University of Ghana http://ugspace.ug.edu.gh

MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF *ANOPHELES MELAS* POPULATIONS IN ENDEMIC AND NON-ENDEMIC LYMPHATIC FILARIASIS COMMUNITIES IN COASTAL GHANA

BY

DORCAS ATIBILLA (BSc. HONS)

(10442997)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY (ENTOMOLOGY) DEGREE

JULY, 2015

INTEGRI PROCEDAMUS

***JOINT INTER-FACULTY INTERNATIONAL PROGRAMME FOR THE TRAINING OF ENTOMOLOGISTS IN AFRICA**

COLLABORATING DEPARTMENTS: ANIMAL BIOLOGY AND CONSERVATION SCIENCE AND CROP SCIENCE, COLLEGE OF BASIC AND APPLIED SCIENCES

University of Ghana http://ugspace.ug.edu.gh

DECLARATION

This is to certify that, this thesis is the result of research undertaken by Dorcas Atibilla towards the award of Master of Philosophy (M.Phil.) in Entomology at African Regional Postgraduate Programme in Insect Science (ARPPIS), University of Ghana.

…….. **.. ……..**

DORCAS ATIBILLA (BSc Hons) **DATE**

(STUDENT, ID: 10442997)

…….. **……**

PROFESSOR MICHAEL DAVID WILSON **DATE**

(SUPERVISOR)

…….. **……**

DR DZIEDZOM KOMI DE SOUZA **DATE**

(SUPERVISOR)

…….. **……**

DR FRED ABOAGYE-ANTWI **DATE**

(SUPERVISOR)

…… **…..……**

DR ROSINA KYEREMATEN **DATE**

(ARPPIS COORDINATOR)

University of Ghana http://ugspace.ug.edu.gh

DEDICATION

To my parents Mr Emmanuel Kwame Atibilla and Mrs Agnes Atibilla, and my siblings Justice, Linda, Ishmael and Prosper. To the Adare and Amidini Families and to my dear husband, Mr Isaac Yakubu Butias.

University of Ghana http://ugspace.ug.edu.gh

ACKNOWLEDGEMENTS

I thank God for how far he has brought me, may his name be praised. I would never have been able to finish my dissertation without the guidance of my supervisors, and support from my family and my husband.

I would like to express my deepest gratitude to my supervisor Professor Michael D. Wilson for his excellent guidance, care, patience and being very supportive and guiding my understanding of this research project. I really appreciate every little effort and arrangements made throughout this work. From that first discussion about the project topic to the very last, he has been patient and always has his door open for a quick chat. I could not have asked for a better supervisor

I would like to also thank Dr Dziedzom de Souza for training me on my laboratory analysis and coaching me and tolerating all the questions asked during this period, I am most grateful. My thanks also go to Dr Fred Aboagye who gave his best suggestions to writing a good thesis despite his busy schedules he still gave some contributions to making this work a success.

Many thanks to Millicent Opoku and Joseph Harold Nyarko for your immense help and assistance during the start of the analysis, species identification, SOP development and optimization of the PCR reagents for analysis, Dr Jewelna Osei-Poku for quantifying my DNA samples. I thank Eddy Grand, Amelema and Mabel for their assistance in the grinding of my mosquitoes, and to Dora, thanks for assisting me to troubleshoot for the presence of *An. funestus* in my samples and Mba Tihsomah Mosore for training me on cibarial mounting and observation. I thank staff of Parasitology Department for making me feel welcome during my stay; I will surely miss this family.

University of Ghana http://ugspace.ug.edu.gh

My thesis was funded by Carnegie Corporation of New York through the University of Ghana under the Next Generation of Academics in Africa programme. I would like to acknowledge the management of Kintampo Health Research Centre for giving me the opportunity to pursue this degree. I thank ARPPIS lecturers for impacting the knowledge which helped me in the understanding of my work. I thank my family for their unfailing support and prayers through it all. I love you

I also thank Mr Joseph Otchere for your diverse contribution to making this work possible. Last but not least I want to thank Mr Sampson Otoo for his assistance in the collection of the mosquitoes in the field and Mr Danso for agreeing to drive me round the communities for the field work which would not have reached this stage but for his assistance, God bless him.

Finally, I thank my dear husband, Mr Isaac Yakubu Butias for his unfailing support, patience and prayers through it all.

University of Ghana http://ugspace.ug.edu.gh

TABLE OF CONTENTS

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

LIST OF TABLES

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

University of Ghana http://ugspace.ug.edu.gh

LIST OF PLATES

University of Ghana http://ugspace.ug.edu.gh

LIST OF APPENDICES

University of Ghana http://ugspace.ug.edu.gh

LIST OF ABBREVIATIONS

Bp	Base pair
NTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylene diamine tetra-acetate
EtBr	Ethidium bromide
H_2O	Water
KAc	Potassium acetate
HCl	Hydrochloric acid
Kg	Kilogram
M	Molar
mtDNA	Mitochondrial DNA
mg	Milligram
NaCl	Sodium chloride
PCR	Polymerase chain reaction
pH	Hydrogen-ion exponent
Ml	Millilitre
Rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
s.s	Senso strict
s.l	Senso lato
sddH_2O	Sterile double distilled water
GPELF	Global Programme for the Elimination of Lymphatic Filariasis

University of Ghana http://ugspace.ug.edu.gh

TAE	Tris-Acetate EDTA
LF	Lymphatic filariasis
ML	Maximum likelihood
ICT	Immuno-chromatographic test
MDA	Mass drug administration
PSC	Pyrethrum spray catches
UV	Ultra violet
WHO	World Health Organization

University of Ghana http://ugspace.ug.edu.gh

ABSTRACT

Several studies have revealed that towards the east of Accra, there is no lymphatic filariasis (LF) but it occurs to the west of Accra. *Anopheles melas* is known to be an efficient vector of LF and it occurs along the coastal part of Ghana, especially in estuarine areas. The present study therefore aimed at seeking explanation to why this different patterns of LF distribution occur along coastal Ghana. The hypothesis was that the eastern *An. melas* populations could be different from those occurring to the west of Accra at both morphological and molecular levels. At the molecular level, (cytochrome oxidase I (COI)) gene sequences were analysed and at the morphological level the number of cibarial teeth were studied. Two sites representing communities to the east of Accra; Azizanya and Otropke in the Ga Adangme District and three communities; Asemko, Butre and Mpataano in the Ahanta West district were selected for the study. Pyrethriod spray catches was used to collect indoor mosquitoes. The mosquitoes obtained were morphologically identified as *An. gambiae* sensu lato and An. *melas* molecularly identified. Deoxyribonucleic acid (DNA) of each identified *An. melas* was then used as the template for COI analysis. Polymerase chain reaction products obtained were purified and shipped to Macrogen Inc. in the Netherlands for sequencing. Sequenced data was analysed using Bio Edit and MEGA V6 software for construction of phylogenetic trees as well as statistical inferences *In-silico* restriction enzyme digest of the sequences was done to determine restriction site differences between two *An. melas* populations. Similarity index was determined using Sørensen's formula. Cibarial armatures of the two mosquito populations were compared using Student T-test. The study found no significant differences between the sequences of the two *An. melas* populations (Z-test of

University of Ghana http://ugspace.ug.edu.gh

neutrality = 0.33). Results of the phylogenetic analysis though not significant, revealed a geographic relationship only between two eastern populations clustering together, with Asemko branching off on a different node. Analysis of the *in-silico* showed eight mutational differences thus giving a QS value of 0.99. The restriction site analysis however revealed 16 unique site differences between them. The mean numbers (n, median and range) of cibarial teeth were 14.54 (n = 13, 15, 11 = 18) and 14.1 (n=13, 14, 11-19) for Azizanya and Asemko respectively, which were not statistically different (t=1.53, P = 0.14). In conclusion the two *An. melas* populations could be said to be similar in all aspects, however restriction enzyme differences could be potential markers for distinguishing the two populations. Further studies involving wider sampling of the areas and experiments with identified restriction enzymes should be undertaken to enable a firm conclusion to be drawn. Furthermore, to determine whether the eastern population are really refractory to *W. bancrofti*, xeno- experiments could be conducted using reared *An. melas* mosquitoes.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Lymphatic filariasis (LF) or elephantiasis is a severe and disfiguring disease and incapacitates the sufferer. LF is caused by infection with the filarial parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (World Health Organization, 2002, World Health Organization, 2013)

Out of the estimated 120 million infections world-wide and *Wuchereria ebancrofti* is responsible for 90% cases and it is the only etiologic agent in the African Region while *Brugia malayi* accounts for 10% (Singh and Prakash 2009). The disease is transmitted through the bites of infective female mosquito vectors belonging to the genera *Anopheles*, *Culex*, *Mansonia* and *Aedes*, depending on the geographic region. In West Africa, only the *An. gambiae* and *An. funestus* are the most important vectors (Ottesen 2000). The most common clinical manifestations of LF include lymphoedema and scrotal hydrocele which adversely affect personal and social life, and limit occupational activities, making LF the second leading cause of chronic disability worldwide (Ottesen 2000). The disease has a high psychological and social impact (Gyapong, Gyapong et al. 1996). Due to its disfiguring and socioeconomic impacts on affected individuals and communities, the disease has been targeted for elimination by the year 2020, through the Global Programme for Elimination of Lymphatic filariasis (GPELF) (Organization 2002). The elimination programme is based on mass drug administration (MDA), through a regular treatment with a single-

University of Ghana http://ugspace.ug.edu.gh

dose of albendazole and diethylcarbamazine, or albendazole and ivermectin, to reduce microfilaraemia in populations at risk, for a period long enough to ensure that microfilaraemia remain below levels necessary to sustain transmission.

Globally, 1334 million people live in 81 countries where the disease is known to be endemic.

Africa and South-East Asia regions harbour 95% of the population (Organization 2010) living in endemic areas, with 98% of infected persons. Of the endemic population, 874 million (65%) live in the South-East Asia Region (9 endemic countries) and 396 million (30%) live in the African Region (39 countries). The Mekong Plus area (6 countries) accounts for 3%; and the Americans, Eastern Mediterranean Region and Oceania (with 7, 3 and 17 countries, respectively) together account for 2% (Organization 2010).

West Africa has an LF distribution pattern of endemicity which is separated by a continuous non-endemic area spanning from east of Ghana through Togo to Central Benin (Gyapong, Kyelem et al. 2002, de Souza 2010).This distribution in Ghana extends from the middle forest belt (2º.5´W 6ºN), to the east of Accra, and covers the whole of the Volta Region. However, *Anopheles gambiae s.l.* which is the main vectors of LF is present in both endemic and non-endemic areas, raising questions about this distribution.

1.2 Justification

The GPELF has two main strategies to meeting the goal of eliminating LF. The first is the interruption of the transmission of *W. bancrofti* that causes LF through annual MDA to all population at risk in endemic areas. Secondly, the programme was going

University of Ghana http://ugspace.ug.edu.gh

to manage morbidity and prevent disability among people already affected by the disease. The fact that *Anopheles* mosquitoes exhibit the phenomenon of facilitation makes the GPELF strategy seemingly a laudable one. However, if the transmission cycle involves culicines, it would be difficult to achieve elimination of the disease using MDA alone. This is because culicines are capable of transmitting LF even at low Mf (< 1%). In Ghana, recent studies have indicated that some vectors particularly *An. melas* (Amuzu *et al.*, 2010) and *Mansonia spp* (Ughasi *et al.*, 2010, 2012) could transmit at low levels of microfilaraemia thus keeping a residual transmission of the disease even after more than seven rounds of LF MDA.

Moreover, the programmatic issues of drug distribution (Gyapong and Twum-Danso 2006) and the perpetual threat of drug resistance (Schwab, Palatnik et al. 2005) coupled with the diversity of vectors and their differing abilities to transmit low level parasitaemia (Gyapong and Twum-Danso 2006), may lead to a failure to stop transmission in some regions even if the MDA coverage is achieved (Gyapong and Twum-Danso 2006).

Transmission of LF in endemic sentinel communities in Ghana is expected to have halted after 2006. But this has not been the case since there is still evidence of residual prevalence of the disease in these communities (FILARIASIS 2010) Integrated vector control is therefore considered an important and integral part necessary to achieve elimination in specific areas where MDA alone will not provide the solution. This is especially important, with the report of *Mansonia* spp. being very efficient vectors of LF in some Ghanaian communities (Ughasi, Bekhard et al. 2012), where they were previously thought to be non-vector species.

University of Ghana http://ugspace.ug.edu.gh

The presence and number of cibarial armature in a mosquito determines its efficiency as a vector (McGreevy, Bryan et al. 1978) of a disease. *Anopheles melas* have been shown to possess less cibarial armature than *Anopheles gambiae* and therefore making it a more efficient vector, (Amuzu, Wilson et al. 2010) than other LF vectors. In furtherance of the above, recent studies have showed significant variation among *An. gambiae s.s.*, one of the *An. gambiae* complex across the country between the M and S forms (de Souza 2010). It is therefore important to morphologically and molecularly characterize and document *An. melas* which happens to be one of LF vectors exhibiting limitation, to assist the entomological surveillance team incorporate all strategies involved for the control of LF.

Dunyo *et al*. (1996) carried out a survey in 6 villages and 3 towns along the coast of Ghana showed few filarial infections observed in the towns and the villages east of Accra. However, *Wuchereria bancrofti* microfilaraemia was common in the four western villages, with overall prevalence rates of 9.2% – 25.4% and overall microfilariae (mf) geometric mean intensities of 321 – 1172 mf/mL of blood (Dunyo, Appawu et al. 1996). This however, did not establish why the disparities in infectivity between the studied eastern and western communities. This study therefore aims at investigating whether two distinct populations of *Anopheles melas* with different vectorial roles exist to the east and to the west of Accra in Ghana.

1.2.1 Main objectives

The objective of this study is to investigate the molecular and morphological differences in *An. melas* that could account for differences in LF transmission in the east and west of Ghana.

University of Ghana http://ugspace.ug.edu.gh

1.2.2 Specific objectives:

i. To molecularly characterize two populations of *An. melas* species using COI DNA sequences

ii. To determine the morphological differences potentially influencing transmission of lymphatic filariasis by comparing the cibarial armature of the two different populations of *An. melas*

iii. To determine the degree of divergence between the two populations using phylogenetic and statistical analysis.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER TWO

LITERATURE REVIEW

2.1 Lymphatic Filariasis

Lymphatic filariasis (LF) is caused by a group of parasitic worms *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (Organization 2002). However, *W. bancrofti* is the most widespread of human filariae in the world. The parasite is transmitted through the bites of infected female mosquito vectors belonging to the genera *Anopheles, Culex, Mansonia* and *Aedes*, depending on the geographic region. The parasite lodges in the lymphatic system and lives for 4 - 6 years, producing millions of immature microfilariae (mf) that circulate in the blood (Ottesen 2000).

2.1.1 Global burden of LF

LF is estimated to affect more than 120 million people worldwide, with 98% living in the tropical and subtropical regions of Africa and Asia (Ramaiah, Das et al. 2000). It is estimated that 1.2 billion people are at risk of infection (Ramaiah, Das et al. 2000). Of the infected individuals, a third live in India, one-third live in Africa and the rest is distributed among Asia, the Pacific and Latin America (Ottesen 2000). In sub-Saharan Africa, it is estimated that about 512 million people are at risk of infection, with almost 40 million men at risk of developing hydrocoele (Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization 2002)(Organization, 2002a) Whilst about 28 million are already infected. Of this number, there are 4.6 million cases of lymphoedema and over 10 million cases of hydrocoele. These represent about 20% of

University of Ghana http://ugspace.ug.edu.gh

the global burden of the disease (Michael, Bundy et al. 1996). Approximately one third of the infected individuals have physical manifestations of elephantiasis with LF estimated to result in the loss of almost US$1.3 billion per year in productivity.

2.1.2 Lymphatic filariasis in Ghana

In Ghana, most febrile illnesses are treated presumptively as malaria and examination of thick films of night blood for microfilariae is not routinely done. Studies conducted in the Kasena-Nankana District (Gyapong, Adjei et al. 1996) and in Ahanta West District (Gyapong, Gyapong et al. 1996) suggest that the disease is common in the northern sector of the country, as well as in some coastal communities in the Western and Central Regions.

Filariasis was known to be moderately common and widespread in the northern territories of the then Gold Coast. During 1936 and 1937, among 55000 inhabitants admitted to hospital in the whole northern territory, there were 147 cases of hydrocele and 134 of elephantiasis. It was also observed that in villages outside Accra, 9 out of 28 adults were carrying *Wuchereria bancrofti* microfilariae and 4% of wild *Anopheles gambiae* and 1.7% of wild *A. funestus* contained matured larvae of the parasite (Muirhead-Thomson 1954).

The national prevalence of microfilaraemia was 3.0% (95% confidence interval [CI] 25 - 35) in 1996 with regional variation between zero in Brong Ahafo and Greater Accra Regions to 20.0% in the Upper West Region (Gyapong, Adjei et al. 1996). Infection is present in 8 of the 10 administrative Regions with variations in the prevalence of parasitaemia within the Regions. People of all ages and sexes are affected but more females are known to have elephantiasis than males (Gyapong, Magnussen et al. 1994). Mass chemotherapy is the advocated primary control for LF

University of Ghana http://ugspace.ug.edu.gh

with ivermectin(Dunyo, Nkrumah et al. 2000), diethylcarbamazine (DEC) and albendazole in control programmes. The main vector is *An. gambiae* and *An. funestus* with about 4 million people estimated to be living in endemic areas (Gyapong, Kyelem et al. 2002). Recently *Mansonia* species have been implicated in the disease transmission (Ughasi *et al*., 2012)

2.1.3 Symptoms of the disease

Although majority of people infected with these parasites are asymptomatic, it causes slow damage to the lymphatic system and other organs, from chronic infection to a variety of pathologies ranging from sub-clinical damage to severe disfigurement (Mahanty and Nutman, 1995). The most recognized manifestation of LF is elephantiasis, the swelling of the limbs or genitals with lymph fluid that results from total blockage of the lymphatic system by adult worms (Taylor, Hoerauf et al. 2010). LF is painful and disfiguring, and one of the leading causes of permanent disability worldwide. Generally not fatal, secondary infections due to bacterial and fungal invasion of compromised lymphatic tissues cause more progression and physical destruction associated with elephantiasis, which if not treated may lead consequently to death (King and Nutman 1991, Ramaiah, Das et al. 2000).

Other symptom of the disease is tropical pulmonary eosinophilia (TPE) or Weingarten‟s syndrome. This differs from other forms of the disease and appears to be related to an immediate hypersensitivity response to the filarial antigens. It is characterized by recurrent nocturnal cough and wheezing, recticulonodular densities on chest radiographs, peripheral blood eosinophilia >3,000mm, extreme elevations of

University of Ghana http://ugspace.ug.edu.gh

serum IgE and specific anti-filarial antibodies (Ottesen and Nutman 1992). Usually, there is dramatic improvement following anti-filarial chemotherapy.

2.1.4 Biology and life cycle of *Wuchereria bancrofti*

Wuchereria bancrofti belongs to the family of filarial worms, *Filariidae*. Other members of this family *include Onchocerca volvulus, O. gutturosa, Brugia malayi, B. timori, Loa loa, Litomosoides carinii, Dirofilaria immitis, Dipetalonema perstans* and *D. viteae* (Mehlhorn 1988).

In general, the length of filarial worms measures between 20mm and 700mm. The female of each species is longer in length than their corresponding males (40mm - 700mm in females and 20mm - 180mm males).

Females produce a lot of larvae that may be sheathed or unsheathed. Larvae of filarial worms are found in circulation and inhabit subcutaneous tissues, the eye, pleural cavity, pulmonary artery, lymph vessels and lymph nodes. These parasites have both intermediate hosts (*Simulium* spp., *Odagmia* spp., *Aedes* spp., *Anopheles* spp., *Culex* spp., *Mansonia* spp., mites (*Bdellonyssus*), *Culicoides* spp. and *Ornithodorus moubata*) and definitive hosts (humans, cattle, rats, dogs, cats and *Meriones sp*).Their shape and structure may be used as identification keys because they are species specific (Mehlhorn 1988). *Wuchereria bancrofti* male and female adult worms (4cm and 10cm respectively) live in the lymph vessels and nodes. After copulation the female adult worm produces thousands of microfilariae (Mf) that could be found in peripheral blood during the night.

Mosquito vectors (*Aedes*, *Anopheles*, *Culex* and *Mansonia*), the intermediate hosts, may ingest these Mf after a blood meal. Mf moult in the gut to form the first larval

University of Ghana http://ugspace.ug.edu.gh

stage (L1) that penetrate the intestine, then enter abdominal cavity and thoracic muscles. They moult again to form the second larval stage (L2) that could be described as a stumpy or sausage shape. The third and final moult forms the filariform infective stage (L3). The L3s (1.5mm in length) migrate to the proboscis and escape into pool(s) of blood that surround the wound formed as a result of the piercing of the stylet of the proboscis. The L3s eventually enter circulation and migrate to the lymph vessels and lymph nodes where they mature into adults after two moults within a year.

2.1.5 Vectors of lymphatic filariasis

The vectors responsible for the transmission of *W. bancrofti* in West Africa are the *Anopheles gambiae* s.l. and *An. funestus* complexes (Appawu, Dadzie et al. 2001). The same species are responsible for the transmission of the disease in rural parts of East Africa (cited from(Kwansa-Bentum, Aboagye-Antwi et al. 2014). However, in urban East Africa and Asia, *Culex species*, especially *Culex quinquefasciatus* are responsible for the transmission of *W. bancrofti* (Thomson 1946, Kuhlow and Zielke 1978) Mosquitoes belonging to the genera *Aedes* and *Mansonia*, also transmit the disease in the Pacific Islands, Southeast Asia and South America (Ottesen 2000).

2.1.6 Biology and life cycle of the LF vectors

The vectors and carriers of *W. bancrofti* parasites include *Aedes*, *Anopheles*, *Culex*, *Coquilletidia*, *Mansonia* and the *Ochlerotatus* genera (cited from de Souza et al., 2012).Mosquitoes may be diurnal, crepuscular or nocturnal and this to a great extent influence their blood feeding times in the wild. Mosquito in general is holometabolous with four distinct stages in their life cycle (Rueda 2008) [Figure 1].

2.1.6.1 Life cycle of *Aedes species*

University of Ghana http://ugspace.ug.edu.gh

Aedes species exhibit holometabolism (complete metamorphosis). This involves both aquatic and terrestrial phases. Adult females may lay as many as several hundred single eggs per batch. Eggs may be laid on the surface of shallow temporary/permanent pools, natural containers of plant, animal and artificial containers. Examples of these include tree holes, nuts, pitchers, pods, shell of snail, clams, arboreal and ant nest, crab holes, tires, cans, flower vases, bottles, tanks, troughs, drums, and gutters among others (Laird 1988). The eggs of *Aedes* species are smooth. They are long and ovoid measuring about 1mm long. Adult females may also lay eggs on dump surfaces most likely to get temporarily flooded. In some species eggs are laid on moist substrates, in and around depressions of breeding sites with tidal wetlands. A batch of eggs may be laid over a few hours or days depending on availability of suitable substrate. Eggs may also be laid at varying distance above water line. This is to ensure that this batch of eggs is dispersed over a number of breeding sites. The eggs would have to dry for about 24 hours before they become viable. These desiccant resistant eggs may remain viable for up to about 5 years. The eggs may usually sink to the bottom of the water. *Aedes* species prefer clean water for larval development. Development to emergence may take 4 - 5 days or more depending on water temperature, availability of food and predators. In very cold temperatures, the larvae may not develop for months (diapauses). Hatching occurs once there is adequate amount of water at the breeding site. The various larval stages hangs head down just below the water surface breathing through their siphons.

They feed on microbes and phytoplankton in the water. They dive to the bottom of the water when disturbed and wiggles back to the surface. Fourth instars moult into pupae. Adult development occurs in the pupal case within 2 days. The pupa does not feed and rest mainly at the surface of the water. They dive to the bottom when

University of Ghana http://ugspace.ug.edu.gh

disturbed and floats back to the surface of the water. Developed adult in the pupal case ingest air to expand their abdomen. This split opens the pupal case dorsally for the adult mosquito to emerge head first. Males usually emerge before females. They wait till their exoskeletons and wings dry up before flying away.

Adult *Aedes* species feed on nectar for survival. Only the adult female blood feed to mature its eggs. It takes about 2 - 7 days for the blood meal to be digested. They bite around the twilight periods of dusk and dawn (crepuscular). They may also bite during the day (diurnal). These mosquitoes are known to exhibit both zoophily and anthropophily in their feeding behaviour (Thompson, Hayes et al. 1963). They may also be described as being predominantly endophagic and endophilic in nature.

2.1.6.2 Life cycle of *Anopheles* species

This vector also undergoes complete metamorphosis (egg, larva, pupa and adult). Its life cycle involves both aquatic and terrestrial phases. The first three stages are aquatic and the last stage is terrestrial. Adult female after mating lay between 50-200 single brown/black boat shaped eggs having lateral floats. The floats aid dispersal of the eggs on the surface of the water. The eggs may measure 1mm long and 2 - 5mm wide. After oviposition, viable eggs hatch within 2 - 3 days but 4 - 7 days in the temperate zones (Osei 2013). It takes roughly 12 - 14 days to complete a life cycle. *Anopheles* species may breed in cool clean rivers with shades, streams, pools having water lettuce (*Pistia stratiotes*), rivers with grass at the edges, rice fields, open exposed ground pools, brick pits, foot-prints, tire ruts, wheelbarrows, mortar-pans, and rarely in domestic ant-traps (Gillett, 1972). According to Sattler *et al.* (2005) they have adapted to breeding in urban settlements with stagnant drains, railway lines,

University of Ghana http://ugspace.ug.edu.gh

shallow wells, irrigation channels, organically polluted water, large drains, swamps, and puddles (Sattler, Mtasiwa et al. 2005).

In these various breeding sites, the larvae usually lay flat (parallel) just below the surface of the water with the help of its palmate (abdominal setae) and forage for microbes and phytoplankton. Larvae are filter feeders. They may pupate in about 6 - 9 days after four moults. This may however depend largely on water temperature, nutriments and predators. They grow bigger after every moult. After 2-3 days the non-feeding pupa may split open its pupal case dorsally for emergence of adult mosquito.

Every generation of mosquitoes has the males from that batch of eggs emerging first and maturing within 24 hours to be ready for mating by the time the females emerge. After mating, most males may die. Adult males and females survive on nectar. The females take blood meal to develop and mature their eggs (Gillies 1955). A combination of cues such as carbon dioxide, temperature, moisture, odour, host movement and colour from a vertebrate may attract a female mosquito to take a blood meal (Zimmerman, Galardo et al. 2006).

Anopheles are nocturnal so their blood feeding, mating, egg laying and even emergence of adults from pupae is usually from the evening to early morning. Some *Anopheles* species are exophagic (bite outdoors) biting after sunset to about 21:00 hours and others are endophagic (bite indoors) biting usually after 21:00 hours (Greenwood 1997). They may be exophillic (rest outside) or endophillic (rest indoors) or both. Most may also be endophilic, exophilic, endophagic and exophagic with this behaviour not exclusively exhibited. A few *Anopheles* feed exclusively on humans

University of Ghana http://ugspace.ug.edu.gh

and others are mostly zoophilic. Anthropophilism and zoophilism varies according to availability of host and the species of mosquitoes that feed on both humans and animals (Greenwood 1997).

2.1.6.3 Life cycle of *Culex species*

Culex species undergo complete metamorphosis requiring both aquatic and terrestrial phases. Adult females after mating lay eggs in cluster. This may be several hundred per batch on the water surface. Apart from shallow temporary pools, *Culex* species may oviposit in flowing streams (creeks, irrigation ditches, drainage), pond streams (flooded stream beds, chlorophyta-rich habitats, polluted ponds), lake edges, swamps and marshes (coastal marshes, mangrove swamps, irrigated fields), shallow permanent ponds (fishponds, duckweed ponds), intermittent ephemeral puddles, natural containers of plant origin (treeholes, nuts, pitchers, pods), natural containers of animal and other origins (shell of snail, clams, arboreal and ant nest, crab holes), and artificial containers such as tires, cans, flower vases, bottles, tanks, troughs, drums, gutters and others (Laird 1988, PRENDERGAST, MIYAGI et al. 2005). Some *Culex* species may breed in the most stinking water (Gillett 1972). Females lay eggs one after the other, sticking the eggs together to form a raft with about 200 - 300 eggs. This egg raft stays afloat using the air trapped underneath the raft. The approximately 6.25 mm long and 3.13 mm wide eggs may hatch and the larvae escape from the bottom of the egg raft into the water in about 1-2 days. Larvae hang at an angle just below the surface of the water with their siphons (air tubes) to breathe. These filter feeders feed on microbes and phytoplankton.

University of Ghana http://ugspace.ug.edu.gh

The development of the wigglers (larvae) involves four stages with each of the stages referred to as instars. After development through the four larval stages the fourth instars metamorphose into pupae. The pupae do not feed. They breathe through their pair of trumpets at the surface of the water. When disturbed, they dive in a jerking tumbling motion and float back to the surface. It takes approximately 1-4 days depending on the species and importantly water temperature for adult mosquitoes to emerge from the pupal case. Adult male mosquitoes may emerge 24 hours before females to mature in readiness for mating with females after their emergence. *Culex* mosquitoes feed on nectar. Adult females, however, take blood meals in other to mature their ovaries and lay viable eggs. It may take about 2-7 days for the blood meal to digest. Some prefer biting around dusk outdoors and throughout the night indoors. *Culex* may be zoophagic (both zoophilic and anthropophilic). Some mostly prefer domestic and wild birds (ornithophagic) to man, cattle horses and other mammals (Kent, Juliusson et al. 2009). In the temperate, they may hibernate in the late summer until early spring before finding water bodies to oviposit. Their feeding and resting behaviour may be described as endophagic and endophilic respectively.

2.1.6.4 Life cycle of *Mansonia species*

The genus *Mansonia* could be described as holometabolous. Adult female mosquitoes search for water bodies with emergent vegetation. They then lay eggs in the part of the water with a high population of emergent vegetation (Carpenter and La Casse 1955). Some temperate species may even enter diapause (over-winter) at the egg stage when conditions are not favourable (Urquhart, Armour et al.). Eggs are laid side by side to form a raft. About a hundred eggs or more may be laid per batch (Urquhart, Armour et al. , Urquhart, Armour et al. 1987, Moore and Pratt 1993). Each egg in the raft is vertically arranged with the anterior end pointing downwards towards the water

University of Ghana http://ugspace.ug.edu.gh

surface (Soulsby 1982). Eggs may hatch after a few days when the temperature of the water is quite suitable (Urquhart, Armour et al. 1987). There are four larval stages (1st – 4th instar). The larvae reach the 4th larval stage after three moults. Development of larvae is dependent on availability of food (Pratt and Moore, 1993). The larvae stay attached to the roots and stem of emergent plants in the water with specialized/modified siphons (Carpenter and LaCasse, 1955) with which they breathe whiles foraging for microbes and phytoplankton. The fourth instars moult to become pupae. Pupae do not feed. The pupae attach themselves to roots or stems of emergent vegetation with specialized trumpets and remains at that part of the plant until the adults emerge (Carpenter and LaCasse, 1955).

Adult development occurs within the pupal case before emergence. It takes a few hours for the pupae of certain species in dry climates to emerge. In the tropical and temperate species, however, it may take about 2 days and several weeks respectively (Urquhart *et al.* 1987; Pratt and Moore 1993; Bowman 1999). The male mosquitoes usually emerge first before the females as already discussed in the genera above. The adults feed on nectar. Females bite to acquire blood to mature their eggs. Host preference for most species may vary seasonally depending on their availability. The various hosts may include amphibians, birds and mammals. They are known to be savage biters (Pratt and Moore 1993

2.1.6.5 Life cycle of *Ochlerotatus* species

They have a holometabolous life cycle. *Ochlerotatus* species lay single eggs on damp soil. Several hundred eggs may be laid per batch. The eggs would hatch only when flooded with water. The water may either be fresh or brackish. The temperature of the water is also important for hatching and especially larval development. Breeding sites

University of Ghana http://ugspace.ug.edu.gh

of these mosquitoes include irrigated pastures, tree holes, vinyl tarpaulins covering swimming pools, rain pools, rock pools, wood piles, catch basins, bottom of flooded streams, and depressions flooded by salt water during high tides (Andreadis, Anderson et al. 2001).

The larvae hang just below the surface of the water breathing atmospheric air through their siphons. They are filter feeders foraging on microbes and phytoplankton. There are four larval stages referred to as the instars. The first instars moult three times to get to the fourth instars. They get bigger after each moult. The fourth instars then moult becoming pupae. The pupa may be found resting at the surface of the water breathing through a pair of trumpets. When disturbed the pupa may dive to the bottom of the water in a jerking motion. The pupa may then float back to the surface of the water (Pratt and Moore 1993). Adult development commences in the pupal case. Depending on the water temperature the pupa may take about 1-3 days to split open dorsally for the emergence of the adult mosquitoes.

The adult *Ochlerotatus* species feed on nectar. Only the females take blood meals to mature their eggs. They are known to give very painful bites and are a nuisance because of their persistent biting behaviour. They are diurnal, mostly anthropophilic and exophilic. They are known to be very strong fliers and could be found miles away from their breeding sites.

University of Ghana http://ugspace.ug.edu.gh

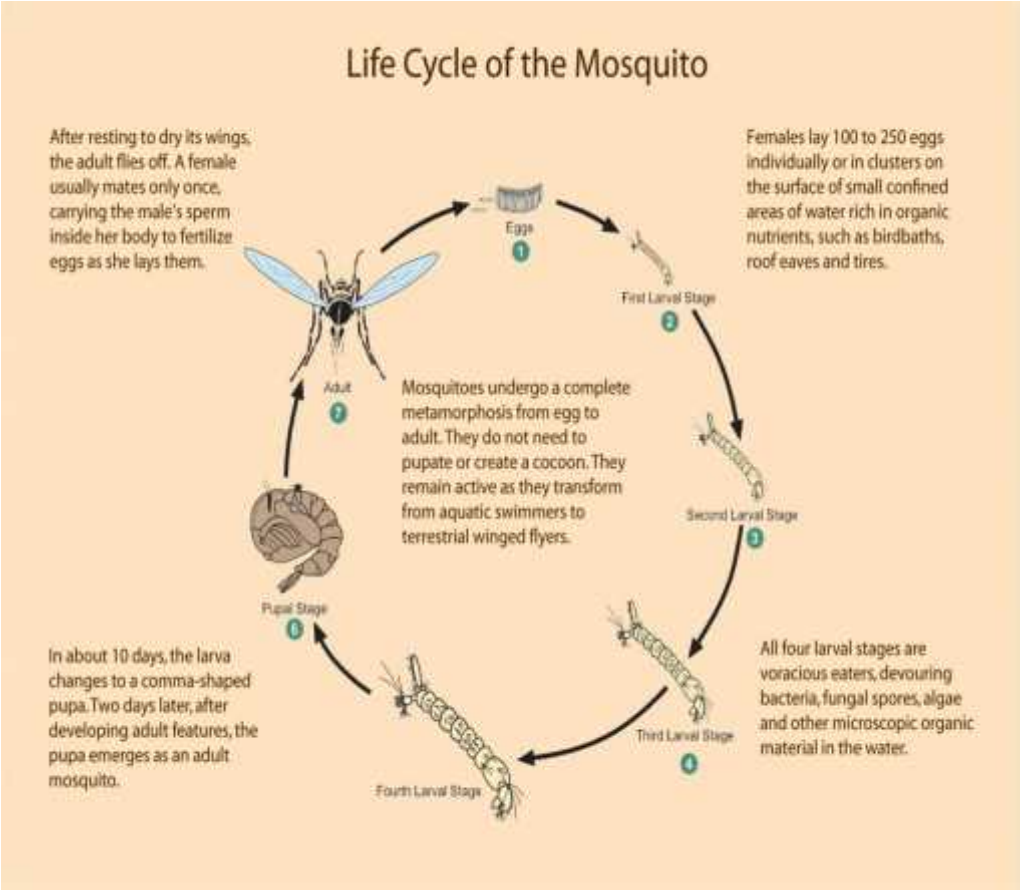

Figure 1: A diagram showing the life cycle of a mosquito (Source: www.deltapestcontrolandlawnservice com)

University of Ghana http://ugspace.ug.edu.gh

2.2 Factors Influencing the Transmission of *Wuchereria bancrofti*

The factors affecting transmission of *Wuchereria bancrofti* are basically determined by the nature of the vectors (vectorial role and capacity, etc.) and the relationship existing between the parasite, vector and human host.

2.2.1 Vector composition

This refers to the number of mosquitoes known to be involved in the transmission of a particular disease in a given geographical area or study sites. The local vector for the transmission of LF in endemic communities informs control programmes the kind of strategies to use in managing or even eliminating the disease in that community. Knowing the vector composition of a study site, one may use the information and the biology of incriminated vectors in endemic areas to control the transmission of LF.

2.2.2 Facilitation, limitation and proportionality

The phenomenon of "facilitation" refers to a process where ingested Mf below a certain threshold, called the Webber's Critical Point (Pichon 2002), would not support the transmission of LF by *Anopheles* vectors (Webber 1991, Southgate and Bryan 1992). "Limitation" is a process where there is a stable transmission of LF even when there is lower than the threshold parasitaemia in the human population (Subramanian, Krishnamoorthy et al. 1998, Duerr, Dietz et al. 2005) and this phenomenon is usually exhibited by culicines. These vectors when found transmitting in endemic areas would have serious implications on the transmission dynamics. Even with several rounds of MDA, such vectors may maintain residual transmission of the disease in endemic communities (Amuzu, Wilson et al. 2010, de Souza, Koudou et al. 2012). Vectors may also exhibit "proportionality" whereby a constant number of Mf develop to the

University of Ghana http://ugspace.ug.edu.gh

infective (L3) stage after the ingestion of blood meal (Duerr, Dietz et al. 2005). This implies that when such vectors are involved in transmission, they would constantly be contributing a good percentage to the annual transmission potential (ATP) in a given endemic area.

2.2.3 Vector species complexes and their importance in the disease transmission and control

Certain mosquito species are recognized as species complexes or groups consisting of morphologically identical (isomorphic) but reproductively isolated, which most often occur in sympatry in the same area (Service, Lane et al. 1993, de Souza 2010) Species-specific, ecologic and behavioural differences among members of these complexes can significantly affect disease transmission and control (de Souza 2010).

A*nopheles* species belong to the order Diptera, family Culicidae and subfamily Anophelinae. They have a worldwide distribution and occur in both tropical and temperate zones (de Souza 2010). Chromosomal data suggest that the *Anopheles* is primitive within the family Culicidae (Besansky and Collins 1992). Existing evidence, including the unique possession of dimorphic sex chromosomes and long-period interspersion of repetitive sequences in the genome (Black and Rai 1988), smaller chromosomes and lower nuclear DNA content (RAO and Rai 1990), support the extensive divergence of the *Anopheles* from the other mosquitoes. The most distinguishing features of anopheline mosquitoes are the long palps present in both males and females and the characteristic pattern of blocks of dark and pale scales on the wing veins, especially along the costa.

In Africa, two major *Anopheles* species complexes exist. These are the *Anopheles gambiae* and *Anopheles funestus* complexes. *An. gambiae* sensu lato (s.l.) occurs in

University of Ghana http://ugspace.ug.edu.gh

sympatry with *An. funestus* in many parts of Africa and shows high adaptability to changing environments (Coluzzi 1984). In West Africa however, *An. gambiae* s.l. populations dominate during the rainy season, while *An. funestus* become more abundant in the dry seasons (Coluzzi, 1984). There is high proportion of *An. gambiae* s.l. which happens to be one of the important vectors of LF and malaria (Mekuria, 1983).

2.2.3.1 *Anopheles gambiae* Giles complex

In Ghana and West Africa, *An. gambiae s.l.* is considered as one of the most important LF vectors (Dzodzomenyo and Simonsen 1999, Appawu, Dadzie et al. 2001). The complex was thought to be one species but studies by Ribbands in West Africa, and Thomson in East Africa provided the initial evidence for the specific distinctive nature of salt water breeding *An. gambiae* (cited from de Souza, 2010). The complex comprises of six different species not morphologically distinguishable but collectively referred to as *An. gambiae s.l.* The complex was recognized as comprising three fresh-water and one mineral-water breeding species designated as species A, B, C and D (Coetzee, Hunt et al. 2013) and the two salt-water breeders retaining the early names of *An. merus* for the East African species and *An. melas* for the West African species (Davidson, Paterson et al. 1967). All could be distinguished on the characteristics of inter-species hybridization resulting in sterile male progeny (Davidson, Paterson et al. 1967) and the banding patterns of the giant polytene chromosomes found in the larval salivary glands and the female ovaries (Bryan and Coluzzi 1971).

An. gambiae complex include *An. gambiae s.s* (*sensu stricto*) Giles, *An. arabiensis* Patton (Paterson 1964), *An. quadriannulatus* Theobald (Hunt, Coetzee et al. 1998) *An.*

University of Ghana http://ugspace.ug.edu.gh

bwambae White (Davidson and Hunt 1972), *An. melas* Theobald and *An. merus* Donitz (Paterson 1964, Mahon, Green et al. 1976). A seventh species within the *An. gambiae* complex has been identified and named *An. quadriannulatus* B because of its close resemblance to this species (Hunt, Coetzee et al. 1998). Based on strong genetic evidence, the *An. gambiae* M and S molecular 'forms' and *An. quadriannulatus* sp. B are assigned formal names:with the M form as *An. coluzzii*, S form to be *An. gambiae and An. quadriannulatus* sp.B as *An. amharicus* (Coetzee, Hunt et al. 2013).

The M form of *An. gambiae*, now *An. coluzzii* is believed to be more associated with *W. bancrofti* (Hunter 1992) is a relatively poor vector of malaria compared with other species such as the Savanna form (Coluzzi 1993).

2.2.3.2 Factors influencing the distribution of *Anopheles* vectors

While many *Anopheles* vectors may exist in the same aquatic habitat, they occupy different niches (Kirby and Lindsay 2009), *Anopheles melas* is known as the West African saltwater breeder while *An. gambiae s.s.* is a freshwater breeder. Factors that affect the distribution of mosquito species include; temperature, relative humidity, vegetation, rainfall, nutrients, water sources and quality, and the presence or absence of other mosquito species and predators (Stresman 2010). The distribution of various *Anopheles* vector species of LF, however, depends on the degree of variation among these factors, which may also in turn be dependent on seasonality. These factors can be grouped into physicochemical and biological factors (de Souza 2010).

Physicochemical factors including larval habitats, their number and their productivity determine the density and the distribution of *Anopheles* vectors (Shililu,

University of Ghana http://ugspace.ug.edu.gh

Ghebremeskel et al. 2003). Mosquito larval habitats contain nutrients and deterrents (Fisher, Bradshaw et al. 1990, SOTA 1993) as well elevated concentrations of organic material which may lead to high bacterial growth (Costerton, Cheng et al. 1987). The chemical properties of the habitat can be influenced by pH, and the concentration of ammonia, nitrate, sulphate and chlorophyll, all of which affect larval development and survival (Carpenter 1982, Mutero, Wekoyela et al. 2004, Mwangangi, Mbogo et al. 2007). Water temperature is considered an important factor affecting the distribution of *Anopheles* species (Kirby and Lindsay 2009). Thus, larvae of *Anopheles* species, like many other insect larvae, exist within temperature limits defined as critical thermal maximum and minimum (Kirby and Lindsay 2009)

Biological factors such as predators, parasitoids, certain bacteria and fungi affect the density of emergent mosquitoes (Mwangangi, Mbogo et al. 2007). Examples of such predators include; Anuran larva, Odonata nymphs, Hemiptera, and Coleoptera (Mwangangi, Mbogo et al. 2007) all feed on *Anopheles* larvae. Competition also exists between closely related larval mosquito species inhabiting the same breeding sites. Thus, while the presence of *An. arabiensis* has no effect on the survival of *An. gambiae* s.s larvae, *An. arabiensis* suffers reduced survival when reared with *An. gambiae* (Kirby and Lindsay, 2009). The presence of bacteria such as *Bacillus thuringiensis B.israelensis* and *B. sphaericus* (Fillinger, Knols et al. 2003), nematodes such as *Romanomermic culicivorax (Zaim, Ladonni et al. 1988),* microsporidia such as *Nosema algerea* (Undeen and Dame 1987) and entomopathogenic fungi such as *Metarhizium anisopliae* (Federici 1995) in breeding sites may also affect the survival of mosquito vector species. As such, these have been evaluated as biological control agents against larval stages of mosquitoes.

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

2.3 Methods of Identification of *Wuchereria bancrofti* and LF Vectors

2.3.1 Identification of *Wuchereria bancrofti*

Current existing methods for the identification of *W. bancrofti* parasite (microfilaria) in blood include microscopy which is considered the 'gold standard', for detection of antibodies and circulating filarial antigen in blood (immunodiagnostic test,) and detection of DNA (polymerase chain reaction).

Microscopy requires night collection of blood to detect the presence of the circulating microfilariae. Blood smears are prepared and stained with Geimsa after drying for observation under the microscope. Then also, 2% saponin can be added to the night collections to lyse the erythrocytes for easy identification and counting of the mobile microfilaria under the microscope. An immune-chromatographic test (ICT) card is another method used for the detection of circulating filarial antigens in blood samples (Weil, Lammie et al. 1997) .This method is instant and mostly used for screening purposes and is known to show a high sensitivity and specificity requiring as little as 100ul of blood for testing (Pani and Das 2012).

Enzyme linked immunosorbent assay test kits that detect antigens of *W. bancrofti* parasites from blood collected during the day uses a monoclonal antibody, Og4C3, to detect the LF parasite (Itoh, Gunawardena et al. 1998, Pani, Hoti et al. 2000) The results of this test could take quite some time to be ready but able to test for many samples at a time.

WB123 is the latest which uses similar principles as the Og4C3 but the incubation time is shorter compared to the former.

University of Ghana http://ugspace.ug.edu.gh

A molecular technique which uses purified genomic DNA from blood or mosquito vector samples could be used in a PCR reaction to detect *W. bancrofti* parasite DNA (Zhong, McCarthy et al. 1996, Boakye, Baidoo et al. 2007). PCR can now be used to determine infection rates as well as transmission of LF in a study area (Laney, Ramzy et al. 2010).LAMP assays, which uses principles similar to PCR. After samples are run in the thermo cycler, PCR product observed under the UV light instead of loading on a gel like PCR and this method can be used in the field during surveys unlike PCR method.

The mosquito vectors can be detected by dissecting the carcasses and observed on slides for the presence of any of the parasite stages. This is done using the morphological characteristics of the different stages of the parasite (Boakye, Wilson et al. 2004).

2.3.2 Identification of *Anopheles* vectors of LF

Correct and timely identification of vectors is important in the transmission of diseases. Various techniques have been developed for their identification, especially for discriminating between very closely related species. *Anopheles* vectors in particular have been given much attention due to their importance as vectors of LF and malaria. These methods are based on morphological features, chromosomal inversion arrangements, cuticular hydrocarbon components, electrophoretic separation of enzyme variants of different populations and molecular biology methods based on unique sequences in the vectors DNA composition (Jinbo, Kato et al. 2011).

University of Ghana http://ugspace.ug.edu.gh

2.3.2.1 Morphology and morphometrics

Morphological characters used in the identification of mosquitoes include; the length of the maxillary Palps and proboscis, the presence of speckles and banding pattern on the legs and wings and presence or absence of scales on the thorax. Generally, *culicines* can be distinguished from *Anopheles* mosquitoes by observing their resting postures. a*nophelines* rest at an angle between 45° and 90° to the surface whereas *culicines* rest parallel to the surface. Also, the length and shape of the palps of female *anophelines* have about the same length as the proboscis, in contrast to culicines, where the palps are shorter. Various techniques are employed with different degrees of specificity in vector identification (Collins, Kamau et al. 2000). These techniques are based on characters common to sibling species across geographical zones.

Morphological identification keys for *Anopheline* mosquitoes have been provided by Gillies and colleagues (Gillies and De Meillon 1968, Gillies and Coetzee 1987) . Adult females of the *An. gambiae* complex are identified by their smooth palps with 3 pale bands on the 3rd, 4th and 5th segments; the wing field is pale with yellowish or creamy markings and has pale fairly long costal spots. The femora, tibia and 1st tarsal segments are speckled to a variable degree. The abdomen is pale brown and hairy with scales on the 8th tergite and on the cerci. The adult females of the *An. funestus* are identified by the observation of three pale bands on the 2nd, 3rd and 5th segments of the palps. The dark wings bear characteristic pale scales, with the costa having four pale spots usually shorter than the intervening dark areas. The abdomen is dark brown and lacks scales, whilst the legs are usually dark with a small apical white spot on the tibia.

University of Ghana http://ugspace.ug.edu.gh

2.3.2.2 Cytotaxonomy

Sibling species are usually suspected if there exist differences in the behaviour, ecology, seasonality and susceptibility to parasites in the field (Adler 1987). Species complexes are defined as the existence of morphologically similar species which are reproductively isolated (Mayr 1942). The cytotaxonomic principles involve the classification of organisms based on the detection of differential banding patterns of the polythene chromosomes. The chromosomal banding patterns from the ovarian nurse cells of semi-gravid females have been used to distinguish between *An. gambiae* s.s. and *An. arabiensis* of the *An. gambiae* complex (Hunt, Coetzee et al. 1998). Cytotaxonomy has been used to identify all six members of the *An. gambiae* complex (Coluzzi, Sabatini et al. 1979).

Until recently, it was the only tool that could be reliably used to differentiate all members of the *An. funestus* group (Green and Hunt 1980). A cytogenetic map has been developed for *An. funestus* and compared to the polytene chromosome arrangement for *An. gambiae (Sharakhov, Sharakhova et al. 2001)*. Frequencies of chromosome inversion arrangements have been utilized to investigate population structure and estimate population sizes (TAYLOR, TOURE et al. 1993, Kamau, Hunt et al. 2002). In addition, chromosomal inversions have been used to address phylogenetic issues regarding the origin, maintenance and introgression of inversions between sympatric populations for many *Anopheline* species group (Coetzee, Estrada-Franco et al. 1999). Species genetic polymorphism due to paracentric inversions has also been detected using this technique (Coluzzi 1993). This technique, however, is

University of Ghana http://ugspace.ug.edu.gh

limited by the skill and experience required for its wide application and routine field analysis. Polytene chromosomes must be prepared from ovarian tissue of the fourth instar larvae and this limits the identification of adult blood-fed female mosquitoes or late instar larvae.

2.3.2.3 Cuticular hydrocarbon analysis

The presence of cuticular waxes by insects, serves as protection against desiccation and in chemical communication (Carlson, Mayer et al. 1971, Jones, Lewis et al. 1971). The major components are fatty acids, sterols, esters and hydrocarbons. Cuticular hydrocarbons of closely related species are known to differ and form the basis of their use in taxonomy. This technique is based on utilizing the variation in composition of carbon content of cuticular waxes among different species via chemical analysis.

Using cuticular hydrocarbon analysis Carlson and Service (Carlson 1979) were able to identify members of the *An. gambiae* s.l. By using discriminate analysis Anyanwu *et al.* (2000), observed high segregation of hydrocarbon content among four strains of *An. gambiae* s.s. larvae. Carlson and Walsh (1981) have used similar techniques in the identification of *Simulium squamosum* and *S. sirbanum (Walsh, Molyneux et al. 1993)*. This method for identification requires highly skilled workers and sophisticated equipment, therefore not practical for routine fieldwork regardless of the successes attained by cuticular hydrocarbon analysis, and its relative ease in distinguishing geographic variants. Its use also requires a prior identification by other methods. However, it is best suited for dead and dried samples, provided unnecessary contact with plastic is avoided.

University of Ghana http://ugspace.ug.edu.gh

2.3.2.4 Allozyme/isozyme analysis

This technique is based on the electrophoretic separation of enzyme variants which allows for the quantification of gene differentiation among populations, showing the evolutionary diversity up to species level (Bullini and Coluzzi 1978). The use of this technique led to the discovery of diagnostic allozyme foci for wild sympatric populations of *An. quadriamaculatus* A and B and a dichotomous electrophoretic taxonomic key for three species within the *An. quadrimaculatus* complex (Narang, Kaiser et al. 1989, Lanzaro, Narang et al. 1990), and allowed broader use of these tools for the population genetics of natural vector populations (Hii *et al*., 1998). The problem with this technique however, is that fresh specimen and a large amount of sample material is required compared to the few nanograms of DNA required for PCR (Norris 2002, Richardson, Baverstock et al. 2012). The cost of this technique is also high and it is tedious and requires a lot of time to perform large scale studies.

2.3.2.5 Molecular methods of identification

In the identification of species, the analysis of DNA is well suited since it involves the use of genomic material which is not influenced by environmental and life-stage differences. DNA analysis has been used successfully for the identification of several vector and parasite species.

2.3.2.6 Polymerase chain reactions (PCR)

The development of the PCR method has been very instrumental in the identification of mosquito vectors of medical importance, especially where sibling species that are morphologically indistinguishable exist. The identification of members of the

University of Ghana http://ugspace.ug.edu.gh

Anopheles gambiae complex can be carried out based on specific DNA nucleotide differences in the intergenic spacer (IGS) of the ribosomal DNA (rDNA) on the X chromosome (Scott, Brogdon et al. 1993). Mosquitoes identified as *An. gambiae* s.s. are further analysed for molecular forms using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of (Fanello, Santolamazza et al. 2002), which involves a combination of the protocols established by (Scott, Brogdon et al. 1993) and (Favia, Della Torre et al. 1997). This method allows for simultaneous identification of *An. gambiae* s.l. as well as the M and S forms within the *An. gambiae* s.s.

2.3.2.7 Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) analysis is a PCR-based technique that allows a virtually unlimited number of genetic markers to be amplified in individual organisms. RAPD markers have been extensively used in distinguishing between members of cryptic species (Williams, Kubelik et al. 1990). This technique targets specific regions of repeat gene families, such as ribosomal deoxyribonucleic acid (rDNA). The rDNA genes occur as 150-1,000 tandem repeats in arthropods. Each repeat contains 18s, 5.8s, and 28s rRNA genes alternating with a large intergenic spacer (IGS) region. Located between the 18s and 5.8s genes is the first internal transcribed spacer (ITS1) and between the 5.8 s and the 28S genes is the ITS2. A universal primer in the 28s gene and the species reverse primers which are located in the IGS region are used for *An. gambiae* complex identification (Scott, Brogdon et al. 1993, Collins and Paskewitz 1996, Favia and Louis 1999).

University of Ghana http://ugspace.ug.edu.gh

The RAPD technique is fast, easy and requires very little material. Markers can be readily identified for a variety of taxonomic levels. Compared to DNA sequencing, the cost is modest and many individuals can be assayed. Also, there are numerous commercially available primer kits which can be used to screen populations. RAPD markers however reveal continuous variations between sample populations and their dominant nature makes it impossible to distinguish between homozygous and heterozygous alleles (Carlson *et al*., 1991).

2.3.2.8 Cytochrome oxidase subunit I (COI)

Several genetic approaches have targeted at the nuclear genes in identifying and determining the population structure of mosquito species, especially the *An. gambiae* (Koekemoer, Lochouarn et al. 1999, Kent, West et al. 2004, Smith and Fonseca 2004, Cywinska, Hunter et al. 2006), but very few studies have targeted the mitochondrial genes to identify mosquitoes species using the cytochrome C oxidase subunit 1 (Sallum, Schultz et al. 2002, Cywinska, Hunter et al. 2006, Oshaghi, Yaaghoobi et al. 2006). The inclusion of molecular information into taxonomy is expected to lead to the use of DNA data to distinguish between closely related species and most importantly, to discover new species.(de Souza 2010). Cytochrome c oxidase I (COI) also known as mitochondrial encoded cytochrome c oxidase I (MT-CO1) is a gene that is often used as a DNA barcode to identify animal species. MT-CO1 gene sequence is suitable for this role because of its high mutation rate which is often fast enough to distinguish between closely related species and the sequence also conserved among conspecifics (García-Horsman, Barquera et al. 1994) , More than 2% sequence divergence has been detected between organisms, proving the barcode is effective (Hebert, Ratnasingham et al. 2003). In addition to high copy-number per cell

University of Ghana http://ugspace.ug.edu.gh

which has made them attractive and more tractable targets for characterization, population genetics and phylogenetic studies (Jehle, Lange et al. 2006). Regions within the mitochondrial DNA (*mt*DNA) have been proven useful in biology, epidemiology and diagnosis of several parasitic infections of human and veterinary importance (Kong, Bandelt et al. 2006, Traversa, Costanzo et al. 2007)

University of Ghana http://ugspace.ug.edu.gh

CHAPTER THREE

MATERIAL AND METHODS

3.1. Study Area

The study was conducted in five communities in two different districts, Otrokpe (N.05.78333) and Azizanya (N.05.77009) in Ada in the Dangme East District (Greater Accra Region) and, Mpataano (N.04.84261), Asemko (N.04.83002) and Butre (N.04.82296) in the Ahanta West District representing non endemic and endemic districts respectively. The sites were selected with consideration to communities which have been established to have *An. melas* based on an earlier survey that was conducted by Boakye *et al*, (unpublished data). Most of the houses in the study areas were built with sandcrete blocks or clay with the rooms having no ceilings (**Plate 1 E**), with eaves of the houses having gaps in between them serving as hideouts for mosquitoes. People living in the study communities were predominantly fisher folks.

Ada has a monthly mean temperature of 18°C in the year and typically a pronounced dry season, with the driest month being January, which has a precipitation of less than 60mm. Mpataano, Butre and Asemko on the other hand have high temperatures throughout the year and range between 23°C - 33°C. Rainfall is heavy during the major season between March and September. Humidity records in Mpataano, Butre and Asemko is high, mostly about 60% due to the proximity of the sea and other water bodies. The vegetation is characterized by short Savannah grass interspersed with shrubs and short trees. Along the coast, stretches of coconut trees and patches of coconut groves could be seen. A few stands of the mangrove trees can also be found

University of Ghana http://ugspace.ug.edu.gh

around the estuaries where rivers empty into the sea and tributaries where the soil is waterlogged and salty.

University of Ghana http://ugspace.ug.edu.gh

3.2 Mosquito Collection and Identification

Ten houses were randomly selected in each community. Pyrethrum spray catch (PSC) was employed in sampling mosquitoes between 05:00 hours and 08:00 hours GMT each morning, in a onetime collection. Two trained persons were involved in the collection process as volunteer vector collectors.

Gray baft sheets (Plate **1 D**) were laid on the entire floor of the room and sometimes, over furniture and other items that were too heavy to move. The spray guns were filled with Raid® insecticide (Pynamin Forte-0.05%, Neopynamin-0.05%, Deltamethrin-0.05%, solvent, fragrance-99.885%). The eaves of the buildings or houses to be sampled were sprayed first from the outside windows, doors and eaves before spraying inside the room. After spraying, the gray baft sheets were carefully lifted from the room, folding them from the edges so that the knockdown insects would collect in the middle of the gray baft sheets. The knockdown mosquitoes were collected and sorted with forceps outside the rooms, in the open after ten 10 - 15 minutes. This was done to separate all genera of mosquitoes caught from the other haematophagous insects. Mosquitoes were transported to the laboratory for processing in well labelled Petri dishes lined with wet filter paper to prevent desiccation of the samples (identification and molecular characterization). The label information included the house number, geographic coordinates of locations and the date of sampling.

All *Anopheles* mosquitoes were morphologically identified using presence of the pale and dark banding patterns on the costal margins of their wings while culicines were also identified by the absence of the pale and dark band patterns on the costal margins of their wings (Gillies and de Meillon, 1968),(Daniiav 1983),(Gillies and Coetzee

University of Ghana http://ugspace.ug.edu.gh

1987); to separatethe mosquitoes into anophelines (*Anopheles* mosquitoes) and culicines (*Aedes,Culex,*and *Mansonia* mosquitoes). Generally, the male mosquitoes were distinguished from the females by the presence of their plumose (bushy).

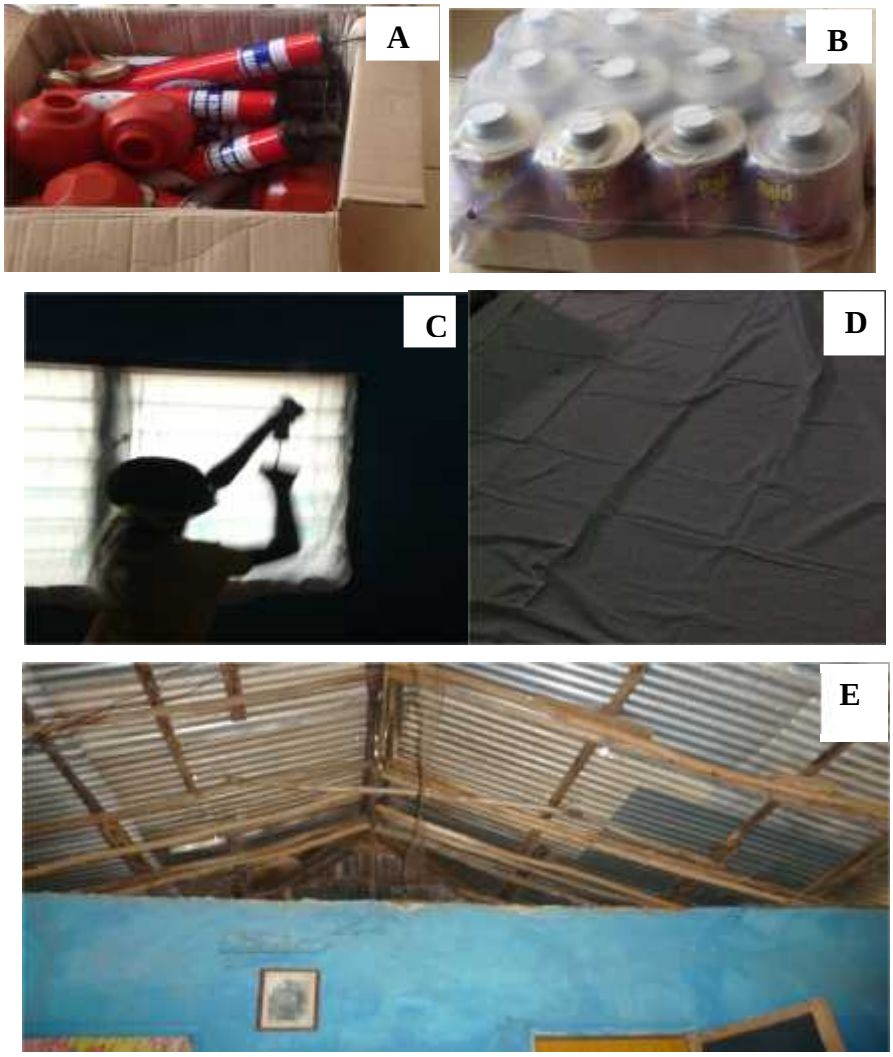

Plate 1: Plate A is the type of spraying gun used for the spraying. Plate B represent the type of insecticide spray used. Plate C is a room been sprayed with insecticide for knockdown collections. Plate D is a room laid with gray baft sheets in preparation for spraying and visible collections of mosquitoes. Plate E is a typical sand Crete block house without ceiling.

University of Ghana http://ugspace.ug.edu.gh

3.3 Molecular Analysis

The genomic DNA of identified *An. gambiae* s.l. mosquitoes were extracted and used for species identification and for the COI sequence analysis.

3.3.1 Extraction of DNA from *An. gambiae* s.l.

The DNA extraction method of Collins *et al*. (1987) was used. Briefly, legs and abdomen of each mosquito were placed in a 1.5 ml Eppendorf tube containing 100µl Bender buffer (0.1M Nacl,0.2M Sucrose,0.1M Trish HCl,0.05M EDTA,0.5% SDS) and homogenized using a sterile polypropylene rod. The homogenate was incubated in a water bath at 65ºC for 30 minutes followed by the addition of 15µl of pre-chilled 8M potassium acetate to each tube and mixed well by tapping the tube. This was followed by incubation at 4ºC for 30 minutes after which the tubes were centrifuged at 14000 rpm for 10 minutes and the supernatants transferred into fresh tubes. A volume of 100µl of pre-chilled absolute ethanol was then added to each supernatant and mixed well by inverting the tubes several times and left to stand for 5mins, followed by centrifugation at 14000 rpm for 10 minutes to pellet the DNA. The supernatant was discarded and the DNA pellets washed with 70% ethanol (100µl) followed by centrifugation at 10000 rpm for 10 minutes. The supernatants were discarded and the tubes inverted over a paper towel and left to dry by evaporation. The dried DNA pellets were dissolved in 50µl TE + RNAse and stored in a -20ºC freezer until ready for use.

University of Ghana http://ugspace.ug.edu.gh

3.3.2 Molecular identification of *An. melas*

The extracted DNA was used as template for PCR runs using modifications of Scott *et al.* (1993) method. The cocktail PCR master mix included all the *gambiae* s.l sibling member primers except *An. quadriannulatus* and *An. bwambae*. The PCR reaction mix contained a total volume of 20µl involving, 2.5 µl 10X Dream taq PCR Buffer containing 20mM of $MgCl_2$, 0.25 µl 10mM dNTP mix, X10mM of primers, containing and 0.3µM each of primers UN, GA, AR and ME (see table 1 below for details of the primers) and 0.05µl of 0.5U Dream *Taq* polymerase (Thermo Fisher Scientific Inc.USA®), 14µl of deionized water and 2µl of a concentration of 15-20 ng/µl template DNA for the PCR reaction.

The PCR was performed and the amplification carried out using a PTC 100 thermal cycler (MJ Research Inc., USA). This had an initial pre-heating step of 5 minutes at 94 °C to activate the DNA polymerase followed by 30 cycles each consisting 30s denaturation at 94°C, 30s annealing at 50°C and 1min extension at 72°C; and the final cycle products are extended for 5 minutes at 72 °C. After amplification, about 5µl amplicon was mixed with 2µl orange G dye and loaded into wells of 2% agarose gel stained in ethidium bromide. The electrophoresis was run in a Mupid®-2 plus submarine electrophoresis system for 1hr with a X 0.5TBE buffer and the results visualised and photographed using a TOYOBO transilluminator gel imaging device (Model TM-20 - TOYOBO FAS-III monitor system). Band size was estimated by comparison with the mobility of a 100bp DNA molecular weight ladder with the diagnostic band size of *A. melas* a s 464bp.

University of Ghana http://ugspace.ug.edu.gh

Table 1: Diagnostic primers sequences and amplicon size for the identification of the members of the *An. gambiae* complex (Scott *et al*., 1993) and primers used for the COI amplifications (Folmer *et al*., 1994)

Primer Name	Species	Primer sequence	Diagnostic Size bp
UN	Universal	GTGTGCCCCTTCCTCGATGT	
GA	*An. gambiae*	CTGGTTTGGTCGGCACGTTT	390
AR	*An. arabiensis*	AAGTGTCCTTCTCCATCCTA	315
QD	*An. quadriannulatus*	CAGACCAAGATGGTTAGTAT	153
LCO1490	COI (Forward primer)	GGTCAACAAATCATAAAGATATTGG	700
HCO2198	COI (Reverse primer)	TAAACTTCAGGGTGACCAAAAAATCA	700

3.3.3 Amplification of COI DNA sequences

Amplification of the COI segment of the mitochondrial gene was performed according to the method of Cywinska *et al*. (2006). The PCR assay was performed using two oligonucleotide primers, LCO1490 and HCO2198 (Folmer *et al*., 1994). The primer sequences for the COI amplification are shown in Table 1 above. Each amplification reaction was done in a final volume of 25µL containing 13.05 µL sterile distilled water, 5 µL of 10x PCR buffer containing $MgCl_2$, 0.5 µL of 10mM dNTPs, 10mM of each primer (1.6 µL), 0.25 U/L *Taq* polymerase (0.25 µL), and 3 µL of 15-20 ng/ul DNA template. The thermal cycling profile was as follows, an initial denaturation at 98°C for 30seconds, followed by 35 cycles of denaturation at 98°C for 10seconds, annealing at 55°C for 1.5 minutes, and extension at 72 °C for 1.5 minutes.

University of Ghana http://ugspace.ug.edu.gh

This was followed by a final cycle at 72°C for 7 minutes. A diagnostic band of approximately 700bp was expected for each reaction.

3.3.4 Sequencing and analysis of amplified COI DNA sequences

The amplified PCR products were purified using Qiagen DNA purification kit and sent to a commercial house Macrogen BV in Netherlands for sequencing. Each sequence was edited and the sequence alignments and construction of consensus sequences were performed using BioEdit version 7.2.5 a free software programme for sequence alignment.

3.3.4.1 Phylogenetic analyses

The consensus sequences from different communities were plotted to reveal the relationship between sequences. The Neighbourhood-Joining analysis based on the Kimura 2 Parameters of the sequences was performed. This was tested using the equality of evolutionary rate between the consensus sequences of *An. melas* from the various community areas using *An. gambiae* as an out- group in Tajima' relative rate test (Tajima, 1993). The equality of evolutionary rate between sequences A (*ASEMKO Consensus*) and B (*AZIZANYA Consensus*), with sequence C (*Anopheles gambiae COI*) and D (*An. melas*) used as an out-group in Tajima's relative rate test (Tajima, 1993). The χ^2 test statistic was used with *P*-value less than 0.05 to reject the null hypothesis of equal rates between lineages. A z-test of neutrality of evolution between Ada and Azizanya sequences, to establish the null hypothesis for equal evolution between sequences was performed.

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei 1987). Evolutionary distances were computed using the Kimura 2-parameter

University of Ghana http://ugspace.ug.edu.gh

method (Kimura 1980) and in the units of the number of base substitutions per site. The analysis involved four nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 637 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura, 2013).

The construction of the phylogenetic tree used the consensus sequences, and the out-group sequences were *gambiae* isolate GM84 cytochrome oxidase subunit I (COI) gene (accession number DQ465336) and *An. melas* (accession number DQ792580.1) obtained from the DNA database GenBank.

3.3.4.2. *In-silico* restriction analyses of COI sequences

There are multiple computational tools used for performing restriction digestion and for the analyses of a genome sequence. Insilco restriction analysis can be used to differentiate or compare between sequences for the presence of mutations. Consensus sequences generated had few ambiguous characters like K, S, and N which were all checked and corrected to the right nucleotide before the analysis was done. A restriction-map data was generated using *in silico* analyses of the two consensus sequences using BioEdit version 7.2.5. *I*n silico analyses were performed on the two groups of sequences together with the reference *An. melas* homologous sequences (accession number DQ792580). Then the outputs were examined for difference and shared restriction sites. This information was then used to calculate similarity/dissimilarity indices using Sørensen's formula which was applied to presence/absence data, as stated below:

University of Ghana http://ugspace.ug.edu.gh

$$QS = \frac{2C}{A+B} = \frac{2|A \cap B|}{|A| + |B|}$$ (Salako, Adebanji et al. 2013)

Where *A* and *B* are the number of sequences (bp) in population A and B, respectively, and *C* is the number of sequences shared by the two populations; QS is the quotient of similarity and ranges between 0 and 1(Salako, Adebanji et al. 2013)

University of Ghana http://ugspace.ug.edu.gh

3.4 Mounting of Cibarial Armature of Mosquitoes

The heads of *An. melas* mosquitoes were dissected from mosquitoes that were collected from the study sites for clearing and mounting. The head of each mosquito was placed in an appropriately labelled 0.5 ml Eppendorf tube and three drops of clearing medium (1:1 volumes of chloral hydrate and phenol) added and kept in the dark at a room temperature of 15^{o}C-30^{o}Cfor one month (this varied depending on the concentration prepared and the species of mosquito to be cleared). After clearing, the head was removed and placed in a drop of Puri's medium on a microscope slide; the position of the mount manipulated until the ideal position that facilitated counting of the cibarial teeth was obtained before covering the slide with a cover slip. The cibarial armature was then viewed under a compound microscope at 400× magnification. The number of cibarial armature of each head was then counted and recorded.

A two sample t-test was used to estimate the means, medians, ranges and standard deviations of the number of cibarial armature counted using GENSTAT software version 12.1 (VSN International Ltd, UK) A two sample t-test was used to compare the cibarial armature obtained for two communities ‘Ada’ (Otrokpe and Azizanya) and Asemko with significance set at $p < 0.05$).

University of Ghana http://ugspace.ug.edu.gh

3.5 Ethical Clearance

Ethical clearance was obtained from the IRB of the Noguchi Memorial Institute for Medical Research (IRB #0000176, dated November 5, 2014). The study was explained to the inhabitants of the communities and sprayers consent sought at both community and individual levels. Household's participation was strictly voluntary and only persons who agreed to be part of the study had their rooms sprayed. All the sprayers were trained and nose masks given to them to protect them from inhaling the insecticide.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER FOUR

RESULTS

4. 1 Composition of Mosquito Species in the Study Area

A total of 552 mosquitoes were caught in all the study communities during the study period. *Culex* mosquitoes were the highest in number with 164 (29.7%), *Aedes* 138 (25%), *Anopheles gambiae* 133 (24.1%), *An. funestus* 116 (21%) and *Mansonia* 1 (0.2%). Table 23 shows the summary of mosquito composition by genus, collected from the study area.

Table 3: A summary of mosquitoes collected in the various communities.

Community	Date (2014)	Coordinates	*An. gambiae*	*An. funestus*	*Culex*	*Mansonia*	*Aedes*	Total
Otrokpe	12-Nov	N.05.78333	19	0	48	0	2	69
Azizanya	13-Nov	N.05.77009	54	0	114	0	136	304
Mpataano	25-Nov	N.04.84261	31	116	2	1	0	150
Asemko	26-Nov	N.04.83002	28	0	0	0	0	28
Butre	27-Nov	N.04.82296	1	0	0	0	0	1
Total			**133**	**116**	**164**	**1**	**138**	**552**

University of Ghana http://ugspace.ug.edu.gh

4.1.1 Identification of sibling species of *Anopheles gambiae* complex

Polymerase chain reaction (PCR) was carried out on the 133 morphologically identified *Anopheles gambiae* s.l. mosquitoes to identify the sibling species. Ninety-four (70.7 %) were identified as *An. gambiae s.s* and 39 (29.3%) mosquitoes were *An. melas.* Figure 3 shows an example of gel electrophoregram for the identification of members of *An. gambiae* species complex. Table 3 also displays a summary of sibling species identification results for the various communities.

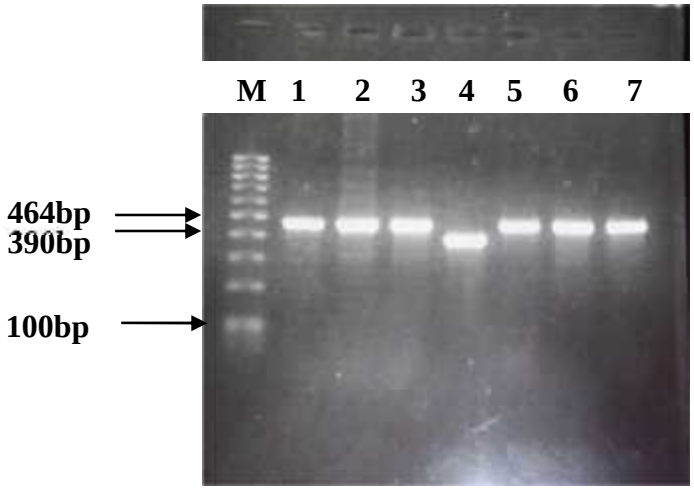

Figure 23: Gel electrophoregram for the identification of members of the *An. gambiae* species complex. Lane M is 100bp molecular weight marker, Lane 1 is positive control for *An. melas*, Lanes 2, 3, 5, 6 and 7 are *An. melas*, and Lane 4 *An. gambiae* s.s

University of Ghana http://ugspace.ug.edu.gh

4.4 Analyses of *An. melas* Sequences

A total of 32 amplified *An. melas* COI PCR products were sequenced. Figure 5 shows an example of a gel electrophoregram obtained for the COI PCR analysis.

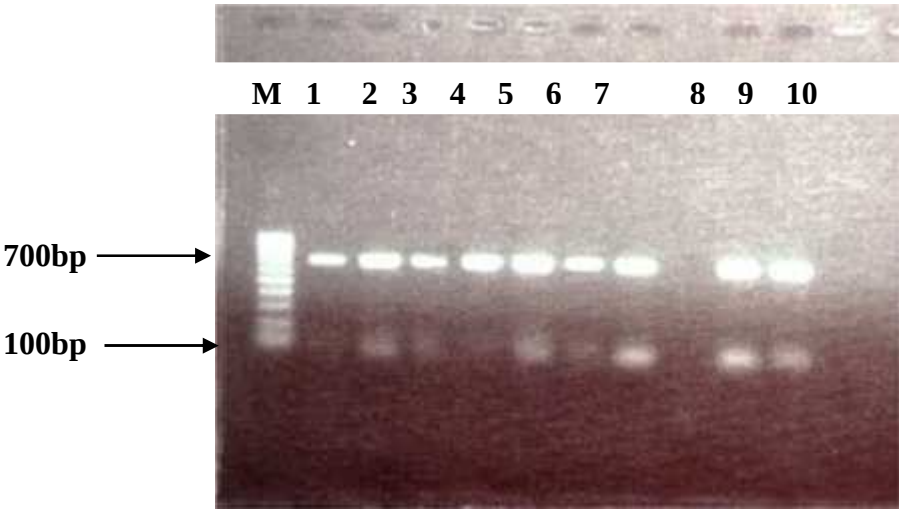

Figure 35: A gel electrophoregram of COI PCR products for sequenced samples. All products amplified at approximately 700bp.Lane M=100bp molecular marker; Lane 1-9= positive samples and Lane 10=negative control.

Following the sequence analyses of the COI PCR products, a chromatogram of the sequences of *An. melas* was obtained as depicted in Figure 6 below

University of Ghana http://ugspace.ug.edu.gh

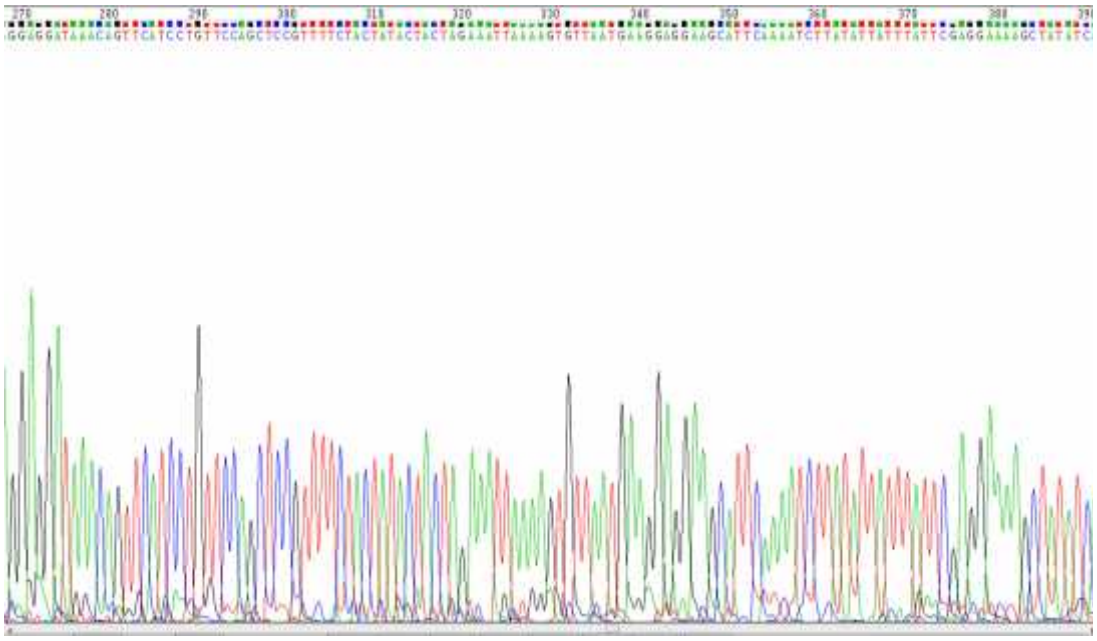

Figure 46: An example of a chromatogram of sequence analysis from *An. melas* samples. The peaks represent the various bases. Colour blue is Cytosine, red is Thymine, green is Adenine and black represent Guanine

4.4.1 Nucleotide usage patterns

Sequence records for 32 *An. melas*, comprising 15 from Azizanya, 2 from Otrokpe and 15 from Asemko were used respectively to create a consensus sequence for each community. Butre had only one sequence so was omitted for this analysis. The consensus sequences generated was compared and analysed using MEGA 6 software. From the results, the CO1 sequences for *An. melas*, was A + T rich (average of 67.5% for all codons). The A + T nucleotide compositions for the first, second and third positions were 59.5%, 69.8% and 75.6% respectively, suggesting that there is an A + T mutational bias within members of the *An. melas*. Nucleotide composition for the Asemko samples had a G+C content of 31.51% and an A+T content of 68.49% with 642bp while Azizanya had a G+C content of 32.23% and an A+T content of 67.77% with a molecular weight of 633bp.

University of Ghana http://ugspace.ug.edu.gh

DNA sequences with high G + C content are more stable than those with high A +T content due to the 3 hydrogen bonds between Guanine and Cytosine, compared to the 2 hydrogen bonds between Adenine and Thymine.

4.4.2 Z - Test of neutrality of evolution between sequences

The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) is when p-value is less than 0.05 at the 95% confidence level. Consensus sequences from Azizanya and Asemko were assessed for neutrality of evolution between the two ecological zones using the Z-test. The test statistic, dS and dN are the numbers of synonymous and non-synonymous substitutions per nucleotide site, respectively. The variance of the difference was computed using the analytical method for the Z-test. Analyses were conducted using the Nei-Gojobori method (Nei and Gojobori, 1986**).** The analysis involved two nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 90 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6 (Tamura K *et al*., 2013). The p value for the observed z-test of neutrality of evolution between sequences was 0.330 (SD = -0.978), thus failing to reject the null hypothesis of strict neutrality.

4.4.3 Phylogenetic analyses

The consensus sequences from different communities were plotted to reveal the relationship between sequences (Fig. 7). The Neighbourhood-Joining analysis based on the Kimura 2 Parameters of the sequences from the various communities revealed slight differentiations, with Azizanya evolving from the more distant Otropke group. Sequences from Asemko when compared with the other two communities in Ada, was an out-group revealing that slight difference exist between the various study sites

University of Ghana http://ugspace.ug.edu.gh

(Fig.7). This was tested using the equality of evolutionary rate between the consensus sequences from the various communities using *An. gambiae* as an out- group in Tajima' relative rate test (Tajima, 1993). The equality of evolutionary rate between sequences A (*ASEMKO Consensus*) and B (*AZIZANYA Consensus*), with sequence C (*Anopheles gambiae COI*) used as an out-group in was tested using Tajima's relative rate test (Tajima, 1993). The χ^2 test statistic was 1.00 (*P* = 0.31731 with 1 degree[s] of freedom. *P*-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages. But then, Azizanya and Asemko had equal number *An. melas* in creating consensus sequence. A z-test of neutrality of evolution between Asemko and Azizanya sequences, showed a p value of 0.350 failing to reject the null hypothesis for equal evolution between sequences.

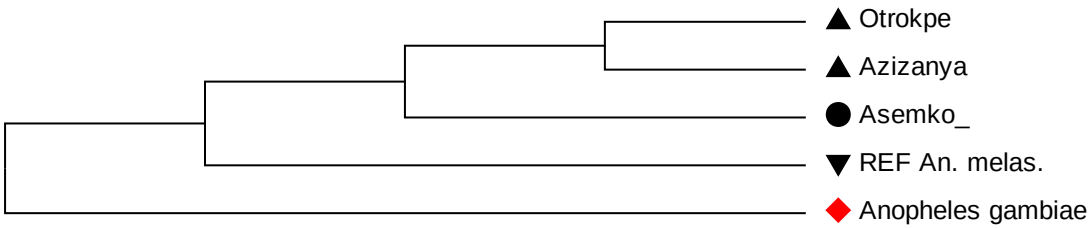

Figure 57: Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 *An. melas* consensus sequences. Phylogenetic tree plotted in Mega 6 software

4.4.4 *In-silico* restriction analyses

After cleaning the initial sequence data of approximately 700 bp the final sequences of 590bp was used for the *in-silico* analysis. After the analysis 16 restriction site differences were observed (Table 2). Details of the restriction map analyses and the enzymes common or unique to each community's population of *An.melas* are shown in Appendix II.

University of Ghana http://ugspace.ug.edu.gh

Table 42: Restriction enzymes digest of the COI sequences with different restriction sites and positions of the two *An. melas* populations.

No.	Enzymes	Azizanya sequence	Asemko sequence
1	AccBSI, BsrBI, MbiI CCG'CTC		405
2	AciI, BspACI, SsiI C'CG_C or G'CG_G		405
3	AclWI, AlwI, BspPI GGATCNNNN'N_	588	
4	Alw21I, BsiHKAI, Bbv12I G_WGCW'C		191
5	Alw26I, BcoDI, BsmAI, BstMAI GTCTCN'NNNN_		70
6	BaeGI, BseSI, BstSLI G_KGCM'C	191	
7	BclI, FbaI, Ksp22I T'GATC_A	74	
8	Bse3DI, BseMI,BsrDI GCAATG_NN'	522	
9	BseYI C'CCAG_C		587
10	BslFI, FaqI GGGACNNNNNNNNNN'NNNN_		26
11	BsmBI, Esp3I CGTCTCN'NNNN_		71
12	BsmFI GGGACNNNNNNNNNN'NNNN_		26
13	BfuCI, Bsp143I, BssMI, BstMBI, DpnII, Kzo9I, MboI, NdeII, Sau3AI 'GATC_	584	
14	BmiI, BspLI, NlaIV, PspN4I GGN'NCC	190	
15	BsaMI, BsmI, Mva1269I, PctI GAATG_CN'		238
16	BseYI C'CCAG_C		587

University of Ghana http://ugspace.ug.edu.gh

4.3 Comparison of Cibarial Armatures of Eastern and Western Populations of *An. melas*

A total of 39 *An.melas* heads had the cibarial armatures mounted ,comprising of 13 from Ada and 26 from Asemko were studied. A typical mount of the cibarial armatures is as shown in Plate 2 below.

The mean number of cibarial teeth of the total 39 *An. melas* mosquitoes was 14.1 (median = 14, range = 11-19, SD = ±1.5), that of Asemko was 14.1 (median = 14 range = 11-19, SD = ±1.5), and of Ada was 14.4 (median = 15, range = 11-18, SD = ±1.51). The observed difference between the two populations was not significant (t = 1.53, d.f. = 1, P = 0.140), thus failing to reject the null hypothesis of no differences in the number of cibarial teeth in the two populations from the study areas.

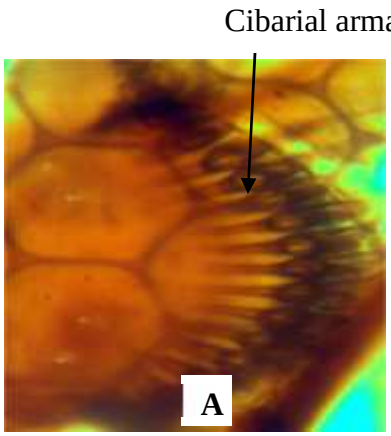

Plate 32: Plate A shows a picture of cibarial armatures of an *An. melas* observed under a microscope.

University of Ghana http://ugspace.ug.edu.gh

CHAPTER FIVE

DISCUSSIONS

5.1 Discussion

This study was aimed at characterizing the LF vector *An. melas* both molecularly and morphologically to determine if differences existed between their populations west of Accra in Asemko an endemic community, and an eastern population in Azizanya a non-endemic community. From the molecular analysis of the consensus sequences of the two populations, mutational differences were observed which resulted in 16 unique and different restriction sites. This difference was nonetheless not significant in telling the two populations apart

However, the phylogenetic studies revealed the closeness of the two populations with a z-test of neutrality of evolution of 0.35 between Asemko and Azizanya sequences of the *An. melas* ($p = 0.350$); thus not significantly different from each other. Furthermore, their similarity was confirmed by a QS value of 0.99 when analysed using Sørensen's formula.

Speciation occurs over time and one population becomes distinct when it can no longer interbreed with the parent population due to either geographical isolation and/or other environmental factors(Coyne and Orr 2004). In order for one population to diverge enough from another to become a new species, there need to be a physical barrier that keeps them from mixing(Mayr 1954). Asemko and Azizanya are two different communities in Ghana that are geographically allopatric, which means there is a possibility of some gradual

University of Ghana http://ugspace.ug.edu.gh
changes occurring within a particular geographical area. However, the observed relatedness of

Azizanyo and Otropke with regards to Asemko *An. melas* populations in the phylogenetic tree suggests geographic distribution and not necessarily different species.

The evidence of the similarity of the two *An. melas* populations is further supported by the cibarial teeth count which also did not reveal any difference between them. The results of the teeth count obtained for *An. melas* were also similar to a mean number of 14.1 (median = 14. obtained by Amuzu *et al*. (2010) for the same species in the Gomoa District along the coast of Ghana east of Accra.

According to Mcgreevy *et al*. (1978) one factor that has been reported to influence the efficacy of vector species is the cibarial armature which is known to have lethal effect on ingested filarial parasites. Amuzu *et al*. (2010) had attributed the high vectorial efficiency of *An. melas* compared to *An. gambiae* s.s., to the comparatively lower numbers of cibarial teeth. From this reasoning the eastern and western populations should have similar vectorial capacities, which begs the question as to why there are differences in LF endemicity pattern along the coast of Ghana. The answer then must lie elsewhere and that needs further investigations. Nonetheless, many factors affect the distribution of mosquito species. Notable among these are temperature, relative humidity, vegetation, rainfall, nutrients, water sources and quality, and the presence or absence of other mosquito species and predators(de Souza 2010). The distribution of various *Anopheles* vector species of LF, however, depends on the degree of variation

University of Ghana http://ugspace.ug.edu.gh

within these factors, which may also in turn be dependent on seasonality (Gyapong, Kyelem et al. 2002).

The limitation to the present study is that not enough samples from more and varied communities were obtained, which is also a valid requirement for further studies.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATION

In conclusion, there were no difference either molecularly or morphologically between the *An. melas* species populations in Azizanya and Asemko but sixteen mutational differences were observed.

The observed restriction site differences potentially represent markers that could be used to distinguish 'eastern' and 'western' populations of *An. melas* but this would require further studies involving wider sampling of the areas and experiments with the identified enzymes.

To determine whether the eastern population are really refractory to *W. bancrofti* it is also recommended that xeno- experiments be conducted by feeding reared *An. melas* mosquitoes from the East coast of Ghana on infected individuals from the West and monitor the development of the parasite in the mosquito.

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

REFERENCES

ADLER, P. H. 1987. Ecology of Black fly Sibling Species.

AMUZU, H., WILSON, M. D. & BOAKYE, D. A. 2010. Studies of *Anopheles gambiae* sl (Diptera: Culicidae) exhibiting different vectorial capacities in lymphatic filariasis transmission in the Gomoa district, Ghana. *Parasit Vectors,* 3, 85.

ANDREADIS, T. G., ANDERSON, J. F., MUNSTERMANN, L. E., WOLFE, R. J. &FLORIN, D. A. 2001. Discovery, distribution, and abundance of the newly introduced mosquito *Ochlerotatus japonicus* (Diptera: Culicidae) in Connecticut, USA. *Journal of medical entomology,* 38, 774-779.

APPAWU, M. A., DADZIE, S. K., BAFFOE-WILMOT, A. & WILSON, M. D. 2001.

Lymphatic filariasis in Ghana: entomological investigation of transmission dynamics and intensity in communities served by irrigation systems in the Upper East Region of Ghana. *Tropical Medicine & International Health,* 6, 511-516.

BESANSKY, N. & COLLINS, F. 1992. The mosquito genome: organization, evolution and manipulation. *Parasitology Today,* 8, 186-192.

BLACK, W. C. & RAI, K. S. 1988. Genome evolution in mosquitoes: intraspecific and interspecific variation in repetitive DNA amounts and organization. *Genetical Research,* 51, 185-196.

BOAKYE, D., WILSON, M., APPAWU, M. & GYAPONG, J. 2004. Vector competence, for *Wuchereria bancrofti*, of the *Anopheles* populations in the Bongo District of Ghana. *Annals of Tropical Medicine and Parasitology,* 98, 501-508.

BOAKYE, D. A., BAIDOO, H. A., GLAH, E., BROWN, C., APPAWU, M. & WILSON, M. D. 2007. Monitoring lymphatic filariasis interventions: Adult mosquito sampling, and improved PCR–based pool screening method for *Wuchereria bancrofti* infection in *Anopheles* mosquitoes. *Filaria Journal,* 6, 13.

BRYAN, J. H. & COLUZZI, M. 1971. Cytogenetic observations on *Anopheles farauti*

Laveran. *Bulletin of the World Health Organization,* 45, 266.

BULLINI, L. & COLUZZI, M. 1978. Applied and theoretical significance of electrohoretic studies in mosquitoes (Diptera: Culicidae). *Parassitologia,* 20, 8-21.

CARLSON, D. 1979. Differentiation between species of the *Anopheles gambiae* Giles complex (Diptera: Culicidae) by analysis of cuticular hydrocarbons. *Annals of Tropical Medicine and Parasitology,* 73, 589-592.

CARLSON, D., MAYER, M., SILHACEK, D., JAMES, J., BEROZA, M. & BIERL,

University of Ghana http://ugspace.ug.edu.gh

B. 1971. Sex attractant pheromone of the house fly: isolation, identification and synthesis. *Science,* 174, 76-78.

CARPENTER, S. J. & LA CASSE, W. J. 1955. *Mosquitoes of North America (North of Mexico),* University of California Press.

CARPENTER, S. R. 1982. Stem flow chemistry: effects on population dynamics of detritivorous mosquitoes in tree-hole ecosystems. *Oecologia,* 53, 1-6.

COETZEE, M., ESTRADA-FRANCO, J. G., WUNDERLICH, C. A. & HUNT, R. H.1999. Cytogenetic evidence for a species complex within *Anopheles pseudopunctipennis* Theobald (Diptera: Culicidae). *The American Journal of Tropical Medicine and Hygiene,* 60, 649-653.

COETZEE, M., HUNT, R. H., WILKERSON, R., DELLA TORRE, A., COULIBALY, M. B. & BESANSKY, N. J. 2013. *Anopheles coluzzii* and *Anopheles amharicus,* new members of the *Anopheles gambiae* complex. *Zootaxa,* 3619, 246-274.

COLLINS, F. & PASKEWITZ, S. 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Molecular Biology,* 5, 1-9.

COLLINS, F. H., KAMAU, L., RANSON, H. A. & VULULE, J. M. 2000. Molecular entomology and prospects for malaria control. *Bulletin of the World Health Organization,* 78, 1412-1423.

COLUZZI, M. 1984. Heterogeneities of the malaria vectorial system ~~in tropical~~in tropical Africa and their significance in malaria epidemiology and control. *Bulletin of the World Health Organization,* 62, 107.

COLUZZI, M. 1993. Advances in the study of Afrotropical malaria vectors. *Parassitologia,* 35, 23-29.

COLUZZI, M., SABATINI, A., PETRARCA, V. & DI DECO, M. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 73, 483-497.

COSTERTON, J. W., CHENG, K., GEESEY, G. G., LADD, T. I., NICKEL, J. C.,DASGUPTA, M. & MARRIE, T. J. 1987. Bacterial biofilms in nature and disease. *Annual Reviews in Microbiology,* 41, 435-464.

CYWINSKA, A., HUNTER, F. & HEBERT, P. D. 2006. Identifying Canadian mosquito species through DNA barcodes. *Medical and Veterinary Entomology,* 20, 413-424.

DAVIDSON, G. & HUNT, R. 1972. The crossing and chromosome characteristics of a new, sixth species in the *Anopheles gambiae* complex. *Parassitologia,* 15, 121-128.

DAVIDSON, G., PATERSON, H., COLUZZI, M., MASON, G. & MICKS, D. 1967.The *Anopheles gambiae* complex in Genetics of Insect Vectors of Disease, edited by JW Wright and R. Pal. Elsevier, Amsterdam.

DE SOUZA, D. K. 2010. *Diversity in Anopheles gambiae ss and Wuchereria bancrofti, and the Distribution of Lymphatic Filariasis in Ghana.*

University of Ghana http://ugspace.ug.edu.gh
Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology.

DE SOUZA, D. K., KOUDOU, B., KELLY-HOPE, L. A., WILSON, M. D., BOCKARIE, M. J. & BOAKYE, D. A. 2012. Diversity and transmission competence in lymphatic filariasis vectors in West Africa, and the implications for accelerated elimination of *Anopheles*-transmitted filariasis. *Parasit Vectors,* 5, 259.

DUERR, H.-P., DIETZ, K. & EICHNER, M. 2005. Determinants of the eradicability of filarial infections: a conceptual approach. *Trends in Parasitology,* 21, 88- 96.

DUNYO, S., APPAWU, M., NKRUMAH, F., BAFFOE-WILMOT, A., PEDERSEN,E. & SIMONSEN, P. E. 1996. Lymphatic filariasis on the coast of Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 90, 634- 638.

DUNYO, S. K., NKRUMAH, F. K. & SIMONSEN, P. E. 2000. A randomized double-blind placebo-controlled field trial of ivermectin and albendazole alone and in combination for the treatment of lymphatic filariasis in Ghana. Transactions of the Royal Society of Tropical Medicine and Hygiene, 94, 205- 211.

DZODZOMENYO, M. & SIMONSEN, P. E. 1999. Bancroftian filariasis in an irrigation project community in southern Ghana. *Tropical Medicine and International Health,* 4, 13-18.

FANELLO, C., SANTOLAMAZZA, F. & DELLA TORRE, A. 2002. Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Medical and Veterinary Entomology,* 16, 461-464.

FAVIA, G., DELLA TORRE, A., BAGAYOKO, M., LANFRANCOTTI, A., SAGNON, N. F., TOURÉ, Y. & COLUZZI, M. 1997. Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Molecular Biology,* 6, 377-383.

FAVIA, G. & LOUIS, C. 1999. Molecular identification of chromosomal forms of*Anopheles gambiae* sensu stricto. *Parassitologia,* 41, 115-118.

FEDERICI, B. 1995. The future of microbial insecticides as vector control agents. *Journal of the American Mosquito Control Association,* 11, 260-268.

FILLINGER, U., KNOLS, B. G. & BECKER, N. 2003. Efficacy and efficiency of new *Bacillus thuringiensis* var. israelensis and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya. *Tropical Medicine & International Health,* 8, 37-47.

FISHER, I. J., BRADSHAW, W. E. & KAMMEYER, C. 1990. Fitness and its correlates assessed by intra-and interspecific interactions among tree-hole mosquitoes. *The Journal of Animal Ecology*, 819-829.

University of Ghana http://ugspace.ug.edu.gh

GARCÍA-HORSMAN, J. A., BARQUERA, B., RUMBLEY, J., MA, J. & GENNIS, R. B. 1994. The superfamily of heme-copper respiratory oxidases. *Journal of Bacteriology,* 176, 5587.

GILLETT, J. D. 1972. *Common African Mosquitoes and their Medical Importance*, London, UK, William Heinemann.

GILLIES, M. 1955. The pre-gravid phase of ovarian development in *Anopheles funestus*. *Annals of Tropical Medicine and Parasitology,* 49, 320.

GILLIES, M. & COETZEE, M. 1987. A Supplement to the Anophelinae of Africa South of the Sahara. *Publications of the South African Institute for Medical Research,* 55, 1-143.

GILLIES, M. & DE MEILLON, B. 1968. *The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region)*.

GREEN, C. & HUNT, R. 1980. Interpretation of variation in ovarian polytene chromosomes of *Anopheles funestus* Giles, *An. parensis* Gillies, and *An. aruni*? *Genetica,* 51, 187-195.

GREENWOOD, B. 1997. The epidemiology of malaria. *Annals of Tropical Medicine and Parasitology,* 91, 763-770.

GYAPONG, J., GYAPONG, M., EVANS, D., AIKINS, M. & ADJEI, S. 1996a. The economic burden of lymphatic filariasis in northern Ghana. *Annals of Tropical Medicine and Parasitology,* 90, 39-48.

GYAPONG, J., KYELEM, D., KLEINSCHMIDT, I., AGBO,K., AHOUANDOGBO, F., GABA, J., OWUSU-BANAHENE, G., SANOU, S., SODAHLON, Y. & BISWAS, G. 2002. The use of spatial analysis in mapping the distribution of bancroftian filariasis in four West African countries. *Annals of Tropical Medicine and Parasitology,* 96, 695-705.

GYAPONG, J. O., ADJEI, S. & SACKEY, S. 1996b. Descriptive epidemiology of lymphatic filariasis in Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 90, 26-30.

GYAPONG, J. O., MAGNUSSEN, P. & BINKA, F. N. 1994. Parasitological and clinical aspects of bancroftian filariasis in Kassena-Nankana District Upper East Region, Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 88, 555-557.

GYAPONG, J. O. & TWUM-DANSO, N. A. 2006. Editorial: Global elimination of lymphatic filariasis: fact or fantasy? *Tropical Medicine & International Health,* 11, 125-128.

GYAPONG, M., GYAPONG, J. O., ADJEI, S., VLASSOFF, C. & WEISS, M. 1996c. Filariasis in northern Ghana: some cultural beliefs and practices and their implications for disease control. *Social Science & Medicine,* 43, 235-242.

University of Ghana http://ugspace.ug.edu.gh

HEBERT, P. D., RATNASINGHAM, S. & DE WAARD, J. R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences,* 270, S96-S99.

HUNT, R. H., COETZEE, M. & FETTENE, M. 1998. The *Anopheles gambiae* complex: a new species from Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 92, 231-235.

HUNTER, J. M. 1992. Elephantiasis: a disease of development in north east Ghana. *Social science & medicine,* 35, 627-645.

ITOH, M., GUNAWARDENA, N. K., QIU, X.-G., WEERASOORIYA, M. V. &KIMURA, E. 1998. The use of whole blood absorbed on filter paper to detect *Wuchereria bancrofti* circulating antigen. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 92, 513-515.

JEHLE, J. A., LANGE, M., WANG, H., HU, Z., WANG, Y. & HAUSCHILD, R.2006. Molecular identification and phylogenetic analysis of baculoviruses from Lepidoptera. *Virology,* 346, 180-193.

JINBO, U., KATO, T. & ITO, M. 2011. Current progress in DNA barcoding and future implications for entomology. *Entomological Science,* 14, 107-124.

JONES, R. L., LEWIS, W., BOWMAN, M. C., BEROZA, M. & BIERL, B. A. 1971. Host-seeking stimulant for parasite of corn earworm: isolation, identification, and synthesis. *Science,* 173, 842-843.

KAMAU, L., HUNT, R. & COETZEE, M. 2002. Analysis of the population structure of *Anopheles funestus* (Diptera: Culicidae) from western and coastal Kenya using paracentric chromosomal inversion frequencies. *Journal of Medical Entomology,* 39, 78-83.

KENT, R., JULIUSSON, L., WEISSMANN, M., EVANS, S. & KOMAR, N. 2009. Seasonal blood-feeding behavior of *Culex tarsalis* (Diptera: Culicidae) in Weld county, Colorado, 2007. *Journal of Medical Entomology,* 46, 380-390.

KENT, R. J., WEST, A. J. & NORRIS, D. E. 2004. Molecular differentiation of colonized human malaria vectors by 28S ribosomal DNA polymorphisms. *The American Journal of Tropical Medicine and Hygiene,* 71, 514-517.

KIMURA, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,* 16, 111-120.

KING, C. L. & NUTMAN, T. B. 1991. Regulation of the immune response in lymphatic filariasis and onchocerciasis. *Parasitology Today,* 7, 54-58.

University of Ghana http://ugspace.ug.edu.gh

KIRBY, M. J. & LINDSAY, S. W. 2009. Effect of temperature and inter-specific competition on the development and survival of Anopheles gambiae sensu stricto and An. arabiensis larvae. ***Acta tropica,*** **109, 118-123.**

KOEKEMOER, L. L., LOCHOUARN, L., HUNT, R. H. & COETZEE, M. 1999. Single-strand conformation polymorphism analysis for identification of four members of the ***Anopheles funestus*** **(Diptera: Culicidae) group.** ***Journal of Medical Entomology,*** **36, 125-130.**

KONG, Q.-P., BANDELT, H.-J., SUN, C., YAO, Y.-G., SALAS, A., ACHILLI, A., WANG, C.-Y., ZHONG, L., ZHU, C.-L. & WU, S.-F. 2006. Updating the East Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. ***Human Molecular Genetics,*** **15, 2076-2086.**

KUHLOW, F. & ZIELKE, E. 1978. Dynamics and intensity of Wuchereria bancrofti transmission in the savannah and forest regions of Liberia. ***Tropenmedizin und Parasitologie,*** **29, 371-381.**

KWANSA-BENTUM, B., ABOAGYE-ANTWI, F., OTCHERE, J., WILSON, M. D.& BOAKYE, D. A. 2014. Implications of low-density microfilariae carriers in ***Anopheles*** **transmission areas: molecular forms of** ***Anopheles gambiae*** **and** ***Anopheles funestus*** **populations in perspective.** ***Parasites & Vectors,*** **7, 157.**

LAIRD, M. 1988. ***The Natural History of Larval Mosquito Habitats,*** **Academic Press Ltd.**

LANEY, S. J., RAMZY, R., HELMY, H. H., FARID, H. A., ASHOUR, A. A., WEIL, G. J. & WILLIAMS, S. A. 2010. Detection of ***Wuchereria bancrofti*** **L3 larvae in mosquitoes: a reverse transcriptase PCR assay evaluating infection and infectivity.** ***PLoS Neglected Tropical Diseases,*** **4, e602.**

LANZARO, G. C., NARANG, S. K. & SEAWRIGHT, J. 1990. Speciation in an anopheline (Diptera: Culicidae) mosquito: enzyme polymorphism and the genetic structure of populations. ***Annals of the Entomological Society of America,*** **83, 578-585.**

MAHON, R., GREEN, C. & HUNT, R. 1976. Diagnostic allozymes for routine identification of adults of the ***Anopheles gambiae*** **complex (Diptera, Culicidae).** ***Bulletin of Entomological Research,*** **66, 25-31.**

MAYR, E. 1942. ***Systematics and the origin of species, from the viewpoint of a zoologist,*** **Harvard University Press.**

MCGREEVY, P., BRYAN, J., OOTHUMAN, P. & KOLSTRUP, N. 1978. The lethal effects of the cibarial and pharyngeal armatures of mosquitoes on microfilariae. ***Transactions of the Royal Society of Tropical Medicine and Hygiene,*** **72, 361-368.**

MEHLHORN, H. 1988. ***Parasitology in focus. Facts and trends,*** **Springer-Verlag.**

University of Ghana http://ugspace.ug.edu.gh

MICHAEL, E., BUNDY, D. & GRENFELL, B. 1996. Re-assessing the global prevalence and distribution of lymphatic filariasis. *Parasitology,* 112, 409-428.

MOORE, C. & PRATT, H. 1993. *Mosquitoes of Public Health Importance and Their Control* (1-43). Atlanta, GA: Centers for Disease Control and Prevention.

MUIRHEAD-THOMSON, R. 1954. Factors determining the true reservoir of infection of *Plasmodium falciparum* and *Wuchereria bancrofti* in a West African village. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 48, 208-225.

MUTERO, C. M., WEKOYELA, P., GITHURE, J. & KONRADSEN, F. 2004. Ammonium sulphate fertiliser increases larval populations of *Anopheles arabiensis* and culicine mosquitoes in rice fields. *Acta Tropica,* 89, 187-192.

MWANGANGI, J. M., MBOGO, C. M., MUTURI, E. J., NZOVU, J. G., KABIRU, E. W., GITHURE, J. I., NOVAK, R. J. & BEIER, J. C. 2007. Influence of biological and physicochemical characteristics of larval habitats on the body size of *Anopheles gambiae* mosquitoes (Diptera: Culicidae) along the Kenyan coast. *Journal of Vector Borne Diseases,* 44, 122.

NARANG, S., KAISER, P. & SEAWRIGHT, J. 1989. Dichotomous electrophoretic taxonomic key for identification of sibling species A, B, and C of the *Anopheles quadrimaculatus* complex (Diptera: Culicidae). *Journal of Medical Entomology,* 26, 94-99.

NORRIS, D. E. 2002. Genetic markers for study of the anopheline vectors of human malaria. *International journal for parasitology,* 32, 1607-1615.

OSEI, J. H. N. 2013. *Assessing transmission of lymphatic filariasis using mosquitoes in communities with at least five rounds of mass drug administration in Ghana.* Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology.

OSHAGHI, M., YAAGHOOBI, F. & ABAIE, M. 2006. Pattern of mitochondrial DNA variation between and within *Anopheles stephensi* (Diptera: Culicidae) biological forms suggests extensive gene flow. *Acta Tropica,* 99, 226-233.

OTTESEN, E. A. 2000. Editorial: The global programme to eliminate lymphatic filariasis. *Tropical Medicine & International Health,* 5, 591-594.

OTTESEN, E. A. & NUTMAN, T. B. 1992. Tropical pulmonary eosinophilia. *Annual Review of Medicine,* 43, 417-424.

PANI, S. & DAS, L. 2012. 2 8 Lymphatic Filariasis. *Emerging and Re-Emerging Infectious Diseases*, 135.

PANI, S., HOTI, S., ELANGO, A., YUVARAJ, J., LALL, R. & RAMAIAH, K. 2000. Evaluation of the ICT whole blood antigen card test to detect infection due to nocturnally periodic Wuchereria bancrofti in South India. *Tropical*

University of Ghana http://ugspace.ug.edu.gh
***Medicine & International Health*, 5**, 359-363.

PATERSON, H. 1964. Direct evidence for the specific distinctness of forms a, b, and c of the *Anopheles gambiae* complex. *Rivista di malariologia,* 43, 191.

PICHON, G. 2002. Limitation and facilitation in the vectors and other aspects of the dynamics of fi filarial transmission: the need for vector control against Anopheles-transmitted fi filariasis. *Annals of Tropical Medicine & Parasitology,* 96, S143-S152.

PRATT, H. & MOORE, C. 1993. Vector-Borne Disease Control: Mosquitoes. *of Public Health Importance and Their Control. US Department of Health and Human Services, Atlanta, GA*.

PRENDERGAST, B. F., MIYAGI, I., PECOR, T. T. J. E. & WILKERSON, R. C. 2005. Habitats and distribution of *Anopheles sinensis* and associated *Anopheles hyrcanus* group in Japan.

RAMAIAH, K. D., DAS, P. K., MICHAEL, E. & GUYATT, H. L. 2000. The economic burden of lymphatic filariasis in India. *Parasitology Today,* 16, 251- 253.

RAO, P. N. & RAI, K. 1990. Genome evolution in the mosquitoes and other closely related members of superfamily Culicoidea. *Hereditas,* 113, 139-144.

RICHARDSON, B. J., BAVERSTOCK, P. R. & ADAMS, M. 2012. *Allozyme electrophoresis: a Handbook for Animal Systematics and Population Studies*, Academic Press.

RUEDA, L. M. 2008. Global diversity of mosquitoes (Insecta: Diptera: Culicidae) in freshwater. *Freshwater Animal Diversity Assessment.* Springer.

SAITOU, N. & NEI, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* 4, 406- 425.

SALLUM, M., SCHULTZ, T., FOSTER, P., ARONSTEIN, K., WIRTZ, R. & WILKERSON, R. 2002. Phylogeny of Anophelinae (Diptera: Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. *Systematic Entomology,* 27, 361-382.

SATTLER, M. A., MTASIWA, D., KIAMA, M., PREMJI, Z., TANNER, M., KILLEEN, G. F. & LENGELER, C. 2005. Habitat characterization and spatial distribution of *Anopheles* sp. mosquito larvae in Dar es Salaam (Tanzania) during an extended dry period. *Malaria Journal,* 4, 4.

SCHWAB, R., PALATNIK, J. F., RIESTER, M., SCHOMMER, C., SCHMID, M. & WEIGEL, D. 2005. Specific effects of microRNAs on the plant transcriptome. *Developmental Cell,* 8, 517-527.

SCOTT, J. A., BROGDON, W. G. & COLLINS, F. H. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *The American Journal of Tropical Medicine and Hygiene,* 49, 520- 529.

University of Ghana http://ugspace.ug.edu.gh

SERVICE, M., LANE, R. & CROSSKEY, R. 1993. Mosquitoes (Culicidae). *Medical Insects and Arachnids*, 120-240.

SHARAKHOV, I. V., SHARAKHOVA, M. V., MBOGO, C. M., KOEKEMOER, L.L. & YAN, G. 2001. Linear and spatial organization of polytene chromosomes of the African malaria mosquito *Anopheles funestus*. *Genetics*, 159, 211-218.

SHILILU, J., GHEBREMESKEL, T., SEULU, F., MENGISTU, S., FEKADU, H., ZEROM, M., GHEBREGZIABIHER, A., SINTASATH, D., BRETAS, G. & MBOGO, C. 2003. Larval habitat diversity and ecology of anopheline larvae in Eritrea. *Journal of Medical Entomology*, 40, 921-929.

SINGH, G. & PRAKASH, S. 2009. Efficacy of *Bacillus sphaericus* against larvae of malaria and filarial vectors: an analysis of early resistance detection. *Parasitology Research*, 104, 763-766.

SMITH, J. L. & FONSECA, D. M. 2004. Rapid assays for identification of members of the *Culex (Culex) pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). *The American Journal of Tropical Medicine and Hygiene*, 70, 339-345.

SOTA, T. 1993. Performance of *Aedes albopictus* and *A. riversi* larvae (Diptera: Culicidae) in waters that contain tannic acid and decaying leaves: is the treehole species better adapted to treehole water? *Annals of the Entomological Society of America*, 86, 450-457.

SOULSBY, E. J. L. 1982. *Helminths, Arthropods and Protozoa of Domesticated animals*. Bailliere Tindall.

SOUTHGATE, B. & BRYAN, J. H. 1992. Factors affecting transmission of *Wuchereria bancrofti* by anopheline mosquitoes. 4. Facilitation, limitation, proportionality and their epidemiological significance. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86, 523-530.

STRESMAN, G. H. 2010. Beyond temperature and precipitation: Ecological risk factors that modify malaria transmission. *Acta Tropica*, 116, 167-172.

SUBRAMANIAN, S., KRISHNAMOORTHY, K., RAMAIAH, K., HABBEMA, J., DAS, P. & PLAISIER, A. 1998. The relationship between microfilarial load in the human host and uptake and development of *Wuchereria bancrofti*microfilariae by *Culex quinquefasciatus*: a study under natural conditions.*Parasitology*, 116, 243-255.

TAYLOR, C. E., TOURE, Y. T., COLUZZI, M. & PETRARCA, V. 1993. Effective population size and persistence of *Anopheles arabiensis* during the dry season in West Africa. *Medical and Veterinary Entomology*, 7, 351-357.

TAYLOR, M. J., HOERAUF, A. & BOCKARIE, M. 2010. Lymphatic filariasis and onchocerciasis. *The Lancet*, 376, 1175-1185.

THOMPSON, E., HAYES, D. & LUDLAM, K. 1963. Notes on the feeding habits of *Aedes sollicitans* in the Chincoteague-Assateague Island area of Virginia. *Mosquito News*, 23, 297-298.

University of Ghana http://ugspace.ug.edu.gh

THOMSON, R. 1946. Studies on the breeding places and control of *Anopheles gambiae* and *An. gambiae* var. *melas* in coastal districts of Sierra Leone. *Bulletin of Entomological Research,* 36, 185-252.

TRAVERSA, D., COSTANZO, F., IORIO, R., AROCH, I. & LAVY, E. 2007. Mitochondrial cytochrome c oxidase subunit 1 (cox1) gene sequence of Spirocerca lupi (Nematoda, Spirurida): avenues for potential implications. *Veterinary Parasitology,* 146, 263-270.

UGHASI, J., BEKHARD, H., COULIBALY, M., ADABIE-GOMEZ, D., GYAPONG, J., APPAWU, M., WILSON, M. & BOAKYE, D. 2012. Mansonia africana and Mansonia uniformis are vectors in the transmission of *Wuchereria bancrofti* lymphatic filariasis in Ghana. *Parasites and Vectors,* 5, 89.

UNDEEN, A. & DAME, D. 1987. Measurement of the effect of microsporidian pathogens on mosquito larval mortality under artificial field conditions. *Journal of the American Mosquito Control Association (USA).*

URQUHART, G., ARMOUR, J., DUNCAN, J., DUNN, A. & JENNINGS, F. 1987. *Veterinary Parasitology*. Longman Scientific & Technical. Published in USA by Churchill Livingstone Inc., New York.

WALSH, J., MOLYNEUX, D. & BIRLEY, M. 1993. Deforestation: effects on vector-borne disease. *Parasitology,* 106, S55-S75.

WEBBER, R. 1991. Can anopheline-transmitted filariasis be eradicated? *Journal of Tropical Medicine and Hygiene,* 94, 241-244.

WEIL, G., LAMMIE, P. & WEISS, N. 1997. The ICT filariasis test: a rapid-format antigen test for diagnosis of bancroftian filariasis. *Parasitology Today,* 13, 401-404.

WILLIAMS, J. G., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. & TINGEY, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* 18, 6531-6535.

ZAIM, M., LADONNI, H., ERSHADI, M., MANOUCHEHRI, A., SAHABI, Z., NAZARI, M. & SHAHMOHAMMADI, H. 1988. Field application of *Romanomermis culicivorax* (Mermithidae: Nematoda) to control anopheline larvae in southern Iran. *Journal of the American Mosquito Control Association,* 4, 351-355.

ZHONG, M., MCCARTHY, J., BIERWERT, L., LIZOTTE-WANIEWSKI, M., CHANTEAU, S., NUTMAN, T. B., OTTESEN, E. A. & WILLIAMS, S. A. 1996. A polymerase chain reaction assay for detection of the parasite *Wuchereria bancrofti* in human blood samples. *The American Journal of Tropical Medicine and Hygiene,* 54, 357-363.

ZIMMERMAN, R. H., GALARDO, A. K. R., LOUNIBOS, L. P., ARRUDA, M. & WIRTZ, R. 2006. Bloodmeal hosts of *Anopheles* species (Diptera: Culicidae) in a malaria-endemic area of the Brazilian Amazon. *Journal of Medical Entomology,* 43, 947-956

University of Ghana http://ugspace.ug.edu.gh

APPENDICES

Appendix I: **Nomenclature for incompletely specified bases in nucleic acid sequences**

Generally, Guanine, adenine, thymine, cytosine: G, A, T, C are symbols used

But the following are symbols previously recommended

Name of Nucleotide	Symbol Previously Recommended
Adenine or Guanine	R
Thymine or Cytosine	Y
Adenine or Thymine	W
Guanine or Cytosine	S
Adenine or Cytosine	M
Adenine or Thymine or Cytosine	H
Guanine or Cytosine or Thymine	B
Guanine or Adenine or Cytosine	V
Guanine or Adenine or Thymine	D
Guanine or Adenine or Thymine or Cytosine	N

University of Ghana http://ugspace.ug.edu.gh

Appendix II: Field Collections

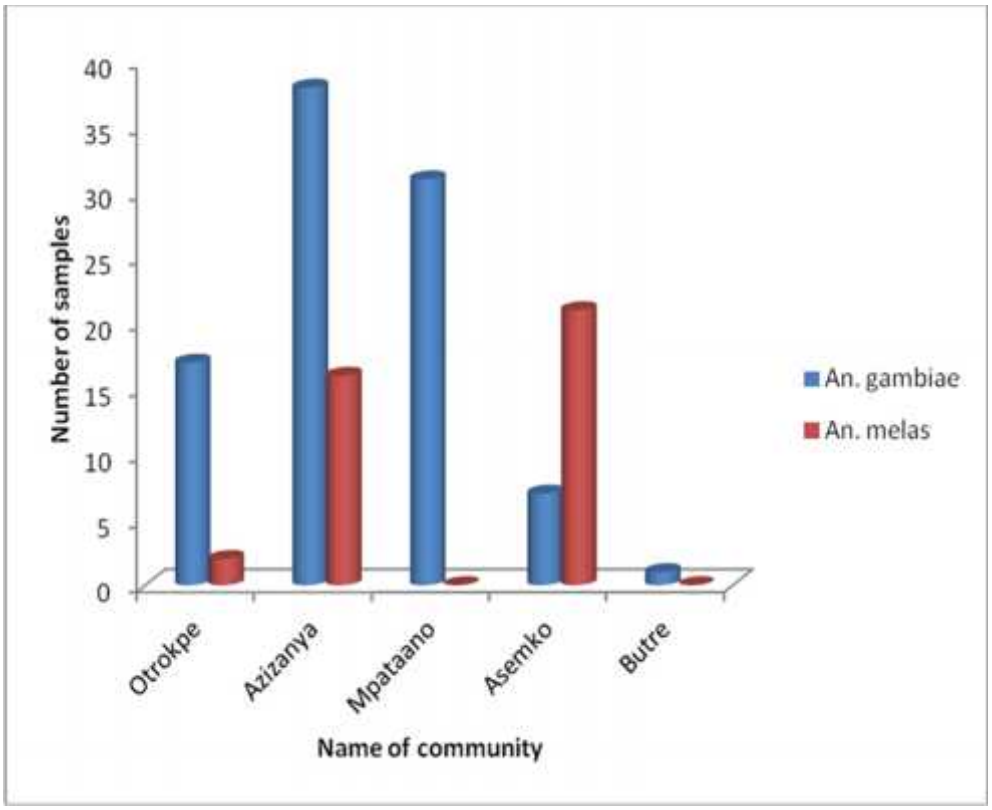

Figure 64: Relative abundance of sibling species of *An. gambiae s.l*

Table 53: A summary of mosquitoes collected in the various communities.

Community	Date (2014)	Coordinates	*An. gambiae*	*An. funestus*	*Culex*	*Mansonia*	*Aedes*	Total
Otrokpe	12-Nov		19	0	48	0	2	69
Azizanya	13-Nov		54	0	114	0	136	304
Mpataano	25-Nov	N.04.84261	31	116	2	1	0	150

University of Ghana http://ugspace.ug.edu.gh

Asemko	26-Nov	N.04.83002	28	0	0	0	0	28
Butre	27-Nov	N.04.82296	1	0	0	0	0	1
Total			**133**	**116**	**164**	**1**	**138**	**552**

Table 64: The sibling species of *An. gambiae* complex identified in the various communities

Community	*An. gambiae*	*An. melas*	Total
Otrokpe	17	2	19
Azizanya	38	16	54
Mpataano	31	0	147
Asemko	7	21	28
Butre	1	0	1
Total	**94**	**39**	**249**

University of Ghana http://ugspace.ug.edu.gh

Apendix: Cibarial teeth observed in *An.melas* between the two study areas

Sample ID	Species	Cibarial Teeth	Species ID	Species	Cibarial Teeth
Ada 1	*An.melas*	15	Asemko 8	*An.melas*	14
Ada 2	*An.melas*	14	Asemko 9	*An.melas*	14
Ada 3	*An.melas*	15	Asemko 10	*An.melas*	13
Ada 4	*An.melas*	14	Asemko 11	*An.melas*	11
Ada 5	*An.melas*	11	Asemko 12	*An.melas*	15
Ada 6	*An.melas*	14	Asemko 13	*An.melas*	15
Ada 7	*An.melas*	15	Asemko 14	*An.melas*	14
Ada 8	*An.melas*	18	Asemko 15	*An.melas*	14
Ada 9	*An.melas*	15	Asemko 16	*An.melas*	14
Ada 10	*An.melas*	15	Asemko 17	*An.melas*	12
Ada 11	*An.melas*	14	Asemko 18	*An.melas*	14
Ada 12	*An.melas*	14	Asemko 119	*An.melas*	14
Ada 13	*An.melas*	15	Asemko 20	*An.melas*	13
Asemko 1	*An.melas*	11	Asemko 21	*An.melas*	15

University of Ghana http://ugspace.ug.edu.gh

Asemko 2	*An.melas*	14	Asemko 22	*An.melas*	15
Asemko 3	*An.melas*	14	Asemko 23	*An.melas*	15
Asemko 4	*An.melas*	14	Asemko 24	*An.melas*	15
Asemko 5	*An.melas*	15	Asemko 25	*An.melas*	15
Asemko 6	*An.melas*	14	Asemko 26	*An.melas*	19
Asemko 7	*An.melas*	14			

University of Ghana http://ugspace.ug.edu.gh

Appendix III

Two-sample t-test for cibarial armature

Variate: *An._melas*
Group factor: Communities

Test for equality of sample variances

Test statistic F = 1.31 on 12 and 12 d.f.

Probability (under null hypothesis of equal variances) = 0.140

Sample	Sample size	Mean	Variance	Standard deviation	Standard error of mean
Ada	13	14.54	2.269	1.506	0.4178
Asemko	13	13.69	1.731	1.316	0.3649

Difference of means: 0.846

Standard error of difference: 0.555

95% confidence interval for difference in means: (-0.2987, 1.991)

Test statistic t = 1.53 on 24 d.f.

Probability= 0.140

University of Ghana http://ugspace.ug.edu.gh

Appendix IV

Restriction enzyme digest of DNA for Azizanya and Asemko

Azizanya sequence.

```
    0 SEQUENCEGT GGGAACATCT TTAAGAATCC TAATTCGAGC TGAACTAGGG CACCCTGGAG CATTCATTGG AGATGATCAA ATTTATAATG TAATTGTTAC
  100 TGCTCATGCT TTTATTATAA TTTTCTTTAT AGTTATACCT ATTATAATTG GAGGGTTTGG AAACTGATTA GTTCCTTTAA TATTAGGGGC ACCTGATATA
  200 GCTTTTCCTC GAATAAATAA TATAAGATTT TGAATACTTC CTCCTTCATT AACACTTTTA ATTTCTAGTA GTATAGTAGA AAACGGAGCT GGAACAGGAT
  300 GAACTGTTTA TCCTCCTCTA TCTTCTGGAA TTGCTCATGC TGGGGCTTCA GTAGATTTAG CAATTTTTTC TCTTCATTTA GCAGGAATTT CTTCTATTTT
  400 AGGAGCAGTA AATTTTATTA CAACAGTAAT TAATATACGA TCTCCAGGAA TTACATTAGA TCGAATACCA TTATTTGTTT GATCGGTAGT TATTACAGCA
  500 GTATTATTAT TATTATCATT GCCAGTATTA GCAGGAGCTA TTACTATATT ATTAACTGAT CGAAATTTAA ATACATCTTT CTTTGATCCA GCAGG
  600
```

Asemko sequence.

```
    0 SEQUENCEGT GGGGACATCT TTAAGAATCC TAATTCGAGC TGAACTAGGG CACCCTGGAG CATTCATTGG AGACGATCAA ATTTATAATG TAATCGTTAC
  100 TGCTCATGCT TTTATTATAA TTTTCTTTAT AGTTATACCT ATTATAATTG GAGGGTTTGG AAACTGATTA GTTCCTTTAA TATTAGGAGC ACCTGATATA
  200 GCTTTTCCTC GAATAAATAA TATAAGATTT TGAATGCTTC CTCCTTCATT AACACTTTTA ATTTCTAGTA GTATAGTAGA AAACGGAGCT GGAACAGGAT
  300 GAACTGTTTA TCCTCCTCTA TCTTCTGGAA TTGCTCATGC TGGGGCTTCA GTAGATTTAG CAATTTTTTC TCTTCATTTA GCAGGAATTT CTTCTATTTT
  400 AGGAGCGGTA AATTTTATTA CAACAGTAAT TAATATACGA TCTCCAGGAA TTACATTAGA TCGAATACCA TTATTTGTTT GATCGGTAGT TATTACAGCA
  500 GTATTATTAT TATTATCATT ACCAGTATTA GCAGGAGCTA TTACTATATT ATTAACTGAT CGAAATTTAA ATACATCTTT CTTTGACCCA GCAGG
  600
```

Alignment

```
    0  SEQUENCEGT GGGAACATCT TTAAGAATCC TAATTCGAGC TGAACTAGGG CACCCTGGAG CATTCATTGG AGATGATCAA ATTTATAATG TAATTGTTAC
       |||||||||| ||| |||||| |||||||||| |||||||||| |||||||||| |||||||||| ||| |||||| |||||||||| |||| |||||
       SEQUENCEGT GGGGACATCT TTAAGAATCC TAATTCGAGC TGAACTAGGG CACCCTGGAG CATTCATTGG AGACGATCAA ATTTATAATG TAATCGTTAC
  100  TGCTCATGCT TTTATTATAA TTTTCTTTAT AGTTATACCT ATTATAATTG GAGGGTTTGG AAACTGATTA GTTCCTTTAA TATTAGGGGC ACCTGATATA
       |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||| || ||||||||||
       TGCTCATGCT TTTATTATAA TTTTCTTTAT AGTTATACCT ATTATAATTG GAGGGTTTGG AAACTGATTA GTTCCTTTAA TATTAGGAGC ACCTGATATA
  200  GCTTTTCCTC GAATAAATAA TATAAGATTT TGAATACTTC CTCCTTCATT AACACTTTTA ATTTCTAGTA GTATAGTAGA AAACGGAGCT GGAACAGGAT
       |||||||||| |||||||||| |||||||||| ||||| |||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
       GCTTTTCCTC GAATAAATAA TATAAGATTT TGAATGCTTC CTCCTTCATT AACACTTTTA ATTTCTAGTA GTATAGTAGA AAACGGAGCT GGAACAGGAT
```

University of Ghana http://ugspace.ug.edu.gh

```
300  GAACTGTTTA TCCTCCTCTA TCTTCTGGAA TTGCTCATGC TGGGGCTTCA GTAGATTTAG CAATTTTTTC TCTTCATTTA GCAGGAATTT CTTCTATTTT
     |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
     GAACTGTTTA TCCTCCTCTA TCTTCTGGAA TTGCTCATGC TGGGGCTTCA GTAGATTTAG CAATTTTTTC TCTTCATTTA GCAGGAATTT CTTCTATTTT
400  AGGAGCAGTA AATTTTATTA CAACAGTAAT TAATATACGA TCTCCAGGAA TTACATTAGA TCGAATACCA TTATTTGTTT GATCGGTAGT TATTACAGCA
     |||||| ||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
     AGGAGCGGTA AATTTTATTA CAACAGTAAT TAATATACGA TCTCCAGGAA TTACATTAGA TCGAATACCA TTATTTGTTT GATCGGTAGT TATTACAGCA
500  GTATTATTAT TATTATCATT GCCAGTATTA GCAGGAGCTA TTACTATATT ATTAACTGAT CGAAATTTAA ATACATCTTT CTTTGATCCA GCAGGG
     |||||||||| ||||||||||  ||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||||||| |||||| ||| ||||||
     GTATTATTAT TATTATCATT ACCAGTATTA GCAGGAGCTA TTACTATATT ATTAACTGAT CGAAATTTAA ATACATCTTT CTTTGACCCA GCAGGG
600
```

Number	Restriction enzyme	Azizanya Seq	Asemko Seq
1	AccB1I, BanI, BshNI, BspT107I G'GYRC_C	49 188	49
2	AccBSI, BsrBI, MbiI CCG'CTC		405
3	AciI, BspACI, SsiI C'CG_C or G'CG_G		405
4	AclWI, AlwI, BspPI GGATCNNNN'N_	588	
5	Alw21I, BsiHKAI, Bbv12I G_WGCW'C		191
6	Alw26I, BcoDI, BsmAI, BstMAI GTCTCN'NNNN_		70
7	BaeGI, BseSI, BstSLI G_KGCM'C	52 191	52
8	BclI, FbaI, Ksp22I T'GATC_A	74	

University of Ghana http://ugspace.ug.edu.gh

9	BfuCI, Bsp143I, BssMI, BstMBI, DpnII, Kzo9I, MboI, NdeII, Sau3AI 'GATC_	74 438 458 480 557 584	74 438 458 480 557
10	BmiI, BspLI, NlaIV, PspN4I GGN'NCC	51 190	51
11	BsaMI, BsmI, Mva1269I, PctI GAATG_CN'	65	65 238
12	Bse3DI, BseMI, BsrDI GCAATG_NN'	522	
13	BseYI C'CCAG_C	339	339 587
14	BslFI, FaqI GGGACNNNNNNNNNN'NNNN_		26
15	BsmBI, Esp3I CGTCTCN'NNNN_		71
16	BsmFI GGGACNNNNNNNNNN'NNNN_		26
17	BstKTI G_AT'C	77 441 461 483 560 587	77 441 461 483 560
18	DpnI,MalI GA'TC	76 440 460 482 559 586	76 440 460 482 559
19	GsaI C_CCAG'C	343	343 591
20	MluCI, Sse9I, TasI, Tsp509I, TspEI 'AATT_	31 79 91 118 145 259 328 361 385 410 427 448 563	31 79 118 145 259 328 361 385 410 427 448 563

University of Ghana http://ugspace.ug.edu.gh

21	MluCI, Sse9I, TasI, Tsp509I, TspEI AATT_	31 79 91 118 145 259 328 361 385 410 427 448 563	31 79 118 145 259 328 361 385 410 427 448 563