were furthest apart ($F_{st} = 0.0565$) and lowest in closer populations ($F_{st} = 0.0284$). Accordingly, estimates of gene flow were higher in closer populations and lower in those further apart. Heterozygotes were in excess at the centrally located site ($F_{is} = 0.0435$) but deficient in others suggesting the presence of a possible hybrid zone in the study area.

The study established the presence of two distinct karyotypic populations of *An. gambiae* s.s in the coastal zone of Ghana and also revealed that levels of gene flow between populations decreased after 5km. The importance of karyotyping and microsatellite analysis in the characterization of vector populations is discussed.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Mosquitoes are known vectors of parasites that cause tropical diseases such as malaria, lymphatic filariasis, yellow fever and arboviruses, e.g. dengue fever. Of these diseases, malaria is reported to be the most important worldwide (WHO, 1998) and still remains one of the world's most prevalent vector borne disease and a leading cause of morbidity and mortality in the tropical countries. Malaria is caused by the infection of humans with either one or more of the protozoan parasites *Plasmodium falciparum*, *P. ovale*, *P. malariae* and *P. vivax*, transmitted through the bite of an infected female *Anopheles* mosquito (Service, 1993).

Over 400 species of *Anopheles* mosquitoes are known, most of which are members of species complexes, such as the *An. gambiae* complex and *An. funestus* complex. Out of these, 80 species have been implicated in malaria transmission, but only 40 are important vectors (WHO, 1999). Members of the *An. gambiae* complex, mainly *An. gambiae* sensu stricto and *An. arabiensis* are the most efficient vectors of malaria in sub-Saharan Africa, transmitting *falciparum* malaria which is the most common and the most lethal form of the disease (Coluzzi, 1984). Other known important vectors include *An. pharoensis* in Egypt, *An. culicifacies* in South West Asia, *An. darlingi* in North America and *An. albimanus* in Central America (Service, 1993).

Previously widespread, the disease is now mainly confined to poorer tropical areas of Africa, Asia and Latin America and is endemic in a total of 101 countries, with pockets of transmission in 8 other countries (WHO, 1999). WHO, (1999) estimates that the disease represents 2.3% of the overall global disease burden and 9% in Africa, ranking third among major infectious disease threats after pneumococcal acute respiratory infections (3.5%) and tuberculosis (2.8%). In Ghana, malaria is one of the major health problems. The disease is hyperendemic and accounts for over 43% of all out patient cases seen in health institutions and 44.1% of all clinic attendance. It is the most commonly reported disease responsible for 7-8% of all certified deaths and ranks 5th as the commonest cause of death in the 0-4 year age group (Centre for Health Statistics, 1992).

Approximately, 41% of the world's population is at risk and each year, there are an estimated 300 to 500 million clinical cases of malaria (more 95% of them in Africa), resulting in over 2 million deaths mainly of children under five years of age, especially in remote areas with poor access to health services. With acute disease, a child may die within 24 hours of infection. Pregnant women are four times more likely to suffer malaria attacks causing low weight babies and still births, endangering the health of the women and the prospects for the new born (Lindsay *et al.*, 2000). Outside tropical Africa, malaria deaths occur mainly among non-immune new comers to endemic areas, for example among agricultural workers, miners and settlers in newly colonized areas (WHO, 2000).

Malaria poses as a serious obstacle to the socio economic development of tropical countries and interferes with industrial and agricultural development as it severely diminishes human work capacity, which consequently translates to food shortages and malnutrition. The direct and indirect costs of malaria are therefore very high and these, coupled with the burden of human suffering due to the disease call for its eradication. However, for economic and environmental reasons among others, it is not considered feasible to eradicate the disease from all parts of the world where it now occurs (Zahar, 1984).

Malaria control therefore remains the only reliable option and an essential prerequisite for reducing the magnitude of this serious public health problem and for relieving the suffering of millions of people living in malaria endemic regions. Using current control methods it should be possible to reduce the severity and incidence of malaria to a level at which the disease ceases to be a public health problem. The need to reduce malaria burden has recently led to two global initiatives; Multilateral Initiative on Malaria (MIM) and the Roll Back Malaria initiative (RBM). This need has also motivated research into the development of vaccines and the development of genetic control measures against the vector (Curtis, 1994; TDR, 1998).

Previously, it was believed that the control and eradication of malaria would be easy. This belief was based on the assumption that the relationship between the parasite, the vector and the host was clearly understood, that effective therapeutic and chemoprophylactic agents were available and that insecticides held great promise for

vector control (Beausoleil, 1984). However, decades later and after several pilot projects, reviews and revisions of strategies and various attempts at control, malaria still remains an insidious and ever-present scourge with serious public health, social, and economic consequences that constitute serious obstacles to development especially in rural areas (Rojas *et al.*, 1992). Chief among the factors responsible for this situation are the climatic and ecological characteristics that favour vector survival and disease transmission (MARA, 1998) and the complexity of the vectorial system (Lanzaro *et al.*, 1998; Lochouart and Fontenille, 1999), which consists of several sibling and incipient species.

Since the discovery of the European *An. maculipennis* complex (Hackett, 1937; Bates, 1940), it has been repeatedly discovered that human malaria vectors belong to cryptic or sibling species complexes, and that these, often sympatric species exhibit behavioural and ecological differences that influence their role as vectors (Coluzzi, 1970; Collins and Paskewitz, 1996). Thus, the failure of malariologists to recognize sibling species of *Anopheles* has meant in the past, failure to distinguish an epidemiologically important species from an unimportant one. Moreover, the studies of host preference, infection rate, resting habits, and biting cycle as well as the assessment of control measures were seriously misleading because the morphological species was in reality a mixture of two or more species.

The current approaches to malaria control are chemotherapy, vector control and case management. Unfortunately these have also not achieved their expected levels of success

due to limitations of development of resistance to anti-malarial drugs and chemoprophylactics by the parasites (Peters, 1970), insecticide resistance by the vector (Ranson *et al.*, 2000) and disease resurgence in areas where the disease had been previously eradicated (Krogstad, 1996).

To date, the only malaria control strategy that has achieved some success were those aimed at the vector using insecticides (TDR, 1998). Insecticide application methods used include indoor insecticide spraying, aerial spraying, residual insecticide application on walls and surfaces, the use of insecticide treated materials such as curtains and bednets and the application of larvicides at mosquito breeding sites. Currently, the RBM initiative has adopted the promotion of the use of insecticide treated bednets (ITBNs) using pyrethroids in malaria endemic regions to reduce human-vector contact. This has been shown to provide a remarkable degree of protection against malaria in Africa (Binka *et al.*, 1996, Curtis *et al.*, 1998). Recent studies by Abdulla *et al.* (2001) in Ifakara, Tanzania revealed that the use of ITBNs had a protective efficacy of 62% on the prevalence of parasitaemia. However, reports of reduced susceptibility of *An. gambiae* to permethrin associated with the use of permethrin impregnated bednets and curtains in both East and West Africa (Vulule *et al.*, 1994, 1996; Doannio *et al.*, 1999) may reduce the effectiveness of ITBNs as a vector control tool. Insecticide resistance arising in a local vector population as a result of selection pressure due to insecticide use may spread to adjacent populations by gene flow, widening the distribution of the gene for resistance. For the effective management of insecticide resistance therefore, knowledge

of the range of distribution of vector populations and gene flow patterns is a paramount requisite for any malaria control programme.

Apart from the problem of insecticide resistance, the diversity of the vectorial system has also contributed to the lack of success of most vector control operations (Coluzzi, 1992). Formerly regarded as a single species, the afrotropical vectors of malaria are members of the *An. gambiae* Giles complex, which is now known to comprise of 6 sibling species (Gillies and Coetzee, 1987) one unnamed species (Hunt *et al.*, 1998) and several incipient species (Coluzzi *et al.*, 1979; Favia *et al.*, 1997). These vectors generally cause high parasite inoculation rates and are also remarkably stable in a wide range of bio-ecological and seasonal conditions hence appear to be very flexible, both in exploiting new man-made environments and in their response to malaria control activities (Coluzzi, 1984).

The adaptability to environmental changes leading to marked contrasts in vector bionomics has led to the development of various levels of vectorial efficiency for populations of *Anopheles* species in heterogenous environments within the same locality and has thus become an important factor in the determination of the epidemiology of malaria (Toure *et al.*, 1994). Environmental heterogeneities have arisen mainly as a result of human activities which act as a means of constant evolutionary challenge as they provide a source of environmental change to which anthropophilic *Anopheles* have to respond by developing a highly dynamic vector-host relationship.

The monitoring of processes such as vector movement and distribution requires an intimate knowledge of vector behavioural attributes such as host seeking and choice of resting and oviposition sites that may differ between sibling species yet are a prerequisite for the implementation of vector control (Coluzzi *et al.*, 1979). In this respect, the identification of sibling species is a problem that needs to be solved before the bionomics of these species can be studied. The identification of sibling and incipient species and population characterisation is therefore necessary because vector control strategies must be specific to target the exact vector. A necessary condition for this is reference to taxonomic units; species, sub-species, forms and populations that have biological significance. Various methods exist in *Anopheles* studies that can be applied for all these taxonomic levels. These include the conventional morphological methods, cuticular hydrocarbon analysis, crossing experiments, biochemical methods, cytotaxonomy (Coluzzi, 1968; Coluzzi and Sabatini, 1967, 1968 a; 1969) and molecular methods (Scott *et al.*, 1993, Lanzaro *et al.*, 1995).

To gain a better understanding of the population dynamics, gene flow and possible influence of ecological processes on the genetic variability in *An. gambiae* s.l. species among local populations within and between geographic regions, it is important to identify and characterize the vector populations, as this is important in the interpretation of studies on vector biology.

One of the methods most commonly used for *An. gambiae* species identification is cytotaxonomy which exploits the use of floating chromosomal inversions to delineate geographic distribution of *An. gambiae* s.s. populations (Coluzzi *et al.*, 1979).

Microsatellite DNA analysis has been widely applied in studies of *An. gambiae* population differentiation and assessment of gene flow between populations (Kamau *et al.*, 1998).

The current malaria control activities in Ghana involve the use of chemotherapy and case management for disease control and no systematic use of insecticides for vector control. However, Ghana has adopted the Roll Back Malaria strategies and intends to promote large-scale use of ITBNs. However, there have been reports of pyrethroid resistance in malaria vectors in some parts of West Africa including the neighbouring Cote d' Ivoire (Chandre *et al.*, 1999 a, b, c) and in Accra (Adasi *et al.*, 2001) in the absence of any organised vector control programmes. Although for the Accra study mosquitoes were collected from specific sites, pyrethroid sensitivity tests were carried out on pooled samples. It is therefore likely that pyrethroid resistance in mosquitoes is present at various levels at certain sites and not at others. This however, does not preclude the possibility that eventually pyrethroid resistance will spread to all mosquito populations in Accra through gene flow and human aided movement of the vectors.

The effective management of the existing pyrethroid resistance in Accra will require the characterization of the vector species and populations and also identification of the specific areas where resistance has developed. This will prevent the problem of misdirected vector control efforts, which have previously led to the failure of malaria control programmes based on vector control.

1.2 Objectives

The main aim of this study was to identify and characterise *Anopheles* vector populations within an area covering approximately 25 kms across to determine the levels of population differentiation and gene flow between them. This was to be achieved by the use of karyotyping and microsatellite DNA analysis, to reveal intraspecific differences between *An. gambiae* s.s. populations.

Specific Objectives

The specific objectives were:

1. To collect and identify members of the *An. gambiae* Giles complex from selected sites within a 25 km radius in the coastal savanna area of Ghana.
2. To characterize the *An. gambiae* sensu stricto populations by use of karyotyping
3. To study the *An. gambiae* s.s populations using PCR- based microsatellite DNA analysis.
4. To determine the population structure of the *An. gambiae* s.s. populations.
5. To determine the levels of gene flow between the *An. gambiae* s.s. populations.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria: The disease and symptomatology

Malaria is a febrile illness caused by protozoa of the class sporozoa, genus *Plasmodium* (Farr, 1988). The parasite is transmitted from human to human through the bites of infected female *Anopheles* mosquitoes. In endemic regions where transmission is high, people are continuously infected and some may develop immunity to the disease (Allison, 1984). Malaria is often described as a great imitator of other diseases, sharing various characteristics with other childhood illnesses like influenza, typhoid, brucellosis and urinary tract infections. Even more confusing, malaria may coexist with other diseases. Most of the morbidity due to malaria is caused by cerebral malaria and anaemia (Marsh, 1992). Malaria is diagnosed by the clinical symptoms and microscopic examination of blood films.

The symptoms first appear some 10 to 16 days after the infectious mosquito bite and coincide with the bursting of infected red blood cells. When many red blood cells are infected and break at the same time because of the parasites growth, malaria attacks can recur at regular time periods – every two days for *P. vivax* malaria and *P. ovale* and every three days for *P. malariae* (Berendt *et al.*, 1994). In *P. vivax* malaria, the patient may feel entirely well between attacks. Even without treatment, the paroxysms subside in a few weeks. However, a person with *P. falciparum* malaria is apt to feel very ill

between attacks and without treatment, is in danger of dying. One reason why *P. falciparum* malaria is so virulent is that the parasite can infect red blood cells in all stages of development, and the number of parasites in the blood can reach extremely high levels (Bruce-Chwatt, 1985). In contrast, *P. vivax* parasites infect only young red blood cells which means the number of parasites in the blood does not reach the same high levels as seen in *P. falciparum* infection.

Malaria typically produces a string of recurrent attacks or paroxysms, each of which has three stages: chills, followed by fever, pain in the joints and then sweating (Cheesbrough, 1991). Along with chills, the patient is likely to have headache, nausea and repeated vomiting (Commey, 1989) and within an hour or two, the patient's temperature may rise. One of the commonest causes of death in children and adults with *falciparum* malaria is cerebral malaria, which is due to parasitized red cells and fibrins blocking the capillaries and venules in the brain (Marsh, 1992). Cerebral oedema and peri-capillary haemorrhaging from these small blood vessels may occur, causing irreversible injury to the brain (Warrel *et al.*, 1982). In adults, cerebral malaria often develops after several days of fever and other non-specific symptoms (Warrell, 1983; Molyneux *et al.*, 1989), but in children, the history is usually less than two days. Cerebral malaria often starts with a generalized convulsion followed by persisting unconsciousness. It is frequently associated with major metabolic and physiologic changes, particularly hypoglycaemia, decreased cerebral perfusion/hypoxia and cytokine induction, resulting in tissue damage and generalized seizures, paralysis, speech and

behaviour disorders and hearing impairment (MIM, 2001). Severe anaemia is often the attributable cause of death in areas with intense malaria transmission (Comney, 1989).

2.2 Global Distribution

It is estimated that over 40% of the world's population lives in areas with malaria risk, with the great majority living in Sub-Saharan Africa (WHO, 2000). The prevalence and severity of malaria as well as the magnitude of associated social and economic effects vary widely in different geographical areas where the disease occurs. However, the worst effects of the disease are felt in Sub-Saharan Africa.

The geographical area affected by malaria has shrunk considerably over the past 50 years, with successful eradication and cessation of transmission in large areas of North America, Southern Europe, and the former Soviet Union and some territories of Asia and South America (WHO, 1998). The disease is now confined to poorer tropical areas of Africa, Asia and Latin America because of the tropical climate, which is suitable for vector development and survival (Figure 1).

Malaria is endemic throughout Ghana (Ahmed, 1989) and records show that more cases of malaria occur in the middle forest zone (Ashanti, Brong Ahafo, Eastern and greater parts of Western Regions). This is followed by the coastal zone (Central, Greater Accra and parts of Western regions), which is a terrain of lagoons, creeks, etc and then by the northern savanna zone, which is usually drier than the rest of the country.

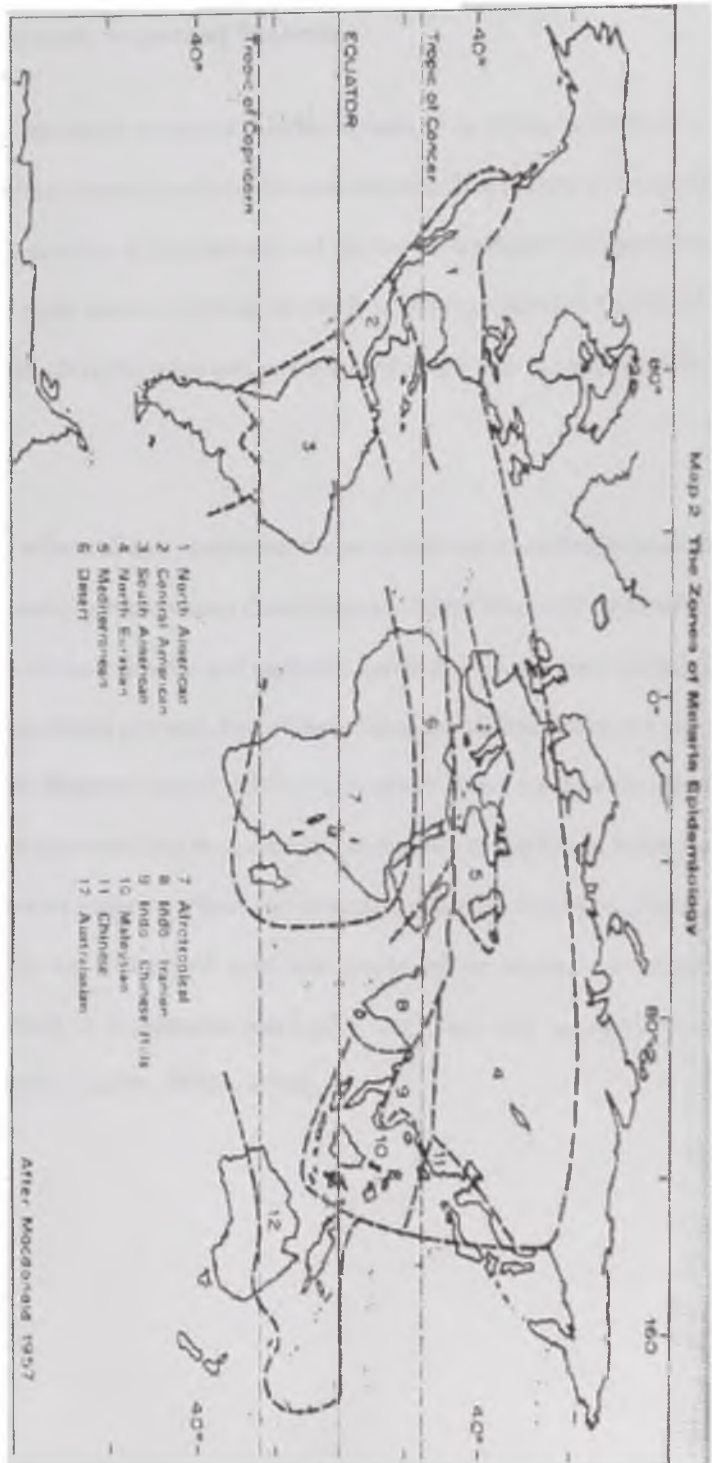


Figure 1: Global distribution of Malaria: The zones of malaria epidemiology (After Mac Donald, 1957)

2.3 Social and Economic Impact of Malaria

Recent estimates put the annual economic burden of malaria in Africa at about \$11.7 billion or 1% of the gross domestic product. The cost of a case from society's view point is \$9.84 or 12 days equivalent of productivity and the cost of treatment per household ranges from \$0.2-\$15 each month in Africa. In 1998, it was reported that 39,000,000 daily adjusted life years (DALYs) were lost as a result of illness due to malaria (WHO, 1999).

The disease generally affects all age groups, but the most affected are children (Breman and Campbell, 1988) and pregnant women (Lindsay *et al.*, 2000). Over 300-500 million people suffer from the disease annually and mortality due to malaria has been estimated to account for 2 million deaths per year, half of these being in children under five years of age in Africa (TDR Progress Report, 1997-98). In cases where the disease affects working adults such as labourers, loss in income is incurred due to lost labour hours and cost of treatment. Workers suffering a bout can be incapacitated for 5-20 days. Malaria stricken family spends an average of over one-quarter of its income on malaria treatment, and prevention. It is estimated that such a family can only harvest 40% of crops harvested by healthy families (WHO, 1990).

2.4 The Life cycle and Transmission of Human *Plasmodium* Parasite

Four protozoan parasites, namely *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax* cause malaria in humans and they all have the similar life cycle (Figure 2).

During the infective bite of a female *Anopheles* mosquito numerous infective sporozoites are injected into the bloodstream, where they may remain for about 30 minutes and then disappear from the blood stream. Most of the sporozoites are destroyed by phagocytes whilst some enter the liver parenchymal cells, develop and undergo asexual multiplication (pre-erythrocytic schizogony). A large unpigmented schizont is formed containing several merozoites, which are released into the bloodstream to invade the erythrocytes. The merozoite attaches to an erythrocyte and is invaginated into the red cell through a parasitophorous vacuole, where it feeds and deposits a pigment called haemozoin as a by product (Aikawa, 1980). The ingested merozoite becomes a feeding trophozoite and in the early stages of an infection, the fully-grown trophozoite multiplies asexually to become a schizont (erythrocytic schizogony), producing a small number of merozoites (Aikawa and Seed, 1980).

Release of the merozoites from the erythrocytes brings on an attack of malaria, and the interval between attacks is the length of the schizogonic cycle. This may last several hours. The released merozoites repeat the cycle and invade other erythrocytes. After a number of cycles of schizogony, the trophozoites do not divide but become gametocytes, which develop no further in man but circulate in the bloodstream until a mosquito takes them up during a blood meal (Kettle, 1992).

When in the *Anopheles* mosquito, microgametocytes exflagellate to become microgametes. The microgametes move away to find and fuse with macrogametes to form zygotes, which remain motionless for 18-24 hours then elongate to form ookinetes (Aikawa and Seed, 1980; Kettle, 1992). The ookinete penetrates the wall of the midgut and forms an oocyst and through sporogony, the oocyst develops into enlarged motile sporozoites, which burst through the ruptured wall of the oocyst and invade the body cavity of the mosquito. The sporozoites, which are now infective then migrate to the salivary glands of the mosquito and are injected into the wound when the mosquito takes the next blood meal.

The effective transmission, high incidence and mortality of malaria is determined by a number of factors which affect the distribution, transmission intensity, disease outcome, small-scale variation and seasonality (MARA, 1998).

The number and type of anopheline mosquitoes determine to a large extent the level of transmission in a given area. Malaria transmission is influenced by climate and geography and often coincides with the rainy season (Craig *et al.*, 1999) when breeding sites are available, ensuring high numbers of *Anopheles*. The climatic conditions in tropical Africa favour intensive transmission of malaria parasites. Temperature affects the sporogonic cycle of the parasite and vector survival. Very low temperatures limit the vector by lengthening the gonotrophic cycle and reducing the survival rate. *Anopheles gambiae* for example, only exists in frost-free regions (de Meillon, 1934) or where

absolute minimum temperatures in winter remain above 5°C (Leeson, 1931). Rainfall provides mosquito-breeding sites and increases humidity, thus enhancing mosquito survival. However, the relationship between mosquito abundance and rainfall is not direct, as a specific amount of rain does not lead to a specific mosquito density. By examining rainfall patterns in known malaria and non-malarious areas, Craig *et al.* (1999) estimated that an average of 80mm per month for at least three to five months is a reasonable requirement for the availability of mosquito breeding sites to sustain malaria transmission.

Poor environmental sanitation, like poor drainage systems also increase mosquito-breeding sites. This, in addition to development projects in pursuit of economic development such as water schemes, construction of dams and bridges, oil drilling and mining activities, urban planning and development, logging activities inadvertently lead to an increase in mosquito breeding sites, thereby increasing their numbers, human-mosquito contact and therefore transmission (Okenu, 1999).

Historically, population movement has also contributed to the spread of the disease (Prothero, 1977). The unprecedented increase in mobility in the last few decades has led to the relationship between mobility and malaria. The movement of infected people from areas where malaria is still endemic to areas where the disease had been eradicated has led to the resurgence of the disease (Gubler, 1998). For example, in 1985, 26 new active foci of malaria were introduced in Brazilian states outside the Amazon region (Marques, 1986). As people move, they increase their risk for acquiring the disease through the

ways in which they change the environment and through the technology they introduce, for example through deforestation for resettlement and irrigation systems (Service, 1991). Such environments create more favorable mosquito breeding habitats and at the same time, workers may have increased exposure to the vector. Furthermore, people can inadvertently transport infected mosquitoes to malaria free areas, reintroducing the disease (Martens and Hall, 2000). Population movement is also intensely implicated in the spread of drug resistance in malaria (Rajagopalan *et al.*, 1986), with people carrying resistant parasites moving from endemic to non-endemic disease areas and accelerating transmission of resistant strains.

Weak health systems, poor strategic development and inadequate funding of control programmes all play a role in the transmission of malaria. Recent rural to urban migration in search of economic and educational opportunities has created densely populated communities, especially in peri-urban areas and weakened health systems with the attendant increase in human activity and adverse effects on the environment, thus creating breeding sites. This promotes vector abundance leading to increased human-vector contact.

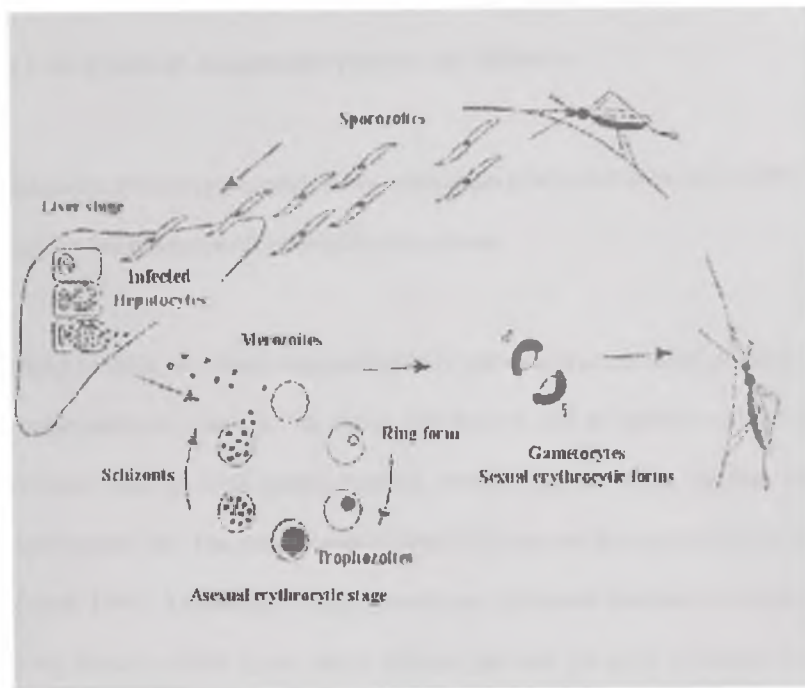


Figure 2: Schematic representation of the life cycle of *Plasmodium* species (After Garnham, 1966)

2.5 The Life Cycle of *Anopheles* Vectors of Malaria

The complete cycle from egg to adult takes a minimum of about 2 weeks or slightly less.

Figure 3 shows the life cycle of *Anopheles* mosquitoes.

The breeding habitats vary from large and usually permanent collections of water, such as fresh water swamps, marshes, rice fields and borrow pits to smaller collections of temporary water such as small pools, puddles, water filled car tracks, ditches, drains, gulleys, hoof prints, etc. The most common breeding sites are the shallow open sun lit pools (Service, 1993). Larvae also occur in wells and manmade container habitats such as clay pots, motor vehicle tyres, water storage jars and tin cans (Chinery, 1984). However *Anopheles gambiae* s.s prefer small and undisturbed temporary pools of water exposed to sunshine (Muirhead Thompson, 1945)

The female *An. gambiae* after mating and blood feeding lays some 50-200 small (1 mm long) brown or blackish boat shaped eggs on the water surface. Viable eggs hatch into larvae within 2-3 days in the tropics, but in cooler temperate regions they may not hatch until after 4 -7 days or longer (Service, 1980). The larvae, while on the water, lie parallel to the surface to allow water intake and surface feeding

At mean water temperatures of 25-28⁰ C the larvae undergo four moults within 6-9 days to reach the pupal stage, which lasts 2-3 days depending on temperature. Thus, the minimum duration for one generation may be as long as 10-11. The pupae bear

respiratory trumpets that are short and broad distally thus appearing conical. The most distinctive characteristic of *Anopheles* pupae is the presence of short peg like spines situated laterally near the distal margins of abdominal segments. The pupal skin splits dorsally and the adult emerges. Careful movements are required to ensure that the adult mosquito does not fall sideways and be trapped in the surface film. This danger is particularly acute when the adult is largely out of the pupal exuviae but the terminal appendages are still not free. Finally the legs become free and spread on the water surface giving stability. The newly emerged adult inflates its wings, and separates and grooms its head appendages before flying away (Kettle, 1992). When the progeny of any one egg batch emerge as adults the males emerge first. The males become ready for mating within 24 hours after emergence such that by the time the females emerge, the males are competent for mating. Mating is often preceded or accompanied by swarming in which the males associate over a marker and fly in a particular manner. Most of the male mosquitoes usually die after mating. The females require a blood meal for ovarian development, followed by the maturation and oviposition of a batch of eggs (Gillies, 1955).

The percentage of the eggs that goes to form the adults is unknown, but there is usually heavy mortality, especially among larvae due to predators, disease, drought, flood, etc. Larval loss due to predation is one of the factors that reduce the numbers of larvae that develop into adults. It is recognized that predation of larvae in established pools is an important factor in limiting their numbers. In some instances, *Culex tigripes* colonizes the same pools as *An. gambiae*, causing a dramatic reduction in larval density (Haddow,

1942). In permanent wells in Tanzania, Christie (1958) concluded that predation pressure was so intense that few larvae survived to pupae. Notonectidae were among the most important predators in these wells. It is possible that the same pressures exist in other types of permanent waters, thus limiting their productivity for *An. gambiae*. It may be noted that the vagility often displayed by *An. gambiae* larvae, in contrast to species such as *An. funestus*, would tend to increase their vulnerability to attack by predators (Service, 1980).

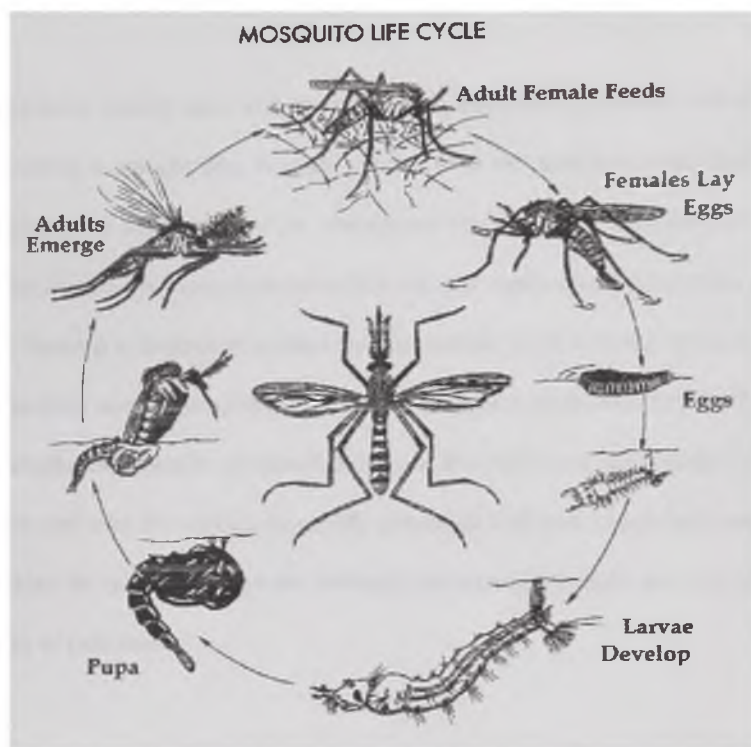


Figure 3: Schematic illustration of the life cycle of *Anopheles* vectors of malaria (After Service, 1980).

Adult *Anopheles* usually rests with the body at an angle to the surface, with proboscis and abdomen in a straight line. In some species, they rest at almost right angles to the surface, whereas in others such as *An. culicifacies* the angles are much smaller (Kettle, 1992). Most *Anopheles* mosquitoes have dark and pale scales on the wing veins arranged in blocks, forming a distinctive spotted pattern, which varies between species. Female mosquitoes have non-plumose antennae while males have plumose antennae. The palps of adult anopheline females are about as long as the proboscis and usually lie closely alongside it and may be marked, especially the apical half with broad and narrow rings of pale scales. In males the palps are distinctly swollen at the ends, and may also have apical rings of pale scales.

Most *Anopheles* are crepuscular or nocturnal in their activities, thus emergence from the pupae, mating, blood feeding and oviposition normally occur in the evenings, at night or early in the morning around sunrise. Some species such as *Anopheles albimanus*, a malaria vector in Central and South America, bite man mainly outdoors (exophagic) from about sunset to 2100 hours whereas in Africa species of the *An. gambiae* complex bite mainly after 2100 hours and mostly indoors (endophagic). Some species will rest in houses (endophilic) in between feeding whereas others will rest outside (exophilic) in a variety of natural shelters, such as amongst vegetation, in rodent burrows, cracks and crevices in trees, under bridges, in termite mounds, and other cracks in the ground. Most *Anopheles* species are not exclusively exophagic or endophagic, exophilic or endophilic, but exhibit a mixture of these extremes of behavior. Similarly, few *Anopheles* feed

exclusively on either man or humans, most feed on both man and animals but the degree of anthropophilism and zoophilism varies according to species (Gillies *et al.*, 2000).

2.6 Malaria Control

The strategy of malaria control is based on breaking the chain of transmission of the parasites between humans and mosquitoes. There are presently two approaches: control of parasites by chemotherapy and chemoprophylaxis and by breaking the contact between human host and vectors by controlling the vector by using insecticides, bednets and environmental management.

2.6.1 Chemotherapy of malaria

Chemotherapy in malaria control was started as early as the 1930's with the discovery of the aetiology and mode of transmission of the disease (Hackett, 1937). When antivector measures failed to significantly reduce the effects of the disease in endemic areas despite some early successes, it became evident that there was still heavy reliance on chemotherapy. Quinine is the drug that was widely used at the time but had undesirous side effects. Thus the advent of new and effective therapeutic drugs particularly the 4-aminoquinolines in the 1940's provided for the first time, reliable and acceptable means for treating malaria and for malaria chemoprophylaxis (Jeffery, 1984). Chloroquine

became the antimalarial treatment of choice because it was safe, inexpensive and highly effective against susceptible malaria parasites (WHO, 1967; Djimde *et al.*, 2001). Thus, malaria chemotherapy assumed a major role in primary health care because of the rationale of preventing mortality and curbing morbidity and suffering of afflicted persons. However, the efficacy of chloroquine in malaria chemotherapy has been compromised with the development of resistance to the drug by malaria parasites. Of the four *Plasmodium* species infective to man that have developed drug resistance, only *P. falciparum* poses a very serious clinical problem. The first documented cases of *P. falciparum* resistance to chloroquine were in South America in the 1950's (Peter, 1970), and confirmed in Thailand in 1959 (Harinasuta *et al.*, 1962). Since then chloroquine resistance spread to almost all the malarious areas of the world with heterogenic distribution both in frequency and degree (Bjorkman and Philip-Howard, 1990). In Africa, resistant strains of *P. falciparum* were first recorded in Kenya and Sudan in 1978 (WHO, 1986) and more recently in West Africa (Cheesbrough, 1991). In many parts of Africa, the drug is no longer used alone for therapy (Brasseur *et al.*, 1998). Chemotherapy has also been complicated further by the development and spread of resistance to other antimalarials such as Fansidar (Watkins and Mosobo, 1993), which is known to be heavily influenced by a combination of factors; Human migration, heavy malaria transmission, severe infection, immune response to malaria and sustained and/or haphazard use of the drug. Currently, drug combination therapy is recommended for malaria control in areas where drug resistance is highly prevalent.

2.6.2 Vector control

Malaria control with a vector control component has a long history in the African region. Prior to the Second World War, control was based mainly on antilarval measures, including source reduction, while pyrethrum spray as an adulticide was tried on a small scale in certain areas with variable results (Zahar, 1984). During the 1940's when the organochlorine insecticide Dichlorodiphenyltrichloroethane (DDT) became available during the Second World War, malaria control by house spraying was initiated on a small scale in certain countries and on a larger scale in Madagascar, Mauritius, South Africa (Natal and Transvaal), Swaziland, and Zimbabwe, formerly Southern Rhodesia (Bruce-Chwatt, 1963). When the global malaria eradication programme was initiated by the WHO in 1955 the WHO African Region was excluded because of the problem of logistics requirement associated with vector control, which were beyond the scope of the vast majority of the African countries and also because of intense transmission of *P. falciparum* by *An. gambiae* s.l., and *An. funestus* (WHO, 1999). To overcome this problem, a reorientation of control activities was undertaken in certain countries and islands, and eradication pilot projects were circumscribed in areas in several countries from the mid-1950's to the early 1960's (Bruce-Chwatt, 1963; NIAID, 2000). These projects, initiated by the WHO Malaria Eradication Programme between 1955 and 1969 were aimed at complete interruption of malaria transmission as a pre-requisite for malaria eradication.

The main approach was residual house spraying using DDT with or without chemoprophylaxis. In some areas, such as Cape Verde, Mayotte, Sao Tome and Principe there was a spectacular drop in parasite rates and malaria incidence, following the use of DDT (WHO, 1983). Malaria was successfully eradicated in large areas of South America, Southern Europe, the former Soviet Union and some territories of Asia and South America. The disease persisted in Latin America, most Asian countries and Africa. In the dry savanna of West Africa complete failure to control malaria transmission was encountered owing to a complex of technical, operational, administrative, logistic and financial factors (Haworth, 1981). In addition, population movements represented a constant threat of re-infection to the areas freed of malaria transmission (Prothero, 1961).

In 1968, DDT resistance in *An. gambiae* s.l. was reported in areas in West Africa where DDT was used in house spraying (Ivorra Cano and Bakri, unpublished report to WHO, 1975). The uses of other pesticides in agriculture coupled with the extensive use of DDT in cotton production areas were believed to have contributed to the selection pressure for resistance (Zahar, 1984). A report of resistance to other classes of insecticides by mosquito vectors has further compromised the use of insecticide spraying in malaria vector control. The evolution of resistance to most insecticides by the vector prompted the need to develop new tools for vector control.

2.4.2.1 Current vector control interventions

Significant progress has been made since the 1980's in research and development of new tools for use in malaria control activities. The use of insecticide treated bed nets (ITNs) and curtains with pyrethroids seems to be the most promising available method of controlling malaria in endemic tropical countries. Studies carried out in Senegal (Alonso *et al.*, 1991) and China (Cheng *et al.*, 1995) demonstrated the efficiency of ITNS for reducing infant mortality. These findings have been confirmed by subsequent large-scale multicenter studies in 6 countries across Africa including Ghana (Nevill *et al.*, 1996; Binka *et al.*, 1996; Lengeler *et al.*, 1996).

However, there have been reports of the emergence of pyrethroid resistance in the *Anopheles* vectors (Curtis *et al.*, 1998). Some cases of such resistance are known, notably in *An. gambiae* in West Africa where the kdr type of resistance has been selected probably due to the use of pyrethroids in cotton production (Zahar, 1984). Moreover, Doannio (1999) reports that despite the known repulsion effect of permethrin treated bednets on mosquitoes, the use of ITNs had no significant impact on transmission. On the other hand, other studies have shown that the use of ITNS is effective in reducing morbidity and mortality due to malaria (Lengeler, 2000).

2.7 The Systematics of *Anopheles gambiae* complex

Anopheles gambiae was thought to be one species until 1944 when studies by Ribbands and Muirhead Thomson (1944; 1945) in West Africa and Muirhead Thomson (1951) in East Africa provided the initial evidence for the specific distinctive nature of salt water breeding *An. gambiae*. The taxonomic status was still not understood but differences in behaviour were recognized. Cross mating studies by Muirhead Thomson (1951) later provided strong evidence of real differences by demonstrating hybrid male sterility between the two forms from East and West Africa.

In separate studies, hybrid male sterility between two fresh water-breeding populations was reported (Davidson, 1962). However, this was not considered to be evidence for interspecific differences between the two. Thus, as regards the fresh water *gambiae*, the familiar concept continued to prevail of a single opportunistic species adapted to a variety of environments. By 1964, three species had been recognized with Paterson (1964) presenting evidence that the three freshwater breeding species did not mate in nature. Holsten, (1952) had earlier suggested that the species consisted of 2 races. However on the basis of studies carried out by Davidson and Jackson (1962), Paterson (1963) concluded that these authors were dealing with sibling species. Paterson *et al.*, (1964) later provided direct evidence for the specific distinctiveness of three freshwater forms which he designated A, B and C that did not mate in nature. Later, another species breeding in heavily mineralized water in Uganda, originally documented by Haddow *et*

al., (1947) was designated species D (Davidson and Hunt, 1973). This brought the *An. gambiae* complex to a total of six species.

Mattingly (1977) proposed the formal names *gambiae* Giles, *arabiensis* Patton and *quadriannulatus* Theobald for species A, B, and C respectively, and species D was named *bwambae* by White (1985). The two recognized salt-water breeding species are *melas* Theobald in West Africa and *merus* Donitz in East Africa (Paterson *et al.*, 1964, Mahon *et al.*, 1976). Recently, Hunt *et al.* (1998) reported a new member of the complex designated *quadriannulatus* species B from Ethiopia.

While the existence of the six formally named species of the *An. gambiae* complex is well established, there is the indication that further subdivision within species of the complex may exist. The evidence for cryptic taxa within *An. gambiae* s.s is the observation that the micromorphology of the second chromosome for different populations differ by inversions. These chromosomal forms, which are far from Hardy Weinberg equilibrium at certain times of the year in West Africa also carry different strain specific combinations of inversions and differ in vectorial capacity. This has been well studied in Mali from where three forms “Bamako”, “Mopti” and “Savanna” have been described (Toure *et al.*, 1983). These forms appear to be fully or partially isolated reproductively in nature. In sympatric areas, hybrids between the “Savanna” and the two other forms have been observed at frequencies lower than expected. Also, no individuals carrying heterozygous complements of the “Mopti” and “Bamako” inversions are seen in nature, even though the two produce viable progeny under laboratory conditions

(Coluzzi *et al.*, 1985). Two other forms of '*gambiae*' namely "Forest" and "Bissau" have also been proposed (Bryan *et al.*, 1982).

2.8 Distribution of *Anopheles gambiae* s.l.

Members of the *An. gambiae* complex have a wide geographical distribution and have been reported from most African countries and adjacent islands including Madagascar, as well as Saudi Arabia and Yemen (Coetzee *et al.*, 2000). *An. gambiae* and *An. arabiensis* have the widest distributions. The two species show dissimilar distributional limits and asynchronous seasonal prevalence, probably due to divergent responses to climatic factors. *An. gambiae* predominates in forest and humid savanna zones whereas *An. arabiensis* is more successful in arid savannas and steppes, including those of the south-western part of the Arabian Peninsula (Coetzee *et al.*, 2000). Shifts in seasonal prevalence are observed showing an increase in the relative frequency of *arabiensis* during the dry season (Coluzzi *et al.*, 1979).

The distribution of *An. arabiensis* is higher in the low rainfall zones, which are usually the drier savanna areas (Gillies and Coetzee 1987; Hunt *et al.*, 1998). In areas where rainfall is less than 1000mm, *An. arabiensis* is recorded more often than *An. gambiae* and the reverse is true where rainfall is greater than 1000mm. *An. quadriannulatus* occurs sympatrically with *An. arabiensis* and less frequently with *An. gambiae* s.s. It appears to have a relict (narrow) distribution occurring in Southeast Africa, Ethiopia and

Zanzibar (Hunt *et al.*, 1998). In Ethiopia, it occurs at localities with mean annual rainfall figures above 1000mm whereas in Southern Africa, the same species occurs in localities where annual rainfall is around 700mm (Coetzee *et al.*, 2000).

Anopheles melas and *An. merus* are mainly salt and brackish water breeders on the West and East African Coast respectively, although *An. melas* has been found breeding in fresh water streams in the Gambia (Chinery, 1984). Evidence points to a short dispersal range from preferred breeding sites by *An. melas* and the adults are usually not found at distances more than 3 km from the saline environment (Bryan, 1987). *An. bwambae* is restricted to the Semliki forest in the Rift valley near the Zaire border where it breeds in geothermal mineral springs (White, 1985).

The larval stages of the “Mopti” form occur mainly in man-made habitats, even during the dry season, while the “Savanna” and “Bamako” forms have a tendency to breed in more natural sites, exploiting rain dependent pools for larval development (Coluzzi *et al.*, 1985). This affects their spatial and seasonal distribution, since the “Mopti” form will breed throughout the year and can therefore displace the other forms in irrigated areas, leading to changes in the patterns of malaria entomology (Toure *et al.*, 1994). “Mopti” is the only form of *An. gambiae* found during the dry season and could be more adapted than *An. arabiensis*, which could be competitively displaced (Toure *et al.*, 1998). “Mopti” is known from Mali, Guinea, Ivory Coast and Burkina Faso. Of the two other forms of ‘*gambiae*’ namely “Forest” and “Bissau” that have been proposed (Bryan *et al.*, 1982), “Forest” refers to forest breeding *gambiae*, occurring in rain forest areas

and in humid or derived savannas. “Bissau” refers to a form of *An. gambiae* s.s recorded in the Gambia and Senegal where it is associated with the rice fields along the banks of river Gambia (Bryan *et al.*, 1982). Unlike the six accepted species of the complex, these forms appear to display no post-mating reproductive isolation, with hybrids between forms actually having higher fecundity under laboratory conditions (Di Deco *et al.*, 1980).

The complexity surrounding the *An. gambiae* complex and presence of incipient species within *An. gambiae* s.s presents a need for accurate characterization of these strains, an exercise which is of utmost importance to malaria epidemiology studies.

2.9 Methods used in the identification of *Anopheles gambiae* sibling species

Although it has long been known that only some members of anopheline species complexes are important vectors of malaria, these sibling species have proven difficult, if not impossible to distinguish by traditional taxonomic measures. The similarity in the morphology of members of the *Anopheles gambiae* complex was a problem in their identification and characterization. The first indications of the presence of more than one species in *An. gambiae* came from crossmating experiments through which species A, B and C of the complex were recognized because of their mating incompatibility with known species (Davidson, 1964; Crampton, 1994). Since then, more techniques have been developed and applied in the differentiation of the cryptic species. These include:

the detection of differential banding patterns of the polytene chromosomes from the ovarian nurse cells of the semi gravid adult females (Coluzzi and Sabatinni, 1967), analysis of iso-enzyme frequencies (Mahon *et al.*, 1976; Miles 1978), analysis of cuticular hydrocarbons (Carlson and Service, 1979; Hamilton and Service, 1983) and the exploitation of species specific differences in the genome by the analysis of DNA sequences for distinguishing adults of *Anopheles gambiae* complex (Gale and Crampton, 1987; Collins *et al.*, 1987; Besansky, 1999).

2.9.1 Morphological identification

The available morphological diagnostic characters although not reliable are of definite taxonomic value for the distinction of the saltwater and the fresh water sibling species of the *Anopheles gambiae* complex. The number of sensilla coeloconica, the value of the palpal index and the shape of the eggs can separate these two groups (Service, 1983). However, none of these characters appears to be completely discriminant as the morphological characters of adults are variable and overlap in many instances (Coluzzi, 1964). No morphological features have been found as yet that can be reliably used for the distinction of freshwater species; *An. gambiae* and *An. arabiensis*; or between these species and *An. quadriannulatus*. The morphological divergences observed among the three fresh water species appears to be much less consistent and the morphological picture portrayed seems characterized by a wide intraspecific variability, presumably implying both polymorphism and polytypicism, which makes the evaluation of most of the taxonomic reliability of most of the characters studied problematic (Davidson, 1964).

2.9.2 Identification of *Anopheles gambiae* based on reproductive incompatibility

Hybridization tests have been exploited to reveal various degrees of genetic incompatibility, typically expressed as hybrid male sterility, which was shown to exist between all the six taxa (Davidson, 1964). In experiments by Paterson, (1964) all crosses produced sterile male hybrids and the females from these hybrids were fertile but in certain crosses, few or no females were produced, meaning that the reproductive isolation is almost complete. However, there are practical limitations to this diagnostic approach, which depends on time-consuming laboratory activity, hence inappropriate for large-scale routine identification. Moreover, while species distinctness is generally indicated by hybrid sterility lack of it does not necessarily indicate or prove conspecificity.

2.9.3 Cuticular Hydrocarbon analysis

This method, first described by Carlson and Service (1979) is based on the quantities of cuticular hydrocarbons of insects and has also been applied in the discrimination of *Simulium* blackflies (Carlson and Walsh, 1981) and in the *Phlebotomine* sand flies (Ryan *et al.*, 1986). Anyanwu *et al.* (2000) used this method in an attempt to separate the larval stages of members of the *Anopheles gambiae* complex. Overlaps in the hydrocarbon

profiles were observed even after the use of discriminant analysis. This is not surprising because discrimination between the strains of *An. gambiae* is based on the quantities of various hydrocarbons and on the relative concentrations of the component chemicals, rather than mere presence or absence. However, Hamilton and Service (1983) observed that, although the fourth instars of *An. gambiae* and *An. arabiensis* had similar cuticular hydrocarbons, there were still differences in the relative level of some of these chemicals. Although the biological implications of variations in cuticular hydrocarbons between very closely related species have not been fully studied, Phillips and Milligan, (1986) and Phillips *et al.* (1987) suggest that within such relationships species specific differences do exist which tend to highlight the effect of geographical variation and possible incipient speciation mechanisms. These chemicals, which serve mainly to prevent desiccation and also assist in chemical communication, may additionally play a role in mate selection/recognition and population divergence. Hydrocarbon differences among sympatric populations of *An. gambiae* s.s. might also reflect a semiological function of the compounds enabling the insect to recognize potential mates in those locations where sibling species coexist (Phillips *et al.*, 1987). In some insects, part of the mate recognition mechanism has been linked with the detection of specific hydrocarbon compounds and other components of the cuticular lipid layer e.g. fatty acids, alcohols, sterols, aldehydes, etc (Jallon, 1984; Pescke, 1987).

2.9.4 Molecular methods for species identification and population studies

During the past twenty years, the use of molecular data in systematics has become very popular mainly because of the development of new biochemical tools such as gene cloning (Sambrook *et al.*, 1989), DNA sequencing (Southern, 1975; Sanger *et al.*, 1977), restriction enzyme analysis methods (McDonnell *et al.*, 1977, Southern, 1979) and PCR (Mullis and Faloona, 1987). DNA analysis is highly suitable for population studies because it is the most direct analysis of genetic material possible and provides an easy to use method (Post *et al.*, 1992). It is applicable to all developmental stages and both sexes and is quite sensitive, requiring only minute amount of material that is easily preserved.

Molecular identification has the advantage of being applicable to adult females independently of their gonotropic state, as well as to adult males and to pupae. A rDNA based method for the identification of 5 members of the *An. gambiae* complex exists (Scott *et al.*, 1993). The rDNA-PCR method and other methods such as RAPD-PCR (Williams *et al.*, 1990, Favia *et al.*, 1994), RFLP (Beebe and Saul, 1995) and microsatellite DNA analysis (Lanzaro *et al.*, 1995) have been applied to study *An. gambiae* s.l populations. All these methods are PCR based.

2.9.4.1 Principles of PCR technology

The PCR is an *in vitro* method developed for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA (Saiki *et al.*, 1985). The reaction utilizes the DNA polymerase enzyme, and occurs in a repetitive series of cycles resulting in the exponential accumulation of large amounts of the target DNA. This process allows the enzymatic amplification of minute quantities of specific DNA sequences. The first experiments with PCR used the klenow fragment of *Escherichia coli* DNA polymerase (Saiki *et al.*, 1985; Mullis and Faloona, 1987). However, the high temperatures used to denature the template DNA diminished the activity of the klenow fragment. Therefore, more enzyme had to be added at the start of each cycle. This was both labour intensive and expensive. The technique was revolutionized by the discovery of a heat stable polymerase isolated from the thermophilic bacteria *Thermus aquaticus* and therefore called *Taq* polymerase (Saiki *et al.*, 1985). The fact that *Taq* polymerase enzyme is added to the reaction once, at the beginning of the reaction has transformed the PCR into a simple and robust reaction, which is now automated by programmable thermal cycling devices. The reaction components (DNA template, oligonucleotide primers, *Taq* polymerase, deoxynucleotides and reaction buffer which contains salts) are all included in a reaction mixture and the amplification reaction carried out by simply cycling the temperature within the reaction tube (Saiki *et al.*, 1988). The PCR reaction involves three steps of repeated cycles of heat denaturation of the DNA, primer annealing to their

complementary sequence at a lower temperature and extension of the annealed oligonucleotide by the DNA polymerase.

The yield and specificity of PCR is dependent on several factors, which include concentration of the polymerase enzyme, primers, DNA template and salts, time allocated to each reaction for annealing and extension, and the total number of cycles. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 20 cycles of PCR yields about one million-fold (2^{20}) amplifications

During PCR, each primer becomes incorporated into the DNA template. Oligonucleotide primers are designed to amplify DNA from specific sequences of organisms. This increases the specificity of the PCR. The annealing temperature of the primers is generally set as high as possible to promote specificity. However, it is recommended that annealing temperatures should be set at 5^o C below the melting temperature (T_m) of the primer. The T_m is calculated using the formula of Thein and Wallace (1986):

$$T_m = [4(G+C) + 2(A+T)]$$

2.9.5 PCR for the identification of *Anopheles gambiae* species complex

2.9.5.1 The ribosomal DNA method

The PCR is currently widely used in the identification of member species of the *An. gambiae* complex. Scott *et al.* (1993) developed the only method currently used for most studies. The method, which is based on species-specific nucleotide sequences in the ribosomal DNA (rDNA) intergenic spacer regions may be used to identify both species and interspecies of hybrids regardless of life stage using either extracted DNA or fragments of a specimen. The non-coding rDNA spacer sequences can be highly variable in length and sequence between closely related species. Concerted evolution acting on rDNA assays maintains sequence homogeneity within species as it drives differentiation between species, a pattern that explains the utility of rDNA for species diagnostic assays (Collins and Paskewitz, 1996). In using this method for the identification of mosquitoes, intact portions of a specimen as small as an egg or the segment of one leg may be placed directly into the PCR mixture for amplification and analysis (Scott *et al.*, 1993). The method uses a cocktail of five 20-base oligonucleotides to identify *An. gambiae*, *An. arabiensis*, *An. quadriannulatus*, and either *An. melas* in Western Africa or *An. merus* in Eastern and Southern Africa.

2.9.5.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) provides a means of discriminating between similar DNA samples at a species or strain level. Extracted DNA is digested using restriction endonucleases, which cleave at specific recognition sites. The resulting restriction fragments are separated according to size by electrophoresis on agarose gels revealing diagnostic polymorphisms with distinct DNA fragments. However, in most cases, the digestion products appear as a smear with fragments ranging up to approximately 25kb in size. To gain useful information on these gels, the DNA must first be transferred to a nylon or nitrocellulose membrane by Southern blotting (Sambrook *et al.*, 1989) and selective fragments visualized by hybridization with a suitable labeled DNA probe (Mills, 1994). Favia *et al.* (1997) designed a polymerase chain reaction (PCR-RFLP) assay, which unambiguously separates the *An. gambiae* Mopti form from Savanna and Bamako. This method, based on the presence of a restriction site length polymorphism has been tested on previously karyotyped sympatric specimens from Mali and Burkina Faso and it is already applied to verify the distribution of other molecular markers, as for example the pyrethroid resistance gene (*kdr*) among these chromosomal forms (Chandre *et al.*, 1999). It should however be noted that this technique has so far, not differentiated between any chromosomal forms (Favia and Louis, 1999).

2.9.5.3 Random Amplification of Polymorphic DNA (RAPD)

A further approach extensively used to distinguish members of cryptic species has been the use of Random amplified polymorphic DNA (RAPD) markers (Williams *et al.*, 1990). The analysis of random amplified polymorphic DNA (RAPDs) provides a novel and effective method for distinguishing *Anopheles* species and other organisms according to the banding patterns of their DNA as well as providing a new means of obtaining genetic markers (Hedrick, 1992). RAPD markers have been used to identify *An. gambiae* from *An. arabiensis* species. The technique is fast, technically easy, and requires little material. Most importantly, no previous nucleotide sequence information is needed for the construction of primers. Many markers can be readily identified for a variety of taxonomic levels and, in comparison with DNA sequencing, the effort and cost are modest so that many individuals can be assayed. However, questions have been raised concerning the validity of the method and these may both hinder the general use of the technique for identification purposes and cause concern for routine identifications (Rollinson and Stothard, 1994). One potential problem associated with this technique is that RAPD-PCR protocols based on short (10-12mer) primers are often difficult to reproduce in different laboratories with different equipment and reagents, or with DNA extracted and stored by different methods (Black, 1993). Since reproducibility is a fundamental element of the monitoring of vector distribution, RAPD markers do not represent the approach of choice.

2.9.6. PCR for the studies of *Anopheles gambiae* populations

2.9.6.1 Microsatellite DNA analysis

Microsatellites are described as DNA fragments consisting of simple short sequences usually of 2-6 nucleotides (nt), tandemly repeated in more or less uniform tracts upto approximately 10^2 nt long (Tautz, 1993; Chambers and Mc Avoy, 2000). Microsatellites are found in the genomes of just about every known organism and organelle. In most eukaryotic organisms, microsatellites are dispersed throughout the genome (Hamada *et al.*, 1982) and can occur as frequently as every 10kb (Tautz, 1989). Polymorphism in microsatellites leads to an increased probability of finding heterozygous individuals. Microsatellite loci are also frequently hypervariable, (i.e. they possess several alleles at relatively high frequency) improving their versatility and hence are ideal tools for molecular characterization of individuals and studies of intraspecific variation (Tautz, 1993; Lanzaro *et al.*, 1995).

They are usually polymorphic in length due to variation in the number of repeats within a given locus as a result of uneven cross over (as in mini satellite refer to Jeffery *et al.*, 1985), slippage of the DNA polymerase during replication or unequal sister chromatid exchange (Tautz, 1989). The high polymorphism of microsatellite loci results from high mutation rates, estimated to range from 10^{-2} - 10^{-5} locus/gamete/generation (Dallas, 1992; Edwards *et al.*, 1992; Weber and Wong, 1993) with most estimates being between the

10^{-3} and 10^{-4} Replication slippage events are considered to be the main process of producing insertion/ deletion mutations of one or infrequently several repeat units (Levinson and Gutman, 1987). Other evidence suggests that forces that shape allele composition include unbiased mutation rate (Garza *et al.*, 1995) and/or selection acting on allele size (Epplen *et al.*, 1993). However, if such forces strongly affect allele composition at these loci, inter-population differentiation will be underestimated and gene flow will be overestimated to an unknown extent (Lehmann *et al.*, 1996).

Microsatellite loci are more common in some organisms than in others and screening may produce a few useful loci in some species, particularly insects (Cooper, 1995). Repeats such as (dG-dT)_n have been found in all eukaryotic organisms, except yeast. In *An. gambiae*, conservative estimates suggest the presence of about 10,000 (dG-dT)_n microsatellites in the haploid genome. In *Aedes aegypti*, the search for simple sequence repeats has not been fruitful. For example, approximately 104 recombinant cosmids were screened with a (dG-dT)₁₅ probe, and microsatellites were detected at a frequency of approximately 1 in 300 colonies that were screened (S.E Brown, unpublished data). Since the average size was >30 kb, it appears that (dG-dT)_n microsatellites are relatively infrequent in the *Ae. aegypti* genome.

2.9.6.2 Applications of microsatellite analysis

Exploitation of microsatellite loci has provided biologists with a set of molecular tools with unsurpassed versatility. The key to this versatility lies in the high levels of variability, which are characteristically found at such loci, coupled with the speed and reliability with which this information can be accessed in the laboratory (Chambers and Mc Avoy, 2000). Their applications range from estimation of the spatial relationships between chromosome segments to the elucidation of temporal relationships between origins of species and genera. Microsatellite loci have also been described as ideal markers for measuring population level phenomena such as population structure due to their high polymorphism, codominance, abundant presence throughout the genome and relative ease in scoring (Bowcock *et al.*, 1994; Buchanan *et al.*, 1994; Scribner *et al.*, 1994; Eustop *et al.*, 1995; Lanzaro *et al.*, 1995).

Other applications include use in the analysis of laboratory and agricultural organisms and human genome diversity projects to map single gene traits rapidly (Hearne *et al.*, 1992), DNA profiling (Gill *et al.*, 1994) and also in forensic work (Chambers *et al.*, 1997). Microsatellites are almost perfect tools for application in determining the pattern of relationships between individuals because they are capable of highly discriminating biparentally inherited co-dominant markers. In the analysis of *An. gambiae* populations, microsatellite DNA analysis has been used to generate a genetic map of the mosquito (Zheng *et al.*, 1993) genetic differentiation of populations (Lanzaro *et al.*, 1995) and in the study of population structure (Donnelly *et al.*, 1999).

2.9.7 Cytotaxonomy

2.9.7.1 Polytene chromosomes and their value in cytogenetic studies

The repeated replication of chromatids without the occurrence of any mitosis-like events (endoreplication) leads to the production of thousands of sister chromatids within the same nucleus. This results in the formation of extended axial parts and contracted chromomeric loops of different sizes. The outcome are polytene chromosomes, appearing as bundles of extended interphase chromatids, characterized by peculiar banding patterns and by an axial length approximately 100-200 times the length of the same chromosomes during mitotic metaphase (Della Torre, 1997). Such chromosomes can be easily observed under a compound microscope and are useful for cytological studies.

The pattern of cross bands and allied features is what makes polytene chromosomes useful for cytological studies. Each chromosome bears a unique pattern of light and dark bands, puffs (sometimes called balbiani rings) and constrictions that are readable. This permits the characterization and identification of species or groups as the patterns of bands and puffs are characteristic for every group or species. Moreover, the course by which closely related species have evolved away from each other may be traced by seeing which chromosome mutations (i.e translocations, deletions, duplications, inversions) are shared between species and which of these changes are distinctive. Inversions, in particular have proved to be important in the study of chromosomal

relationships within sibling species complex for example in the *Simulium damnosum* complex (Dunbar 1969; Dunbar and Vajime 1972; Vajime and Dunbar, 1975; Boakye, 1993), the *Anopheles maculipennis* complex (Kitzmilller *et al.*, 1967) and the *An. gambiae* complex (Coluzzi and Sabatini 1967; 1969 and White, 1973).

In most mosquito genera, the polytene chromosomes are either not suitable for cytological manipulation or only partially replicated. Only those from Anophelinae give excellent preparations, although in some species it is invariably difficult to obtain good polytene preparations. Polytene chromosomes of *An. gambiae* can be obtained from the salivary glands of fourth instar larvae and from the nurse cells of half gravid adult females (Coluzzi 1968). The species can be characterized by one pair of heteromorphic sex chromosomes (X and Y) and two pairs of autosomes that are numbered 2 and 3 (Figure 4). However, since the heterochromatic region of the X chromosome and the whole Y chromosome are under-replicated, the typical anopheline polytene complement is substantially constituted by the euchromatic region of the X chromosome and by the two metacentric or submetacentric autosomes. This means a total configuration made of five polytenic arms (X, 2R, 2L, 3R, 3L) where X represents the sex chromosome, 2R, 2L and 3R, 3L the long and short arms of chromosome 2 and 3 respectively.

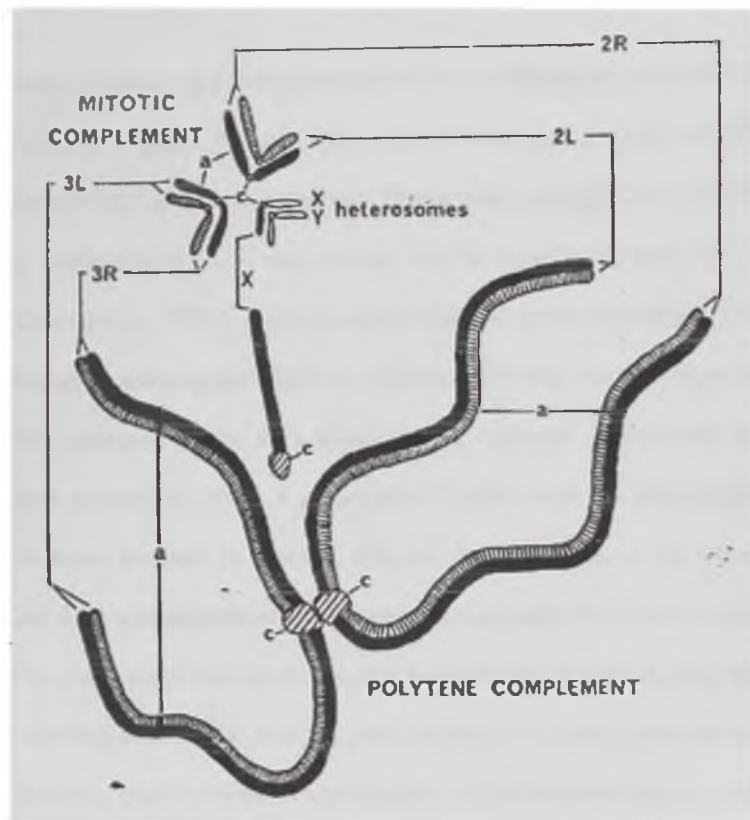


Figure 4: The three chromosome pairs in the mitotic complement of *Anopheles* and the five elements seen in its polytene complement (a=autosomes; c=centromeres, L and R = short and long arms of chromosome 2 and 3) (From White *et al.*, 1975).

2.9.7.2 Chromosomal inversions

A chromosomal inversion is a form of mutation that constitutes an intra-chromosomal reversal of a block of genes. For example, a hypothetical gene arrangement ABCDEF may become ABEDCF after re-arrangement. The inverted genotype persists only if it is intrinsically viable and if so, it may or may not be cross-fertile with the standard genotype (Coluzzi *et al.*, 1985). A new inversion is an important evolutionary step of the sort that frequently accompanies dipteran speciation. When standard inverted gene arrangements interbreed freely in a population, a situation of balanced inversion polymorphism prevails in which a proportion of individuals are heterozygotes. To achieve maximum synapsis in somatic cells of the heterozygote, the homologous chromosomes form a loop configuration. Such loops are easily discerned in cytogenetic polytene slide preparations and have important homeostatic function in suppressing the process of crossing over, which normally gives rise to novel gene combinations during meiosis. Therefore, while inversion heterozygotes exhibit heterotic vigour, which may enhance individual fitness, they reduce random genetic variability, which limits population adaptability.

It was the analysis of inversion polymorphism that led Coluzzi (1982) to propose that the key behavioral and adaptive differences between incipient and sibling species are integral rather than incidental to the speciation process. This idea was actually put forward in the context of chromosomal inversions bearing (and protecting from recombination), the determinants of these species-specific adaptations. Inversions also

act as chromosomal mechanisms that preserve gene associations or supergenes arising in temporary isolates subject to flush and crash in geographically and or ecologically marginal zones (Coluzzi, 1984; Petrarca and Bier, 1992; Coluzzi, 1992). Thus, inversions might express specific adaptive values for the environmental conditions in which they originated and alternative arrangements in a vector population might express ecotypic divergences that can be of interest in malaria transmission. Understanding the basis of chromosomal differences at the molecular level might give some insight into the speciation process and how human activity impinges on this process. In terms of malariology, some of these behavioural distinctions can make the difference between a good and a poor vector, and an understanding of their genetic basis may enable the manipulation of relevant behaviours to control disease transmission (Coluzzi *et al.*, 1985).

2.9.7.3 Inversion polymorphisms in the *An. gambiae* species

Polytene chromosome studies on *An. gambiae* complex show that the rearrangements of the banding pattern are all based on paracentric inversions (Coluzzi, 1984). These occur in the homozygous state in different species (i.e the fixed inversions used for species identification) or constitute intraspecific chromosomal polymorphisms (floating inversions) for studying population variation. Inversion polymorphisms have been recorded in all the six species of the *An. gambiae* complex except *An. merus* and have been found to be particularly frequent in *An. gambiae* and *An. arabiensis*, the two taxa showing the widest distribution and the closest association with man-made breeding places (Favia and Louis, 1999).

Anopheles gambiae karyotypes are recorded according to the nomenclature of Coluzzi *et al.*, 1978 in which standard arrangements for each karyotype are indicated by a + sign followed by a letter referring to the chromosome section and inverted arrangements are indicated by a letter alone, for example, 2L + *a* indicating the standard arrangement of inversion *a* on the short arm of chromosome 2. 2L+*a/a* indicates the heterokaryotype for the same inversion. Figure 5 shows the various karyotypes expected from eight 2R chromosome arrangements.

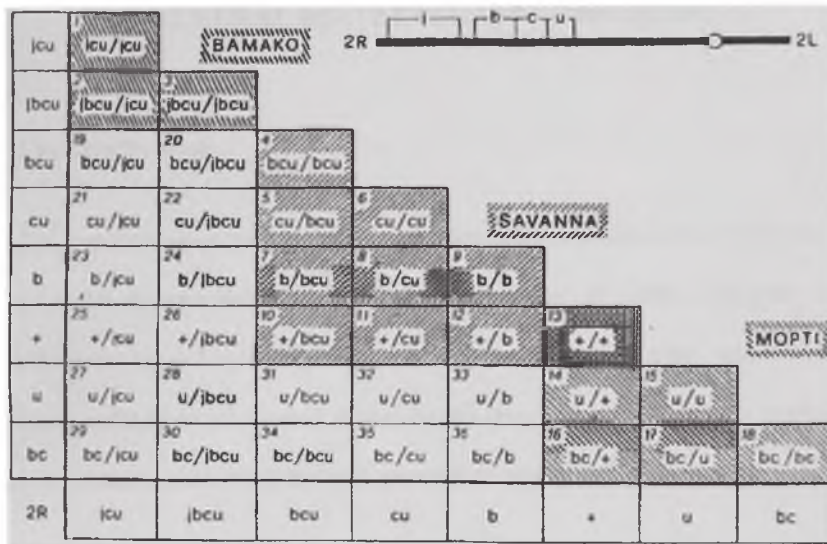


Figure 5: Diagrammatic representation of karyotypes expected from the eight 2R Chromosome arrangements frequently observed in *An. gambiae* (From: Toure *et al.*, 1983)

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 The Study Sites

The *An. gambiae* larvae and pupae used for this study were collected from five breeding sites located within Greater Accra Region, in the southern part of Ghana. One study site, Dodowa is located in the coastal forest region, and the other four study sites, namely Adenta, Madina, Achimota and Legon are located in the coastal savanna region, which is characterized by dry climatic conditions receiving the least amounts of rainfall in Ghana.

The region has two rainfall peaks from April to June and from September to October, with the mean annual rainfall ranging between 740 and 890mm. The highest mean monthly temperature (about 30°C) occurs between March and April and the lowest (about 26°C) in August. Relative humidity is high throughout the year, ranging between 65 and 75% in the afternoon.

The vegetation consists mainly of grass with isolated patches of scrub and sparse trees. The vegetation in Dodowa is described as coastal forest and is located between the coastal savanna and the beginning of the secondary forest areas. Very little of the original forest however remains and most of what is left is a mixture of few large trees with grass covering most of the area. The zone is very humid, with persistent rainfall (Dickson and Benneh, 1977).

Small shallow pools of stagnant rainwater characterized the Dodowa site. In Adenta, Madina and East Legon, the samples were collected from open gutters exposed to sunlight, and containing stagnant water and these contained a mixture of both *Anopheles* and *Culex* larvae. The Achimota site was a stretched pool of water in the open and exposed to sunlight, flowing from a leaking water supply pipe. Photographs of the sample collection sites are shown in Plates 1-5.

The global positioning system (GPS) was used to determine the geographical coordinates of the sites as being: Dodowa 05° 85239N, 00° 07028W; Adenta 05° 70339 N, 00° 15131W; Madina 05° 67444N, 00° 15131W, East Legon 05° 64653 N, 00° 1446W and Achimota 05° 63436N, 00° 23993W. The selection of the study sites was based on distances apart. The furthest distance was between Dodowa and Achimota (25kms), followed by Dodowa and Adenta, 16kms, Achimota and East Legon 11 km. The shortest distance was 4Kms between Adenta and Madina (Fig 6). Sample collection was carried out in the dry season during September and October 2000.

Figure 6: Sample collection sites



3.2 Field collection of mosquito larvae and pupae

The breeding sites of *Anopheles* mosquitoes were identified by random sampling of small pools of stagnant water in the open and in gutters, exposed to sunlight. Small shallow pools of stagnant rainwater characterized the Dodowa and Madina sites. In Adenta and East Legon, the samples were collected from gutters containing stagnant water and these contained a mixture of both *Anopheles* and *Culex* larvae. The Achimota site was a stretched pool of water, flowing from a broken water supply pipe. From all these sites, *Anopheles* larvae were identified by their characteristic horizontal position on the surface of the water. These were carefully collected into small plastic containers by scooping gently to avoid injuring the larvae. The containers were loosely capped to avoid suffocation and immediately transported to the insectary for rearing to adults.



Plate 1: Sample collection site at Dodowa. Shallow pool of stagnant rainwater exposed to sunlight.

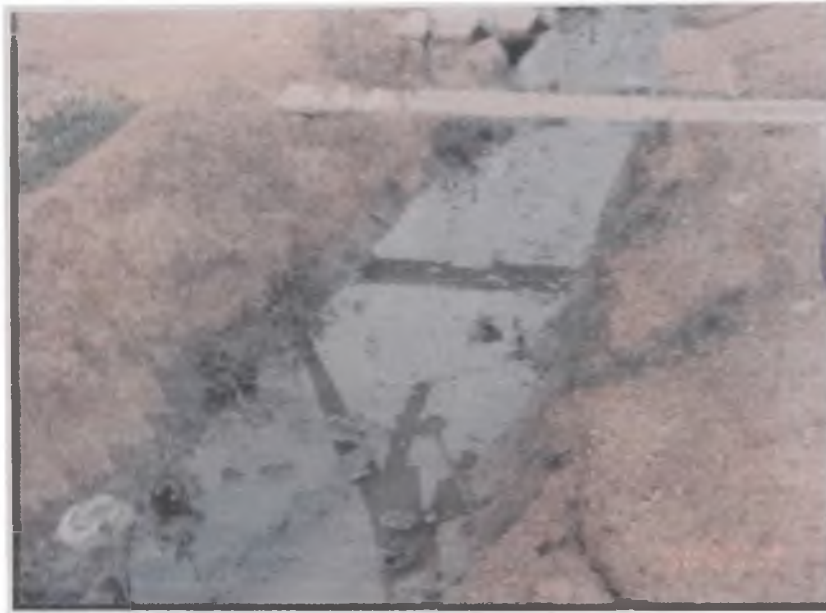


Plate 2: Sample collection site at Adenta. An open gutter exposed to sunlight, and containing stagnant water. This site contained a mixture of both *Anopheles* and *Culex* larvae.



Plate 3: Sample collection site at Madina. Shallow pool of stagnant water flowing from a residential washing area and exposed to sunlight.



Plate 4: Sample collection site at East Legon.



Plate 5: Sample collection site at Achimota. A narrow stretched pool of water flowing from a leaking water supply pipe and exposed to sunlight.

3.3 Laboratory rearing of mosquitoes

The larvae and pupae were transferred into plastic labelled rearing trays containing pond water (Plate 6). In cases of where larvae of *Culex* species were present they were identified by their angular position on the water surface and were removed. The level of water in the rearing trays was maintained at a depth of approximately 2cm. Rearing conditions were maintained at air conditions of 27-30°C and 76±2% relative humidity. A 12h:12h light and dark cycle was maintained. The larvae were fed on finely ground nutrafin goldfish food (Rolf Hagen, USA). Each morning the larvae that hatched to pupae were collected into shallow plastic cups using rubber pipettes, and then placed in appropriately labelled cages for adult emergence (Plate 7). The adults were fed on 10% sugar solution within 24 hours of emergence.

After 3 days, the males and females were assumed to have mated. Most of the males dropped dead at this stage and were picked out of the cage. Any live ones, which were probably yet to mate were left in the cage, presuming that some of the females had not mated. The mated females were then starved for 12 –14 hours prior to blood feeding by removing the sucrose solution from each of the cages. This was followed by aspiration of the females into small paper cups (plate 7) and blood fed, then returned into the cage for ovarian development to the half gravid stage. The development to the half gravid stage took about 12-15 hours. The rate of development differed for each individual female hence the development was regularly monitored after 12 hours by aspirating into tubes and checking each individual female abdomen for the half gravid appearance. The half-gravid females were killed by brief refrigeration at 4^o C.

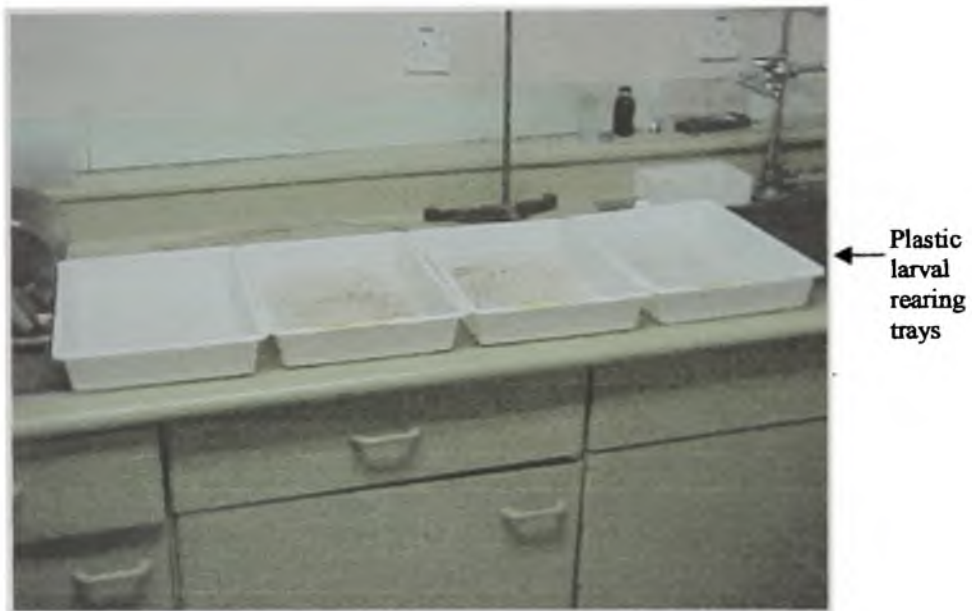


Plate 6: Larval rearing trays used in the laboratory rearing of *Anopheles* larvae and pupae.

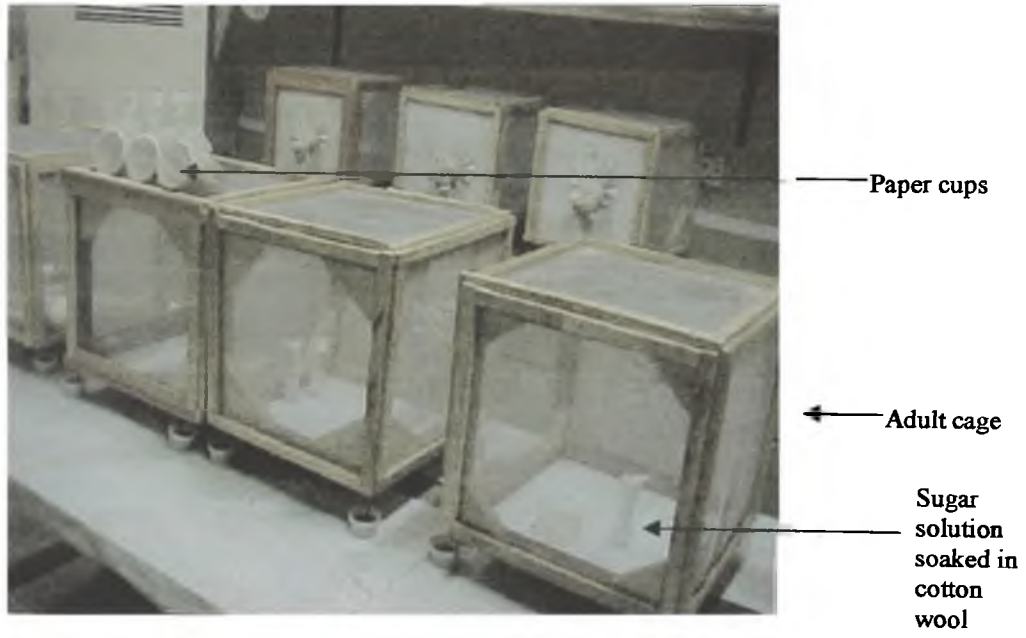


Plate 7: Adult cages for holding emerging adult mosquitoes

3.4 Morphological Identification of Female *Anopheles* Mosquitoes

The identification of adult female *An. gambiae* was made using morphological characters outlined in 3.4.1 below.

3.4.1 Outline of criteria for the morphological features used for the identification of adult *Anopheles gambiae* s.l.

Palps: Smooth, with 3 pale bands: The apical band broad and enveloping the whole of the 5th and apex of the 4th segment; median band narrow, less than half width of apical, involving apex of third and extreme base of 4th segments; basal band as median, usually confined to apex of 2nd segment.

Thorax: Ground colour variable, usually light brown or grayish; mediannally clothed with cream or yellow scales, pointed and moderately broad; laterally scales much broader and often rounded apically; anterior pronotal lobes with scales.

Pharynx: With 12-20 pairs of rods and cones, latter with deep roots and long flattish pointed filaments, which are somewhat fimbriated, crest not bifid in posterior view; rods with short filaments, post pharyngeal ridges with very long hair like processes.

Legs: Femora, tibiae and first tarsal segment speckled to a variable degree, sometimes with speckles fused to form short lines, rarely in the form of complete rings; tibiae rather narrowly pale apically; on fore and mid legs apices of first and fourth tarsi and apices

and bases of tarsi 2 and 3 narrowly but distinctly pale, 5th all dark; hind tarsi similar but basal pale bands on 2 and 3 absent or reduced 5th tarsus usually all dark.

Wing length: 2.8-4.4 mm

Wings: Pale markings, yellowish or cream. Wing field largely pale and with pale costal spots fairly long. The illustration is that of an average type of wing but there is much variation in the relative sizes of the pale and dark markings. Some of this variation may be indicative of inter-specific differences. Fringe spots present opposite veins 3-6; in addition a pale spot present between 6 and 5.2 and between 6 and the base of the wing.

Abdomen: Light brown, mainly clothed with hairs but 8th tergite usually with some scales, which may extend onto 7th tergite. Cerci scaly. Sternites conspicuously marked with symmetrical grey patches.

3.5 Sample Preservation

Each identified *An. gambiae* was divided into two parts by separating the abdomen with the half gravid ovaries from the head and thorax. The ovaries were immediately fixed in freshly prepared modified Carnoys solution (three parts absolute ethanol and one part of glacial acetic acid) for cytotoxicology (Hunt, 1973) and stored at -20^oC. The head and thorax were preserved dry on silica gel until ready to use for DNA extraction.

3.6 Cytotaxonomy

3.6.1 Preparation of chromosomes for cytological studies

The various stains and solutions used for the cytological studies were prepared as outlined in Appendix I.

Each of the fixed abdomens was placed onto a clean (dust-free and grease-free) microscope slide and pierced laterally with a dissection needle. The first three abdominal segments were pulled off directly into a drop of 50% propionic acid. The ovaries were then extracted simultaneously and isolated and then transferred into a drop of 50% propionic acid on a fresh microscope slide. The ovaries were left in the solution until they had swollen to approximately twice their original size and then divided laterally to separate the follicles. A drop of 2% Aceto-lactic orcein stain was added to propionic acid with the follicles and stirred with the needles to ensure homogenous staining. The tissue was left in the stain for 1-5 minutes after which excess fluid was drawn off using absorbent paper. This was followed by washing of the tissue with several changes of propionic acid to give a pale pink colour. A grease free and dust free cover slip was placed on the tissues and carefully squashed by gently tapping on the cover slip with the back of a pen. The preparation was then examined under a compound microscope.

3.6.2 Species identification by karyotyping

Using a phase-contrast microscope (Olympus B201, Japan), inversions on the specific band sequences on the whole chromosome complement were examined. The inversions on X chromosome were used for the identification of *An. gambiae* species (Coluzzi *et al.*, 1977). Full karyotyping was carried out and inversions were scored according to the nomenclature of Coluzzi *et al.* (1988). Standard arrangements for each karyotype were indicated by a “+” sign followed by a letter referring to the chromosome section. A letter alone indicated inverted arrangements. For example, 2L + “a”, indicates the standard arrangement of inversion “a” on the short arm of chromosome 2. Photographs of chromosome preparations were taken using a camera attachment to the microscope with green filter and Ilford Pan F film (Ilford Imaging, UK).

3.7 Molecular Studies

The various buffers and solutions used were prepared as outlined in Appendix II.

3.7.1 The extraction of genomic DNA

DNA extraction was done using the method by Collins *et al.* (1987) with slight modifications. Briefly, each specimen (head and thorax) was placed in a 1.5 ml eppendorf tube and homogenized with a sterile glass rod in 100µl Bender buffer (0.1 M NaCl, 0.2M Sucrose, 0.1M Tris-HCl, 0.05M EDTA pH 8.0 and 0.5% SDS). This was then incubated at 65°C for 30 minutes followed by the addition of 15 µl of pre-chilled 8M KAc and mixed well by tapping the tube, then left on ice for 30 minutes. It was then centrifuged for 5 minutes at 14000 rpm and the supernatant transferred into a fresh tube. To the supernatant was added two volumes of absolute ethanol, mixed well by tube inversion and then incubated at -40°C for 2 hours. This was followed by centrifugation at 14000 rpm to pellet the DNA. The supernatant was discarded and the pellet was left to dry by evaporation after which it was re-dissolved in 25 µL of ddH₂O, and left on ice for 1 hour. The DNA was stored at -20°C until ready for use.

3.7.2 PCR Identification of species of the *Anopheles gambiae* complex

Five sets of primers designed from the DNA sequences of the intergenic spacer region of *An. gambiae* complex ribosomal DNA (rDNA) were used in PCR for the identification (Scott *et al.*, 1993). The sequence details of these primers abbreviated UN, GA, ME, AR and QD and the expected sizes of the PCR products are given in Table 1. The UN primer anneals to the same position on the rDNA sequences of all five species, GA anneals

specifically to *An. gambiae* s.s., ME anneals to both *An. merus* and *melas*, AR to *An. arabiensis* and QD to *An. quadriannulatus*.

The PCR reaction mix of 25 μ l contained 1X PCR buffer supplied by the manufacturer (Sigma, USA), 200 μ M of each of the 4 oligonucleotide triphosphates (dNTPs), 10 μ M of each oligonucleotide primers and 0.125 units of *Taq* Polymerase enzyme (Sigma, USA). 0.5 μ l of the genomic DNA was used as template for the amplification reaction. Sterile double distilled water was used to make up the volume to 25 μ l. The reaction mix was spun down briefly at 14,000rpm and overlaid with mineral oil to avoid evaporation and refluxing during thermo cycling. The amplification was carried out using a PTC 100 thermal cycler, MJ Research Inc., USA

The cycling parameters for the reaction were as follows: 93 $^{\circ}$ C for 3min (initial denaturation), followed by 35 cycles of 93 $^{\circ}$ C for 30s, 50 $^{\circ}$ C for 30s, 72 $^{\circ}$ C for 60s (annealing) and a single extension cycle of 93 $^{\circ}$ C for 30s, 50 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 10 min. For each reaction a positive control with PCR products of *Anopheles* of the same primer set and a negative control that contained no DNA template were included.

Table 1: DNA sequence details of the synthetic oligonucleotide primers used for the identification *An. gambiae* s.l. species and their melting temperatures.

Name of Primer	Sequences (5' –3')	Melting Temperature T_m (°C)	Expected Amplified DNA size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	58.3	468
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390
ME	TGA CCA ACC CAC TCC CTT GA	57.2	464
AR	AAG TGT CCT TCT CCA TCC TA	47.4	315
QD	CAG ACC AAG ATG GTT AGT AT	42.7	153

3.7.3 Microsatellite DNA analysis

Three sets of oligonucleotide microsatellite primers; AGXH7, AG2H26 and AG2H46 (Lanzaro *et al.*, 1995) were used in PCR to amplify microsatellite DNA sequences in the *An. gambiae* s.s. All the three primer sets each consist of a forward primer and a reverse primer. The primer set AGXH7 is designed to amplify a microsatellite locus on the X chromosome, whereas AG2H26 and AG2H46 are designed to amplify microsatellite loci on chromosome 2. The sequence details of these primers and the expected sizes of the PCR products are given in Table 2.

The amplification of the microsatellite sequences was carried out using the method of Zheng *et al.* (1995). The reaction mix of 20 microlitres (μ l) contained 1X PCR buffer supplied by the manufacturer (Sigma, USA), 100 μ M of each of the 4 oligonucleotide triphosphates (dNTPs) (Pharmacia, Sweden), 10 μ M of each oligonucleotide primers and 0.125 units of *Taq* Polymerase enzyme (Sigma, USA). 0.5 μ l of the genomic DNA was used as template for the amplification reaction. Three primer sets AGXH7, AG2H26 and AG2H46 were used respectively in each reaction. Sterile double distilled water was used to make up the volume to 20 μ l. The reaction mix was spun down briefly at 14000 rpm and overlaid with 10 μ l mineral oil to avoid evaporation and refluxing during thermocycling.

The cycling parameters for the reaction were as follows: 95°C for 5 min (initial denaturation), followed by 35 cycles of 95°C for 30s denaturation, 55°C for 30s annealing, 72°C for 2 min (extension) and a final cycle of 95°C for 30⁰ s, 55°C for 30 s and 72°C for 5 mins. The annealing temperature used for each reaction depended on the (T_m) of each primer set. For each reaction a

negative control that contained no DNA template were included. PCR amplification was carried out using a PTC 100 thermal cycler (M.J. Research Inc., USA).

3.7.4 Analysis of PCR products

3.7.4.1 Agarose gel electrophoresis

Following the PCR, the products were electrophoresed on a 2% agarose gel stained with 0.5µg/ml EtBr to detect the presence of amplified DNA fragments. Eight microlitres of each sample was added to 1µl of orange G (5X) gel loading dye for the electrophoresis. The gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system (BIORAD USA) at 100 volts for one hour and the gel photographed over a UV transilluminator (UPC, USA) at short wavelength using a Polaroid camera and film type 667 (Polaroid, USA).

3.7.4.2. Polyacrylamide gel electrophoresis

The microsatellite DNA strands were electrophoresed in a 7 % polyacrylamide gel. The ingredients used in the preparation of the polyacrylamide gel are shown in the Appendix II. Fifteen microlitres of the PCR product from an individual mosquito was mixed with 10µL bromophenol blue dye and loaded into each well. Prior to loading the samples onto the gel, the wells were flushed with ddH₂O to remove any urea from the wells. The gel was run at 13mA for 7 hours, after which the glass plates were separated and the gel carefully transferred into a plastic tray containing 5% EtBr for 5-10minutes. The gel was visualized and photographed as described in section 3.7.4.1 above.

Table 2: DNA Sequence of the synthetic oligonucleotide primers used for the amplification of *An. gambiae* s.s. Each primer is given with its annealing temperature and the size of the expected PCR product for *An. gambiae* s.s. (F) and (R) denote forward and reverse primers respectively.

Name of Primer	Sequences 5' –3'	Annealing Temperature (°C)	Expected Amplified DNA size(bp)
AGXH7	CACGATGGTTTTTCGGTGTGG (F) ATTTGAGCTCTCCCGGGTG (R)	61	99
AG2H46	CGCCCATAGACAACGAAAGG (F) TTGTACAGCTGCAGAACGAGC (R)	63	139
AG2H26	CCGGCAACACACAAACAATCGG (F) GGTTCCTGTTACTTCCTGCC (R)	66	154

3.8 Data Analysis

3.8.1 Karyotype data

The frequencies of the different karyotypes observed were tested for the Hardy - Weinberg equilibrium using the Chi square test for observed and expected frequencies.

3.8.2 Microsatellite data

Each primer set was expected to give specific allele (band) sizes. The banding profile of the alleles of each sample as visualized on the gel was scored for the absence or presence of alleles (bands), depending on the DNA fragment sizes. The different sizes were denoted A, B, C, D, E, F and G. Distinct single bands were scored as homozygotes (e.g. AA) whereas bands which appeared either in pairs and in fours were scored as heterozygotes (e.g. AB). Missing bands were denoted by two dots “.” (for the details of results, see Appendix III). The alphabetic diploid and co-dominant data was analyzed to determine the genetic variation among and within populations using population genetics analysis software, POPGENE, Version 1.31 for Microsoft Windows™ (Yeh *et al.*, 1999). This was used to compute the relevant statistics for single locus in single populations and multiple populations; multilocus in single populations and multilocus in multiple populations. The output statistics included allele frequency, genotypic frequency, genetic distances, differentiation indices (F_{st} and F_{is}) and gene flow (N_m).

Using the algorithm described by Levene (1949) with a null hypothesis of random union of gametes and an alternative hypothesis of heterozygote deficiency, Chi square (χ^2) tests were performed for each locus to test for departure from the Hardy-Weinberg equilibrium at $P < 0.05$. Population differentiation and gene flow were determined by computing Weir and Cockerham's (1984) estimates of F statistics (F_{is} and F_{st}). The F_{is} is an estimate of heterozygote deficiency or excess. This was calculated to determine heterozygote deficiency (Positive F_{is} value) or excess (Negative F_{is} value). F_{st} is an estimate of inter-population genetic differentiation and was considered to be low if the differentiation index (F_{st}) was below 0.05 and high when F_{st} was greater than 0.05 (Wright, 1978). Positive F_{is} values indicate heterozygote deficiency while negative values indicate heterozygote excess (Lehmann *et al.*, 1997). The derived F statistics were used to estimate gene flow (Nm), i.e. the number of migrants per population per generation using the formula $Nm = (1 - F_{ST})/4F_{ST}$ (Slatkin and Barton, 1989). Gene flow was considered low when N_m ranges values are less than 3. Gene flow is high when N_m ranges from 3-30 and very large when N_m is greater than 30 (Donnelly *et al.*, 1999). Estimates of Nei's (1972) genetic distances were also calculated. Based on these distances, phylogenetic trees indicating the genetic relationship between the populations at each locus were constructed using Unweighted Pair Group Mean Average method (UPGMA)

CHAPTER FOUR

RESULTS

4.1 Laboratory Rearing of *Anopheles gambiae*

About 80% of the larvae and pupae that were reared in the insectary completed their growth and emerged as adults. Development from the larval to pupal stage lasted 3-5 days depending on the larval stage from the field whereas adult emergence from pupae lasted 1-2 days. Fourth stage larvae pupated overnight (10-12 hours from time of collection). It was observed that the first batch of mosquitoes that emerged had more males than the subsequent batches, which had more females. The development of blood fed females to the half gravid stage was very successful and lasted 12-15 hours, depending on temperature. Morphological identification revealed that all the specimens that were studied were *Anopheles gambiae* s.l.

4.2 Karyotype analysis of *Anopheles gambiae* s.l.

Out of the 129 samples dissected, only 35 gave readable polytene chromosome preparations and all were *An. gambiae* s.s. as identified by the presence of the diagnostic homozygous inversion *Xag* on the X chromosome (Figure 7). No hybrids or heterozygous inversions on the whole polytene complement were found. No inversions were recorded on the chromosomal arm 2R; hence this karyotype was standard 2R+ in

all the specimens (Figure 8). However, a homozygous floating inversion “*a*” on the chromosomal arm 2L was recorded (Figure 9), hence 2L was polymorphic with the alternative arrangements + *a* and *a*. The inversion 2L*a* was absent in the 10 specimens from Dodowa and in the 10 Adenta samples. In the EastLegon samples, 8 out of 10 were standard for 2R and 2L (2R+, 2L+) whilst the remaining 2 samples possessed the 2R+, 2L*a* chromosomal arrangement. 4 out of 5 samples from Achimota had the chromosome 2L inversion (2L*a*), which was absent in only one sample from this site. Table 3 shows the percent frequencies of the 2L*a* karyotypes observed at the different sites. A significant deviation from the Hardy-Weinberg equilibrium was observed only in the Achimota samples ($P < 0.05$).

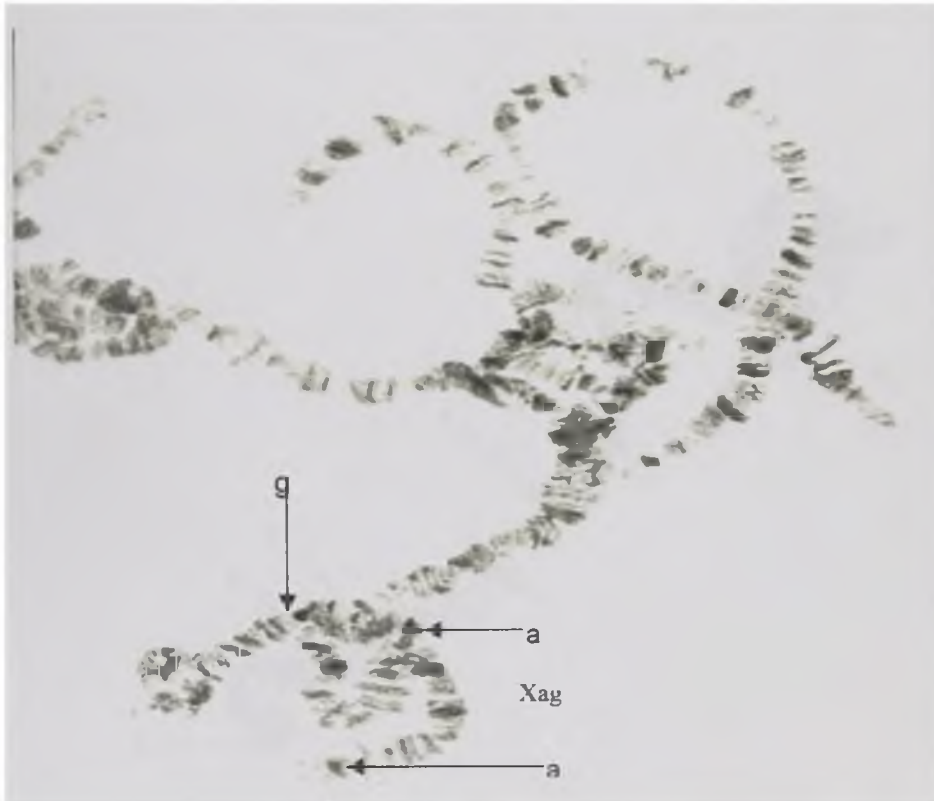


Figure 7: Chromosome complement of *Anopheles gambiae* showing the X chromosome (labelled) and the breakpoints of the diagnostic inversion (Xag)

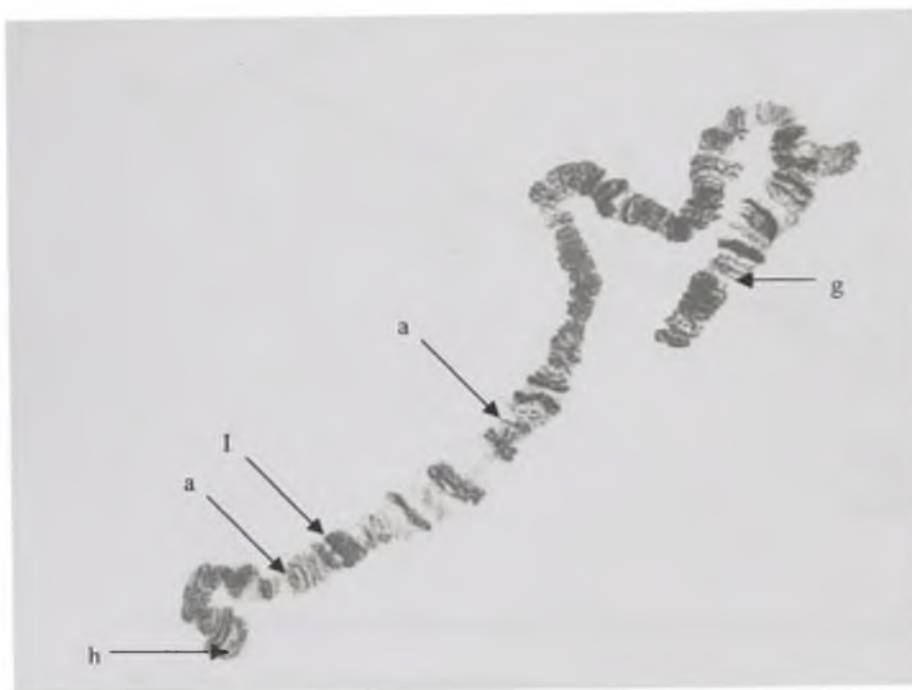


Figure 8: The right arm of chromosome 2 (2R) of *An. gambiae* s.s. showing the standard arrangement.



Figure 9: The left arm of chromosome 2 (2L) showing the floating inversion 2La which is a 180° rotation on section *a*.

Table 3: Frequencies of karyotype 21a observed in the *An. gambiae* s. s

Locality	Co-ordinates	No. of <i>An. gambiae</i> s.s examined	No. of Karyotype (%) +/+	+/a	χ^2
Dodowa	05° 85'23.9" N, 00° 07'02.8" W	10	10 (100)	0 (0)	0.04
Adentia	05° 70'33.9" N, 00° 15'13.1" W	10	10 (100)	0 (0)	0.09
East Legon	05° 64'6.53" N, 00° 14'44.6" W	10	8 (80)	2 (20)	0.12
Achimota	05° 63'43.6" N, 00° 23'99.3" W	5	1 (20)	4 (80)	6.33

χ^2 = Chi square for Hardy-Weinberg equilibrium
 P < 0.05

4.3 PCR identification of *Anopheles gambiae* s.s.

The DNA extraction from the 129 *An. gambiae* specimens was successful in all cases resulting in single bands (Figure 10). PCR amplifications revealed that all the specimens were *An.gambiae* s. s., confirmed by the size of the amplified DNA fragment which is 390 bp (Figure 11). The numbers of mosquitoes identified per site were 30 for Dodowa, 30 Adenta, 22 Madina, 22 East Legon and 25 Achimota.

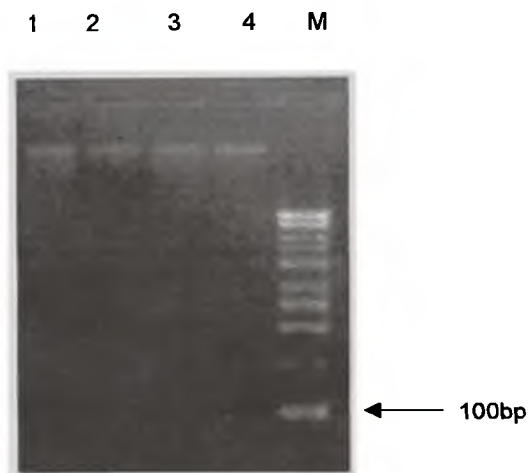


Figure 10: Ethidium bromide stained 1% agarose gel electrophoresis of DNA extracted from *Anopheles gambiae* mosquitoes. Lanes 1-4 = genomic DNA; lane M = 100bp molecular weight marker.

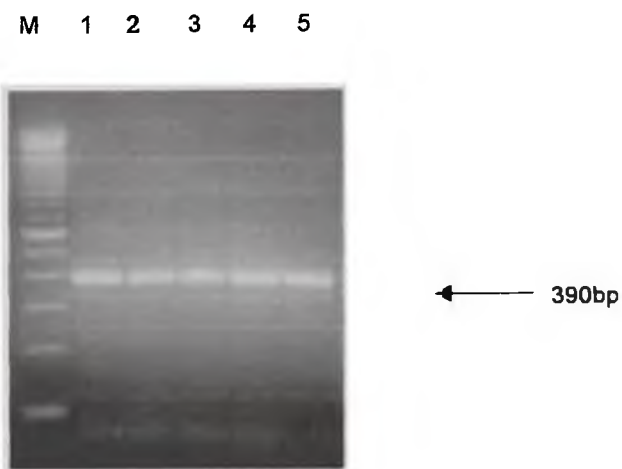


Figure 11: Ethidium bromide stained 2% agarose gel electrophoresis of PCR products obtained from the amplification of *Anopheles gambiae* DNA for species identification. Lanes 1= Dodowa; lane 2 = Adenta; lane 3 = Madina; lane 4 = East Legon; lane 5 = Achimota. M= 100 bp Molecular weight marker.

4.4 Genetic structure of *Anopheles gambiae* populations

Microsatellite DNA analysis was done using the primer sets shown in Table 2. A total of 129 specimens were analysed. Out of these, 80 (62%) were successfully amplified on first attempt. Of the remaining 49 (38%), only 10 (20 %) were successful on re-amplification. The failure of some samples to show any bands even after re-amplification was taken to be due to the possession of null alleles.

Following the convention of Excoffier *et al.* (1992), DNA bands that differed in sizes were taken to be distinct alleles. All the three microsatellite loci studied were polymorphic and a total of 30 alleles were recorded which were not uniformly distributed in all the populations. The distribution was as follows: 12 alleles at locus AGXH7 on the X chromosome and 9 alleles each at the two loci AG2H26 and AG2H46 on chromosome 2. At locus AGXH7, 88 out of the overall total of 129 mosquitoes were heterozygous for the 99bp allele whilst at locus AG2H26, 74 were heterozygous for the 98bp allele. At locus AG2H46 homozygotes were found to be dominant forming 76.7% of the 129 specimens examined.

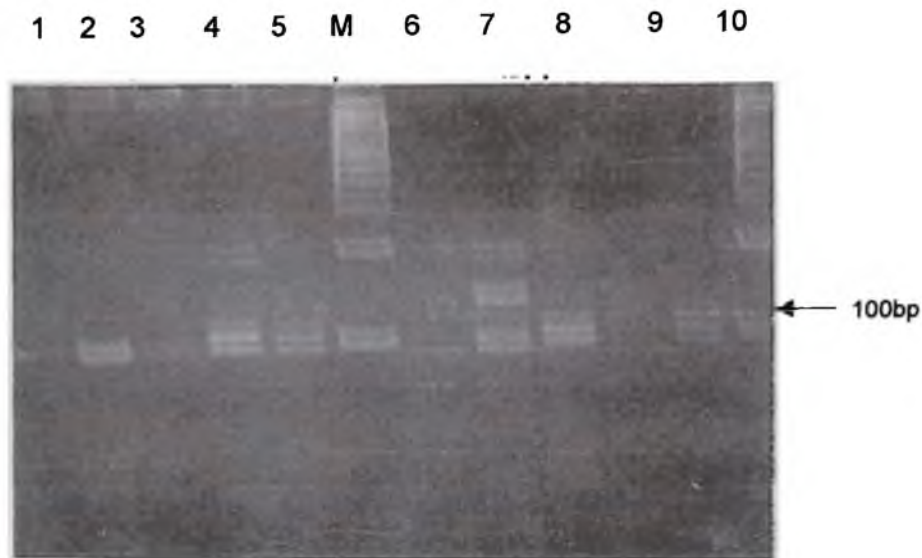


Figure 12: Polyacrylamide gel electrophoresis of PCR products obtained from the amplification of *An. gambiae* s.s. microsatellite DNA with primer AGXH7. Lanes 1 and 3 = Homozygous single bands (AA), Lane 2,5 and 8 = heterozygous double band (AB), Lane 4 and 7 = Heterozygous set of four bands (AB), lanes 6,9 = No bands (.), lane M = 100 bp Molecular weight marker.

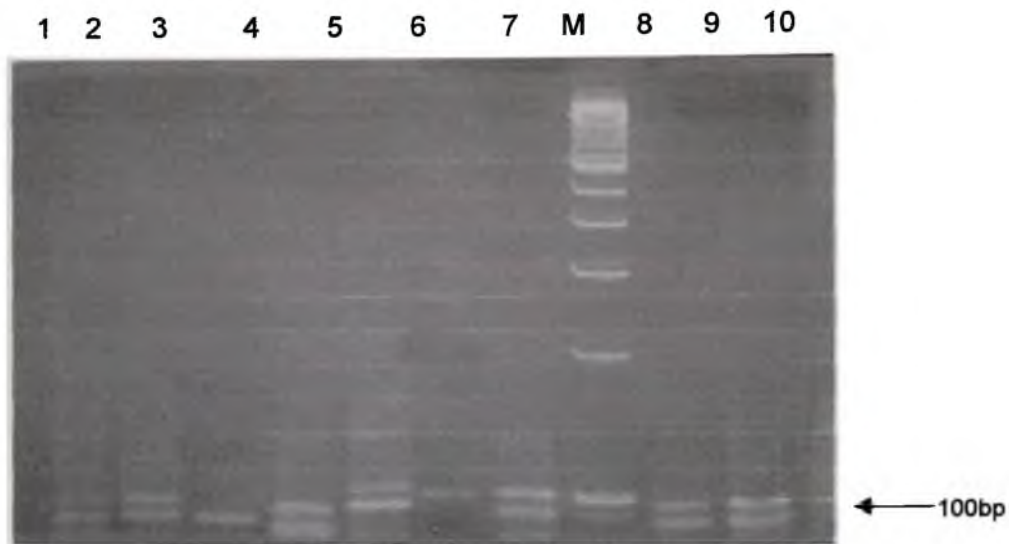


Figure 13: Polyacrylamide gel electrophoresis of PCR products obtained from the amplification of *An. gambiae* s.s microsatellite DNA with primer AG2H26. Lane 3 = Homozygous single band (BB), lane 6 = homozygous single band (DD), lanes 1,2,8,9 = heterozygous double bands (BC), lanes 5, 7 = heterozygous double bands (CD), lane 4 = heterozygous double bands (AC), lane 10 = No bands (.), lane M = 100 bp Molecular weight marker.

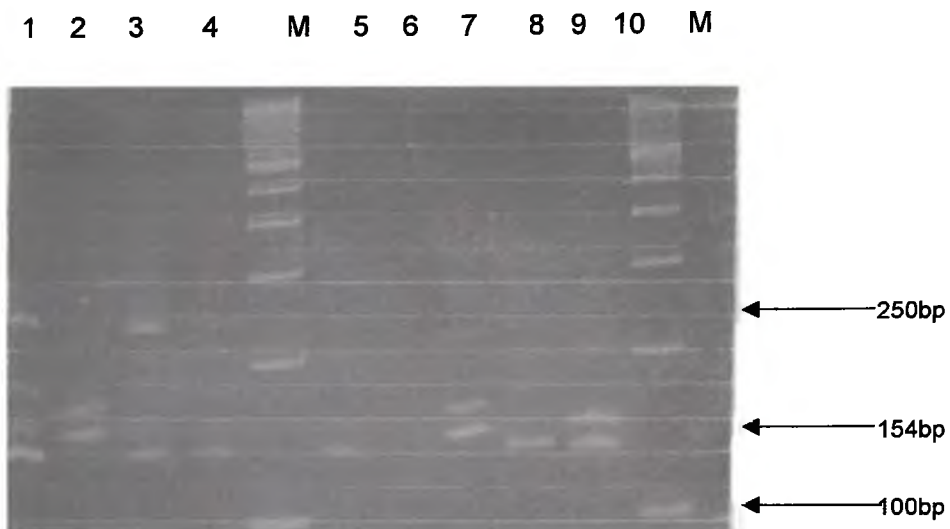


Figure 14: Polyacrylamide gel electrophoresis of PCR products obtained from the amplification of *An. gambiae* s.s. microsatellite DNA with primer AG2H46. Lane 1 = Heterozygous four bands (DE), lanes 2,8,9 & 10 = heterozygous double bands (EF), lanes 3,4 & 5 = Homozygous single bands (DD, EE) lanes 6,7 = No bands (..) M = 100 bp Molecular weight marker.

4.4.1 Allele and genotype frequency distributions

Table 4 and Figures 15-17 show the distribution and frequencies of all the common alleles that were found. Eight alleles were the most common; however alleles 1 and 2 were the most dominant and were present in all the five populations but at different frequencies. The mean allele frequency in the pooled populations was 7.33.

The genotype frequencies for all the loci deviated from the Hardy-Weinberg equilibrium (HWE) at the 0.05 level of significance when pooled population data were analysed (Table 5). When each of the three loci and five populations were tested individually the Dodowa, Madina and Achimota populations were in equilibrium at the AGXH7 locus ($P > 0.05$), whereas Adenta and East Legon populations significantly deviated from it ($P < 0.05$). At the AG2H26 locus only the Achimota population was in HWE ($P > 0.05$), whilst Dodowa, Adenta, Madina and East Legon populations were not ($P < 0.05$). At the AG2H46 locus, Adenta, East Legon and Achimota populations were in HWE ($P > 0.05$) whereas Dodowa and Madina were not ($P < 0.05$).

Table 4: Allele frequencies of most common alleles at all the three loci.

Locus	Sample size	No of alleles	Frequency of the most common alleles							
			1	2	3	4	5	6	7	8
AGXH7	129	12	0.30	0.25	0.18	0.03	0.05	0.05	0.03	0.03
AG2H26	129	9	0.34	0.29	0.13	0.03	0.16	0.00	0.03	0.03
AG2H46	129	9	0.28	0.31	0.08	0.00	0.17	0.06	0.08	0.08

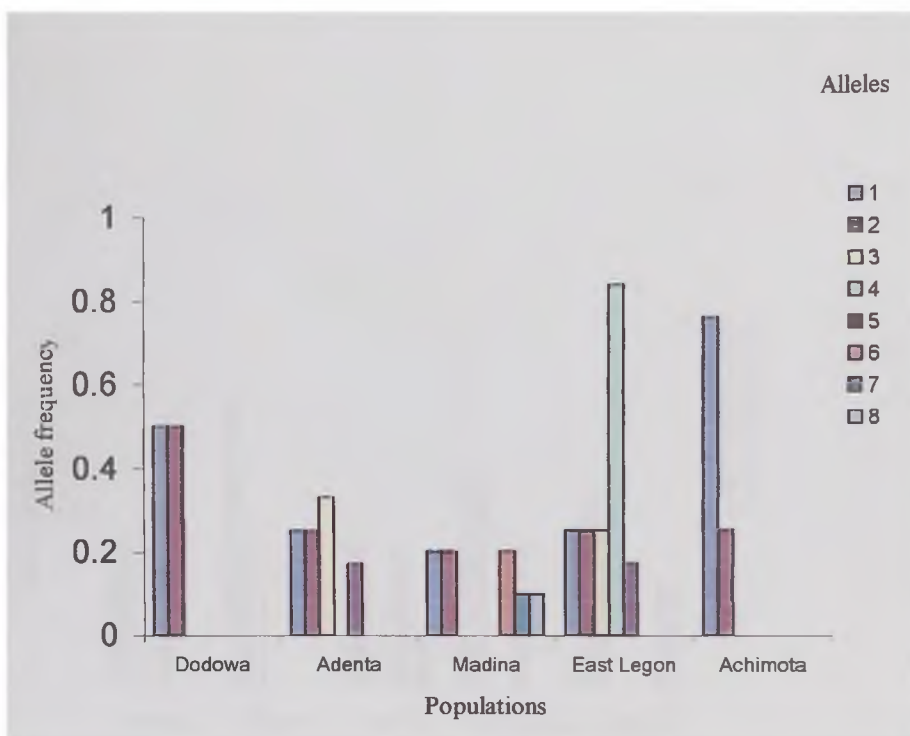


Figure 15: Allele frequency distribution at the AGXH7 locus

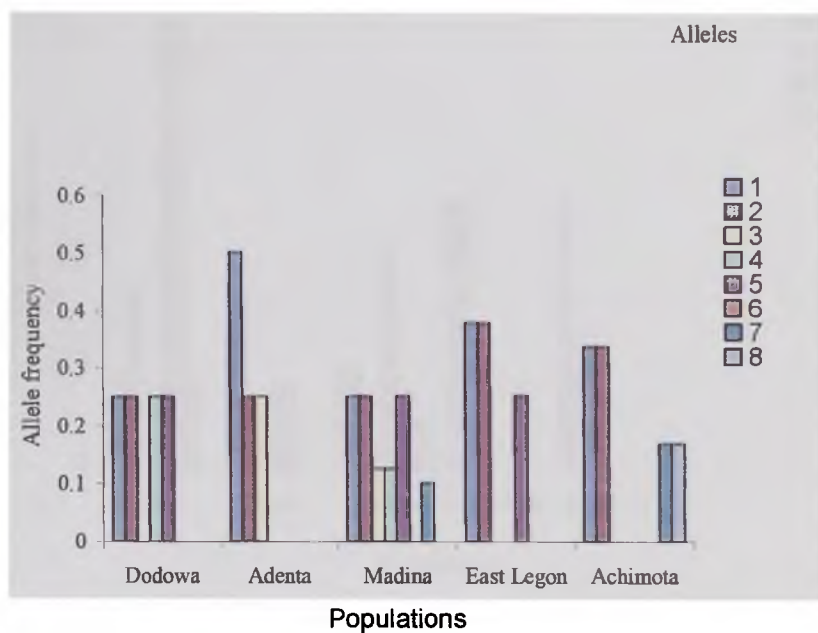


Figure 16: Allele frequency distribution at the AG2H26 locus

Table 5. Contingency χ^2 data for genotype frequencies among the five *An. gambiae* s.s. populations

	Dodowa		Adenta		Madiina		East Legon		Achimota		Pooled
	df	χ^2 p	df	χ^2 p	df	χ^2 p	df	χ^2 p	df	χ^2 p	
AGXH7	1	0 1.00 ^m	6	23.33 0.00*	15	22 0.1 ^m	10	23 0.00*	0	1 1 ^m	0.00*
AG2H26	6	17 0.01*	3	7.67 0.05 ^m	10	17 0.00 ^m	3	10 0.02*	6	7 0.32 ^m	0.00*
AG2H46	15	34 0.00*	6	7.00 0.32 ^m	10	19 0.04*	1	1 0.32 ^m	3	4 0.26 ^m	0.00*

Data were analysed to estimate the effects of collection site and chromosomal form. Loci are grouped by their chromosome location (X, chromosome X; 2, Chromosome 2), df = degrees of freedom.

^m Not significant, * Significant at $P < 0.05$

4.4.2 Population differentiation Index (F_{st})

The differentiation index i.e. F_{st} indicating the genetic relatedness of mosquitoes within each population was zero in all cases for all the loci. However, when pooled loci data were analysed for all the populations together, considerable differentiation between loci was observed (Table 6). The mean F_{st} value over all loci was 0.0984. Estimates of F_{st} were highest for the AGXH7 locus ($F_{st} = 0.1282$) followed by AG2H46 ($F_{st} = 0.0993$) and was lowest at locus AG2H26 ($F_{st} = 0.0664$). F_{st} was significant for all the loci, being greater than 0.05 (see section 3.8.2) but most significant at locus AGXH7 ($F_{st} = 0.1282$) indicating high differentiation at this locus compared to the other two.

Pairwise comparison of genetic differentiation between populations revealed that the highest F_{st} was between the furthest site Dodowa and the rest of the four sites ($F_{st} = 0.0565$). This was followed by a differentiation index of 0.0511 between East Legon and Achimota, separated by a distance of approximately 8 Km. The lower F_{st} values were found between the closer populations being 0.0344 for Madina and Achimota (5km apart) and 0.0284 for Madina and East Legon (2km apart).

4.4.3 Estimate of heterozygote deficiency and excess (F_{is})

The heterozygote deficiency indicated by a positive F_{is} value was obtained in all populations except the Madina population, which had a negative value. The F_{is} values were 0.0204 at Dodowa, 0.1457 at Adenta, -0.0435 at Madina, followed by an increase to 0.4910 in East Legon and 0.5219 in Achimota (Figure 18).

However, when the data were analysed according to loci, the two loci on chromosome 2, Locus AGXH7 and AG2H46 indicated heterozygote excess values of -0.0098 and -0.0654 respectively. Only locus AG2H26 showed a heterozygote deficiency of 0.1427. The mean estimate of heterozygosity (F_{is}) over all loci was an excess value of -0.0655 (Table 6).

Table 6: Estimates of differentiation (F_{st}), gene flow (N_m) and heterozygosity (F_{is}) at each Locus

Locus	F_{st}	F_{is}	N_m
AGXH7	0.1282	-0.1427	1.7004
AG2H26	0.0664	0.0098	3.5152
AG2H46	0.0993	-0.0654	2.2680
Mean	0.0984	-0.0655	2.2900

Table 7:

Pairwise comparison of estimates differentiation F_{st} and gene flow (N_m), and genetic distances between populations based on pooled loci data.

Populations	F_{st}	N_m	Genetic distances
Dodowa vs Adenta	0.0565	4.1773	0.2999
Dodowa vs Madina	0.0565	4.1773	0.2999
Dodowa vs East Legon	0.0565	4.1773	0.2999
Dodowa vs Achimota	0.0565	4.1773	0.2999
Adenta vs Madina	0.0284	8.5672	0.1957
Adenta vs East Legon	0.0284	8.5672	0.1957
Adenta vs Achimota	0.0284	8.5672	0.1957
Madina vs East Legon	0.0344	7.0163	0.1461
Madina vs Achimota	0.0344	7.0163	0.1461
East Legon vs Achimota	0.0511	4.6433	0.1410

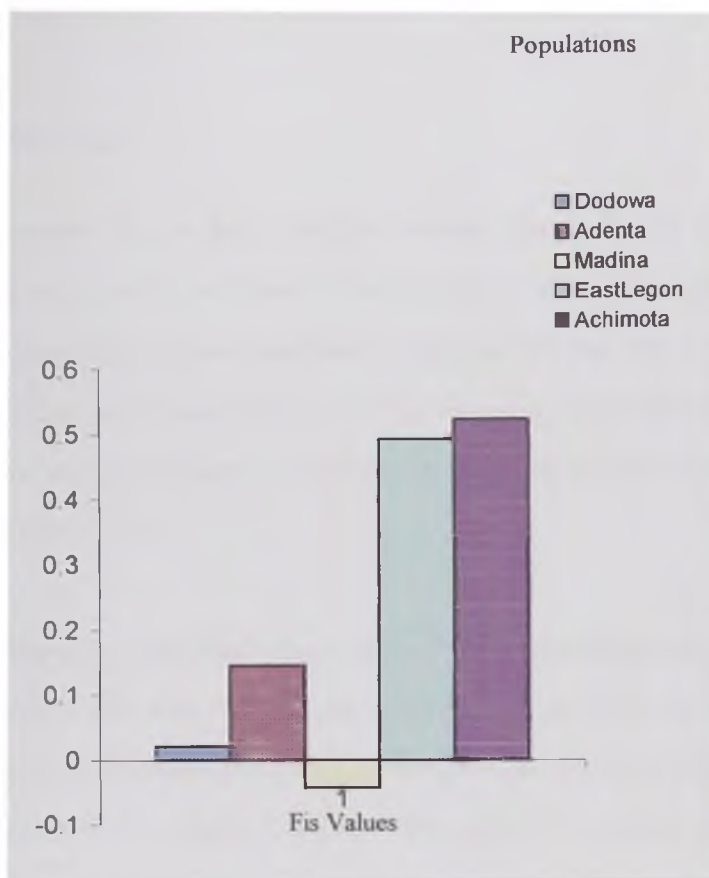


Figure 18: Estimates of F_{is} values showing deficiency and excess of heterozygotes.

4.4.4 Gene flow (N_m)

Fairly large estimates of gene flow (N_m) were derived from F_{st} . Details of results obtained are given in Table 7 and Figure 20. Accordingly, estimates of gene flow (N_m) were highest between the closest populations, Adenta and Madina ($N_m = 8.5672$); followed by Madina and Achimota ($N_m = 7.0163$), East Legon and Achimota $N_m = 4.6433$. The lowest estimate of gene flow was between the furthest populations, Dodowa and Achimota ($N_m = 4.1773$).

A plot of the estimates of gene flow against distances between populations indicates the increase in level of gene flow between populations with a decrease in the physical distances between the populations (Figure 20). Linearization of the plot which is an inverse function ($Y=a/x + b$) resulted in the determination of the two unknowns, a and b ($a = 15$, $b = 3.3$), where a = the point at which the linear function intersects with the straight line and b is the gradient of the straight line. The equation of the inverse function $Y=15/x + 3.3$ was thus derived, where Y = gene flow and x = the distance between populations.

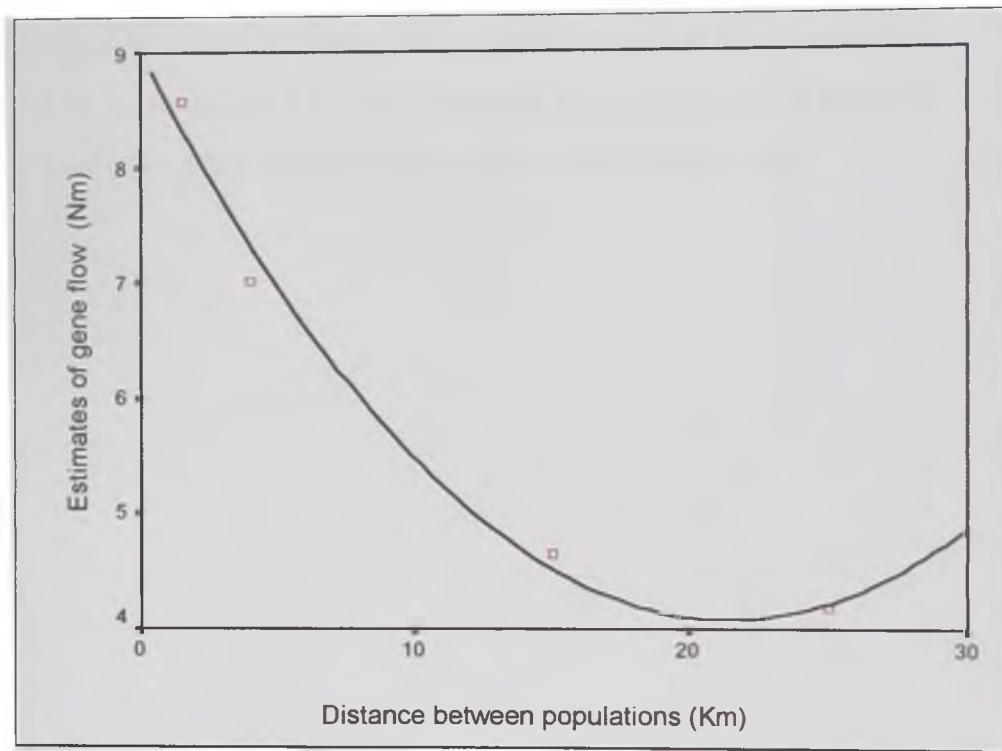
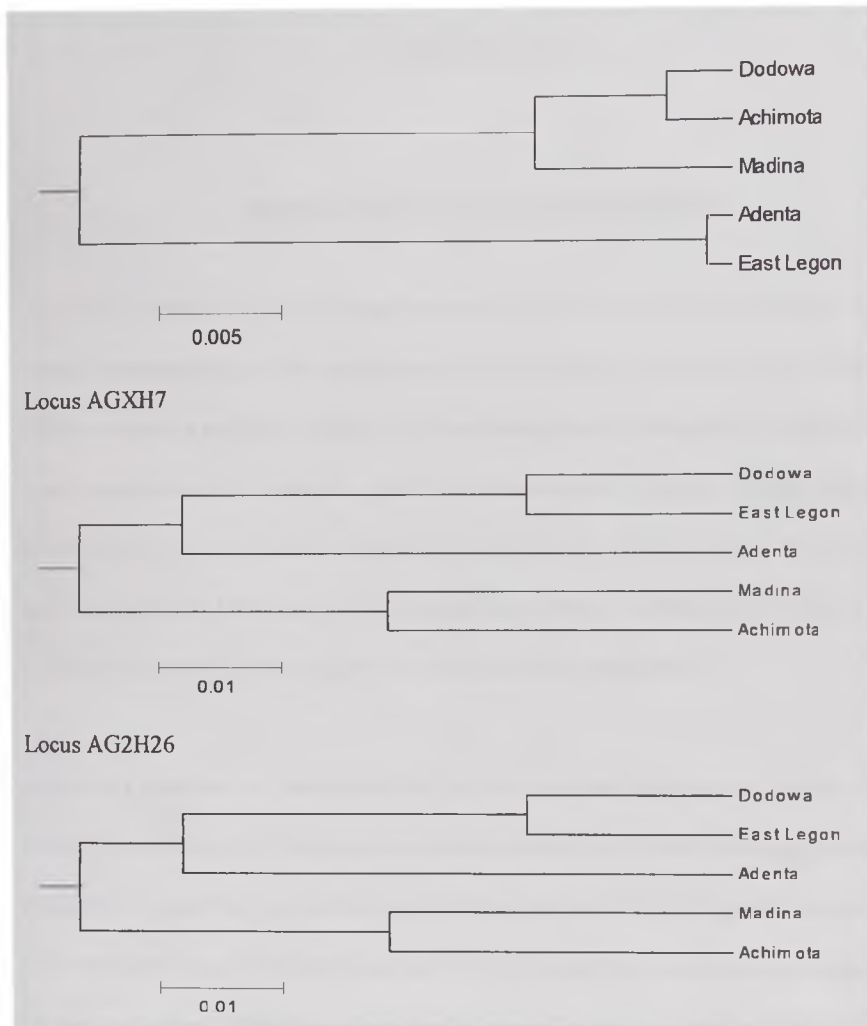


Figure 19: A plot of gene flow between populations against distances between them.

4.4.5 Phylogenetic relationship

To illustrate the phylogenetic relationship among the five *An. gambiae* populations and the three loci, phenograms were constructed for each chromosome separately and for the pooled ensemble using the Unweighted Pair Group Method with Arithmetic averaging of F_{st} values, calculated with the POPGENE software. It was observed that the loci on chromosome 2 provided a phenogram that was most similar to the pooled loci and different from that of the locus on the X chromosome (Figure 20).



All Loci combined (AGXH7, AG2H26 & AG2H46).

Figure 20: Phenograms based on UPGMA to illustrate the phylogenetic relationships among the *An. gambiae* s.s chromosomal forms and populations.

CHAPTER 5

DISCUSSION AND CONCLUSION

Anopheles gambiae is the principal vector of human malaria in sub-Saharan Africa. A better understanding of the species and its distribution is needed for the planning of effective malaria control strategies and the management of insecticide resistance. This study was set to characterize *An. gambiae* species from five sites at various distances up to 25 km within the coastal savanna and coastal forest zones of Ghana by karyotyping and microsatellite DNA analysis. The main aim was to establish the karyotypes in the zone and assess the levels of gene flow between the populations.

Anopheles gambiae s.l. was identified as the dominant *Anopheles* mosquito species. Further identification of these species as *An. gambiae* s.s by both karyotyping and PCR revealed *An. gambiae* s.s. as the major sibling species of the *An. gambiae* complex in this ecological zone. No other species of the *An. gambiae* complex were encountered in the study area. There have been no previous studies on species identification to determine which species of the *An. gambiae* complex occur in Adenta, Madina, East Legon and Achimota. However, Chinery (1984) in a study carried out in Accra (unidentified sites) suggested that *An. arabiensis* had displaced *An. gambiae* s.s. as the predominant species. The presence of only *An. gambiae* s.s. at these sites in Accra as found by the present study clearly contradicts the observation by Chinery (1984). In a more recent study Appawu *et al.* (2001) have reported *An. gambiae* s.s. as the most

important vectors of malaria in forest and savanna areas of Ghana in terms of its distribution and transmission. *An. melas* has also been reported in studies carried out along the coast (Appawu *et al.*, 2001). But this was not encountered in the present study. This is not surprising since *An. melas* breeds in salt water and the study sites were far from the areas with salt pans and lagoons.

The finding that *An. gambiae* s.s. was the major sibling species in the forest area agrees with the general observation by Coluzzi *et al.* (1979) that it is the most prevalent species in the forest belts of West Africa. Although no *An. arabiensis* was encountered Coluzzi *et al.* (1979) have reported that *An. arabiensis* penetrates areas after deforestation and urbanization, but this species was not observed in the study area, especially Dodowa where considerable deforestation and urbanization have taken place.

Cytological analysis was also used in this study to identify the different karyotypes that occur in the study area and to seek evidence for distinctiveness of populations and gene flow. Karyotype analysis revealed the existence of the 'Forest' and 'Savanna' forms of *An. gambiae* s.s. in the study area. Only the 'Forest' form which is characterized by the standard arrangements (2R+, 2L+) on chromosome 2 was found at Dodowa and Adenta. At these two sites, all the 20 specimens studied were the forest form of *An. gambiae* s.s. These findings are also in agreement with Appawu *et al.* (1985) who previously recorded the occurrence of the forest form with inversions 2R+ and 2L+ in *An. gambiae* s.s. populations, from Dodowa, in the coastal forest zone of Ghana. The 'Forest' karyotype was also found at East Legon and Achimota, occurring together with

the 'Savanna' form, which is characterized by the chromosome 2R standard arrangement (2R+) together with inversion *a* on chromosomal arm 2L (2La). At these two sites, the 'Savanna' form was recorded in 2 out of 10 specimens studied at East Legon and in 4 out of 5 specimens studied at Achimota. This karyotype (2R+, 2La), which may be further differentiated by one or more 2R inversions (Toure *et al.*, 1983; Coluzzi *et al.*, 1985) is characteristic of the coastal savanna forms, which may be further differentiated by one or more 2R inversions. However, such further differentiation of the savanna form was not found in all the specimens that were studied.

The observation that two distinct karyotypic populations were found in two different ecological zones is indicative of ecophenotypic plasticity of *An. gambiae* species (Powell *et al.*, 1999), a factor influencing their efficiency as vectors of *Plasmodium*. Moreover, the clinal geographical changes in karyotype frequencies of the 'Savanna' form from 0% to 80% from Dodowa to Achimota correlated with the change in vegetation from coastal forest to coastal savanna. Dodowa represents a degraded forest area whereas Adenta is a peri-urban area followed by Madina, East Legon and Achimota, which are urbanized areas. This trend is indicative of the ecological adaptability/intergradations of karyotypes. The forest form of *An. gambiae* s.s has been found to be monomorphic for the standard 2R arrangement in other studies, whereas the savanna samples form becomes progressively more differentiated by inverted arrangements which increase in frequency eventually becoming fixed in drier savannas with frequencies ranging from 0% to 100% (Coluzzi *et al.*, 1985).

The agreement between the observed and expected karyotypic frequencies according to the Hardy-Weinberg law (HWE) suggests that each population is panmictic (random mating). The evidence for gene flow with respect to use of karyotypes has been reported in other studies (Bryan *et al.*, 1982, Toure *et al.*, 1983, Robert *et al.*, 1990). Moreover, karyotype frequencies have been described as poor indicators of gene flow because their frequencies are apparently strongly affected by natural selection (Lanzaro *et al.*, 1998). Hence the importance of using other methods such as DNA analysis in addition to karyotyping for population studies

Lehmann *et al.* (1997; 1999) have reported that F_{st} values lower than 0.008 indicate high levels of population differentiation, and therefore an evidence for a division of the gene pool. In the present study, it was observed that inter-population differentiation was highest ($F_{st} = 0.0565$) between the furthest population i.e. Dodowa and the four others Adenta, Madina, East Legon and Achimota. Dodowa is separated from the other populations by approximately 25 km. When East Legon and Achimota populations (8km apart) are compared, a lower differentiation index is recorded ($F_{st} = 0.051$), and this decreases further ($F_{st} = 0.0284$) when Adenta and East Legon, which are 2.5 km apart, are compared. It is therefore clear from the F_{st} values obtained from this study that differentiation between these populations is relatively high and increases with distances between them. These estimates of differentiation correspond to effective migration indices (Nm) that also increase with a reduction in distance (Table 7, Figure 19). This observation is similar to previous studies investigating gene flow across the

Rift valley in Kenya covering distances as wide apart as 400-500km and found that increasing distance contributed to differentiation across the Rift valley whereas closer populations were only marginally differentiated (Besansky *et al.*, 1997; Kamau *et al.*, 1998; Lehmann *et al.*, 1996).

The interpopulation differences observed is further supported by the estimates of heterozygote deficiency and excess (F_{is}) values, which indicate non-random mating between all populations except at Madina which geographically, is the centrally located point with respect to the study sites. The evidence for random mating in Madina but not at other populations suggests that it may be a hybrid zone where genetic exchange occurs between it and two adjacent populations, East Legon and Adenta. This evidence is further supported by the levels of gene flow, which are highest amongst these three populations compared to those that are further apart (Dodowa, Achimota).

The distribution of alleles at the three different loci was not uniform in all the five populations and this is indicative of the different levels of association at the different loci. The high differentiation index of $F_{st} = 0.1282$ recorded for the AGHX7 locus suggests that the populations are not related. On the other hand, the low levels of differentiation and heterozygote deficiency observed at the AG2H26 and AG2H46 loci may not necessarily be as a result of random mating but could also be due to natural selection resulting from ecological adaptation hence localizing their existence at certain localities. Another possible reason could be the presence of null alleles. In this study,

the highest number of null alleles and homozygotes was observed at locus 2H46. In a study carried out on various loci including locus 2H46 in *An. gambiae* and *An. arabiensis* in East Africa, Lehmann *et al.* (1996b) inferred that locus 2H46 was under mutational constraint in *An. gambiae*. Null alleles, that is alleles that are not amplified as a result of mutations at the primer binding sites can result in underestimation of heterozygosity because they are detected only in the homozygous state (Donnelly *et al.*, 1999).

At the multi-population level, genotype frequencies at all the three loci showed significant deviation ($P < 0.05$) from the HWE due to lack of heterozygotes. Deviation from HWE is a common finding in studies that utilize microsatellite loci. This deficit is usually attributed to null alleles (Callen *et al.*, 1993; Lehmann *et al.*, 1996a, Garcia de Leon *et al.*, 1997), selection (Garcia de Leon *et al.*, 1997) or grouping of gene pools (so called Wahlund effect) (Gibbs *et al.*, 1997). It is often apparent that pooled populations show more evidence of departure from HWE consistent with a Wahlund effect from pooling subpopulations with unequal allele frequencies (Lehmann *et al.*, 1996; Lanzaro *et al.*, 1998). But in this case, there were still substantial departures between local populations as well. Thus any heterozygote deficit at the loci is unlikely to have resulted from pooling samples from different populations with unequal allele frequencies (Wahlund effect) but could be due to the excess of homozygotes which can be attributed to the presence of null alleles as a result of nucleotide substitutions in the primer annealing sites. It is likely that the presence of null alleles is the cause of the heterozygote deficit observed in this study.

Whereas the use of karyotypes to reveal details of species population differentiation is limited because of selection due to environmental adaptation, microsatellite DNA analysis because of their high polymorphism reveals a higher level of inter- and intra-population variation supporting their description as powerful markers for measuring differentiation (Bowcock *et al.*, 1994; Eustop *et al.*, 1995). Taken together, the two data sets provided by karyotype and microsatellite DNA analysis in this study both support the view that physical distances separating *An. gambiae* populations, coupled with ecological adaptations may greatly influence the genetic structure of these malaria vector populations. The results obtained indicate population subdivision and an inverse relationship between the distance and population genetic relatedness. The higher levels of gene flow ($Nm > 3$) between *An. gambiae* s.s. populations may be attributable to human-mediated and other means of transport, as appetitive flights in anopheline species are usually 1-2 km and this is not readily reconciled with the apparently high levels of gene flow observed.

In this study, the use of karyotyping and microsatellite DNA analysis has provided an insight into the cytotaxonomic status and level of differentiation of *An. gambiae* s.s. populations providing evidence for the existence of two distinct populations and confirming these two methods as useful tools in population studies. However, since no specimens from Madina were karyotyped, it was not possible to determine the presence of heterokaryotypes at this site as was indicated by microsatellite analysis. In view of the possible involvement of different members of the *Anopheles gambiae* complex in

malaria transmission, there is a need to conduct more intensive studies covering a larger geographical area to define in more detail the species composition, distribution and temporal variation of the different chromosomal forms of *An. gambiae* s.s. in Ghana to determine their respective roles in malaria transmission. In effect, this will provide reliable information that can be used in the implementation of disease control interventions and the possible management of insecticide resistance.

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APPENDICES

Appendix I

Schedule of fixative and Stains

Carnoys Fixative

3 parts absolute alcohol

1 part glacial acetic acid

Carnoys fixative should be freshly prepared prior to use.

Lacto-acetic orcein stain

Stock solution

1g orcein

25cc glacial acetic acid

25cc 85% Lactic acid

This can be kept in a well stoppered bottle for long periods of time.

For orcein staining, 1 part of the stock solution is diluted with 3 parts 50% acetic acid.

APPENDIX II

STANDARD SOLUTIONS

The following standard solutions were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121lb/sq in. for 15 minutes in an Eylea Autoclave (Rikikakki Tokyo).

DNA Extraction

Bender buffer

0.1 M NaCl, 0.2 M sucrose. 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA pH 9.1, 0.5% SDS. Stored at 4° C

0.5 M EDTA (pH8.0)

186.1 g/l in water, ph adjusted with NaOH pellets and stored at room temperature.

EtBr (10mg/ml)

1g of EtBr was completely dissolved in 100ml sddw and stored in the dark at room temperature.

KAc (5M K 8M Acetate)

60ml of 5 M KAc and 11.5 ml glacial acetic acid in 28.5 ml distilled water.

TE (pH 8.0), 10Mm Tris-HCl (pH), 1mM EDTA (pH8.0). Stored at room temperature.

Solutions for Electrophoresis

Agarose Gels

10X TAE buffer

242 g Tris base, 57.1 ml glacial acetic acid, 100ml 0.5M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with sddw.

0.5 M EDTA (pH 8.0)

186g of EDTA, dissolved in 800ml ddw, pH adjusted with NaOH pellets, the volume made to 1000 ml with sddw and stored at room temperature.

Urea Polyacrylamide Gels

10X TBE electrophoresis buffer:

108g/l Tris base, 55g/l boric acid, 9.3 g/l Na_2EDTA . Use distilled water added to make up volume to 1l. Stored at room temperature on the bench. Diluted to 1X working solution for electrophoresis.

40% Acrylamide

38g acrylamide, 2g bis-acrylamide in 100ml sddH₂O. Filtered and stored in the dark at 4°C.

The table below gives the required amount of ingredients necessary to prepare the solution for the 7% polyacrylamide running (separation) gel and the plug.

INGREDIENTS	5% POLYACRYLAMIDE GEL SOLUTION
Water (ml)	30.00
Urea (g)	8.25
40% Acrylamide (ml)	11.66
10X TBE Buffer (ml)	5.50

Ten microlitres ammonium persulphate (APS) and 5 μ l of TEMED were added to 2.4ml of the gel to plug the plates to avoid leakage.

Sixty microlitres of APS and 30 μ l of TEMED were added to the rest of the solution (48ml) to form the separating gel, before pouring.

The table below gives the required amount of ingredients necessary to prepare the stacking gel for a 5% polyacrylamide gel.

The solution for the 5% polyacrylamide running (separation) gel and the plug.

INGREDIENTS	4.5% POLYACRYLAMIDE GEL SOLUTION
Water (ml)	31.00
Urea (g)	8.25
30%Acrylamide/0.8%Bis-acrylamide (ml)	7.50
10X TBE Buffer (ml)	5.50
Total	50.4 ml

3.5 ml of this solution were taken per gel, 30 μ l of APS and 10 μ l of TEMED were added and used as stacking gel. The gel was stained in EthBr.

Gel Loading Buffers

6x Bromophenol blue

0.25% bromophenol blue was added to 40% sucrose in water and stored at 4⁰ C.

Bromophenol blue xylene cyanol : 1 volume of bromophenol blue xylene cyanol and 4 volumes of cyanide

5X orange G

20%w/v Ficoll, 25Mm EDTA, 2.5mM EDTA. 2.5%(w/v) orange G. Stored at room temperature.

DNA Molecular weight size markers

The 100 bp DNA molecular weight size marker obtained from Sigma were diluted according to the manufacturers recommendations and used. For the 100 bp ladder, the first band size is 100 bp, the subsequent ones are 200, 300.....1000bp.

APPENDIX III

An example input data format for the population genetics analysis software POPGENE

VERSION 1.31

/*Diploid data of 5 populations each with 3 loci */

Number of populations =5

Number of loci = 3

locus name :

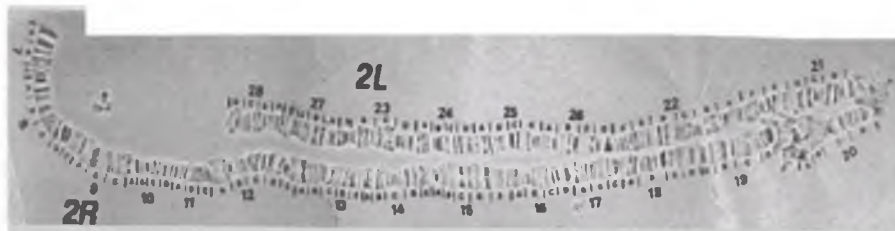
AGXH7 AG2H26 AG2H46

Name = DODOWA

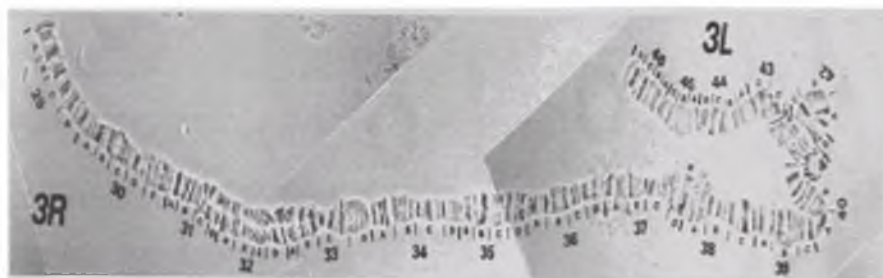
AB.....
AB.....	ABCCEE..
AB.....	AB..EFGG	BBCC...
ABCC....	AB.....	ABCCEE..
ABCC....	AA.....	AB.....
.....	AB..EF..	AB.....
AB..EFGH	AB..EF..
ABCD....	AB..EE..
.....	AB..EEGH	AB..EE..
ABCC....	ABCD....	AB..EE..

APPENDIX IV

Standard Polytene Chromosome maps of *Anopheles gambiae*



Chromosome 2



Chromosome 3



X Chromosome