Diversity in *Anopheles gambiae s.s* and *Wuchereria bancrofti*, and the Distribution of Lymphatic Filariasis in Ghana

BY

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DOCTOR OF PHILOSOPHY

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DECLARATION

I hereby declare that this submission is my own work towards the PhD and that, to the best of my knowledge, it contains no materials previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to my mum, Esther Ame Meme de Souza, who constantly teased me about having to travel all over Ghana to collect mosquitoes because there were not enough mosquitoes in Accra. Thanks for all the efforts invested in me.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

bp	base pair
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
EDTA	Disodium ethylene diamine tetraacetate.
EtBr	Ethidium bromide
H_2O	Water
HCl	Hydrogen chloride
HLC	Human landing catches
KAc	Potassium acetate
Kg	Kilogram
M	Molar
mtDNA	Mitochondrial DNA
mg	milligram
ml	millilitre
NaCl	Sodium chloride
PCR	Polymerase chain reaction
pH	Hydrogen-ion exponent
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolution per minute
sddH_2O	sterile double distilled water
SDS	Sodium dodecyl sulphate
s.l	sensu lato
s.s	sensu stricto
TAE	Tris - Acetate EDTA
µl	microlitre
µM	micromolar

ABSTRACT

Lymphatic Filariasis (LF) is a disease transmitted by the filarial worm *Wuchereria bancrofti*. It is a disease of public health importance, and has been earmarked for elimination by the year 2020 through Mass Drug Administration (MDA) programs. In Ghana, the distribution of LF reveals a pattern of endemicity separated by a continuous area of non-endemicity. As such, this study hypothesized that genetic differences – probably not revealed by current identification methods, within LF vectors and parasite populations, may be responsible for the observed distribution pattern. The main aim of this project was therefore to explain the current observed distribution of LF based on the diversity in the *Anopheles gambiae s.s* and *Wuchereria bancrofti* in Ghana. Mosquito samples were collected from 14 sites across Ghana, divided into 4 ecological zones, and falling into endemic and non-endemic areas. *W. bancrofti* samples were picked from dissected mosquitoes -using dissecting pins, collected from previous studies in Ghana. The samples were morphologically and molecularly identified using routine methods. Using the PCR, the Cytochrome C Oxidase subunit 1 (COI) from *W. bancrofti* and *An. gambiae* s.s was amplified, sequenced and analyzed. To better understand the effects of environmental factors on the diversity in the *An. gambiae* s.s, the spatial distribution of the *An. gambiae* M and S molecular forms and associated environmental factors were examined, and their relationship with disease prevalence was determined. A total of 10274 mosquitoes were collected, out of which 6150 (59.9%) were morphologically identified as *An. gambiae* s.l; 1494 (24.3%) of the *An. gambiae* s.l were further classified into sibling species. The S form of *An. gambiae* s.s

predominated in the middle belt, while the M form predominated in the northern and coastal Savannah belts. Bivariate correlation analysis between the M and S forms indicated that their prevalences were negatively correlated (-0.763). Spatial analyses carried out, indicated a positive spatial clustering for both the *An. gambiae* M (MI =0.19, Z score=4.2, $P \leq 0.01$) and *An. gambiae* S (MI =0.19, Z score=4.2, $P \leq 0.01$) forms. Multiple regression analyses of all data (n=70 sites), indicated that temperature was an important variable for both forms, explaining for *An. gambiae* M, 28% (R_2=0.28, F=25.8, P≤0.001) and for *An. gambiae* S, 36% (R_2=0.36, F=37.9, P≤0.001) of the variance in the model. *An. gambiae* M was significantly correlated with LF, and 2.5 to 3 times more prevalent in the high LF zone than low to medium LF zones. Phylogenetic analyses of *An. gambiae* s.s revealed the samples to be grouped according to endemicity and ecological zones. The equality of evolutionary rate between the consensus sequences from the endemic and non-endemic areas, revealed a χ^2 test statistic of 3.71 (P = 0.054), rejecting the null hypothesis of equal rates between lineages. Seven and 15 *W. bancrofti* specimens from Gomoa District (in the South) and Bongo District (in the North) respectively were sequenced. There was a significant difference in the evolutionary rate between the consensus sequences of *W. bancrofti* from the North and the South, with a χ^2 test statistic of 7.44 (P = 0.00637). The results of this study indicate that environmental factors, especially temperature, play an important role in the distribution of LF and its vectors in Ghana. The observed genetic differences in *An. gambiae* s.s and *W. bancrofti* populations in Ghana may affect the vector-parasite interactions in various areas, and may explain the observed distribution of LF in the country.

CHAPTER I: GENERAL INTRODUCTION

1.1 Introduction

Lymphatic Filariasis (LF) or elephantiasis is a disfiguring disease caused by infection with the filarial parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (World Health Organisation, 2000). It 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. The disease is painful and profoundly disfiguring, and is one of the leading causes of permanent disability in the world. The most common clinical manifestation of LF is the lymphoedema of the legs and genitalia (World Health Organisation, 2000). 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) [World Health Organization, 2000]. The elimination programme is based on Mass Drug Administration (MDA), through a yearly administration of a single-dose of albendazole and diethylcarbamazine, or albendazole and ivermectin, to reduce microfilaraemia (mf) in populations at risk, for a period long enough to ensure that mf remain below levels necessary to sustain transmission.

The distribution of LF in West Africa reveals a pattern of endemicity separated by a continuous area of non-endemicity, spanning from southern Ghana, through the Dahomey Gap (Maley, 1999), to Togo and central Benin (Gyapong *et al*., 2002). In Ghana, this continuum of non-endemicity extends from the middle forest belt (2º.5´W – 6ºN), to the east of Accra, and covers the whole of the Volta region (Figure 1.1). However, the *Anopheles* species (i.e. *Anopheles gambiae s.l*) which are the main vectors of LF and malaria are present in both endemic and non-endemic areas, as is evident from the distribution of malaria. This raises questions about the discontinuous distribution of LF in West Africa.

A study by Kelly-Hope and colleagues, seeking to explain the distribution of LF and malaria, suggested that areas with LF endemicity are less endemic for malaria and vice versa (Kelly-Hope *et al*., 2006). This however may not be entirely true since in Ghana, LF endemicity in the Northern Region is high (Gyapong *et al*., 1996a) and malaria endemicity is also very high (Appawu *et al*., 2004). Also, concomitant infections of malaria and LF have been reported both in human and in mosquito populations in Kenya (Muturi *et al*., 2006). On the other hand, it has been hypothesized that different parasite-vector relationships between *W. bancrofti* and its vectors, may be responsible for the observed distribution of LF (Southgate and Bryan, 1992). There is genetic diversity among the vectors of LF (e.g. *An. gambiae*), which may lead to altered vector-parasite interactions, affecting the distribution of LF. For example, in West Africa, whilst *Culex* species is not a vector for LF, it is the main vector for the disease in urban areas in East Africa (Subramanian *et al*., 1998, Appawu *et al*., 2001). Thus,

Anopheles species may be exhibiting different vector-parasite phenomenon, in LF endemic and non-endemic areas.

The genetic diversity in the *An. gambiae* s.s, that may affect vector-parasite interactions for instance, can be described in terms of chromosomal and molecular forms. Five chromosomal forms namely; "Forest", "Bissau", "Bamako", "Mopti" and "Savannah" have been described (Coluzzi *et al*., 1979, Bryan *et al*., 1982, Coluzzi *et al*., 1985). The Mopti form of *An. gambiae*, for example, is believed to be more associated with *W. bancrofti* (Hunter, 1992), and is a relatively poor vector of malaria compared with other species such as the Savannah form (Coluzzi, 1993, Carnevale *et al*., 1999). There is further genetic diversity in the *An. gambiae s.s.*, in terms of the M and S Molecular forms, identified by sequence differences in the intergenic spacer of rDNA, located on the X chromosome (Favia *et al*., 1997, Gentile *et al*., 2001). Additional evidence suggests the existence of two distinct chromosomal forms within the M form (Slotman *et al*., 2007). The extent of genetic variations further suggest that cryptic taxa may exist within *An. gambiae* s.s due to observed inversions in the micromorphology of the second chromosome for different populations (Powell *et al*., 1999). To add to these, incipient speciation has been reported among members of the *Anopheles species* in West Africa (Della Torre *et al*., 2001, Michel *et al*., 2005), raising further questions as to why these are only reported in West Africa and not elsewhere on the continent (Lehmann *et al*., 1997, Kamau *et al*., 1998).

To further explain the distribution of LF in West Africa, Kelly-Hope *et al.*, (2006) also hypothesized that current LF transmission indices may be affected by seasonality such that in high malaria transmission seasons, LF transmission may be low. These transmission variations may be influenced by seasonal differences in vector species, such that different species may become more important during different seasons and in different ecological zones (Bigoga *et al.*, 2007). Thus, change in abundance of one vector species, for example the Mopti form, may lead to better/increased vector competence for the transmission of LF. Selective effects, due to environmental factors, have also been reported in studies where the frequency of certain inversion arrangements increased during dry seasons and decreased during wet seasons (Coluzzi *et al.*, 1979, Bryan *et al.*, 1982, Coluzzi *et al.*, 1985).

In addition to the inherent genetic variations in the vectors that may influence the transmission of LF, genetic variations within the parasite may also contribute to altered vector-parasite interactions. Three physiological strains of the parasite based on the periodicity of appearance of microfilaria (mf) in the peripheral blood of the human host have been described (Sasa, 1976). The existence of two variants of *W. bancrofti* based on the length of mf (Kumar *et al.*, 2002) and the reported occurrence of two different genetic variants of *W. bancrofti*, high genetic divergence and gene flow in different geoclimatic regions (Kumar *et al.*, 2002, Thangadurai *et al.*, 2006) may all influence the vector-parasite interactions and the successful elimination of the disease.

The resurgence of LF after MDA suspension has been shown in a longitudinal study in India (Sunish *et al*., 2002). Therefore, in view of the current elimination program and MDA, which supposes that the parasite and vector populations are single entities, it is important to conduct further studies, in order to formulate appropriate strategies should there be genetic variability among the vectors of LF and *W. bancrofti*. These observations therefore lead us to the hypothesis that: Genetic differences between LF vectors and parasite populations may be responsible for the observed distribution pattern in Ghana, West Africa. These genetic differences are probably not revealed by current identification methods based on morphology, cytology and ribosomal DNA, used for characterizing genetic variants.

1.2 Rational and Overall Objectives

Whilst various genetic approaches, targeted at nuclear genes, have been used in identifying and determining the population structure of mosquito species, especially the *An. gambiae* (Singh *et al*., 1997, Koekemoer *et al*., 1999, Kent *et al*., 2004, Smith and Fonseca, 2004, Kampen, 2005), very few have targeted the mitochondrial genes to identify mosquitoes and fewer have attempted to study *Anopheles* species using the Cytochrome C Oxidase subunit 1 (COI) (Sallum *et al*., 2002, Lehr *et al*., 2005, Cywinska *et al*., 2006, Foley and Torres, 2006, Oshaghi *et al*., 2006). The inclusion of molecular information into taxonomy is expected to lead to the use of DNA data to distinguish between species and most importantly, to discover new species.

The mitochondrial protein-coding genes generally contain more differences than the ribosomal genes – due to their relatively fast mutation rates, and are more likely to effectively differentiate closely related species (Stoeckle *et al*., 2005). Sequence comparisons among protein-coding genes are easier because they generally lack indels frequently present in ribosomal genes. The cytochrome C Oxidase subunit I gene (COI) contains sequence differences representative of those in other mitochondrial protein-coding genes. The COI region represents about the first half of the gene and is 648 base pairs, a length easy to process in one "grab" with current technology and thus cheap. It is present in hundreds of copies and easy to recover from diverse taxa, readily aligned for sequence comparisons, effective in distinguishing among closely related animal

species from a variety of invertebrate and vertebrate taxa, and thus proposed as a universal method for identifying existing species and discovering new ones (Hebert *et al*., 2003, Hebert *et al*., 2004, Barrett and Hebert, 2005). In common with other protein-coding genes, its third position nucleotides show a high incidence of base substitutions. Unlike other mitochondrial genes, changes in its amino acid sequence occur more slowly, improving the affinity of taxonomic work and primer design (Cywinska *et al*., 2006). Studies revealed that analysis of the (CO1) mitochondrial gene is useful in discriminating closely allied species and their evolutionary history (Hebert *et al*., 2004, Cywinska *et al*., 2006, Foley and Torres, 2006).

The main aim of this project, therefore, is to explain the current observed distribution of LF based on the diversity in the *An. gambiae s.l* and *W. bancrofti* in Ghana. Vector competence plays an essential role in the transmission of LF (Bryan *et al*., 1990, Failloux *et al*., 1995), and this seems to differ among mosquito species and also according to geographic strain (Wharton, 1960, McGreevy *et al*., 1982). Furthermore, the variability in parasite populations in terms of periodicity, species variants and genetic diversity, may be important factors affecting vector competence and the transmission of the disease. The novel component of this research is to look at genetic variability of the vectors and parasite as an explanation to the current observed distribution of LF. Therefore, this project is divided into two parts, aimed at explaining the above objective.

In the first part of this study, sequence variation in the barcode region of CO1 is analyzed to investigate the diversity and phylogeny of LF vectors in endemic and non-endemic areas, as well as in various ecological zones. The genetic variability in *W. bancrofti* populations in Ghana was also studied in this part of the project. Thus, the aim of this part of the project was to explain the current LF distribution based on the diversity of the parasite and its vectors in Ghana.

The presence of physical barriers and the occurrence of major climatic changes which led to the fragmentation of the Guinean-Congo forest belt and its reduction to small refugia might have resulted in the reduction in the spatial distribution of LF and its vectors. Thus, the second part of the study aims at explaining the effects of environmental factors in shaping the distribution of vectors and the transmission of *W. bancrofti* in Ghana.

1.2.1 Specific Objectives

The specific objectives of this study were to:

1. Investigate the *Anopheles gambiae* s.s diversity in LF endemic and non-endemic areas in Ghana, using COI gene.
2. Investigate the *Anopheles gambiae* diversity in various ecological zones in Ghana, using COI gene.
3. Determine the diversity in *W. bancrofti* populations in Ghana, using COI gene.

4. Investigate the effect of environmental factors in shaping the current distribution of LF and its vectors in Ghana.
5. Delineate LF transmission risk areas using vector diversity, in combination with other potential transmission factors.

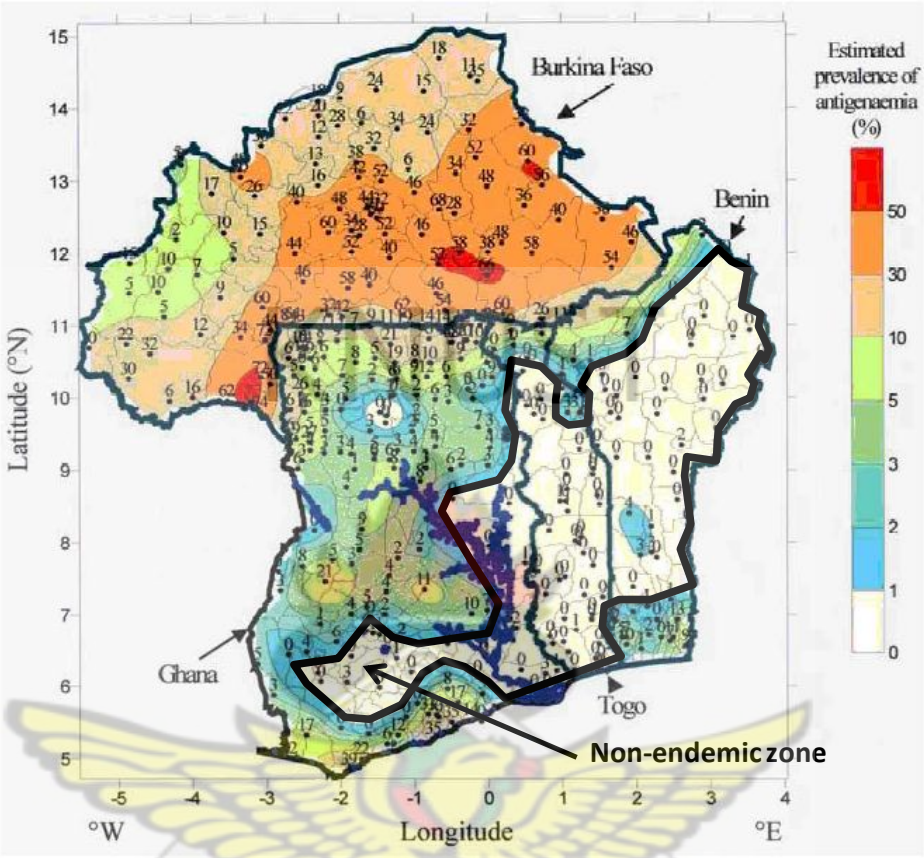


Figure 1.1: Map of the distribution of human infection with *Wuchereria bancrofti* in Burkina Faso, Ghana, Togo and Benin, produced by spatial analysis of the data on filarial antigenaemia in 401 study communities (Gyapong *et al.*, 2002).

CHAPTER II: LITERATURE REVIEW

2.1 Lymphatic Filariasis: The Disease

Most commonly referred to as elephantiasis, Lymphatic Filariasis (LF) is a disease caused by infection with the filarial parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (World Health Organisation, 2000). However, *W. bancrofti* is the most widespread of human filaria in the world. The parasite is acquired by exposure to the bites of infective female mosquito vectors belonging to the genera *Anopheles*, *Culex*, *Mansonia* and *Aedes*, depending on geographic region. It lodges in the lymphatic system and lives for 4-6 years, producing millions of immature microfilariae (mf) that circulate in the blood (World Health Organisation, 2000).

2.1.1 Symptoms and Pathogenesis

LF is painful and profoundly disfiguring, and is one of the leading causes of permanent disability in the world. Though not fatal, secondary infections due to bacterial and fungal invasion of compromised lymphatic tissues cause much more progression and physical destruction associated with elephantiasis, which if not treated, may lead consequently to death (King and Nutman, 1991, World Health Organisation, 2000). The range of clinical manifestations of lymphatic filariasis is broad and the diversity is considered to reflect the intensity and type of immune response to the

parasite (King and Nutman, 1991). However, most individuals in endemic areas are asymptomatic and microfilaria positive (Nutman, 1995), with sub-clinical disease, microscopic haematuria or proteinuria, dilated tortuous lymphatics and, in males, scrotal lymphangiectasia (www.itg.be/.../41_Filariasisp3.htm). The main clinical manifestations of LF are lymphoedema, genital damage and general lymphatic incompetence (World Health Organisation, 2000).

The initial clinical manifestations of lymphatic filarial infection are most frequently fever and or retrograde lymphangitis of the extremities. These signs usually last for a period of one to two weeks and are most common in the second and third decades of life. They may occur with increasing frequency in some individuals and eventually result in persistent lymphoedema of the extremities (Plate. 2.1), genital damage, and general lymphatic incompetence (World Health Organisation, 2000). Although such individuals with chronic disease are usually amicrofilaraemic, microfilaraemia has been observed in residents of endemic areas where transmission is high (Nutman, 1995).

Another 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 >3,000/mm, extreme elevations of

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

2.1.2 Epidemiology

LF was generally thought to occur only in adults (Nutman, 1995, Witte and Ottesen, 2003). However, it has shown that infection occurs in childhood often before age five (Nutman, 1995, Witte and Ottesen, 2003). In such children, lymphatic damage remains sub-clinical until the age of puberty and above, when clinical symptoms are presented. Differential susceptibilities to infection (Subrahmanyam *et al*., 1978) have been reported in LF endemic areas, with females being more resistant to infection than males (Ward *et al*., 1988, Freedman *et al*., 1989, Brabin, 1990). Individuals in all age groups are affected, with men being more predisposed to presenting symptoms (Udonsi, 1988, Pani *et al*., 1991).

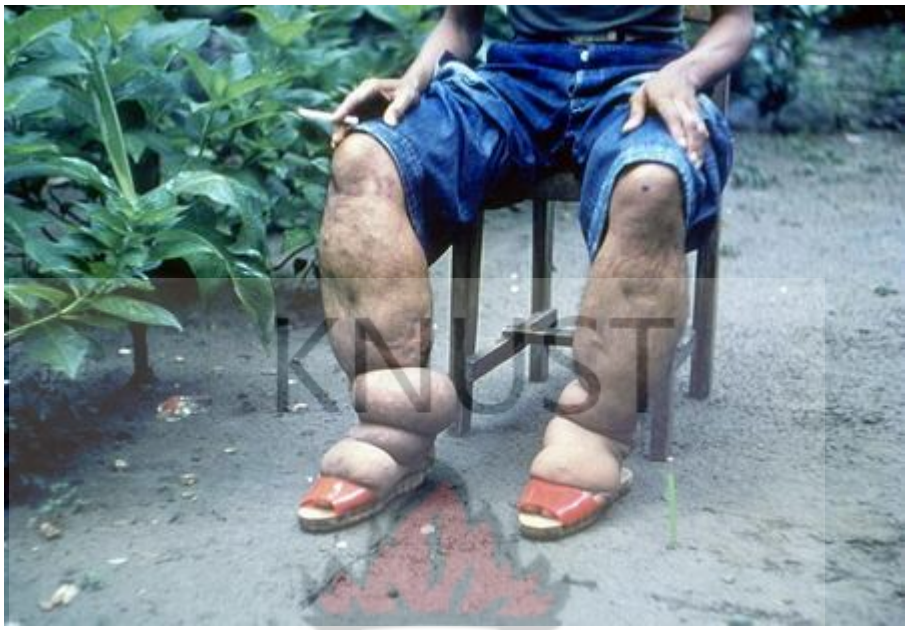


Plate 2.1: Elephantiasis of the leg. Source:

http://www.environmentalgraffiti.com/wp-content/uploads/2009/05/800px-elephantiasis.jpg

2.1.3 Distribution of Lymphatic Filariasis

Lymphatic filariasis is confined to the tropics (Fig. 2.1) because its transmission is limited by the climatic and environmental factors that affect the distribution of its vectors. It is globally known to affect about 120 million people in at least 80 countries, and it is estimated that 1.2 billion people are at risk of infection (World Health Organization, 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 (World Health Organization, 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 (World Health Organization, 2002), 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 the global burden of the disease (Michael *et al*., 1996).

At the micro-geographical level, the distribution of LF is considered patchy and in West Africa, LF distribution reveals a pattern of endemicity separated by a continuous band of non-endemicity (Fig. 1.1 from Gyapong *et al.*, 2002). Until the mid 90s very little studies on LF, had been carried out and documented in Ghana. However, anecdotal clinical reports before then indicated that elephantiasis of the leg and hydrocele were very common, especially in the northern regions and the western part of the coastal belt. In Ghana, studies on LF have shown differences in disease prevalence

and multiplicity of symptoms in two geographically distinct regions. The Northern regions of the country located in the Guinea/Sudan Savannah zones exhibit higher prevalences compared to the Southern regions found in the Coastal Savannah zones (Dunyo *et al*., 1996, Gyapong *et al*., 1996a). In the study by Gyapong and colleagues (1996), the highest prevalence of hydrocele cases was 20.3% and 5.2% in the Northern and Southern parts of the country respectively. High prevalence of elephantiasis and microfilaremia (13.3% and 20% respectively) is found in the Northern part of the country compared to the Southern part (3.6% and 2.1% respectively). Thus, the Northern Savannah regions recorded the highest prevalence of symptoms. The middle forest belt was observed to be relatively free of lymphatic filariasis. While higher prevalence of infection and disease are usually associated with low socioeconomic status, the authors argued that their findings could be a reflection of the biogeographical distribution of the disease (Gyapong *et al*., 1996a).

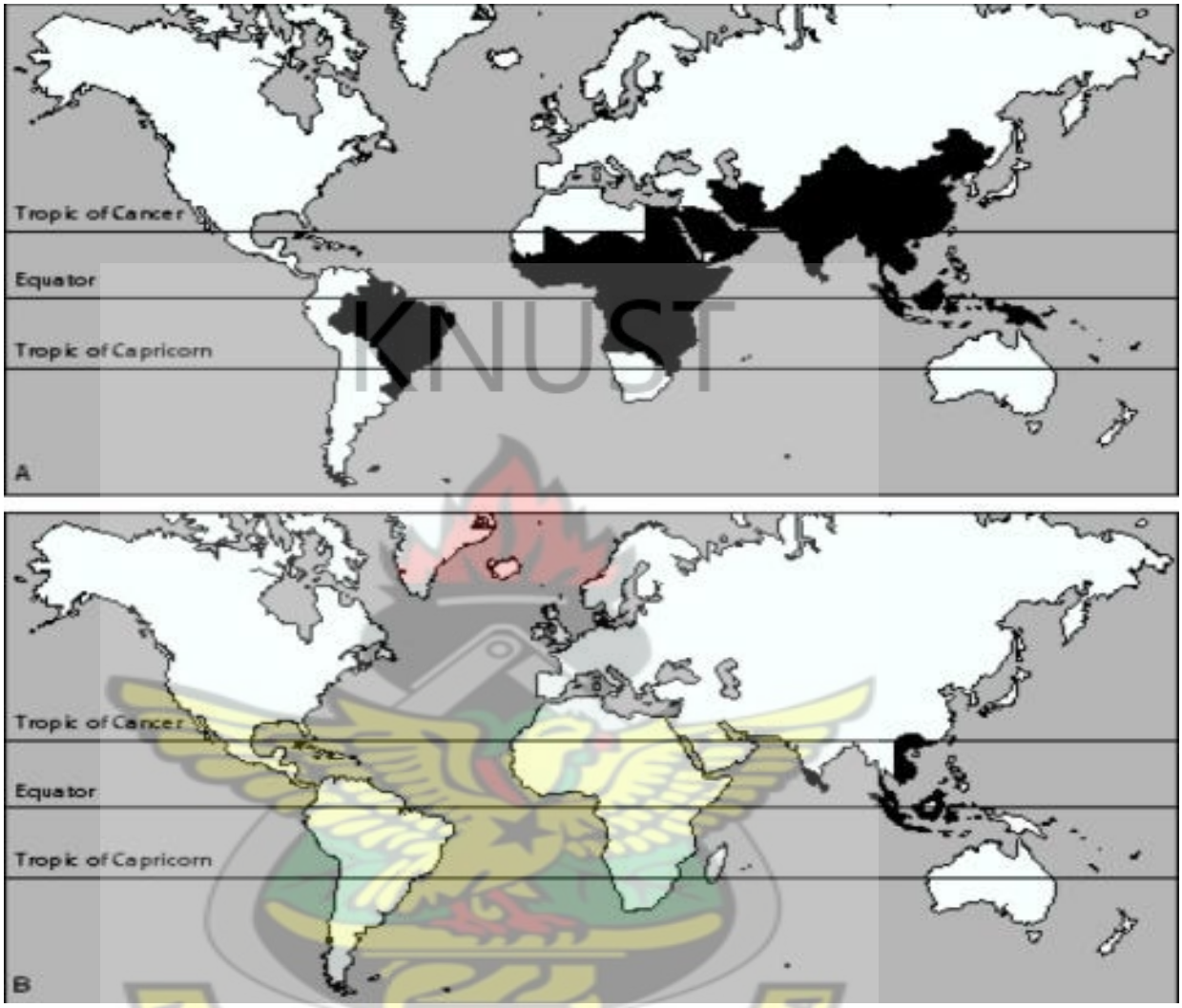


Figure 2.1: The distribution of *W. bancrofti* (A) and *B. malayi* (B). Source: Tropical Medicine Central Resource

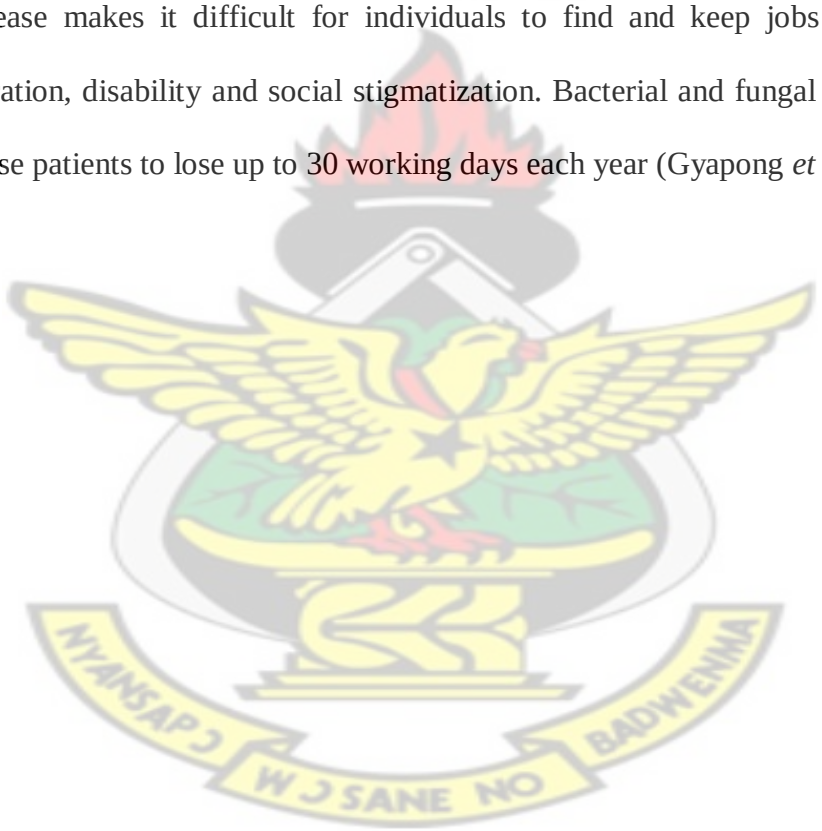
(http://www.isradiology.org/tropical_deseases/tmcr/chapter26/geography2.htm)

2.1.4 Socio-Economic Impacts of Lymphatic Filariasis

Lymphatic filariasis is the fourth leading cause of permanent and long-term disability in the world (World Health Organization, 1998). It is considered a disease of the poor and 80% of endemic countries are classified as low or lower-middle income countries (www.worldbank.org/data/countryclass/classgroups.htm). Besides the associated disability, it causes economic reductions in life opportunities, and is a major burden on direct health and hospital resources especially through the costs for surgical intervention (World Health Organization, 2000). Health care costs are also incurred by patients and their families.

Medical treatment cost for clinical manifestations is estimated to be in the millions of dollars, each year (Ramaiah *et al*., 2000, World Health Organization, 2002). In Ghana for example, it is estimated that close to 25 % of surgical operations is for hydrocele repair in endemic communities (Haddix and Kestler, 2000), which on the average costs $30 per operation and represents over a month of income for the average Ghanaian (Gyapong *et al*., 1996b). The high cost prevents most men from seeking hydrocele surgery (Wegesa *et al*., 1979, Ahorlu *et al*., 2001). The disease imposes a significant financial burden in endemic areas, since the disability afflicts individuals during their most productive years. Reduced productivity from LF disability has been reported in many industries and infected individuals switch to less productive and less labor-intensive activities (Gyapong *et al*., 1996b, Coreil *et al*., 1998, Gasarasi *et al*., 2000).

The disease is estimated to be responsible for over 5.5 million Disability Adjusted Life Years (DALYs) - a measure for comparing the public health impact of disease loss annually. It has the highest loss of all tropical diseases after malaria and tuberculosis (TDR, 2002). India and Africa, together account for 85-90% of estimated burden of the disease in DALYs. The economic burden in terms of productivity loss is estimated at $1.7 billion each year, 83% of which is due to disability in men with hydrocele in this region (World Health Organization, 2002). The associated condition of the disease makes it difficult for individuals to find and keep jobs because of the disfiguration, disability and social stigmatization. Bacterial and fungal infections alone can cause patients to lose up to 30 working days each year (Gyapong *et al.*, 1996a).



2.2 Causative Parasites of Lymphatic Filariasis

Filariasis is caused by nematodes (roundworms) that inhabit the lymphatics and subcutaneous tissues. Eight species of nematodes infect humans: *Onchocerca volvulus*, *Loa loa*, *Mansonella perstans*, *M. streptocerca*, *M. ozzardi*, *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. However, only the last three are responsible for the morbidity associated with LF. *W. bancrofti* (Plates 2.2 and 2.4) is the most widespread and is located in tropical areas worldwide, while *B. malayi* (Plates 2.3 and 2.4) is limited to Asia. *B. timori* (Plate 2.5) is only restricted to some islands of Indonesia.

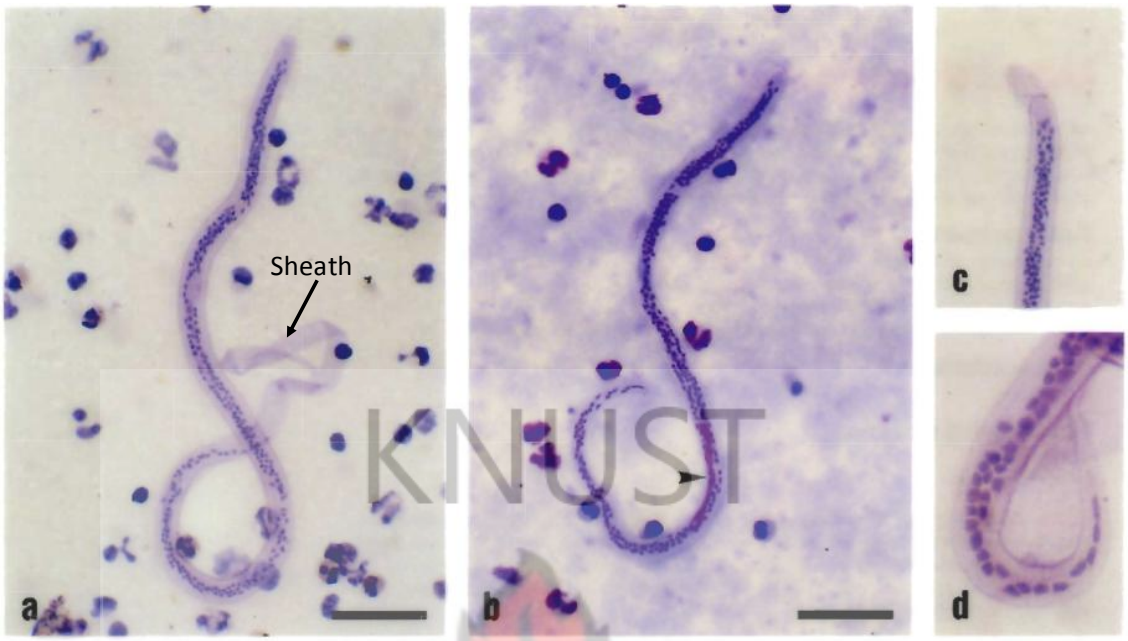


Plate 2.2: ***Wuchereria bancrofti*** **microfilaria in heamatoxylin (a, c, d) and Giemsa (b) stains. Characteristically, the sheath stains lightly with heamatoxylin (a,c) but not with Giemsa stain (b). Key morphological features include a short head space (a, b, c) and a discrete nuclei in the body. The column of nuclei does not extend to the end of the tail (d). The innerbody stains with Giemsa stain (b, arrowhead) but not with heamatoxylin stain. Picture adapted from Bench Aids for the diagnosis of filarial infections. Copyright World Health Organization 1997. Source: http://www.dpd.cdc.gov/DPDx/HTML/PDF_Files/Wbancrofti_Lloa_benchaid_who.pdf**

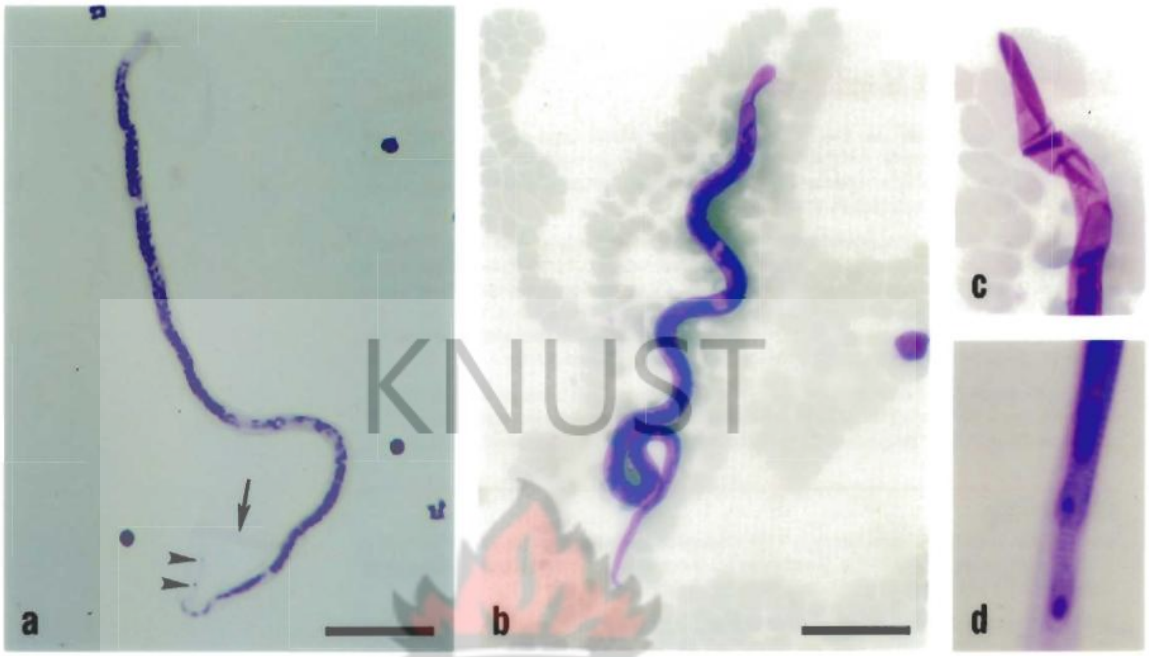


Plate 2.3: *Brugia malayi* microfilariae in heamatoxylin (a) and Giemsa (b-d) stains. In haematoxylin the sheath does not stain but may be faintly visible (a, arrow). This contrasts with the pink stained sheath in Giemsa preparations (b, c). The column of nuclei is compact and the widely separated sub-terminal and terminal nuclei in the tail are key diagnostic features (a, arrowheads; d). Nuclei are sparse in the region of the innerbody (a). Picture adapted from Bench Aids for the diagnosis of filarial infections. Copyright World Health Organization 1997. Source:

http://www.dpd.cdc.gov/DPDx/HTML/PDF_Files/Brugia_benchaid_who.pdf

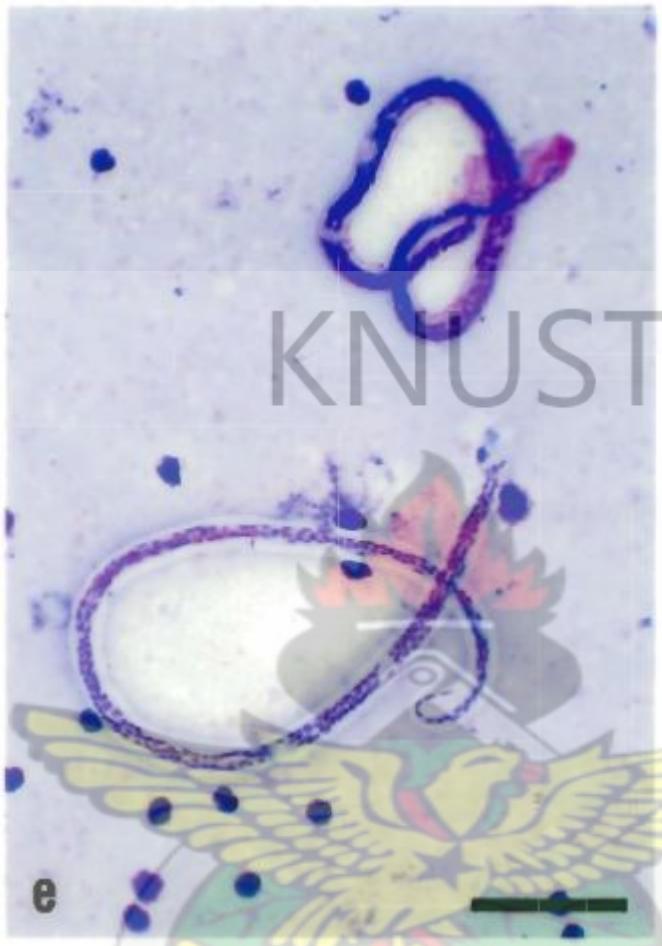


Plate 2.4: *B. malayi* (upper) and *W. bancrofti* (lower) microfilariae in the same field of Giemsa-stained blood film (e). The pink-stained sheath and the darkly stained, compact column of nuclei identify *B. malayi* and distinguish it from *W. bancrofti*. Picture adapted from Bench Aids for the diagnosis of filarial infections. Copyright World Health Organization 1997. Source:

http://www.dpd.cdc.gov/DPDx/HTML/PDF_Files/Brugia_benchaid_who.pdf

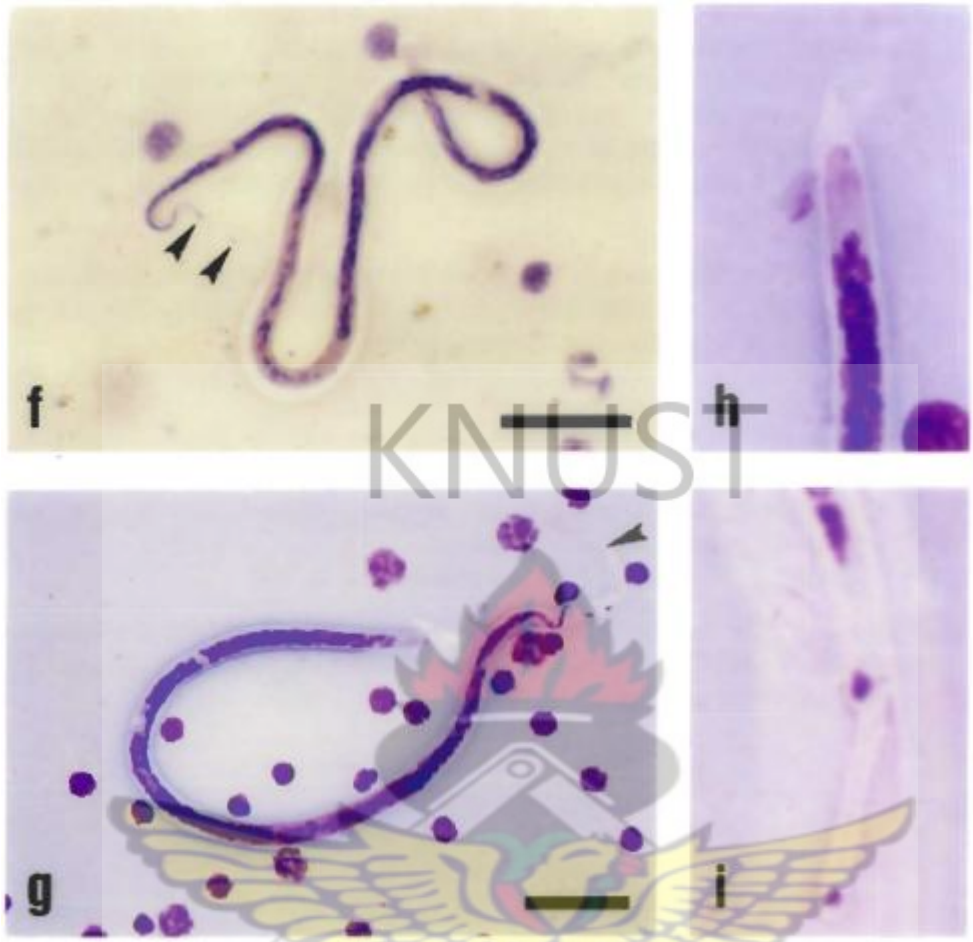


Plate 2.5: *Brugia timori* microfilariae in heamatoxylin (f) and Giemsa (g-i) stains. *B. timori* is larger than *B. malayi* and the sheath does not stain pink (g, arrowhead) with Giemsa stain. The long head space and the subterminal and terminal nuclei are conspicuous features (f-i). Picture adapted from Bench Aids for the diagnosis of filarial infections. Copyright World Health Organization 1997. Source: http://www.dpd.cdc.gov/DPDx/HTML/PDF_Files/Brugia_benchaid_who.pdf

2.2.1 The Life Cycle and Transmission of *W. bancrofti*

The life cycle of the parasite takes place in both human and the vectors, as the definitive and intermediate hosts respectively. Depending on the geographic location, the vectors may be mosquitoes of the genus *Anopheles, Aedes, Mansonia* or *Culex*. The life cycle is shown in Fig. 2.2. The periodicity of *W. bancrofti* microfilariae corresponds to the peak feeding time of the vectors. Circulating mf in the blood are picked up when a female mosquito takes a blood meal. Within the mosquito, the development of the mf depends on the morphological, physiological and biochemical compatibility of the vector and the parasite.

After ingestion by the mosquito, mf travel from the mouthpart to the foregut and midgut of the mosquito. Within hours, the mf passes through the single cell layer of the midgut epithelium to enter the haemolymph. In the midgut mf can exsheath (Chen and Shih, 1988), or during the migration across the midgut, the sheath is damaged, facilitating exsheathment in the haemocoel (Christensen and Sutherland, 1984). However, of the parasites in the midgut, it is only those that migrate to the thoracic muscles that develop successfully. In the thoracic muscles the mf differentiates into the first stage larvae (L1). They then develop into the characteristic sausage-shaped second stage larvae (L2) (Smyth, 1996). It is believed that interrupting the development at this stage can result in the break of transmission (Omar and Zielke, 1978). The parasite subsequently develops into the third stage larvae (L3), also called the infective stage. At

this L3 stage, there is a 4 to 6 times increase in size and the whole development process may last between 10-12 days, under optimal conditions, mainly temperature (Lardeux and Cheffort, 1997). The infective larvae migrate into the head tissues and proboscis of the mosquito to be transmitted subsequently during blood feeding. During feeding, the infective larvae escape via the proboscis from haemolymph and enter the puncture wound made by the mosquito, hair follicles, or other abrasions. Thus, the transmission of filarial worms is highly inefficient (Bartholomay, 2002) and requires many successful bites from infective mosquitoes.

In humans, the infective larvae migrate to the nearest lymph gland where they mature into the thread like adult worms in about 3 months. Development to a sexually mature worm requires about nine months in the host (Smyth, 1996) while the average incubation time before patency is about 15 months. The mature adults can live in the human host for 5 to 10 years. After mating, the viviparous female produces mf which move through the circulatory system and collect in arterioles of the lungs during the day and emerge into the peripheral blood at night, when night biting mosquitoes are most active (Nutman, 1995).

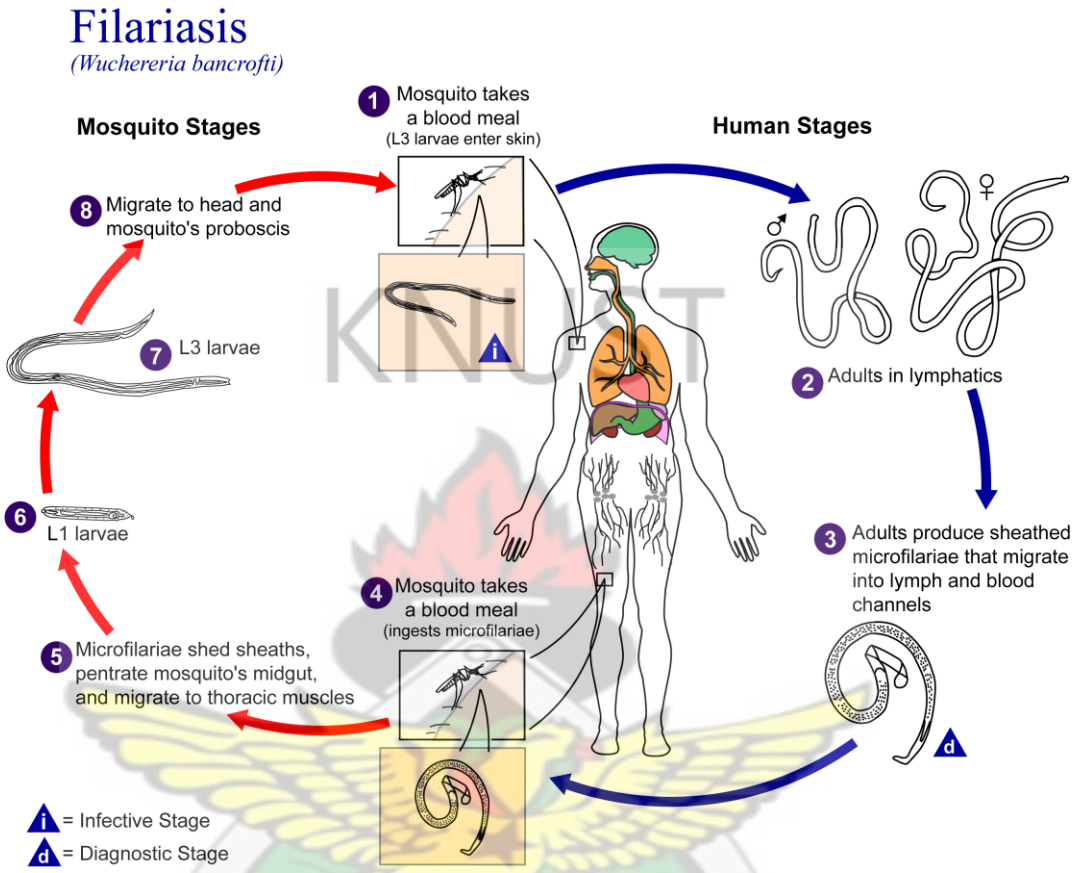


Figure 2.2: The life cycle of *Wuchereria bancrofti* showing the various developmental stages of the parasite in the mosquito and in human. Source: http://phil.cdc.gov/phil/home.asp

2.2.2 Identification Methods for *W. bancrofti*

Various methods exist for the identification of *W. bancrofti* in the blood. Until recently, the method for the diagnosis of filarial infection was through the direct demonstration of the parasite in blood. As such, microscopy is considered the gold standard for filarial diagnosis. However, other methods based on detection of antibodies by immunodiagnostic tests, Circulating Filarial Antigen (World Health Organization, 2000) and DNA detection methods based on the Polymerase Chain Reaction (Zhong *et al.*, 1996) exist.

2.2.2.1 Morphological identification

The gold standard for the detection of microfilaria in the blood is through microscopy. This is a simple technique for examining blood and other fluids for mf. Dry or wet smears are prepared and the slides are examined under the microscope, to identify the parasite. In the method for the dry smear, 20µl of blood is evenly spread over a clean slide, dried and then stained with Giemsa stain (Mak, 1989). The wet smear may be made by diluting 20-40 µl of anticoagulated blood with water or 2% saponin, which lyses the red blood cells but allow the mf to remain mobile and thus more readily identifiable (Mak, 1989). The sensitivity of microscopy can be improved using the Knott's concentration technique. This can be used to examine 1ml volumes of anti-coagulated blood by mixing the blood with 10ml of 2% formalin, centrifuging the preparation and examining the sediment using the dry or wet slide preparation methods (Mak, 1989).

There are many drawbacks to the use of microscopy in infection diagnosis. The first is the parasite's nocturnal periodicity. Thus, for optimum efficiency and identification, night blood collection, preferably from 22.00 to 02.00 hrs, is required for such assessments. Also, large blood volumes may be required for assessment, especially in individuals with low blood parasitaemia. Another drawback is the fact that mfs are non-mobile and can be easily mixed with other artifacts if not examined properly. Membrane filtration of venous blood, although more sensitive, is expensive and still requires night blood collection.

The morphological identification is done through the examination of the sheath (Plate 2.2), the most striking characteristic identification markers of mfs of filarial parasites. It is a delicate, closely fitting membrane, probably the egg-capsule, which is often only detectable when it projects beyond the head or tail of the microfilaria (Smyth, 1996). The mfs of *W. bancrofti* measure between 240-300 µm in stained blood smears. Their body is gently curved and they have a tail that is tapered to a point. The nuclear column (the cells that constitute the body of the microfilaria) is loosely packed and the cells can be visualized individually and do not extend to the tip of the tail. *B. malayi* mfs measure between 175-230 µm in stained blood smear. Their tail is tapered, with a significant gap between the terminal and subterminal nuclei. *B. timori* mfs measure on average 310 µm in stained blood smears. Mfs of *B. timori* differ from *B. malayi* by having a longer cephalic space, a sheath that does not stain with Giemsa, and

a larger number of single-file nuclei towards the tail (http://www.dpd.cdc.gov/dpdx/html/filariasis.htm). The position of certain fixed points, G1-G4 (Newton and Wright, 1956) which represent genital rudiments, have also been used for identification but this method is open to technical difficulties such as the correct identification and location of these structures (Smyth, 1996).

2.2.2.2 Molecular identification

Initial methods for the molecular identification of filarial parasites were based on the development of specific radioactively labeled deoxyribonucleic acid (DNA) probes (Williams *et al.*, 1988). This method, however, required the use of radioactive materials and therefore was not very practical (Dissanayake *et al.*, 1991). However, the advent of the polymerase chain reaction (PCR) presented opportunities for improved diagnostic methods. The PCR is an enzyme-catalysed biochemical reaction in which small amounts of a specific DNA segment are amplified into large amounts of linear double-stranded DNA, using two oligonucleotide primers and a DNA polymerase (Mullis, 1990).

Using the PCR technique, Zhong *et al*. (1996) developed a method for *W. bancrofti* identification based on the *Ssp*I repeat, specific to *W. bancrofti*. This method yields a 188 bp fragment and could detect 0.1 pg of *W. bancrofti* genomic DNA, representing approximately less than 1% of the DNA found in one microfilaria (Zhong *et al.*, 1996). This method was then adopted and tested on blood samples (Williams *et*

al., 1996, Ramzy *et al*., 1997) and also adapted and improved for field testing on pools of mosquitoes (Bockarie *et al*., 2000, Williams *et al*., 2002, Boakye *et al*., 2007).

The PCR method also has limitations among which are the risks of contamination, high cost of reagents and equipments, and the requirement of purified starting DNA material. Also, there is the need for some sequence information to enable synthesis of the specific primers, which delimit the ends of the PCR fragment (McManus and Bowles, 1996). The PCR method is frequently chosen for conducting experiments, such as cloning, making mutations, sequencing, detecting, typing and species identification. Since the PCR amplification reaction uses products from one cycle as a template in the next cycle, mis-incorporations accumulate during the course of amplification. The impact of replication errors on the reliability of PCR-based analysis is small, however, when large numbers of starting templates are used in the reaction it becomes significant (Reiss *et al*., 1990).

2.2.2.3 Immunological identification methods

Immunoassays for antigen detection of circulating filarial antigens constitute a useful diagnostic approach, especially in very low mf levels. Enzyme link immunosorbent assay (ELISA) methods, based on the detection of circulating filarial antigens have been developed for rapid testing of bancroftian filariasis (Weil *et al*., 1987, Weil *et al*., 1997). Other methods for antigen detection, such as the Og4C3 and AD12-ICT have also been developed and tested (Rocha *et al*., 2009). The detection of

specific immunoglobulin G4 and monoclonal antibodies (Chandrashekar *et al*., 1994, Fischer *et al*., 2005, Janardhan *et al*., 2010) have also been exploited as diagnostic tools. However, antibody detection is of limited value, due to the substantial antigenic cross reactivity between filaria and other helminths, and positive serologic tests do not distinguish between past and current infection.

2.2.3 Genetic Diversity in *W. bancrofti*

Not much work has been done in the area of *W. bancrofti* diversity. However, few studies have pointed to genetic and morphological variations in *W. bancrofti* populations. Kumar *et al*. (2002) reported the existence of two variants of *W. bancrofti* based on morphology of the microfilariae in India. The occurrence of two different genetic variants of the parasite with high genetic divergence and gene flow in different geoclimatic regions in India have also been reported (Kumar *et al*., 2002, Thangadurai *et al*., 2006). Similarly, genetic polymorphisms have been identified between the Thai and Myanmar strains of *W. bancrofti* and Randomly Amplified Polymorphic DNA profiles revealed a significant diversity between the two strains (Nuchprayoon *et al*., 2007). The study also revealed that the Thai strain of *W. bancrofti* was nocturnally subperiodic, and significantly larger than the Myanmar strain of *W. bancrofti* that was nocturnally periodic.

These observed genetic variations between strains and different climatic zones may affect the distribution of the disease and the severity and prevalence of symptoms.

In Ghana, higher prevalence of LF is found in the Northern part of the country compared to the Southern part (Gyapong *et al.*, 1996a) and climate variability between the two regions may affect the genetic variability of the parasite in these regions and therefore explain the distribution of the disease.

2.3 Vectors of Lymphatic Filariasis

In West Africa the vectors responsible for the transmission of *W. bancrofti* are the *Anopheles gambiae* s.l. and *An. funestus* complexes (Appawu *et al*., 2001). The same species are responsible for the transmission of the disease in rural parts of East Africa (McMahon *et al*., 1981). However, in urban East Africa and Asia, *Culex species*, especially *Culex quinquefasciatus* Say are responsible for the transmission of *W. bancrofti* (Muirhead-Thomson, 1954, Brinkmann, 1976, 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 (World Health Organization, 2000).

2.3.1 Vector Species Complexes and Their Importance in Disease Transmission and Control

Certain mosquito species are recognized as species complexes or groups consisting of morphologically indistinguishable (isomorphic) but reproductively isolated members, which most often live together in the same area (i.e. they are sympatric) (Service, 1993). Species-specific, ecologic and behavioral differences among members of these complexes can significantly affect disease transmission and control.

Anopheles species belong to the order Diptera, family Culicidae and subfamily Anophelinae. They have a worldwide distribution and occur not only in tropical areas but also in temperate zones (Service, 1980). 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 (Plate 2.6).

Plate 2.6: The wing of an *Anopheles* mosquito. The arrow points to a dark identification patch along the costa. Source:

www.co.rockland.ny.us/.../freshwater.htm

The genus *Anopheles* consists of 437 recognized species, divided into six sub-genera: *Anopheles* (185 species), *Cellia* (200 species), *Lophopodomyia* (6 species), *Kertezia* (12 species), *Nyssorhynchus* (29 species) and *Stethomyia* (5 species) (Sallum *et al*., 2000).

In Africa, two major *Anopheles* species complexes exist. These are the *Anopheles gambiae* and *Anopheles funestus* complexes. *An. gambiae* sensu lato (s.l.) lives in sympatry with *An. funestus* in many parts of Africa and shows high adaptability to changing environments (Coluzzi, 1984). In West Africa *An. gambiae* s.l. populations dominate during the rainy season, while populations of the *An. funestus* become more abundant in the dry seasons (Coluzzi, 1984). However in African urban centres, the proportion of anopheline mosquitoes is largely made of *An. gambiae* s.l. Apart from transmitting *W. bancrofti*, the members of *An. gambiae* complex and *An. funestus* are the most important vectors of *P. falciparum* (Mekuria, 1983).

2.3.1.1 *Anopheles gambiae* Giles complex

The *Anopheles gambiae* complex is the most important malaria vector complex in the world. In Ghana and West Africa, it is considered the most important LF vector (Dzodzomenyo *et al*., 1999, Appawu *et al*., 2001). Initially, the complex was thought to be just one species. However, 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* (Ribbands, 1944, Thomson, 1951). Presently, six formally named

species, not morphologically distinguishable, have been identified (Davidson *et al*., 1967). These are: *An. gambiae s.s* (*sensu stricto*) Giles, *An. arabiensis* Patton (Paterson, 1964), *An. quadriannulatus* Theobald (Hunt *et al*., 1998), *An. bwambae* White (Davidson and Hunt, 1973), *An. melas* Theobald and *An. merus* Donitz (Paterson, 1964, Mahon *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 *et al*., 1998). Collectively, all the members of the complex are referred to as *Anopheles gambiae s.l.* (*sensu lato*).

Among the *An. gambiae s.s.* five forms namely; "Forest", "Bissau", "Bamako", "Mopti" and "Savannah" have been described (Bryan *et al*., 1982, Toure *et al*., 1983). The Mopti form of *An. gambiae,* is believed to be more associated with *W. bancrofti* (Hunter, 1992), and is a relatively poor vector of malaria compared with other species such as the Savannah form (Coluzzi, 1993). Within the *An. gambiae* s.s, it is believed that cryptic taxa may exist as observed from inversions in the micromorphology of the second chromosome in different populations (Powell *et al*., 1999). *An. gambiae s.s.* is also divided into two Molecular forms, namely; M and S by sequence differences in the intergenic spacer of rDNA, located on the X chromosome (Favia *et al*., 1997, Gentile *et al*., 2001). Within the M form, there is further evidence to suggest the existence of two distinct chromosomal forms (Slotman *et al*., 2007). In Mali and Burkina Faso, the M form corresponds to the Mopti chromosomal form, whereas sympatric populations of Savannah and Bamako belong to the S molecular form (Favia *et al*., 1997, della Torre *et al*., 2001). However, the association between chromosomal and molecular forms does

not exist elsewhere in West Africa, especially where the Forest chromosomal form is found (della Torre *et al*., 2001, Gentile *et al*., 2001).

2.3.1.2 *Anopheles funestus* group

Anopheles funestus (Giles) has also been shown to be an important LF vector in Ghana, second to *Anopheles gambiae* s.l. (Dunyo *et al*., 1996, Dzodzomenyo *et al*., 1999, Appawu *et al*., 2001). The *Anopheles funestus* group comprises at least nine members, the adults of which are morphologically very similar and can only be differentiated at specific stages in their life cycle (Gillies and De Meillon, 1968, Gillies and Coetzee, 1987). The members of this complex are *An .funestus* s.s., ***An. vaneedeni*** Gillies and Coetzee, *An. parensis* Gillies, *An. aruni* Sobti, *An. confusus* Evans and Leeson, *An. rivulorum* Leeson, *An. fuscivenosus* Leeson, *An. leesoni* Evans, and *An. brucei* Service. Traditionally, the only method for distinguishing members of the *An. funestus* group has been by chromosomal inversion karyotypes (Green and Hunt, 1980, Green, 1982). More recently, single-strand conformation polymorphism analysis, a PCR based method, has been used to identify four members of this group (Koekemoer *et al*., 1999). Of the nine species in the complex, *An. funestus* s.s. has the widest distribution. This mosquito is also highly anthropophilic (Gillies and De Meillon, 1968).

2.3.2 The Life Cycle of the *Anopheles* Species

The vector has a four stage life cycle which involves aquatic and terrestrial stages and undergoes complete metamorphosis (egg, larva, pupa and adult). Except the adult stage all other stages are aquatic. The life cycle of a mosquito is shown in Fig. 2.3. Only the female mosquitoes bite, requiring blood for the maturation of their eggs. After mating, the adult females lay about 50-200 single, small (1mm long and 2-5mm wide) brown or blackish boat shaped eggs with lateral floats scattered on the water surface. Viable eggs hatch 2-3 days after oviposition, which may take 4-7 days or longer in temperate zones (Service, 1980). The life cycle of *Anopheles species* takes about 12-14 days. The mosquito vectors breed in a wide range of habitats, varying from mostly permanent and large collections of water such as fresh water swamps, marshes, rice fields and burrow pits to smaller collections of temporary water such as small pools, puddles, water filled car tracks, ditches, drains, gullies, hoof prints, etc. According to Service (1993), the most preferred breeding sites are the shallow open sun lit pools. Wells and man-made container habitats such as clay pots, motor vehicle tyres, water storage jars and tin cans may also be ideal for larvae (Chinery, 1984). However *Anopheles gambiae* s.s prefers small and undisturbed temporary pools of water exposed to sunshine (Muirhead-Thomson, 1945), hence their predominance in irrigated and forested areas.

The larva is a filter feeder and forages for microscopic organisms and plants whilst lying parallel to the water surface. Depending on the availability of nutrients, temperature conditions and other competing organisms within the habitat, the larvae reach the pupal stage after four moults within 6-9 days (Gimnig *et al*., 2001). The pupa remains in the water for 2-3 days depending on temperature, but does not feed. The pupal case splits dorsally and the adult emerges. The duration from an egg to the adult stage is thus in a range of 7-14 days. After emerging the adult mosquito extends its wings, separates and grooms its head appendages before flying away. High population densities of developing *An. gambiae* s.l larvae, in most cases, may result in emergence of small females due to prolonged larval developmental stages (Gimnig *et al*., 2001). Predation, disease, drought, flood and desiccation account for the usually heavy mortality among larvae, leading to a drastic reduction in the number of eggs developing into adults.

The males of a progeny of any egg batch emerge first as adults and are ready for mating within 24 hours, such that by the time the females emerge, they are competent for mating. Most male mosquitoes usually die after a single mating. Adult males feed on plant nectar and do not bite. The females on the other hand, require a blood meal for ovarian development, followed by the maturation and oviposition of a batch of eggs (Gillies, 1955). The feeding of the females on a vertebrate is however stimulated by a combination of carbon dioxide, temperature, moisture, smell, colour and host movement (Service, 1980, Rebollar-Tellez, 2005, Zimmerman *et al*., 2009).

Anopheles are mostly nocturnal in their activities, thus, emergence from the pupae, mating, blood feeding and oviposition normally occur in the evening to early morning. Some species bite mainly outdoors from about sunset to 2100 hours, in which case they are described as exophagic, while others bite mainly after 2100 hours and mostly indoors, described as endophagic (Gilles, 1999). Whereas some species, e.g. *An. dirus*, rest outside (exophilic) in a variety of natural shelters, others, such as the *An. gambiae* s.s, rest indoor (endophilic). Mixtures of these extremes of behaviour are exhibited by most *Anopheles* species, which are neither exclusively exophagic or endophagic, exophilic or endophilic. Though predominantly zoophilic, few *Anopheles* feed exclusively on humans. Most feed on both man and animals but the degree of anthropophilism and zoophilism varies according to species and host availability (Gilles, 1999).

Figure 2.3: Life cycle of a mosquito, revealing the aquatic and terrestrial stages.

Source: www.epa.gov/pesticides/health/mosquitoes

2.3.3 Factors Influencing the Distribution of *Anopheles* Vectors of Lymphatic Filariasis

While many *Anopheles* vectors may exist in the same aquatic habitat, they occupy different niches (Kirby and Lindsay, 2009). 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. The distribution of various *Anopheles* vector species of LF, however, depends on the degree of variation within these factors, which may also in turn be dependent on seasonality. These factors can be grouped into physicochemical and biological factors.

2.3.3.1 Physicochemical factors

Larval habitats, their number and their productivity determine the density and the distribution of *Anopheles* vectors. Mosquito larval habitats contain both nutrients, deterrents (Fisher *et al*., 1990, Sota, 1993) and elevated concentrations of organic material which may lead to high bacterial growth (Costern *et al*., 1987). Also, the chemical properties of the habitat can be influenced by pH, and the concentration of ammonia, nitrate, sulphate and chlorophyll a, all of which affect larval development and survival (Carpenter, 1982, Victor and Reuben, 2000, Mutero *et al*., 2004, Mwangangi *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). Other physical factors such as the permanence of breeding sites may determine the mosquito species assemblage as is evident from the distribution of the M molecular form of *An. gambiae* s.s, associated with permanent breeding conditions provided by irrigation facilities (Toure *et al*., 1998).

2.3.3.2 Biological factors

Non-mosquito invertebrates such as Naucorids, Notonectids may serve as predators of, or competitors to, *Anopheles* and other mosquito vectors, affecting the density of emergent mosquitoes (Mwangangi *et al*., 2007). Competition also exists between closely related larval mosquito species inhabiting the same breeding sites. Thus, while the presence of *An. arabiensis* does not affect 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 israelensis* and *B. sphaericus* (Fillinger *et al*., 2003), nematodes such as *Romanomermic culicivorax* (Zaim *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.

2.4 Identification of *Anopheles* Vector of LF

Due to the importance of vectors in the transmission of diseases, various techniques have been developed for their identification, especially for discrimination between very closely related species. *Anopheles* vectors of diseases in particular have been given much attention due to their importance as vectors of 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.

2.4.1 Morphology and Morphometrics

Adult anopheline mosquitoes can be distinguished from culicine mosquitoes by observing their resting postures. Anophelines rest at an angle between 45° and 90° to the surface whereas culicines rest more or less parallel to the surface. Another distinguishing feature of anophelines is the length and shape of the palps. In female anophelines, the palps have about the same length as the proboscis, in contrast to culicines, where the palps are shorter.

In the identification of vectors, various techniques are employed with different degrees of specificity (Collins *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.

2.4.2 Cytotaxonomy

Sibling species are usually suspected if there exist differences in the behavior, 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 (Coluzzi and Sabatini, 1967). Cytotaxonomy has been used to identify all six members of the *An. gambiae* complex (Coluzzi *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 *et al*., 2001). Frequencies of chromosome inversion arrangements have been utilized to investigate population structure and estimate population sizes (Taylor *et al*., 1993, Petrarca *et al*., 2000, Kamau *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 *et al*., 1999). Species genetic polymorphism due to paracentric inversions has also been detected using this technique (Coluzzi, 1993). This technique, however, is limited by the skill and experience required for its wide application and routine field analysis. Polytene chromosomes must be prepared from ovarian tissue or fourth instar larvae and this limits the samples to adult blood-fed female mosquitoes or late instar larvae.

2.4.3 Cuticular Hydrocarbon Analysis

The possession of cuticular waxes by insects, serves as protection against desiccation and in chemical communication (Carlson *et al*., 1971, Jones *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 (1979, 1980) were able to identify members of the *An. gambiae* s.l. By using discriminant 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 *Simulium sirbanum*. 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.

2.4.4 Allozyme/Isozyme Analysis

For several years, the identification of sibling and cryptic species in mosquitoes has been carried out using allozyme analysis (Pasteur *et al*., 1981, Cianchi *et al*., 1985). The 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 *et al*., 1989a, Narang *et al*., 1989b, Lanzaro *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 (Richardson *et al*., 1986; Norris, 2002). The cost of this technique is also high and it is tedious and requires a lot of time to perform large scale studies.

2.4.5 Molecular Methods

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.

2.4.5.1 Polymerase chain reactions

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 *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 *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 *et al* (2002), which involves a combination of the protocols established by Scott *et al.* (1993) and Favia *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.

2.4.5.2 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 *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 *et al*., 1993, Collins and Paskewitz, 1996, Favia and Louis, 1999).

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).

2.4.5.3 Restriction fragment length polymorphisms

In DNA strands, sequence variations lead to Restriction Fragment Length Polymorphisms (RFLPs) which are changes in the length of DNA between specific enzyme cutting sites. The DNA can be cut using one or more restriction endonucleases that recognize sites on the DNA template. The resulting restriction fragments are then separated according to size by electrophoresis on agarose gels to reveal the diagnostic polymorphisms with distinct DNA fragments. A PCR-RFLP assay has been developed, which separates the *An. gambiae* Mopti form from the Savannah and Bamako forms (Favia *et al*., 1997). It is based on the fact that GCG^C restriction site for *Hha*1 enzyme (Favia *et al*., 1997) lies within the *An. gambiae* specific fragment (Scott *et al*., 1993) making it possible to digest this fragment directly in order to differentiate the chromosomal forms. This method has been tested on previously karyotyped specimen and used to verify the distribution of other molecular markers, such as the pyrethroid resistance gene (kdr) among chromosomal forms (Chandre *et al*., 1999). Analyses of the IGS and the internal transcribed spacer (ITS) regions of the rDNA in the *An. gambiae*, showed nucleotide substitutions which enable the identification of two forms of the *An.*

gambiae s.s, designated as the M and S form in the case of the IGS (della Torre *et al*., 2001) and Types I and II in the case of the ITS (Gentile *et al*., 2001). As such PCR-RFLP methods that allow for the simultaneous identification of members of the *An. gambiae* complex and the determination of the M and S molecular forms of the *An. gambiae* s.s have been developed (Fanello *et al*., 2002).

2.5 Vector-Parasite Relationships and their Importance in the Transmission of Lymphatic Filariasis

The species and the geographic strain of mosquitoes are believed to affect the vector competence in the transmission of LF (Wharton, 1960, McGreevy *et al*., 1982, Failloux *et al*., 1995). Vector competence refers to the ability of mosquito vectors to ingest microfilariae and to support their development after ingestion as well as the rate of mosquito survival until parasite maturation (Failloux *et al*., 1995, Bryan *et al*., 1990). The transmission dynamics of LF is believed to be dependent on the relationship between the parasite's behavior, the mf density in the human blood, the ingestion of mf and production of L3 larvae (Brito *et al*., 1997, Calheiros *et al*., 1998).

2.5.1 The Phenomena of Proportionality, Facilitation and Limitation

Vector competence is determined by the uptake of mf from the human host, the development of mfs to the infective-stage larvae (L3) and the transmission of L3 to human (Subramanian *et al*., 1998). Studies have established that the uptake of mf by mosquitoes is dependent on the density and distribution of mf in the human host (Samarawickrema *et al*., 1985, Bryan and Southgate, 1988). The processes of proportionality, facilitation and limitation have been described for the number of L3 larvae developing from a number of mf ingested. In proportionality, there is a constant

ratio (≤ 1) of L3 to ingested mf. In facilitation there is an increase in this ratio (>1), while limitation is the opposite of facilitation (Pichon *et al*., 1974, Southgate and Bryan, 1992, Pichon, 2002). In facilitation, the vector ingests mf at high-density human microfilaremia and not all may develop to the L3 stage. However, the number of L3 is more in this case than for limitation and proportionality, accounting for vectors exhibiting such a vector/parasite relationship appearing to produce infection and disease much more effectively than others. On the other hand, in limitation the vector ingest mf at low-density human microfilaraemia and almost all develop to the infective L3 stage. This may account for vectors exhibiting limitation observed to ingest and develop low-density mf much more readily as against others.

2.5.2 Potential of the Various Vector Species in Disease Transmission

The elimination of LF is dependent on the potential of various vectors to transmit *W. bancrofti*. The threshold levels of microfilaraemia for efficient transmission of *W. bancrofti* differ according to vector species (Pichon *et al*., 1974, Southgate and Bryan, 1992, Pichon, 2002) and have diverse epidemiological impact and implications for the control of the disease. The process of facilitation has been observed between *W. bancrofti – Anopheles gambiae* and limitation between for *W. bancrofti – Cx. quinquefasciatus* (Southgate and Bryan, 1992). *Anopheles* appears to produce infection and disease more effectively than *Culex* and *Aedes* in transmitting *W. bancrofti* (Southgate, 1992a). For *An. gambiae* feeding on a person heavily infected with *W.*

*bancrof*ti, the more microfilariae ingested the greater the proportion that entered the haemocoel (Bartholomay, 2002) subsequently leading to more L3 development. For *Cx. quinquefasciatus* and *Aedes aegypt*i, just the opposite occurs. With more worms ingested, there is less success in penetrating the vector's gut. Southgate (1992b) also observed that *Mansonia*, *Culex* and *Aedes* species vectors ingest and develop low-density microfilariae readily as against *Anopheles* species because they exhibit limitation or proportionality.

Various factors are believed to be responsible for the three vector-parasite relationships. These include the maintenance of a delicate balance of microfilariae ingested, organ barriers in the mosquito, presence of dental armatures, speed of blood clotting, temperature and relative humidity. The ingestion of too many microfilariae can damage the vector and may lead to a shortened life span and insufficient time for microfilariae to mature to infective third stage larvae (Bartholomay, 2002). The presence of organs in the mosquito also serves as barriers to further development. These barriers affect the compatibility of the vector-pathogen association (vector competence). In some mosquito species, the cibarial and pharyngeal pumps are lined with denticulate structures and spines that can fatally damage passing mf (McGreevy *et al*., 1978). These spines and ridges may be present in some *Anopheles*, absent in *Aedes* and *Mansonia*, and weak in *Culex* (Denham and McGreevy 1977). Coagulation of the blood meal can hinder the migration of microfilariae across the midgut. As a result, the anti-coagulant potency of the mosquito saliva in the blood meal can influence vector competence (Bartholomay, 2002). In the haemocoel and thoracic muscles of some Liberian strains

of *Cx. quinquefasciatus* the immune response to the presence of mf leads to either partial or complete encapsulation of the developing larvae- via a process called melatonization, thus limiting vector competence (Omar and Zielke, 1978). As in malaria, temperature is important for the development of the mf to the infective stage. Relative humidity higher than 70% is required for worm development in mosquitoes (Smyth, 1996). The above-mentioned factors may account for the different vector-parasite relationships exhibited by the various vector species.

Facilitation has been advocated as the reason for the possible elimination of the LF, because *Anopheles* appears to produce infection and disease much more effectively than *Culex* and *Aedes* in the transmission of *W. bancrofti*, and at low microfilaraemia levels in the human population, transmission cannot be sustained (Subramanian *et al*., 1997). Thus, in facilitation, there is possibly a threshold of microfilaraemic level below which there would be no transmission (Bartholomay, 2002). On the other hand, *Culex*, *Mansonia* and *Aedes* species vectors ingest and develop low-density microfilaria more readily because they exhibit limitation or proportionality (Southgate, 1992a, b). As a result, it will be very difficult to totally interrupt transmission in areas where *Culex* is implicated as the vector even when control programs reduce mf prevalence and intensity to very low levels (Subramanian *et al*., 1997), unless supplemented with vector control. Thus, the determination of the role of *Culex* and *Anopheles* species in transmission is important, in defining strategies and implementing control programs. This will also contribute to a better understanding of the vector-parasite relationship and the efficiency of the vectors of the disease in the study area. Genetic variation between

vector populations suggests that the same strategy cannot be adopted for all areas. A study in the Bongo District of Northern Ghana showed a probable relationship of limitation between *W. bancrofti* and *An. gambiae* s.l., *An. funestus* or both taxa (Boakye *et al*., 2004). Also, the phenomenon of limitation was observed in *An. melas*, in the Western Region of Ghana (Clement 2006).

2.6 Control of Lymphatic Filariasis

The control of LF is mainly based on two methods targeting the morbidity associated with the disease and the transmission of the disease. The approach to disease transmission can either target a reduction in the vector-host contact or the reduction in the microfilaria load in the population, to levels beyond which the vectors can no longer transmit the parasite. These approaches to control are discussed below.

2.6.1 Morbidity Control

The aim of morbidity control is to reduce the suffering of infected individuals, making them as functional as possible, as well as preventing the progression of their condition to worse states. Until recently, intervention activities were inhibited in most instances by a sense of hopelessness. However, the antihelminthic drugs: diethylcarbamazine (DEC), ivermectin and albendazole, have been shown to have enormous effects on microfilariae levels in the bloodstream (Gyapong 2000). The administration of these drugs to patients, even in a single dose, helps to halt the progression of the disease. Opportunistic bacteria and fungi may gain entry into the patient's tissues through cracks in swollen skin resulting in secondary infections which, if not attended to, may lead to a fatality. It has been suggested that the maintenance of strict personal hygiene by regular washing (twice daily) of elephantoid limbs with soapy water and the application of antibiotic topical ointments reduces the incidence of acute attacks and the progression of elephantiasis (Shenoy *et al.*, 1998, Dreyer *et al.*, 1999).

The wearing of shoes, keeping the nails clean, raising the limbs at night, regular prescribed physical exercise to promote circulation and the formation of self-help groups all help in the promotion of this hygienic regimen as well. The relieving and prevention of suffering and disability is therefore one of the targets of the Global Programme for Elimination of Lymphatic filariasis (GPELF) [World Health Organization, 2000].

Surgical intervention for men with hydrocoele is also an important component of morbidity control (World Health Organization, 2000). The GPELF hopes to disseminate as broadly as possible the most practical surgical techniques appropriate for the care of patients with hydrocoele (World Health Organization, 2002).

2.6.2 Transmission Reduction

Transmission reduction is aimed at the reduction of vector density and vector-host contact, which will subsequently lead to the control of the disease. Efforts at filariasis control in populations through reducing the numbers of mosquito vectors have proven largely ineffective (World Health Organization, 2000), as is the case of Samoa and The Cook Islands, in the 1940's, where vector control programs were aimed at eliminating breeding sites such as coconut shells and other containers (Webber, 1991). Even when good mosquito control are in place, the long life-span of the parasite estimated at between (4-6 years) means that the infection remains in the community for a long period of time, generally longer than intensive vector control efforts can be

sustained (World Health Organization, 2000). Despite this, vector control has traditionally played an important role in the control of LF, especially in areas where *Anopheles* species are involved as vectors of both malaria and LF (Gyapong, 2000) (World Health Organization, 2002). In the 1970's DDT spraying in the Solomon Islands for malaria control brought filariasis down to 0% prevalence. Since the transmission of filarial larvae is less efficient than that of malaria sporozoites, house spraying with residual pyrethroid insecticide has even greater impact on filariasis transmission (Bartholomay *et al.*, 2001). In Liberia the use of residual insecticide spraying in homes reduced LF considerably (Zielke and Chlebowsky, 1980).

Other, new improved techniques for enhancing the effectiveness of vector control include the use of floating layers of expanded polystyrene beads (EPBs), insecticide-treated materials (ITMs) and residual spraying. EPBs create a physical barrier to egg-laying adult *Culex* while suffocating larvae and pupae (Maxwell *et al*., 1990, Reuben *et al*., 2001, Sunish *et al*., 2002). The use of ITMs for protection against malaria transmission (Lengeler *et al*., 1996) also serves as a limit to filarial transmission, especially in areas where both diseases share the same vectors (World Health Organization, 2002). In view of the rapidly expanding ITM coverage as a major intervention of the roll back malaria (RBM) program in most malarious countries, epidemiological investigations have been advocated to assess the impact on LF incidence, prevalence and clinical symptoms (World Health Organization, 2002). Non-treated bednets have also been shown to provide some protection against filariasis, as has been observed in Papua New Guinea where habitual users of bednets show

significantly lower rates of microfilaraemia and hydrocoele (Bockarie *et al*., 2002). The use of these techniques has enabled the elimination of LF in Japan, Taiwan, Solomon Islands, South Korea and some parts of China (Webber, 1991). These methods aimed at reducing human-vector contact, provide useful supplements to treatments, to reduce transmission (Gyapong, 2000) and should be combined with other control strategies to achieve optimum success.

2.6.3 Interruption of Transmission

The LF elimination programme is based on mass chemotherapy to reduce microfilaraemia in at risk populations, for a period long enough to ensure that microfilaraemia levels remain below that which is necessary to sustain transmission. This is to be achieved through a yearly administration of a single-dose of albendazole [400 mg] and diethylcarbamazine [DEC; 6 mg/kg], or albendazole [400 mg] and ivermectin [200 mg/kg].

However, there are challenges, one of which is to deliver treatment to all endemic communities and to maintain high levels of coverage for a long enough period to ensure the cessation of transmission. Reports have suggested 80% coverage over a period of 4-6 years, based on the life span of the parasite (Vanamail *et al*., 1996) and WHO estimates the global elimination program to last 20-25 years. Another challenge is determining the level below which microfilaremia levels should fall, before the cessation of MDA. The daily intake of DEC-fortified salts has been shown to clear

microfilaremia within 6 to 12 months. In some parts of the world, specifically China, it was used as a medicated salt with great success (Shantung Department of Filariasis, 1976, Gelband, 1994, Meyrowitsch *et al*., 1996). The use of DEC is however limited in communities in which onchocerciasis and loasis are endemic due to the severe side effects in people infected with both parasites (World Health Organization, 2000). In those areas, ivermectin and albendazole or one drug regimen of ivermectin alone is required for administration (World Health Organization, 2000).

Developing an effective drug distribution mechanism is a key requirement to the elimination programme, especially in developing countries, since most health services on their own, may not be able to deliver drugs to entire populations on a yearly basis. In Kenya for example, 46% of the population were treated in Health Service-directed program but this went up to 88% when community directed treatment was instituted (World Health Organization, 2000). The Ghana LF control programme uses a community directed treatment approach that involves other partners in the drug distribution (Gyapong *et al*., 1996a). The community takes responsibility and selects its own distributors. The Public Health Service introduces the concept and trains the distributors. The mass treatment of all eligible people avoids the necessity for individual diagnosis and covers both symptomatic and asymptomatic individuals. The only group of people excluded from the treatment is the sick or the infirm, children below the age of 5 years and pregnant or lactating women.

The interruption of transmission and thus the elimination of LF may however face challenges if drug tolerance by *W. bancrofti*, or recrudescence occurs as a result of mechanisms which might stabilize the persistence of the parasite in the host population. The existence of three physiological strains of the parasite based on the periodicity of appearance of mf in the peripheral blood of the human host (Sasa, 1976), the ecological differences in the vector species transmitting the parasite (Sasa, 1976), the existence of two variants of *W. bancrofti* based on the length of mf (Kumar *et al*. 2002) and the reported occurrence of two different genetic variants of the parasite, high genetic divergence and gene flow in different geoclimatic regions (Kumar *et al*. 2002; Thangadurai *et al*. 2006) may all act against the successful elimination of the disease. In view of the current elimination program and mass drug administration, which supposes the parasite and vector populations to be single entities, it is important to conduct further studies, at the global scale and in different regions, in order to formulate appropriate strategies should there be genetic variability among the vectors and *W. bancrofti*, leading to the resurgence of LF following the suspension of MDA.

2.7 Spatial Analytical Methods for Studies of Vector-borne Diseases

Everything can be given a locational attribute, in forms of latitude and longitude. That is, everything at every time can be found in a particular place. Thus, much of the world's information is geographic in nature. People, animals, plants, etc., are dispersed in space and interact in that space, and their interactions can change with time. Linking geographic coordinates to living organisms can therefore be beneficial in understanding factors that influence their distribution in nature. In the past, geographic information was mainly used for navigational purposes. However, there was a gradual realization of the importance of geographic location in shaping the diversity and distribution of organisms. The earliest examples of this can be found during Darwin's travel on the Beagle. Darwin noticed differences in the size of the beaks of the chaffinch birds living on different islands in the Galapagos (Darwin, 1989), which led him to conclude that the birds evolved to fit the environment in which they were living. Thus, begun the use of geographic location in understanding the diversity in living organisms.

2.7.1 Geographic Information Systems

In the quest to better understand factors influencing the distribution of organisms, various tools have been developed. One of these is the Geographic Information Systems (GIS), which is an automated system for the capture, storage, retrieval, analysis, and display of spatial data (Clarke, 1995). It provides means of analyzing epidemiological data, revealing trends, dependencies and inter-relationships

that would be more difficult to discover in tabular format. At the same time, it provides a mechanism to integrate various data sets, analyze their spatial and statistical components, and model possible scenarios, thereby supporting interdisciplinary research.

GIS are potentially powerful resources for health research and promotion for many reasons including their ability to integrate data from disparate sources to produce new information, and their inherent visualization (mapping) functions, which can promote creative problem solving and sound decisions with lasting, positive impacts on people's lives (Buckeridge *et al*., 2002, Gavin, 2002). GIS enables the evaluation of potential disease outbreaks and a more effective allocation of sparse resources, through multivariate spatial statistical modeling of disease processes. It also assists users in better understanding the potential harmful effects of environmental pollutants, e.g., exhaust fumes from vehicles, and even in understanding the occurrence of car accidents. GIS can combine environmental measures of air and water quality, solar irradiation, radon gas levels, and other exposures potentially deleterious to human health, which can be spatially referenced and integrated analytically with other predictor variables and outcome data. In fact, any health-related phenomenon that can be defined spatially (atmospheric, aquatic or terrestrial) can lead to GIS analysis (Croner *et al*., 1996).

A number of studies have used GIS to explain the distribution of various infectious diseases with application to disease control (Hay *et al*., 2000) and in the

implementation of national control programs for onchocerciasis (Ngoumou *et al*., 1994) and filariasis (Lindsay and Thomas, 2000). Many malaria vector studies have also employed the use of GIS and spatial analytical approaches to characterize and predict the patterns of vector distribution (Wood *et al*., 1991, Sithiprasasna *et al*., 2003a, Sithiprasasna *et al*., 2003b, Sogoba *et al*., 2007, Sogoba *et al*., 2008).

2.7.2 Remote Sensing

In the tropics and developing countries, the collection of meteorological data is highly inadequate due to the lack of infrastructure. As such, the use of remote sensing (RS) in the collection of meteorological data can greatly enhance the applications of GIS through the provision of proxy environmental information derived from satellite sensors (Hay *et al*., 2000). Satellite instruments only measure radiations from earth (reflected light or emitted energy) and the information derived from these satellite measurements include: vegetation index, surface temperature and cloud temperature. These parameters are generally used to determine the ecological conditions on the ground (Tucker *et al*., 2001) and assess information on ecological variables relevant to planning of malaria control (Roberts *et al*., 1996, Sithiprasasna *et al*., 2005).

Satellite sensors provide low, high or very high resolution data, the use of which depends on the level of information and analyses appropriate for a particular application. NOAA (National Oceanographic and Atmospheric Administration) and Meteosat satellites, provide low resolution data that are available at scales covering

large remote areas. NOAA satellite data are commonly used to monitor changing ecological conditions over multiple years and provide an inter-calibrated normalized difference vegetation index data set (http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/). High spatial resolution satellite data (such as LANDSAT, IKONOS, and QuickBird) can be used for up-to-date detailed mapping of objects that can be used as a source of spatial baseline mapping for breeding sites of malaria vectors (Sithiprasasna *et al.*, 2003a, Sithiprasasna *et al.*, 2003b, Sithiprasasna *et al.*, 2005). These high-resolution data can be used to create precise land-use maps that can serve as basis for the interpretation of both low-resolution satellite data and other geographical socio-economic data. The access to remotely sensed data, and their analyses (http://iridl.ldeo.columbia.edu/) along with other data sources within a GIS, have created new avenues for the spatial analyses of disease vectors and their distribution.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Description of Study Sites

Site selection required an understanding of climate history of Ghana, necessary for the interpretation of the results. Sites were selected to cover all areas that are a result of major climatic changes/ecological zones (Fig. 3.1), as well as areas endemic and non-endemic for LF (Fig. 3.2). Fourteen sites were selected for the study. Of these, 9 sites were LF endemic and 5 were non-endemic. The map of sampling sites is shown in Fig. 3.3. The communities where samples were collected are mostly farming and fishing communities. Information on the climate and vegetation of the sites was obtained from the Ghana Districts website (http://www.ghanadistricts.com/districts/), while LF prevalence rates were obtained from the Ghana National Lymphatic Filariasis Elimination Program.

Gowrie and Tempane are in the Bongo and Garu-Tempane districts respectively, in the Upper East Region of Ghana, in the Sudan Savannah zone. They are both LF endemic communities with the 2007 district prevalence rates of 70.6% and 69.3% respectively. The climate is usually dry, with sparse grass vegetation and short deciduous trees sparsely distributed. There is one rainy season in the year. However, the amount of rainfall in the district is usually offset by the intense drought that precedes the rain and by the very high rate of evaporation.

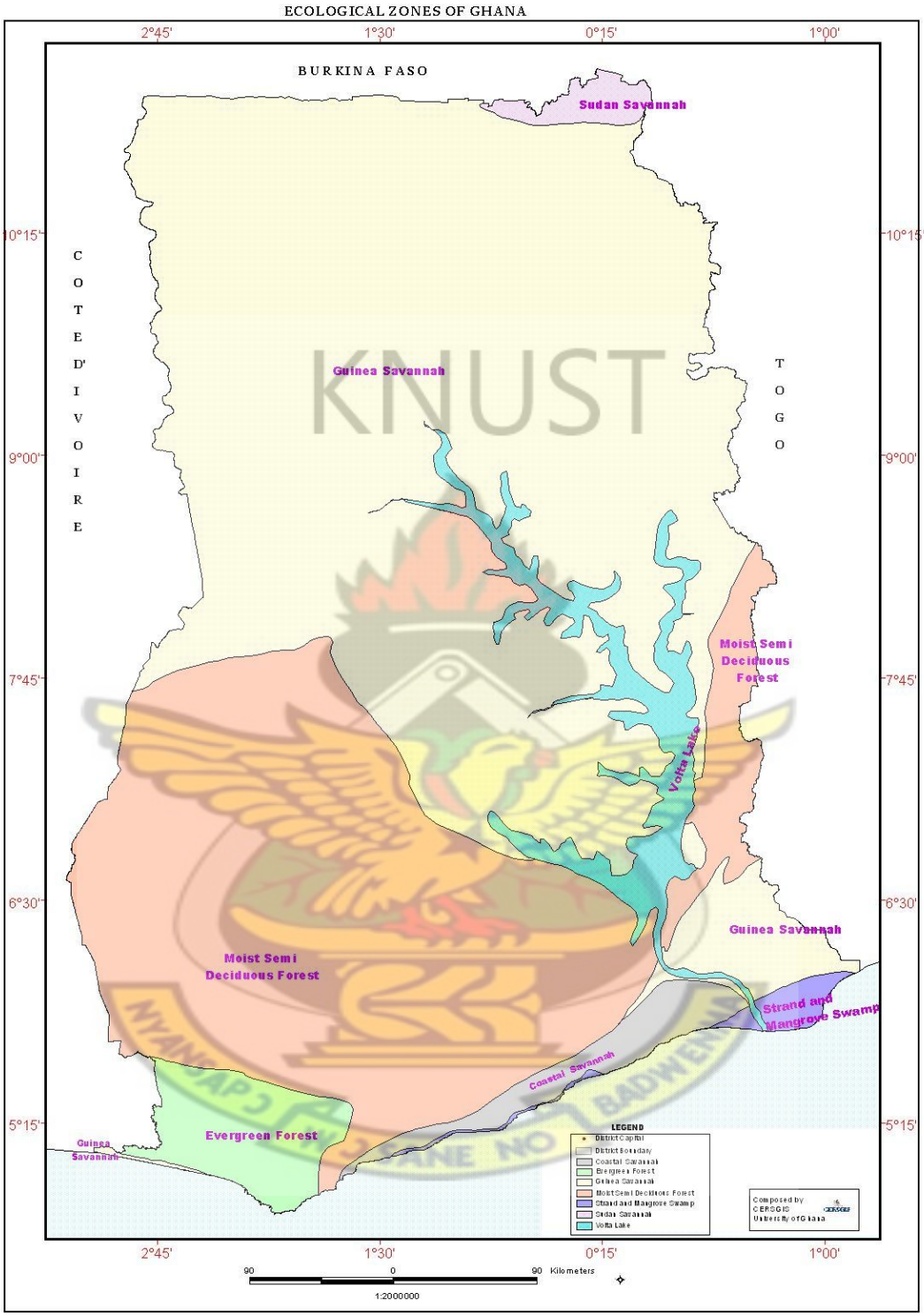


Figure 3.1: Ecological zones in Ghana. Date Source: Centre for Remote Sensing and GIS, University of Ghana.

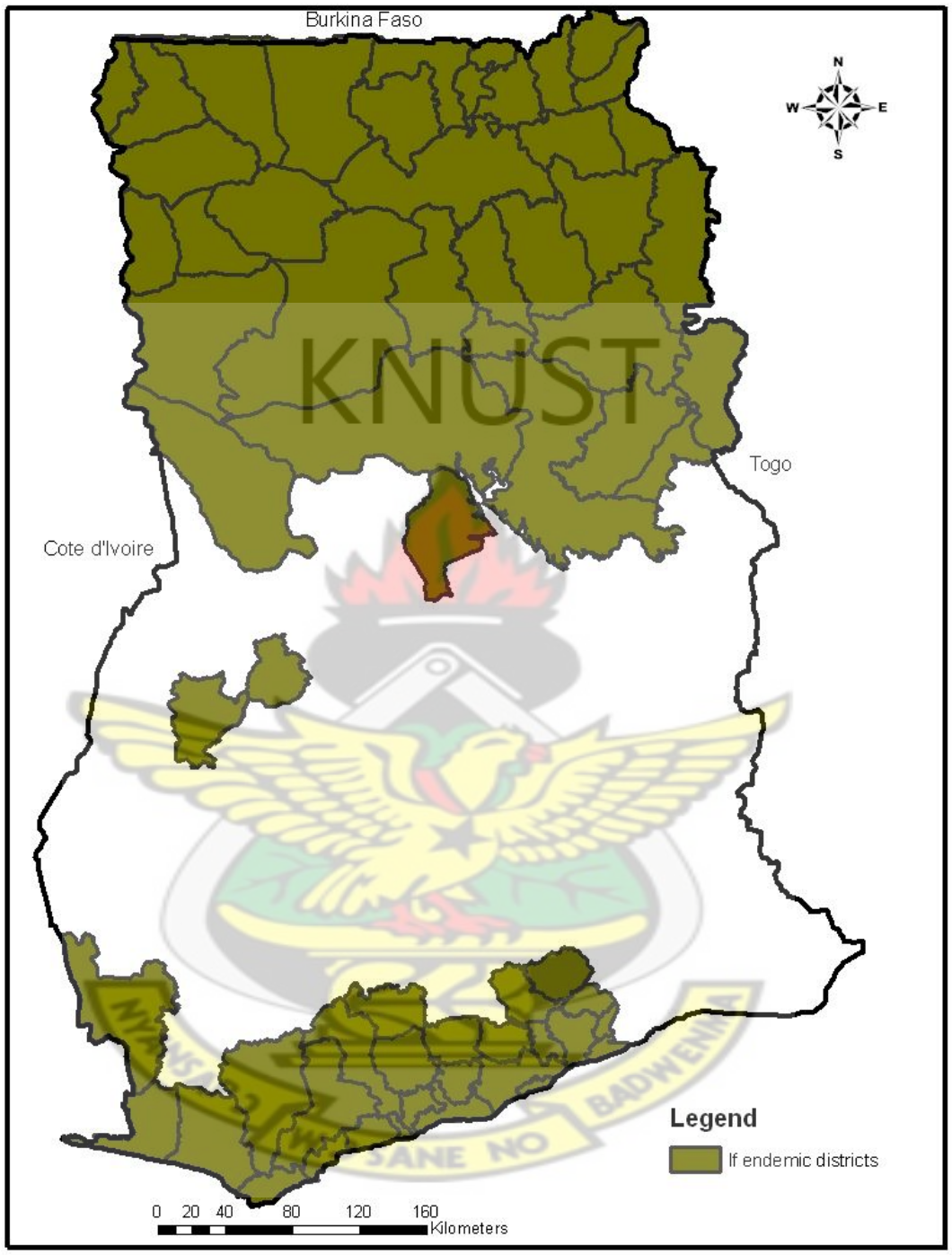


Figure 3.2: Map of Ghana showing the LF endemic districts. Data source: Ghana National Lymphatic Filariasis Control Program, Ministry of Health.

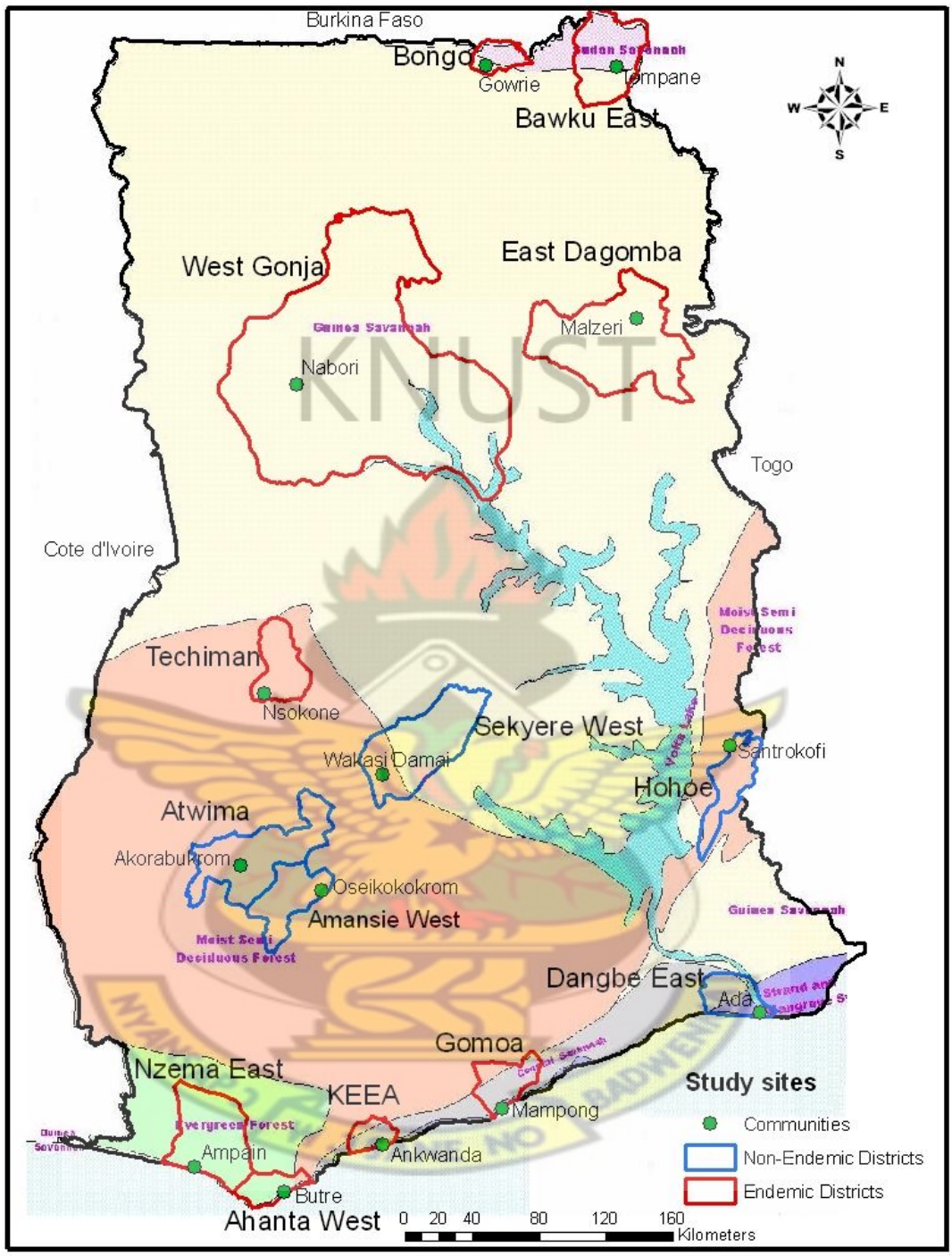


Figure 3.3: Sample sites overlaid on ecological zones and LF endemic districts in Ghana. Maps digitized and drawn in ArcGIS version 9.

Nabori and Malzeri are in the Northern Region of Ghana and located in the West Gonja and Yendi districts respectively, in the Guinea Savannah zone. Temperatures are generally high with the maximum occurring in the dry season, between March/April and are lowest between December/January. Evapo-transpiration is very high causing soil moisture deficiency. Rainfall is bimodal within the Guinea Savannah zone. The vegetative cover is dictated by the soil types and human activities, such as shifting cultivation, slash and burn method of land preparation. Trees are scattered except in few places where isolated woodland or forest are found. Most trees are deciduous, shedding their leaves during the dry season in order to conserve water. Grass grow in tussocks. Both Nabori and Malzeri are LF endemic, with the 2007 district prevalence rates of 74.3% and 78.9% respectively.

Nsokone, Akorabukrom, Oseikokokrom, Wakasi Damai and Sankofi are located in the Moist Semi-deciduous Forest region. This zone experiences the semi-equatorial type of climate, characterized by double maxima rainfall. The major rainfall season begins in March and ends in July, whilst the minor season starts from September and ends in November. The relative humidity ranges between 70 and 80 percent in the dry season. It is, however, important to note that Nsokone and Wakasi Damai lie at the forest-Savannah transition zone and therefore has dual vegetation types. Nsokone is located in the Techiman District, Akorabukrom in the Atwima Mponua District, Oseikokokrom in the Amansie East District, Wakasi Damai in Sekyere West District and Sankofi in the Hohoe District. The Techiman district had an LF prevalence of

79.3% in 2007. The other districts are non-endemic and had no prevalence records, especially since the forest zone is considered to be non-endemic from the study by Gyapong *et al*., (2002).

The remaining 5 sites fall in the Coastal Savannah zone. These are Ampain, Butre, Ankwanda, Mampong and Ada. This zone is one of the hottest in the country. Temperatures are high throughout the year and range between 23°C - 33°C. Rainfalls are heavy during the major season between March and September. Humidity is very high, 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 around the estuaries where rivers empty into the sea and tributaries where the soil is waterlogged and salty. Ampain is located in the Nzema East District and, Butre in the Ahanta west District, all in the Western Region, Ankwanda is situated in the KEEA District (Central Region), Mampong in the Gomoa District (Central Region) and Ada in the Dangme East District (Greater Accra Region). With the exception of Ada, all the other sites are LF endemic. The district LF prevalence rates for Nzema East, Ahanta West, KEEA and Gomoa in 2007 are 47.7%, 72.3%, 65.3% and 78.8% respectively.

3.2 Samples

3.2.1 Mosquito Collection

Each village was divided into four sections and a house selected from each section for mosquito collection. Hourly collections were done from 18:00hrs to 06:00hrs. Two collectors were involved in the hourly collections and are replaced after six hours, to correct for the varying degrees of attractiveness and their ability to catch the mosquitoes. There were a total of sixteen well trained collectors per community.

Mosquitoes were collected using the Human Landing Catches (HLC) method (World Health Organization, 2003). Briefly, two collectors were made to sit in a dark room, with their legs exposed. Torch lights, switched on intermittently, were used to locate host-seeking mosquitoes. Landing mosquitoes that are ready to take a blood meal were then trapped using a test tube. Trapped mosquitoes were then released into paper cups covered with mosquito netting and labeled to indicate the hour of collection. In the field, the mosquitoes were killed and identified. All mosquitoes identified as *An. gambiae* s.l. were stored in isopropanol, and given a unique identification number, for later species identification by PCR. Coordinates of all sampling locations were obtained using a hand-held GPS equipment.

All procedures required for use of human subjects (in mosquito sampling) were strictly followed. Ethical clearance was obtained from the IRB of the Noguchi Memorial Institute for Medical Research (See Appendix I for IRB certificate). The study was explained to the communities and mosquito collectors and consent sought at both community and individual levels. Participation was strictly voluntary and their rights to withdraw from the study without any retribution were ensured. All the collectors were trained and a signed consent form was obtained from each of them (See Appendix II for a copy of the information sheet and consent form).

3.2.2 *W. bancrofti* Specimen

Parasites were dissected out from infective *An. gambiae* s.l. and *An. funestus* collected during two entomological studies that were conducted concurrently in the Bongo district (Boakye *et al.*, 2004) and the Gomoa Districts (Boakye *et al.*, unpublished). The observed *W. bancrofti* parasites, identified under a microscope, were picked using the dissecting pin and transferred into individual tubes containing Isopropanol solution and were stored at 4°C until ready to use .

3.3 Morphological identification of *Anopheles* Species

Anopheles mosquitoes are generally characterized by dark coastal spots on the wings, and palps which are about the same length as their proboscis. The mosquitoes were morphologically identified to the genus level and species level where possible using the keys of Gillies and de Meillon (1968) and Gillies and Coetzee (1987).

An. gambiae s.l. can be distinguished by the presence of smooth palps, with three pale bands that are about the same length as the proboscis. The femur, tibiae and first tarsal segment of the legs have short, white lines. The wings have five distinctively pale spots on the costal margins. There are some variations in the relative sizes of the pale and dark markings, which may be indicative of inter-specific differences.

3.4 Molecular Analysis

Genomic DNA was extracted and was used for species identification, using PCR-based methods. The identified samples were observed through agarose gel electrophoresis. Identified samples were subjected to dye terminator cycle sequencing reactions and sequenced on an automated sequencer.

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

The boil preparation DNA extraction method was used for the extraction of genomic DNA from each mosquito. Briefly, two to three legs from each mosquito was placed in a 1.5 ml eppendorf tube, containing 100µl ddH_2O, and homogenized with a sterile plastic pestle. The homogenate was incubated at 90°C for 10 minutes. This was briefly centrifuged (5-10 seconds) and stored at -20°C until ready to use.

3.4.2 Identification of Sibling Species of *An. gambiae s.l*

The extracted DNA was used for species identification (Fanello *et al.*, 2002) and further analysed for molecular forms using PCR and Restriction Fragment Length Polymorphism (PCR-RFLP) (Favia *et al.*, 1997, Scott *et al.*, 1993). This allows for simultaneous identification of members of the *An. gambiae* s.l. as well as the M and S forms within the *An. gambiae* s.s. It is based on the fact that GCG^C restriction site for *Hha*1 enzyme (Favia *et al.*, 1997) lies within the *An. gambiae s.s* specific fragment

(Scott *et al.*, 1993) making it possible to digest this fragment directly in order to differentiate the M and S molecular forms.

Five sets of oligonucleotide primers designed from the DNA sequences of the intergenic spacer region of *An. gambiae* complex ribosomal DNA (rDNA) were used in PCR for identification of members of the species complex (Scott *et al.*, 1993). The primer sequence details and their expected sizes of the PCR products are given in Table 3.1. The PCR reaction was performed in a total volume of 20µl, containing 1X PCR buffer supplied by the manufacturer (Sigma, USA), 200µM of each of the four deoxynucleotide triphosphates (dNTPs), 10µM of each of the five oligonucleotide primers and 0.125 units of *Taq* Polymerase enzyme (Sigma, USA). One microlitre of the extracted DNA was used as template for the amplification reaction. Sterile ddH_2O was used to make up the volume to 20µl. The reaction mix was centrifuged briefly and overlaid with mineral oil to avoid evaporation and refluxing during thermo-cycling. The amplification was carried out using a PTC 100 thermal cycler (MJ Research Inc., USA). The cycling parameters for the reactions were as follows: 94°C for 10 min (initial denaturation), followed by 30 cycles at 94°C for 30 sec (denaturation), 50°C for 30 sec (annealing), 72°C for 30s (extension) and ended with a final extension cycle at 72°C for 7 min. For each reaction, a positive control and a negative control were added.

Table 3.1: DNA sequence details of the synthetic oligonucleotide primers used for the PCR-based methods for the identification of the members of the *An. gambiae* s.l (Scott *et al*., 1993).

***An. gambiae* s.l.**	**Primer Sequences**	**Product Size (bp)**
Universal (UN)	5' GTG TGC CCC TTC CTC GAT GT 3'	
***A. gambiae* (GA)**	5' CTG GTT TGG TCG GCA CGT TT 3'	390
***A. melas* (ME)**	5' TGA CCA ACC CAC TCC CTT GA 3'	464
***A. arabiensis* (AR)**	5' AAG TGT CCT TCT CCA TCC TA 3'	315
***A. quadriannulatus* (QD)**	5' CAG ACC AAG ATG GTT AGT AT 3'	153

3.4.3 Molecular Identification of *W. bancrofti*

W. bancrofti genomic DNA of each specimen was extracted using the DNeasy Tissue Kit following the manufacturer's protocol for DNA extraction from animal tissues (QIAGEN Inc., USA). The protocol for molecular identification of *Wuchereria bancrofti* described by Ramzy *et al*. (1997) was used. With this PCR-based method a diagnostic band size of 188bp was obtained for the amplified DNA fragment.

3.4.4 Amplification of the Cytochrome Oxidase I Gene

Amplification of the COI segment of the mitochondrial gene and cycle sequencing reactions were performed according to the method of Cywinska *et al*., 2006. The PCR assay was performed on samples using two oligonucleotide primers, LCO1490 and HCO2198 (Folmer *et al*., 1994). The primer sequences for the COI amplification are shown in Table 3.2 below. Each amplification reaction was done in a final volume of 25 μL containing 15.7 μL sterile distilled water, 10x PCR buffer (2.5 μL), 1.5 mM $MgCl_2$ (1.5 μL), 200 μM dNTP's (0.5 μL), 200 nM of each primer (0.8 μL), 0.025 U/μL Taq polymerase (0.2 μL), and 3.0 μL DNA template. The thermal cycling profile was as follows: an initial denaturation at 95 °C for 1 minute, then 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1.5 minutes, and extension at 72°C for 1.5 minutes. This was followed by a final cycle at 72 °C for 7 minutes. A diagnostic band of around 700 bp was expected for each reaction.

Table 3.2: DNA sequence details of the synthetic oligonucleotide primers used for the COI amplifications (Folmer *et al.*, 1994)

COI primers	**Primer Sequences**
LCO1490 (COI Forward)	5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'
HCO2198 (COI Reverse)	5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'

3.4.5 Analysis of PCR Products

The obtained products were visualized on agarose gel stained with 0.5µg/ml ethidium bromide to detect the presence of amplified DNA fragments. The *A. gambiae* species identification products were visualized on a 2% gel, and the COI amplification products were visualized using a 1% gel. Five microlitres of each PCR product was added to 1µl of gel loading dye (orange G (5X)) for the electrophoresis. The gel was prepared and electrophoresis was performed using 1X TAE in a mini gel system (BIORAD USA) at 100 volts for one hour. The gel was visualized over a UV transilluminator (UPC, USA). The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp DNA molecular weight ladder.

3.4.6 Sequencing and Sequence Analysis

The COI products were purified using a QIAQUICK PCR Purification Kit (QIAGEN, USA) following the manufacturer's protocol. Cycle sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on purified PCR products, in a DNA thermal cycler (PTC-100, MJ Research Inc., USA). Each amplification reaction was done in a final volume of 20 µL containing 8 µL Big Dye, 0.3 µL 200 nM primer, 6.7 µL sterile distilled water, and 5.0 µL DNA template. The thermal cycling profile was as follows: an initial step at 96 °C for 1 minute, then 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60 °C for 4 minutes. The post-cycling purification of samples was done using the ethanol precipitation method (http://bitesizebio.com/articles/the-basics-how-ethanol-

precipitation-of-dna-and-rna-works/). The purified samples were allowed to dry and 10 μL Hi-Di formamide was added, to re-suspend the sample, and analyzed in a genetic analyzer (Applied Biosystems Genetic Analyzer 3130).

3.5 Data Analysis

3.5.1 Data Management Structure

All data collected were stored in a relational database in order to facilitate querying of data for analysis. The database contains, field data, molecular data as well as spatially referenced information. Topographic and climatic data for the sampling period was also included in the database. The topo-climatic data was used to discriminate between regions and provide indicators through the sampling areas. The data management structure is shown in Fig. 3.4. Sequence data was analyzed at three levels of aggregation (Fig. 3.5) in order to bring out any differences between the *An. gambiae*.

The parasite sequence data was analyzed within and between the north and south for differences in sequence composition.

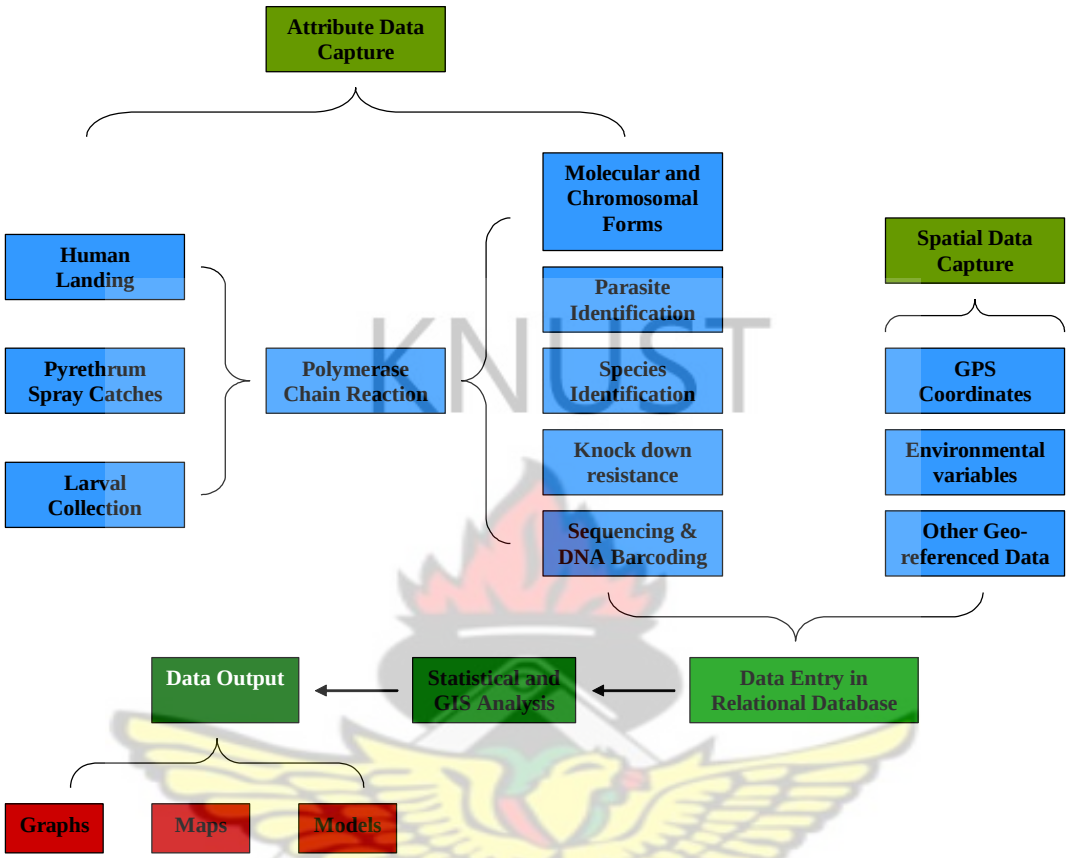


Figure 3.4: Geo-molecular data management structure, designed for the study.

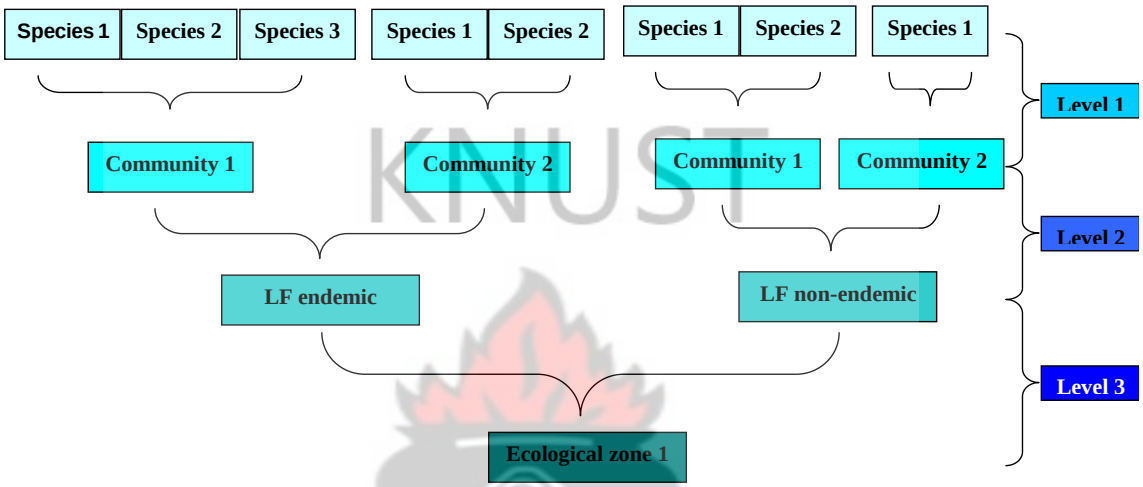


Figure 3.5: Data analysis aggregation levels, designed for the study.

3.5.2 Sequence Analysis

The *An. gambiae* sequences obtained were aligned, to the complete *An. gambiae* COI genome (Accession number L20934), using BioEdit (Hall, 1999). Pairwise nucleotide sequence divergences were calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980), and Neighbour-Joining (NJ) analysis (Saitou and Nei, 1987) was used to examine relationships among taxa, in Mega 4 (Tamura *et al.*, 2007). For phylogeny construction, consensus sequences were generated for groups of sequences. The bootstrap consensus tree inferred from 5000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the dataset using the complete deletion option. The sequences obtained were also analyzed together with other GenBank sequences. The equality of evolutionary rate between Consensus sequences from endemic and non-endemic areas was tested using *An. melas* as an outgroup in Tajima's relative rate test (Tajima, 1993).

The *W. bancrofti* sequences were aligned together with other COI sequences of *W. bancrofti* from GenBank (Accession numbers CD455366.1 and AM 749236.1), and

Filaria martis –used as an outgroup (GenBank Accession number AJ544880.1). The mean evolutionary diversity between the grouped Northern and Southern *W. bancrofti* COI sequences was computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). The equality of evolutionary rate between COI South Consensus and COI North Consensus was tested using *F. martis* as an outgroup in Tajima's relative rate test (Tajima, 1993).

3.5.3 GIS and Meta-Analysis

3.5.3.1 Entomological data and mapping

All entomological studies on *An. gambiae* s.l in Ghana since the year 2000 were identified from various sources including published articles in peer-reviewed journals, unpublished work from MPhil and PhD theses held at the Noguchi Memorial Institute for Medical Research (NMIMR), Accra-Ghana, as well as on-going studies at the NMIMR. In order to produce a comprehensive record of *An. gambiae* s.s, data on the location, study period, sample size, mosquito species, molecular form and *kdr* mutation from each study were collated into a database. Data collected from the same location in different years were considered separately. The collated data is shown in Appendix III.

To examine the geographical distribution of *An. gambiae* s.s in Ghana, each location in the database was geo-referenced using the latitudinal and longitudinal coordinates obtained from the GEOnet Names Server (http://earth-

info.nga.mil/gns/html) and Directory of Cities and Towns in the World (http://www.fallingrain.com/world) databases. All data were imported into and mapped using the geographical information systems software ArcGIS 9.2 for mapping (ESRI, Redlands, CA). The geographical patterns of the i) *An. gambiae* s.l, ii) the M and S molecular forms within *An. gambiae* s.s were assessed.

3.5.3.2 Environmental data and analysis

To examine environmental factors associated with the distribution of *An. gambiae* M and S forms, in *An. gambiae* s.s in Ghana, specific data on elevation, vegetation, rainfall, temperature and humidity were examined. Elevation data were derived from the U.S Geological Survey's (USGS) ETOPO2 Digital Elevation Model available from ESRI Redlands CA, while data on vegetation cover, rainfall, temperature and humidity were obtained from the best available sources, accessed via the IRI/LDEO Climate Data Library of the International Research Institute for Climate and Society (http://iridl.ldeo.columbia.edu/index.html). Vegetation cover was based on Normalized Difference Vegetation Index (NDVI) satellite data extracted from the LandDAAC MODIS version 005 West Africa from USGS (http://iridl.ldeo.columbia.edu/SOURCES/.USGS/.LandDAAC/.MODIS/.version_005/.WAF/.NDVI; Huete *et al.*, 2002). Rainfall (mm), temperature (C°) and specific humidity (qa) measures were obtained from satellite data from the National Oceanic and Atmospheric Administration (NOAA) and based on daily mean readings taken 2 meters above the ground

(http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/.NCEP/.CPC/.FEWS/.Africa/.DAILY/.RFEv2/.est_prcp; http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/.NCEP-NCAR/.CDAS-1/.DAILY/.Diagnostic/.above_ground/.temp; http://iridl.ldeo.columbia.edu/SOURCES/.NOAA/.NCEP-NCAR/.CDAS-1/.DAILY/.Diagnostic/.above_ground/.qa; Love 2002; Kalnay *et al.*, 1996).

The elevation of each geo-referenced location was determined by importing the digital elevation map into ArcGIS 9.2 and extracting the underlying value (metres), while for the NDVI and climate variables, the mean annual values for the specific year of study were extracted from the IRI/LDEO Climate Data Library web interface (http://iridl.ldeo.columbia.edu/index.html) in the 'Expert Mode' using a series of queries, detailing the coordinates of the points, the monthly and yearly averages. All data were compiled into the database, and descriptive and statistical analyses undertaken in Microsoft Excel and SPSS 16.0 (SPSS, Inc, Chicago, IL). The relationship between the *An. gambiae* M and S forms, (and other species), and each environmental variable, were examined using bivariate correlations, Pearson's correlation coefficient (2 tailed P values ≤ 0.05 significance), and by comparing means in different environmental groupings.

3.5.3.3 Relationship with disease prevalence

To examine the relationship between *An. gambiae* M and S distributions and the distribution of disease, two maps on the LF prevalence (Gyapong *et al.*, 2002) and *P.*

falciparum prevalence (Kleinschmidt *et al*., 2001; 2007) for West Africa were imported into ArcGIS 9.2 and geo-referenced. The LF map was modeled from the *W. bancrofti* seroprevalence data collected from 401 villages throughout Benin, Burkina Faso, Ghana and Togo, in 2000 (Gyapong *et al*., 2002). The *P. falciparum* malaria prevalence map was modelled on extensive data obtained from children aged 2–10 years in non-epidemic periods, using a generalized linear mixed model (Kleinschmidt *et al*., 2001).

The *An. gambiae* s.s collection sites were used as focal points, whereby the underlying disease prevalence data could be compared with the entomological data (i.e. *An. gambiae* M and S distributions). In ArcGIS 9.2, LF prevalence data corresponding to the latitude and longitude of each mosquito collection site, were extracted and exported for descriptive and statistical analysis.

Further, it was of particular interest to explore the entomological and environmental characteristics in different LF transmission zones based on the prevalence data map in Gyapong *et al*., 2002. The prevalence distributions ranged from 0 to 30%, and were classified into three transmission zones (i.e. low, medium, high), which were digitized in ArcGIS 9.2. The entomological and environmental data from each of the mosquito collection locations within each zone were summarized.

CHAPTER FOUR: RESULTS

4.1 Mosquito Sampling and Identification

An initial sampling was done in June 2007, in Ada and Ankwanda, during the rainy season, in order to familiarize with the field sampling, identification and laboratory techniques. This was followed by a second and third sampling in September-October 2007 and April 2008 respectively. These two collections were done at the beginning of the dry season and the beginning of the rainy season respectively. However, due to the similarity in species composition between sites the number of sites was reduced to 7 from 14, for the second collection. A final collection was done in the 7 sites, in January-February 2009, and this corresponded to the peak dry season.

A total of 10274 mosquitoes were collected during 30 collection nights between June 2007 and February 2009 (Table 4.1). Mosquitoes were identified as belonging to the following species: *An. gambiae s.l, An. funestus, An. coustani, An. pharaoensis, An. rufipes, Culex spp, Mansonia spp*. and *Aedes spp*. The Table shows the abundance of the *An. gambiae* between sites. There was a general reduction in the number of *An. gambiae* from the rainy to the dry season. Also, there was an apparent increase in the number of *Culex* and *Aedes spp*., with a decrease in the number of *An. gambiae*. *Anopheles* and *Culex* species were found in all areas, whereas other potential LF vectors such as *Mansonia* and *Aedes* species are mostly found in the southern sites, compared to the northern sites. Further studies on these potential vectors –especially climate change

effects, may shed more light on their vectorial importance in the transmission of LF in Ghana.

The peak biting time for *An. gambiae* also varied between sites (Fig. 4.1), with some showing a unimodal and others, bimodal distributions. The nocturnal biting rates showed an increased night time peak between 11 pm and 5 am. An important observation is that in most sampling communities, 5% or more of mosquitoes were collected between 5am to 6 am in the morning.

Table 4.1: Summary of field mosquito collections

Community	Date	Weather	*An. gambiae*	*An. funestus*	*Culex sp.*	*Mansonia sp.*	*An. coustani*	*Aedes sp.*	*An. pharaoensis*	*An. rufipes*	Total
Ada	Jun-07	Rain	404	0	620	1	0	25	21	1	1072
Ada	Oct-07	Dry	120	0	283	39	0	8	1	0	451
Ada	Apr-08	Rain	284	0	358	12	0	61	1	0	716
Ada	Feb-09	Dry	109	0	217	8	0	2	0	0	336
Akorabukrom	Sep-07	Dry	92	1	1	0	0	0	0	0	94
Ampain	Oct-07	Rain	858	0	1	143	0	0	0	0	1002
Ampain	Apr-08	Rain	337	0	16	3	0	4	0	0	360
Ampain	Jan-09	Dry	537	0	0	22	0	0	0	0	559
Ankwanda	Jun-07	Rain	358	2	131	35	0	41	0	0	567
Ankwanda	Oct-07	Dry	246	1	38	61	0	10	0	0	356
Ankwanda	Apr-08	Rain	87	0	1	0	0	24	0	0	112
Ankwanda	Jan-09	Dry	39	0	0	0	0	4	0	0	43
Butre	Oct-07	Rain	852	0	21	30	3	1	0	0	907
Gowrie	Sep-07	Rain	200	0	104	0	0	5	1	0	310
Mampong	Sep-07	Rain	1131	133	108	460	1	1	15	0	1849
Malzeri	Oct-07	Rain	31	8	32	16	1	11	0	0	99
Nabori	Oct-07	Rain	99	61	4	4	0	46	0	0	214
Nabori	Apr-08	Dry	7	0	1	0	0	3	0	0	11
Nabori	Feb-09	Dry	3	1	0	0	0	0	1	0	5
Nsokonee	Sep-07	Rain	248	5	2	1	0	1	0	0	257
Oseikokokrom	Sep-07	Rain	388	0	2	9	2	0	0	0	401
Oseikokokrom	Apr-08	Dry	56	18	5	2	0	0	0	0	81
Oseikokokrom	Feb-09	Dry	43	2	1	6	0	0	0	0	52
Santrokofi	Oct-07	Rain	45	5	10	0	0	0	0	1	61
Santrokofi	Apr-08	Dry	7	3	114	0	0	4	0	0	128
Santrokofi	Feb-09	Dry	1	0	5	0	0	0	0	0	6
Tempane	Oct-07	Rain	28	2	91	0	1	9	0	0	131
Tempane	Apr-08	Dry	15	0	0	0	0	0	0	0	15
Tempane	Feb-09	Dry	2	0	3	0	0	0	0	0	5
Wakasi Damai	Sep-07	Dry	47	3	24	0	0	0	0	0	74
Total			6674	245	2193	852	8	260	40	2	10274

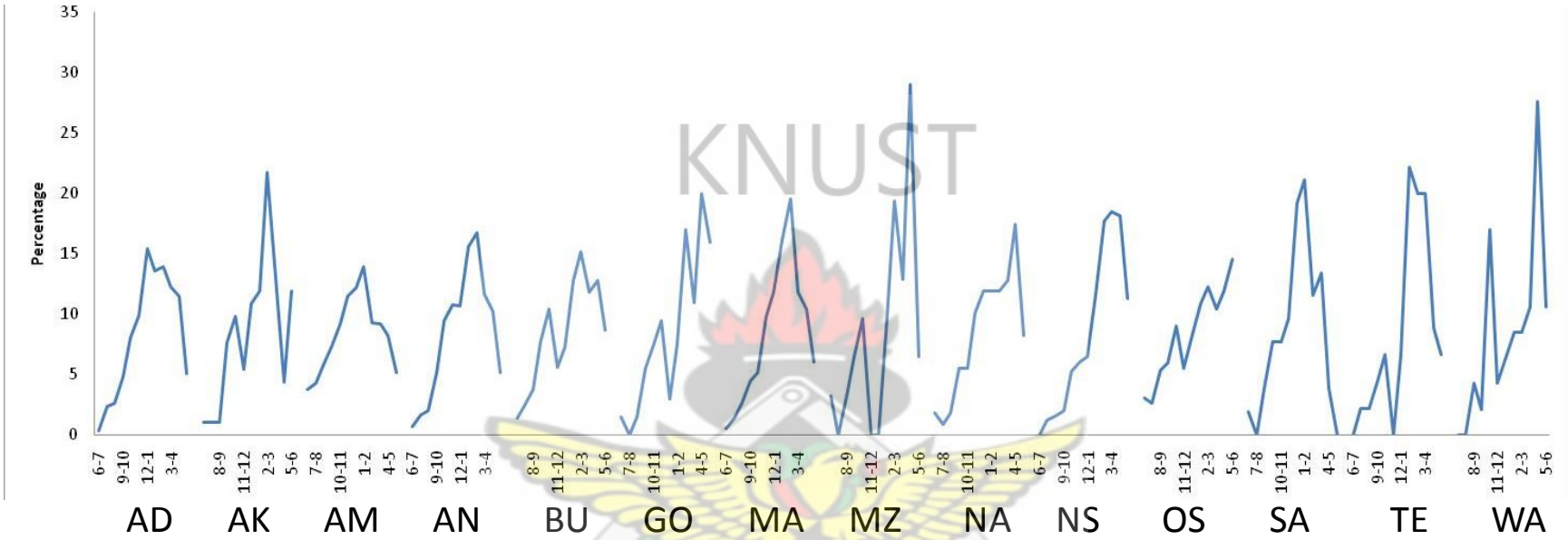


Figure 4.1a: Biting pattern of *An. gambiae* at the various sites, from 6pm to 6am.

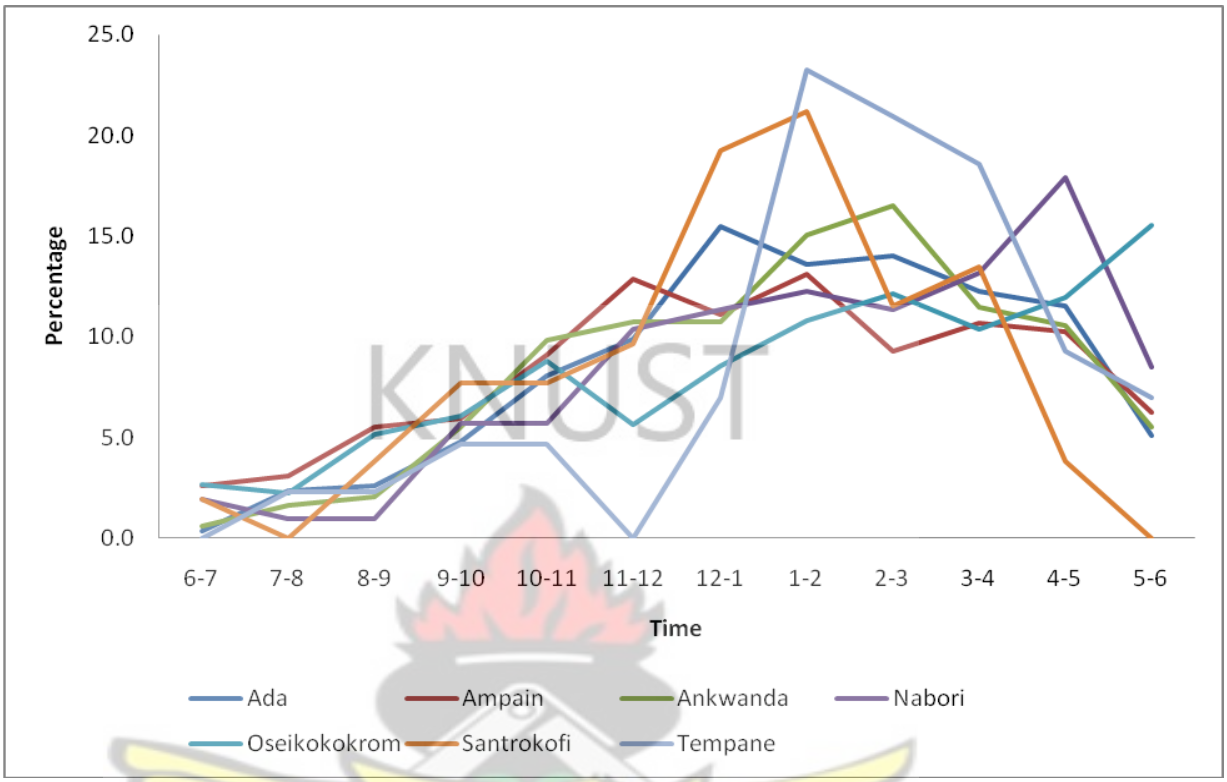


Figure 4.1b: Biting pattern of *An. gambiae* between selected sites

4.2 General Vector Distribution

The results indicate *An. gambiae* to be the major human biting mosquito species in most study communities, followed by *Culex* and *Mansonia species* (Fig. 4.2). *An. gambiae* species is the major vector of LF in Ghana. However, with the exception of *Culex sp*. that has been shown to play no vectorial role in the transmission of LF (Aboagye-Antwi, 2003) all other species collected could be potential vectors of LF in Ghana since they are vectors of LF in other parts of the world. Determining their vectorial importance in Ghana will therefore be very important for the elimination program.

Of the *An. gambiae* collected, a total of 1494 (24.3%) were identified using molecular methods (Table 4.2). Out of these, 1004 (67.2%) were *An. gambiae* s.s, 414 (27.7%) were *An. melas* and 46 (3.1%) were *An. arabiensis*. *An. melas* was found along the coastal Savannah zones, mainly due to the marshy environment in which they breed. *An. arabiensis* was only found in the northern sites in the Sudan Savannah zone (Fig. 4.3). The observed geographical distribution of these mosquitoes indicates the role of the environment in shaping their distribution.

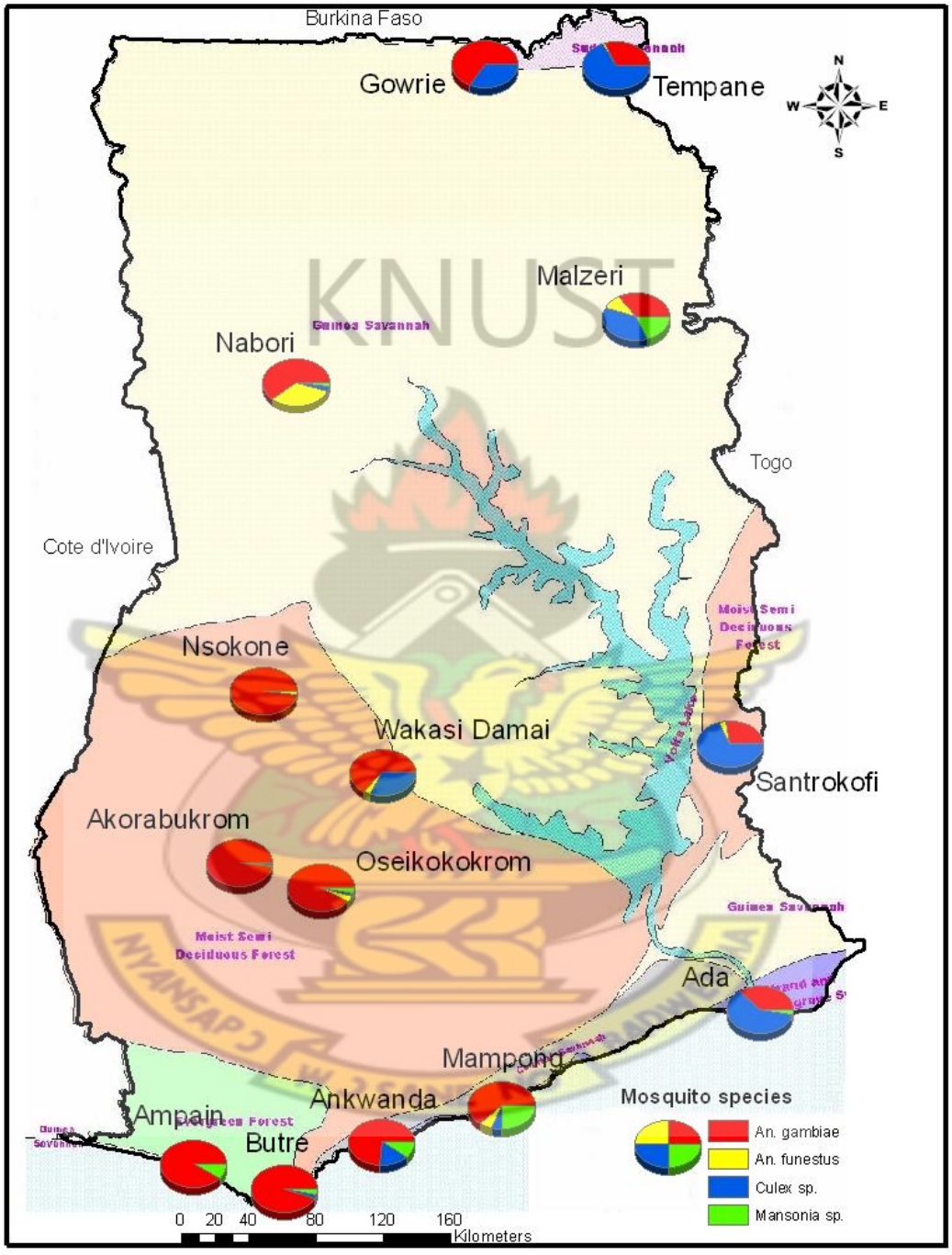


Figure 4.2: Distribution of mosquito species in Ghana. Map drawn in ArcGIS version 9

Table 4.2: Species identification results

Community	*An. gambiae ss*	*An. melas*	*An. arabiensis*	Unidentified	Total
Ada	133	20	2	5	**160**
Akorabukrom	79	0	0	7	**86**
Ampain	3	193	0	0	**196**
Ankwanda	98	96	5	0	**199**
Butre	0	100	0	0	**100**
Gowrie	139	0	33	0	**172**
Malzeri	32	0	0	0	**32**
Mampong	74	5	0	0	**79**
Nabori	86	0	1	0	**87**
Nsokone	83	0	0	0	**83**
Oseikokokrom	166	0	0	0	**166**
Santrokofi	47	0	0	0	**47**
Tempane	36	0	5	0	**41**
Wakasi Damai	28	0	0	18	**46**
Total	**1004**	**414**	**46**	**30**	**1494**

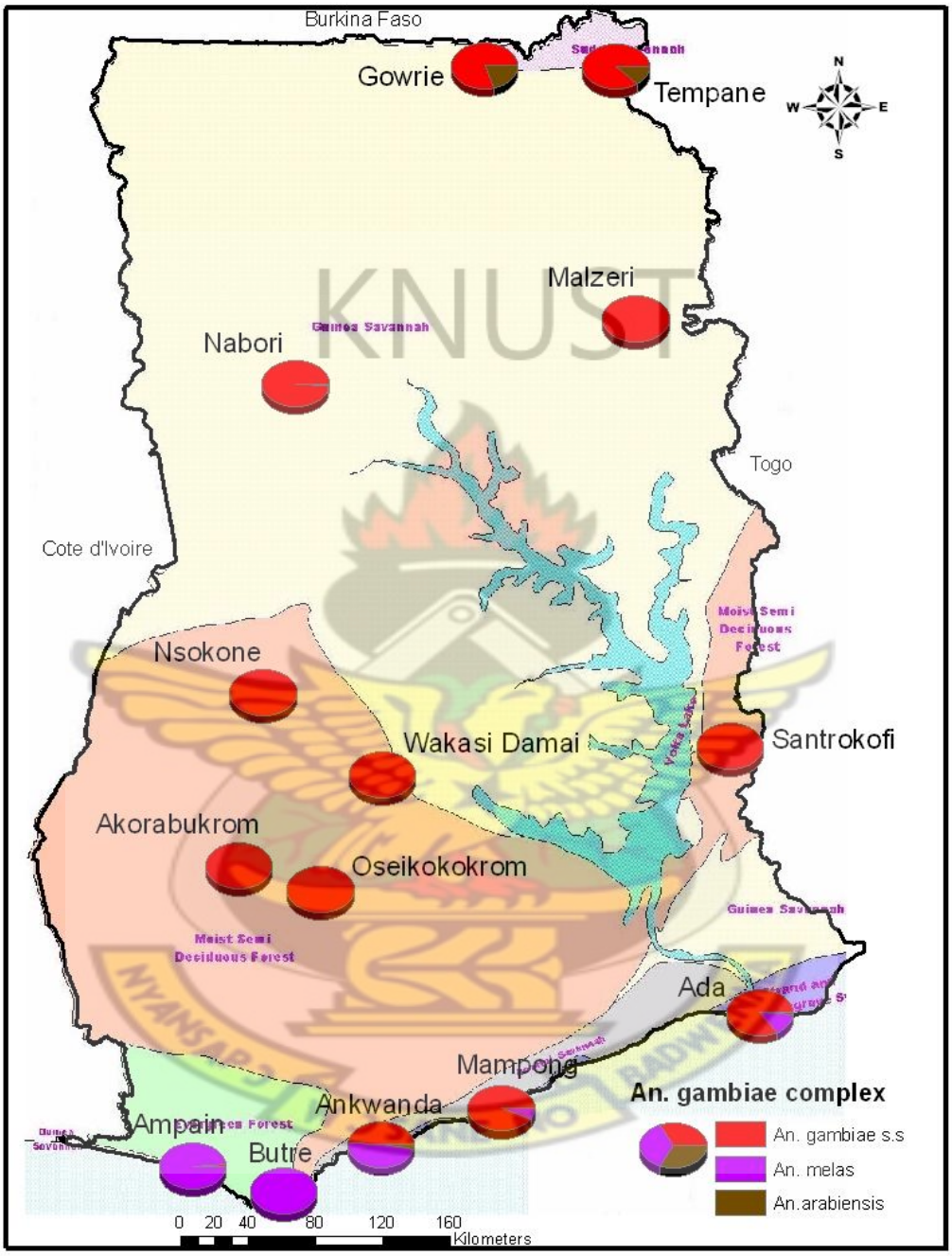


Figure 4.3: Distribution of members of the *An. gambiae* complex in Ghana. Map drawn in ArcGIS version 9.

RFLP analysis was performed on 952 *An. gambiae* s.s. Of these, 485 (51%) were the M molecular form, 439 (46.1%) were S molecular forms and 28 (2.9%) were identified as M/S hybrids (Table 4.3). The distribution of the molecular forms of the *An. gambiae s.s* varies across the country in different proportions and combinations (Table 4.3). Along the coastal areas, the M molecular form was predominant, while the S molecular form was most common in the middle belt (Fig. 4.4). High proportions of M & S hybrids have been observed in few areas, and the implications of these have been determined through analyses with environmental parameters, in order to help determine factors responsible for the non-uniform distribution.

Fig. 4.5 is a gel photograph of the species identification and Fig. 4.6 is a gel photograph of the RFLP.

Table 4.3: Summary of the molecular forms of *An. gambiae* from each site.

Community	M Form	S Form	M/S Form	Total
Ada	131	0	0	**131**
Akorabukrom	0	68	0	**68**
Ampain	0	0	0	**0**
Ankwanda	79	8	0	**87**
Butre	0	0	0	**0**
Gowrie	71	66	0	**137**
Malzeri	2	28	0	**30**
Mampong	59	6	0	**65**
Nabori	12	67	0	**79**
Nsokone	81	0	0	**81**
Oseikokokrom	28	135	0	**163**
Santrokofi	7	25	15	**47**
Tempane	15	9	13	**37**
Wakasi Damai	0	27	0	**27**
Total	**485**	**439**	**28**	**952**

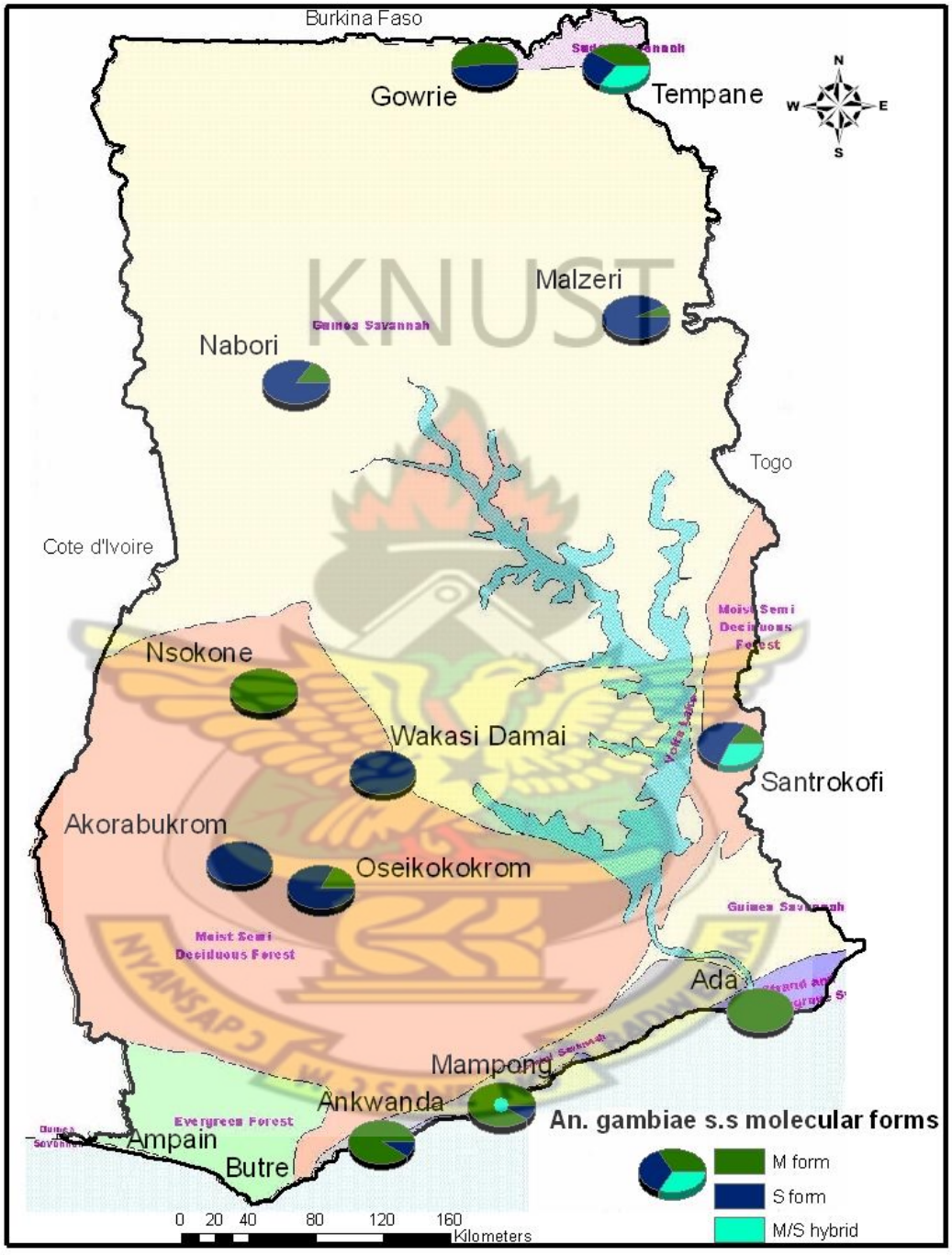


Figure 4.4: Distribution of the molecular forms of *An. gambiae* s.s in Ghana. Map drawn in ArcGIS version 9.

Figure 4.5: Gel electrophoregram for the identification of members of the *An. gambiae* species complex. Lane M = 100bp molecular weight marker, +ve = positive control, Lane 1-3, 5 and 6 = *An. gambiae* s.s, Lane 4 = *An. melas*, -ve = negative control.

Figure 4.6: Gel photograph after RFLP. M = 100bp molecular weight marker; Lanes 1-3 and 5 = *An. gambiae* S Forms showing two visible bands; Lanes 4 and 6 = *An. gambiae* M Form showing a single visible band, and Lane 7 = M/S Hybrid.

4.3 GIS and Environmental Analyses

4.3.1 Entomological Mapping

The collated *An. gambiae* species complex database contained 143 records with a total of 12,607 mosquitoes. From this, the distribution of *An. gambiae* s.l was mapped (Fig. 4.7). The most dominant species was *An. gambiae* s.s, which was found at 114 sites (total n = 10,028), followed by *An. melas* (6 sites, total n = 469) and *An. arabiensis* (8 sites, total n = 240). The map indicates that the distribution of *An. gambiae* s.s was widespread, while *An. melas* was primarily found along the coastal Savannah zones, a predominantly marshy environment, and *An. arabiensis* mainly in the northern Savannah zone. *Anopheles funestus*, the second most important vector of malaria and lymphatic filariasis in Ghana, was also recorded in 9 sites with a total of 1,825 mosquitoes. Appendix III provides further information on *An. funestus* and other *Anopheles* mosquito species and their collection methods.

Of the *An. gambiae* s.s data, a total of 70 sites had information on the M form (n= 2,826) and S form (n= 4,098). The distribution of *An. gambiae* M and S forms was not even and varied across the country in different proportions (Fig. 4.8). Overall, *An. gambiae* M and S were sympatric in most locations. However, *An. gambiae* M form was more prevalent in the northern Savannah, and coastal Savannah areas of the country, and in four sites it was the only species observed. In contrast, *An. gambiae* S form was more prevalent in the middle region of the country, and in seven locations it

was the only species collected. Interestingly, bivariate correlation analysis between the two forms indicated that their prevalences were negatively correlated (-0.763). These trends are supported by the spatial analyses carried out, indicating a positive spatial autocorrelation or clustering for both the *An. gambiae* M (MI =0.19, Z score=4.2, $P \leq$ 0.01) and *An. gambiae* S (MI =0.19, Z score=4.2, $P \leq$ 0.01) forms. The resultant Z scores of the Getis-Ord Gi* hot spot analyses (using inverse-distance weighting), indicated similar trends with significantly different clustering of high and low prevalence of both the *An. gambiae* M and S forms. These spatial trends were overlaid on a density distribution surface map shown in Fig. 4.9, highlighting the high to low patterns of each species.

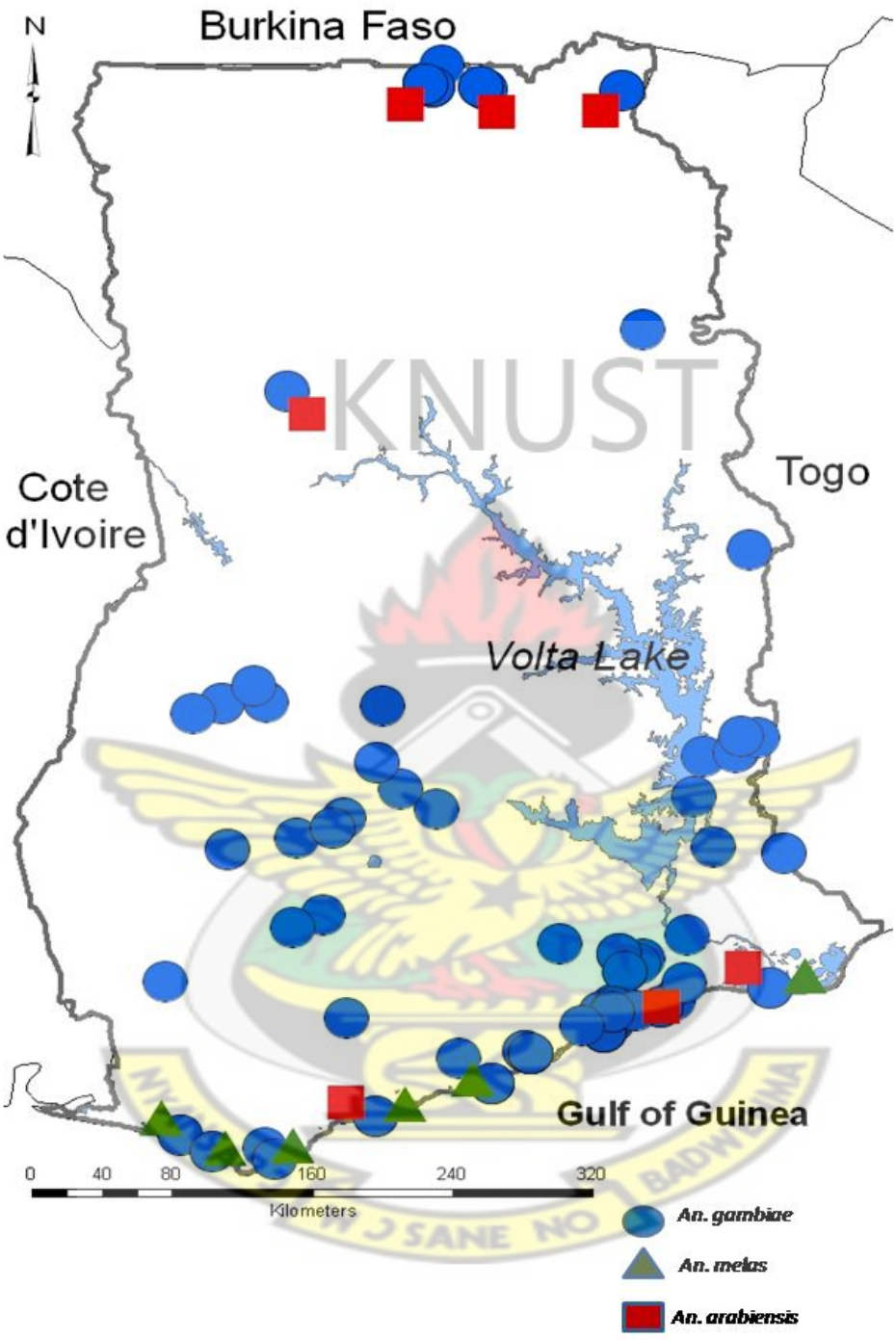


Figure 4.7: Distribution of the *An. gambiae* complex in Ghana. Map drawn in ArcGIS version 9

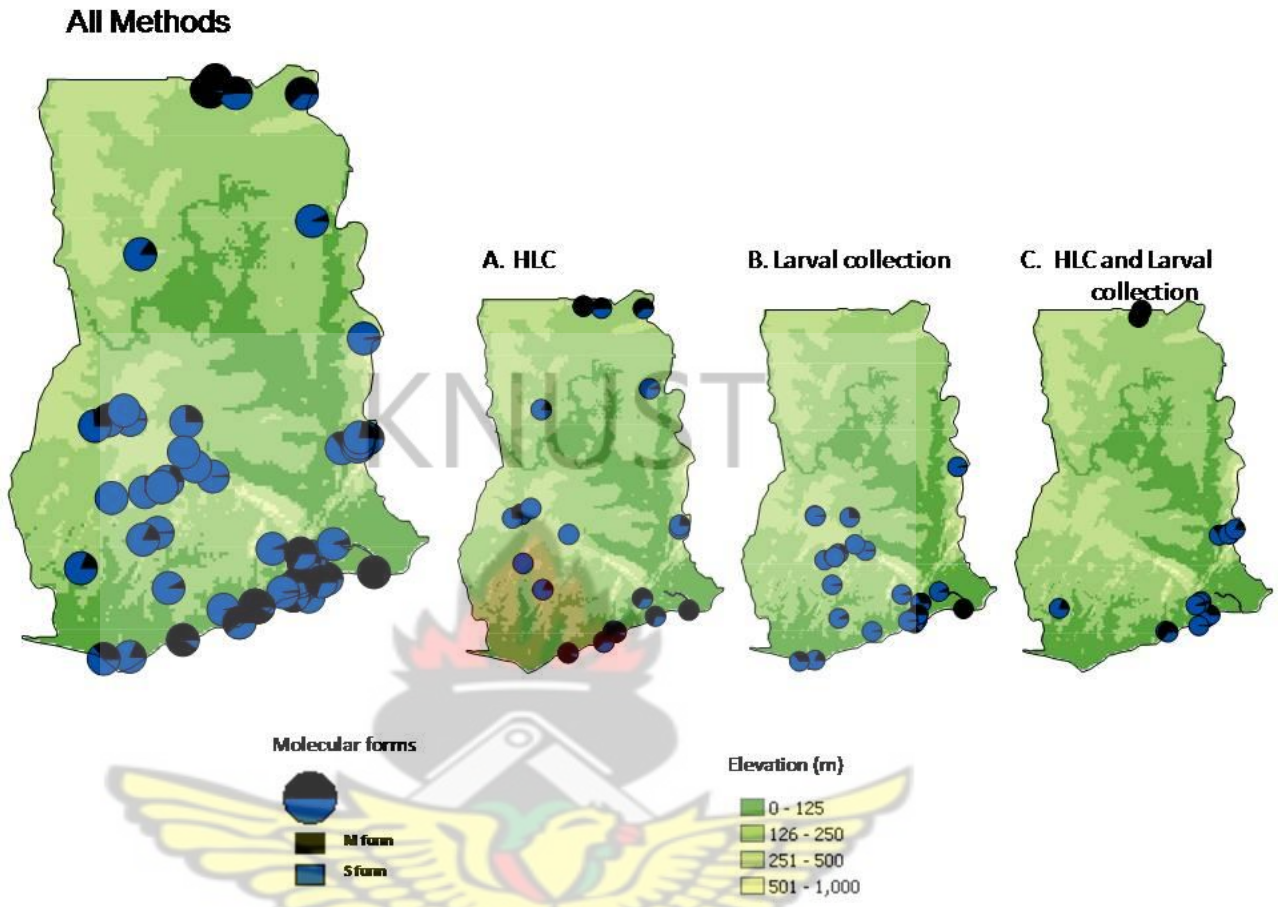


Figure 4.8: Distribution of the M and S Forms of the *An. gambiae* s.s, by collection methods from the various literature sources. Map drawn in ArcGIS version 9.

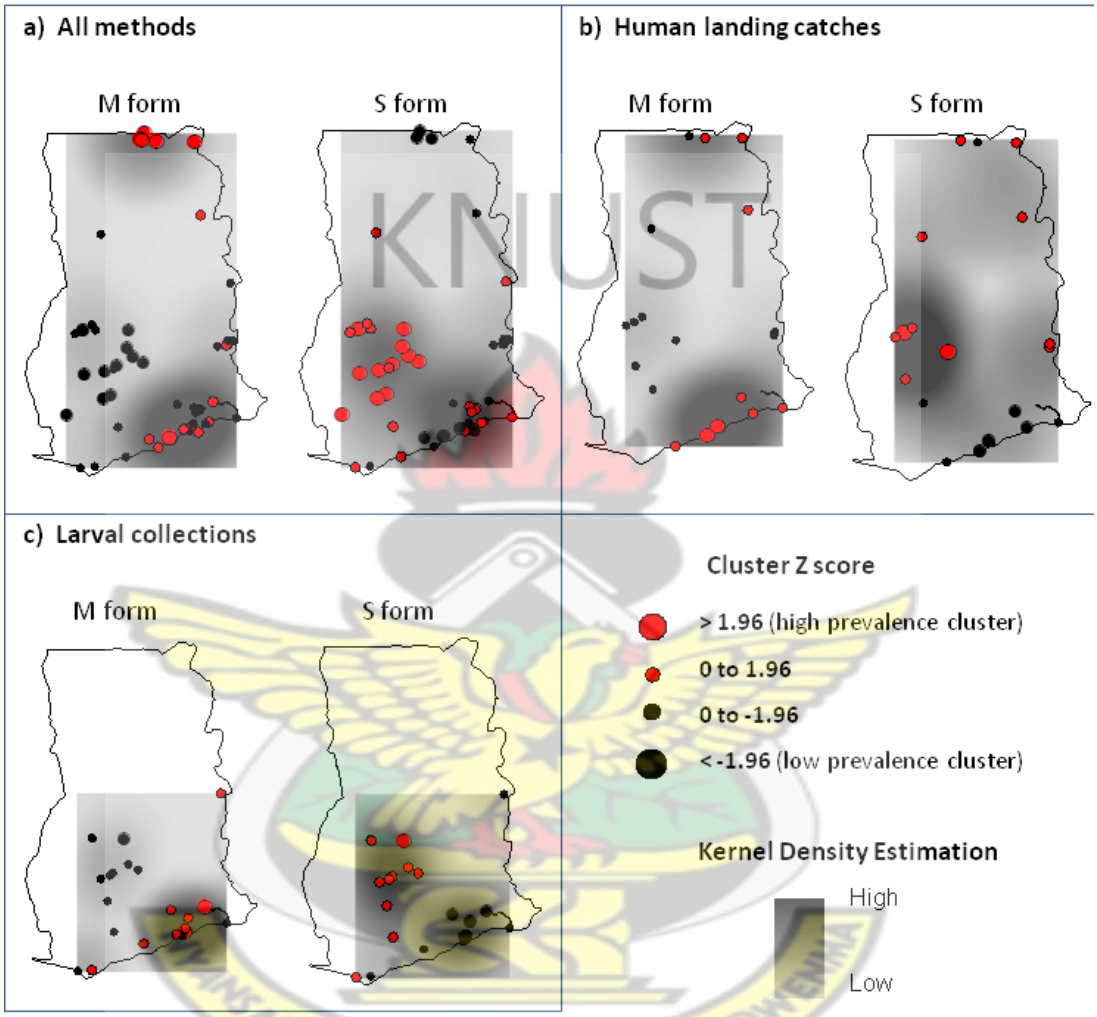


Figure 4.9: Spatial clustering trends and density distributions of *An. gambiae* s.s molecular forms. Maps drawn in ArcGIS version 9.

4.3.2 Environmental Analysis

The relationship between the prevalence of *An. gambiae* M and S forms, and the environmental variables are shown in Table 4.4a. Overall, bivariate correlation analysis indicated that the *An. gambiae* M form was significantly positively associated with temperature (r=0.51), and negatively with elevation (r= -0.28), precipitation (r=-0.33), and humidity (r=-0.26). This contrasts with the *An. gambiae* S form, which was found to be significantly negatively associated with temperature (r=-0.58), and positively with elevation (r=0.30) and rainfall (r=0.41). Interestingly, elevation, precipitation and temperature correlations increased when data were stratified by the two main collection methods, HLC and larval collections (Table 4.4a). Multiple regression analyses of all data (n=70), indicated that temperature was an important variable for both molecular forms, explaining for *An. gambiae* M, 28% (R_2=0.28, F=25.8, P≤0.001) and for *An. gambiae* S, 36% (R_2=0.36, F=37.9, P≤0.001) of the variance in the model (Table 4.5).

For each molecular form, comparisons of environmental measures between locations with significantly high and low prevalences, defined by positive Z scores (≥ +1.96) and negative Z scores (≤ -1.96) respectively, are shown in Table 4.6a. Overall, locations with high *An. gambiae* M prevalences had higher NDVI and temperatures, but lower elevation, precipitation and humidity measures than those locations with lower prevalences by these species and/or where the prevalence of *An. gambiae* S form was higher. Statistical comparisons indicated significant differences (*P* value <0.004

Bonferroni corrected) between elevation, precipitation and temperature for both *An. gambiae* M and S forms between the high and low prevalence areas.

To further explore the differences in elevation, precipitation and temperature, the mean prevalence of *An. gambiae* M and S forms was plotted across a range of environmental groupings (Fig. 4.10). *An. gambiae* M prevalences were found to be high at elevations of 0-50m, and where mean daily precipitation ranged between 1.0-2.5mm, and mean daily temperatures ranged between 26.1ºC - 27.6ºC. In contrast, *An. gambiae* S prevalences were found to be higher at elevations >50m, and where mean daily precipitation ranged between 2.6-3.8mm, and mean daily temperatures ranged between 24.5ºC -26.0ºC.

Table 4.4. Bivariate correlations between *An. gambiae* s.s. molecular forms and environmental and epidemiological variables.

a.) Environmental

	All Methods n=70		Human Landing Catch n=26		Larval Collection n=28	
	M form	S form	M form	S form	M form	S form
Elevation	-0.28*	0.30*	-0.54**	0.58**	-0.42*	0.51**
NDVI	-0.14	0.22	-0.18	0.19	-0.18	0.25
Rainfall	-0.33**	0.41**	-0.63**	0.75**	-0.39*	0.60**
Temperature	0.51**	-0.58**	0.61**	-0.72**	0.16	-0.42**
Humidity	-0.26*	0.08	-0.07	-0.10	0.19	-0.43

b.) Epidemiological

Variable	*An. gambiae* s.s	
	M form	S form
LF	0.46**	-0.48**
Malaria	-0.14	0.26*

*. Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Table 4.5: Multiple regression model for environmental variable predicting the presence of the *An. gambiae* M and S forms.

Species/predictor variable	Standardized Coefficient Beta	T statistic	P value
An. gambiae M (Constant)		-4.76	<0.0001
Temperature	0.527	5.08	<0.0001
An. gambiae S (Constant)		6.67	<0.0001
Temperature	-0.601	-6.16	<0.0001

Table 4.6. Comparison of mean environmental and epidemiological measures between high and low prevalence areas of *An. gambiae* s.s molecular forms

a.) Environmental

Variable	*An. gambiae* s.s			
	M Form		S Form	
	High	Low	High	Low
Elevation	147	276**	272	107**
NDVI	0.42	0.50	0.49	0.44
Precipitation	2.1	3.1**	3.1	2.0**
Temperature	27.0	24.9**	24.9	26.8**
Humidity	0.0161	0.0181	0.0181	0.0174

b.) Epidemiological

Variable	*An. gambiae* s.s			
	M Form		S Form	
	High	Low	High	Low
LF	20.0	2.4**	2.2	17.1**
Malaria	55.9	47.0	55.8	33.9

Note. High = Z score $\geq$+ 1.96, Low= Z score $\leq$-.1.96

** Significant at the 0.004 level after Bonferroni Correction.

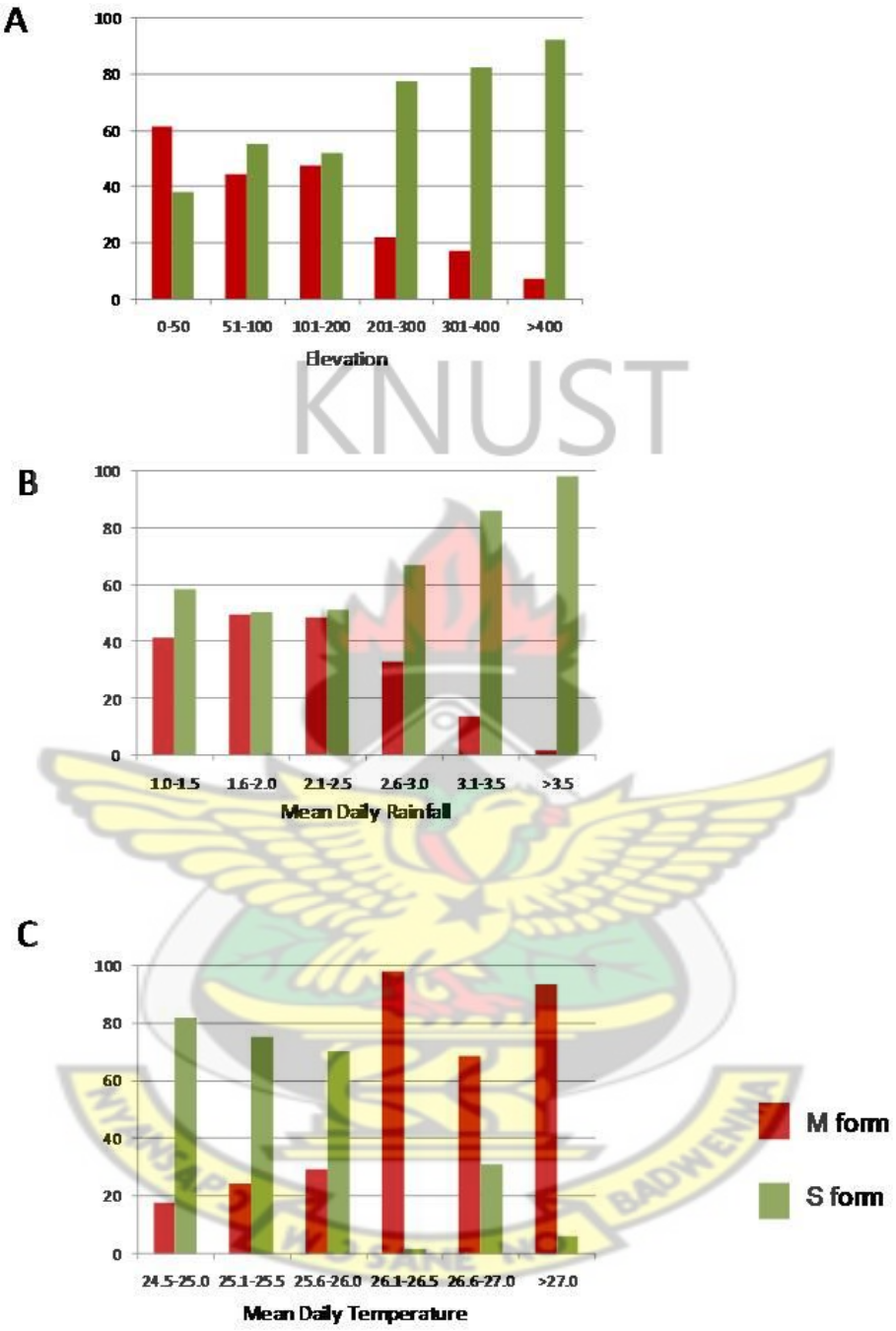


Figure 4.10: Mean prevalence of *An. gambiae* M and S forms plotted against elevation, precipitation, and temperature groupings

4.3.3 Disease Association

The relationship between the *An. gambiae* M and S, and LF and malaria prevalences were first examined using bivariate correlation analysis. Results shown in Table 4.4b, indicate that *An. gambiae* M was significantly positively associated with LF (r=0.46), while *An. gambiae* S form was significantly negatively associated with LF (r= -0.48), but positively with malaria (r= 0.26). Also, correlation analysis between the M and S forms, and each disease indicated significant negative associations between *An. gambiae* M and *An. gambiae* S (r=-0.76), and between LF and malaria prevalence (r=-0.41).

Second, disease prevalence between high and low *An. gambiae* M and S sites (as described above) were compared. Overall, locations with high *An. gambiae* M prevalences (Z scores $\geq$ +1.96), were found to have significantly higher LF prevalences (20%) than those locations with low prevalences (Z scores $\leq$-1.96), by these species (2.4%) and/or where the prevalence of *An. gambiae* S form was significantly high (2.2%). No significant differences were found between malaria prevalence and each mosquito species (Table 4.6b).

Finally, the LF data in Gyapong *et al*. (2002) was examined, and three main transmission zones were identified i.e. zero/low (< 1.0%, n=19), medium (1.0-10.0%, n=32) and medium/high (10.1-30.0%, n=19). Graphical presentation of these three

zones is included in Fig. 4.11, which summarizes mean entomological and environmental measures for the sites within each zone. In the medium/high LF transmission zone, the mean *An. gambiae* M prevalence (53.2%) and temperatures (26.8°C) were found to be significantly higher (+ 2SE), and *An. gambiae* S prevalence (25.6%) significantly lower, than those found in medium transmission (21.1%; 64.7%; 25.5°C) and low transmission (21.4%; 64.4%; 25.3°C) zones, for the M form, S form and temperature respectively. In the zero/low transmission zone, precipitation measures (2.83mm) were found to be significantly higher (+ 2 SE), than those in the medium transmission (2.34mm) to high transmission (1.99mm) zones (Fig. 4.11).

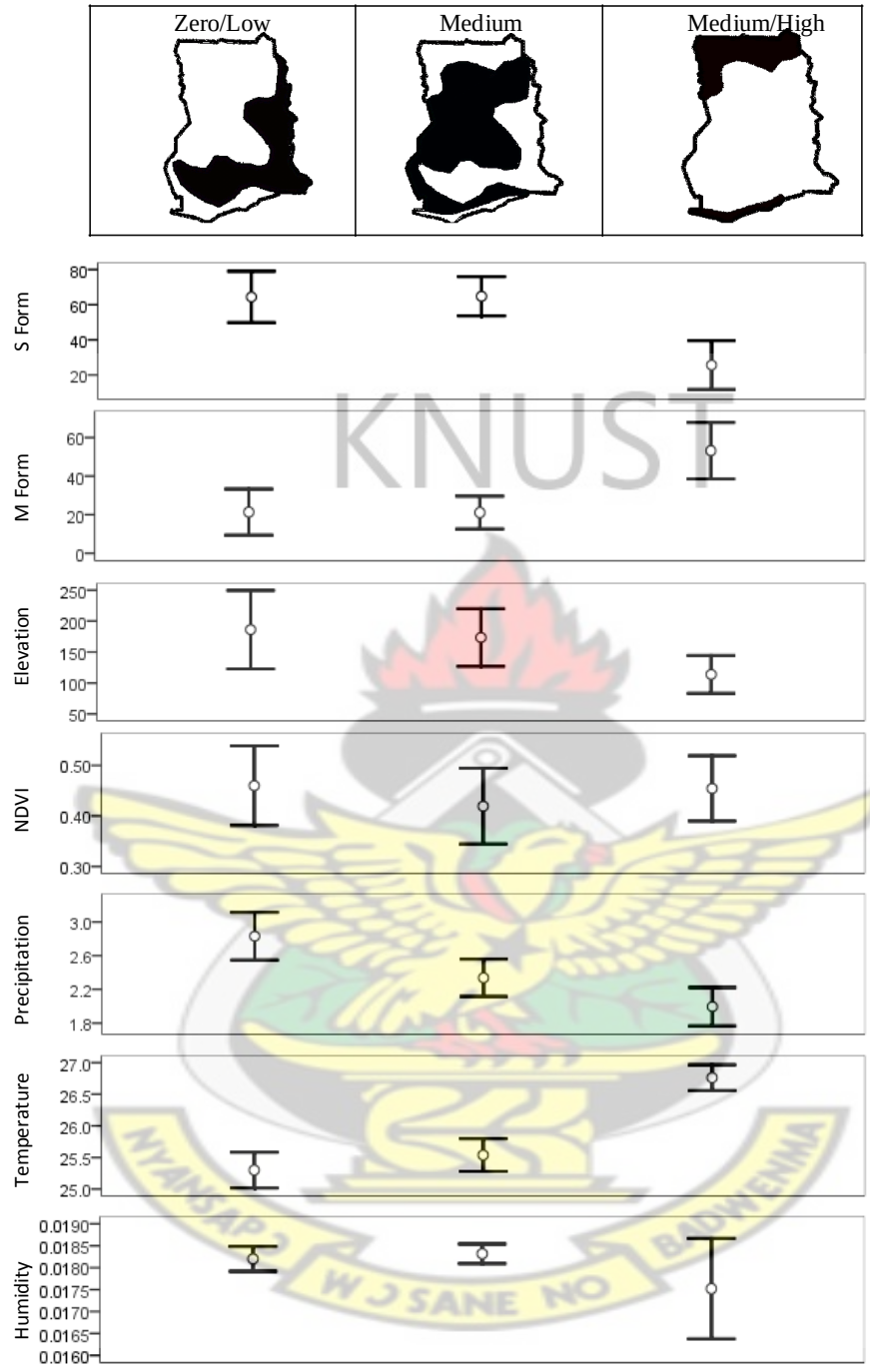


Figure 4.11: Summary of entomological and environmental variables in different LF transmission zones.

4.4 Analyses of *An. gambiae* Sequences

COI sequencing reactions were performed on 65 *An. gambiae* s.s and 5 *An. melas* samples. The obtained sequences were cleaned and the collection localities and other specimen information, have been entered in the 'Ghana Mosquitoes' file in the Project section of the Barcode of Life website (http://www.barcodinglife.org). Fig. 4.12 is a gel photograph of the COI amplification and Fig. 4.13 shows the electrophoregram of the sequence analysis. The sequence alignment was done in Bioedit (Fig. 4.14). The sequences obtained were also analyzed together with GenBank sequences from Nigeria (Accession numbers DQ465336-DQ465318) in order to identify differences in population structure between the two countries.

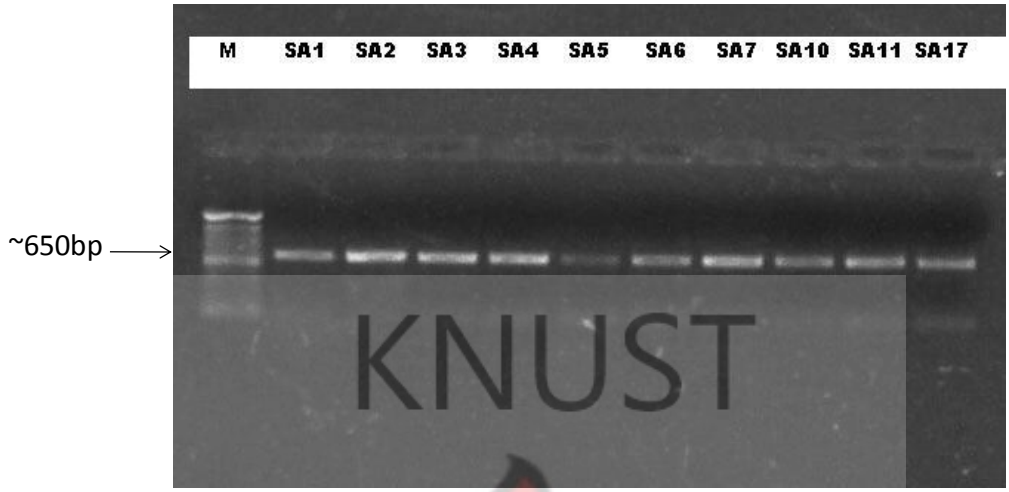


Figure 4.12: A gel image of COI PCR products for sequenced samples. All products amplified at approximately 650bp.

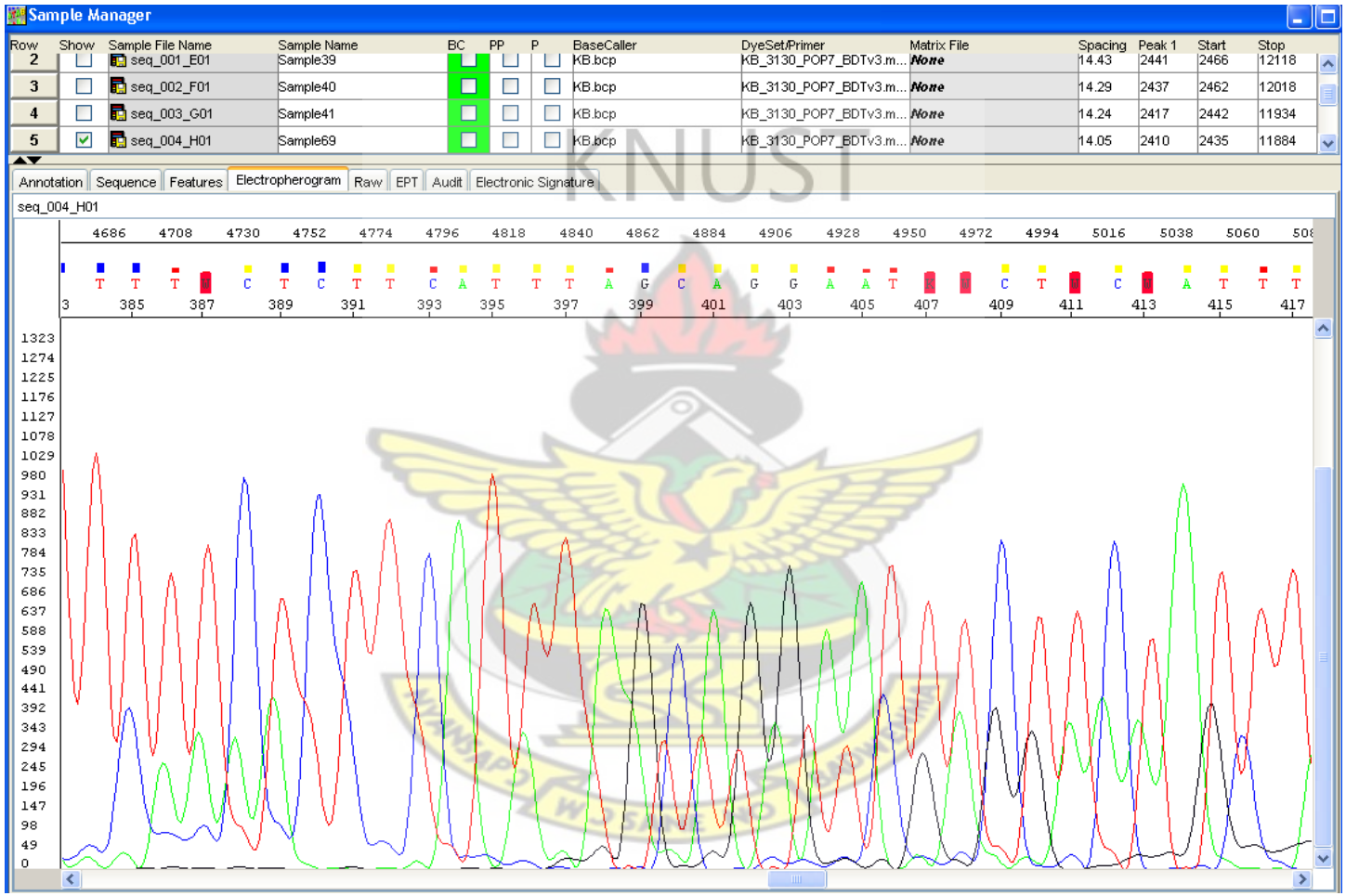


Figure 4.13: An electrophoregram of sequence analysis of *An. gambiae* samples

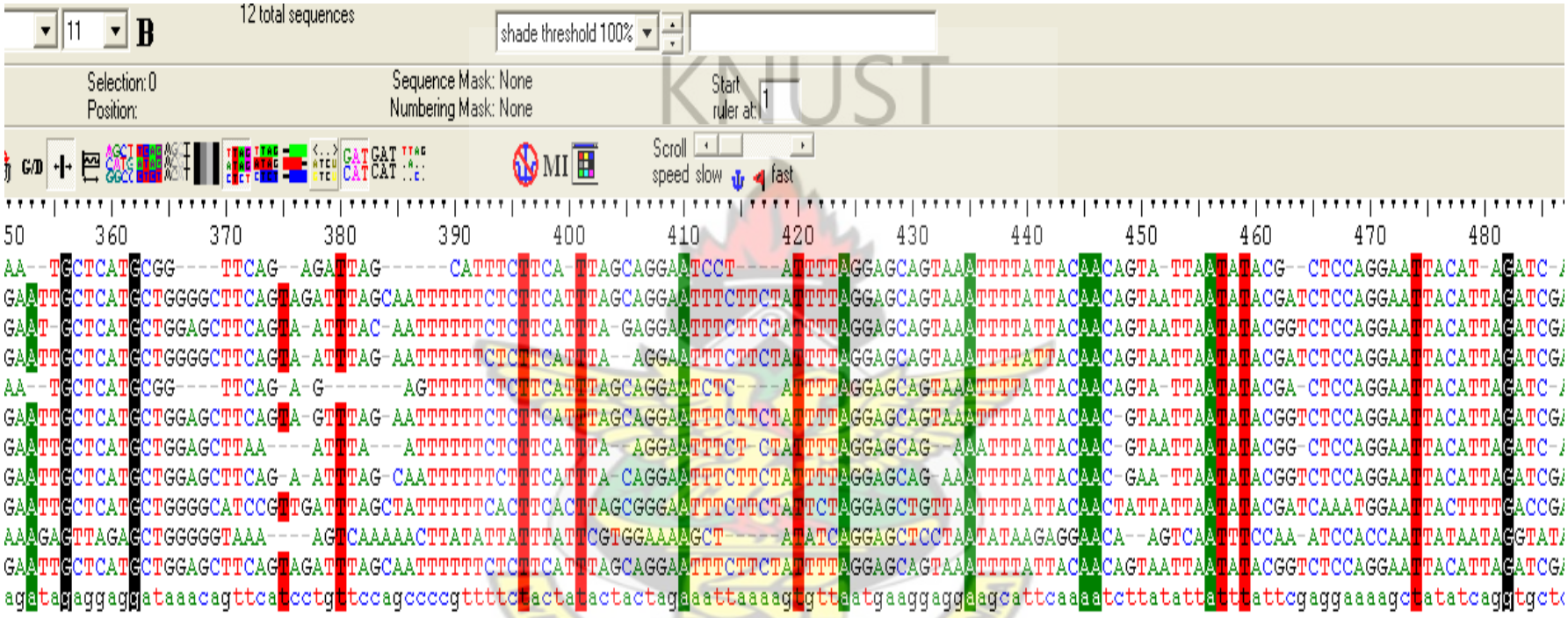


Figure 4.14: A snapshot of the alignment of *An. gambiae* sequences in BioEdit.

4.4.1 Nucleotide Usage Patterns

Sequence records for 70 members of the *An. gambiae* complex, from the study sites, were compared and analyzed. The CO1 sequences are A + T rich (average of 67.4% for all codons). The A + T nucleotide compositions for the first, second and third positions are 71.6%, 54.4% and 76.1% respectively, suggesting that based on the pattern of nucleotide usage; there is an A + T mutational bias within members of the *Anopheles gambiae* s.s. DNA sequences with high G + C content are more stable than those with low G + C content due to the 3 hydrogen bonds between Guanine and Cytosine, compared to the 2 hydrogen bonds between Adenine and Thymine.

Table 4.7 shows the Maximum Composite Likelihood Estimate of the pattern of nucleotide substitution using the method of Tamura and colleagues (Tamura *et al.*, 2004). The substitution matrices generated here show that the rates of substitution from a C or G to an A or T are always higher than rates of substitution in the opposite direction (e.g., the rate of C→T is higher than the rate of T→C, and so on). The nucleotide frequencies are 0.212 (A), 0.364 (T), 0.144 (C), and 0.28 (G). Since the frequency of T is greater than the frequency of A, the transversional rate that involves nucleotides A→T is greater than that of T→A. The transitional rates are far higher than the transversional rates and are more biased towards C→T and G→A. The transition/transversion rate ratios are k_1 = 2.04 (purines) and k_2 = 6.73 (pyrimidines). The overall transition/transversion bias is R = 1.817, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)].

Table 4.7: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

A	T	C	G
-	*5.67*	*2.24*	**8.89**
3.3	-	**15.09**	*4.36*
3.3	**38.15**	-	*4.36*
6.73	*5.67*	*2.24*	-

Note: Each entry in Table 4.7 shows the probability of substitution from one base (row) to another base (column) instantaneously. Only entries within a row should be compared. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). All calculations were conducted in MEGA 4 (Tamura *et al.*, 2007).

4.4.2 Phylogenetic Analyses

The consensus sequences from different communities were plotted to reveal the relationship between sequences (Fig. 4.15). Consensus sequences were generated for the various ecological zones. The Neighbourhood-Joining analysis based on the Kimura 2 Parameters of the sequences from ecological zones revealed ecological differentiations, with the Savannah cluster evolving from the more distant Forest group (Fig. 4.16A). Further classification, into M and S forms from the various environmental zones, showed the Forest S form to be the most distant form, out of which the other environmental branches evolved (Fig. 4.16b). To further test the ecological classification and isolation by distance, some of the sequences were tested with sequences from Nigeria (Fig. 4.17). The results revealed the Ghanaian and Nigerian sequences clustering separately.

Neighbourhood-Joining analysis based on the Kimura-2-Parameters of the COI consensus sequences from endemic and non-endemic areas were also performed (Fig. 4.18). *An. melas* was included as a third sequence since phylogenetic construction requires at least 3 sequences. The results revealed the endemic group of mosquitoes clustering together with *An. melas* which is also a very efficient vector of *W. bancrofti*. The equality of evolutionary rate between the consensus sequences from the endemic and non-endemic areas was tested using *An. melas* as an outgroup in Tajima' relative rate test (Tajima, 1993). The χ^2 test statistic was 3.71 (P = 0.054) at 0.05 confidence

interval, rejecting the null hypothesis of equal rates between lineages. The phylogenetic trees showed that the *An. gambiae* s.s mosquitoes separated into distinct clusters when analyzed according to ecological and endemic zones, implying an evolutionary relationship between the various clusters.

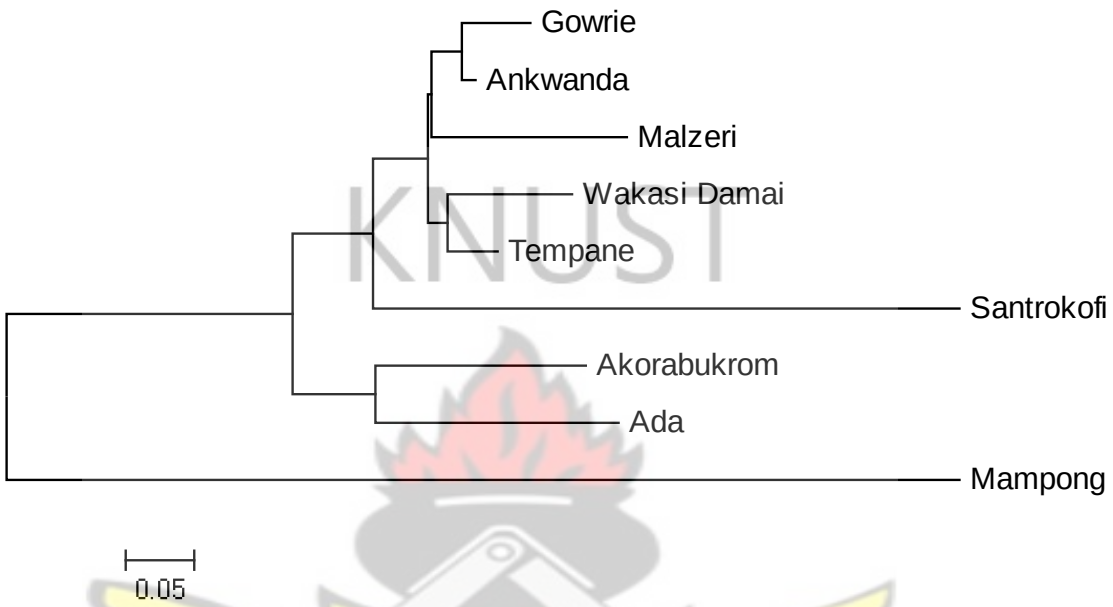


Figure 4.15: Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 *An. gambiae* s.s consensus sequences. Phylogenetic tree plotted in Mega 4 software.

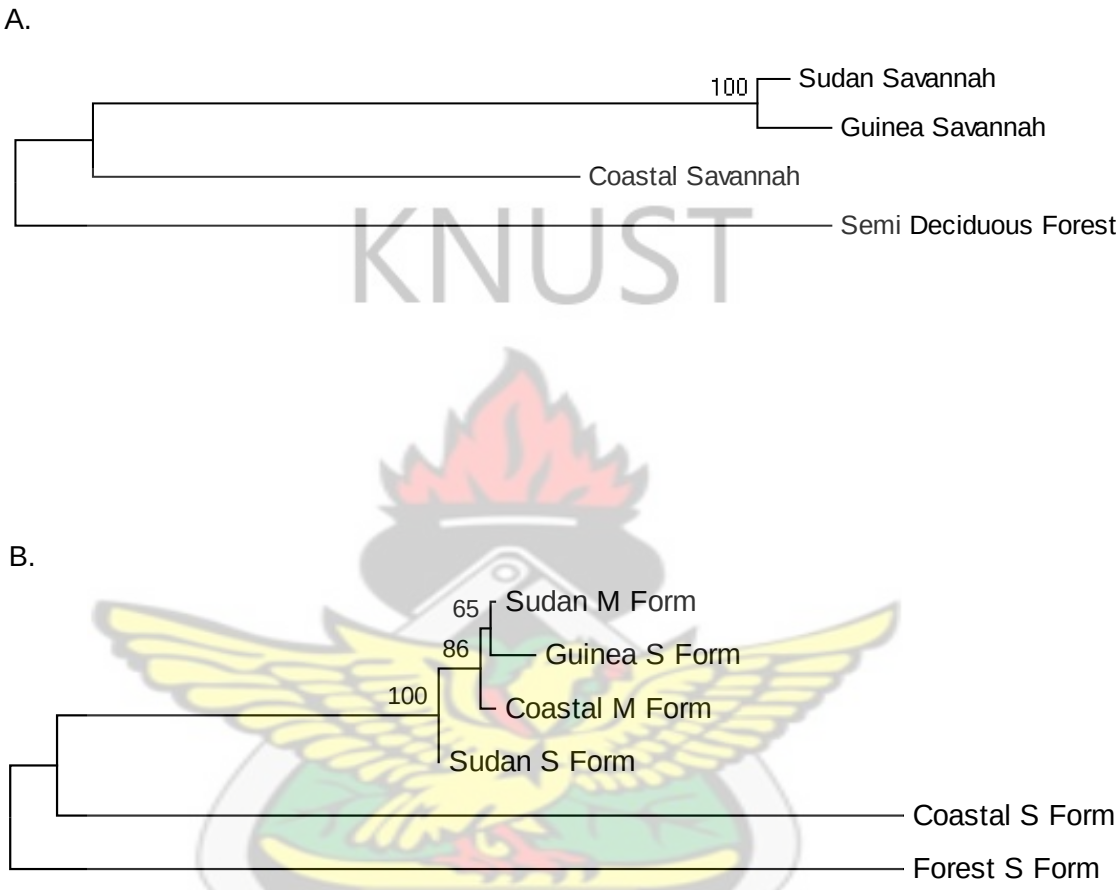


Figure 4.16: Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 *An. gambiae* s.s consensus sequences from different ecological zones. A. shows the relationship between zones and B. shows a further distinction in molecular forms between zones. Phylogenetic trees plotted in Mega 4 software.

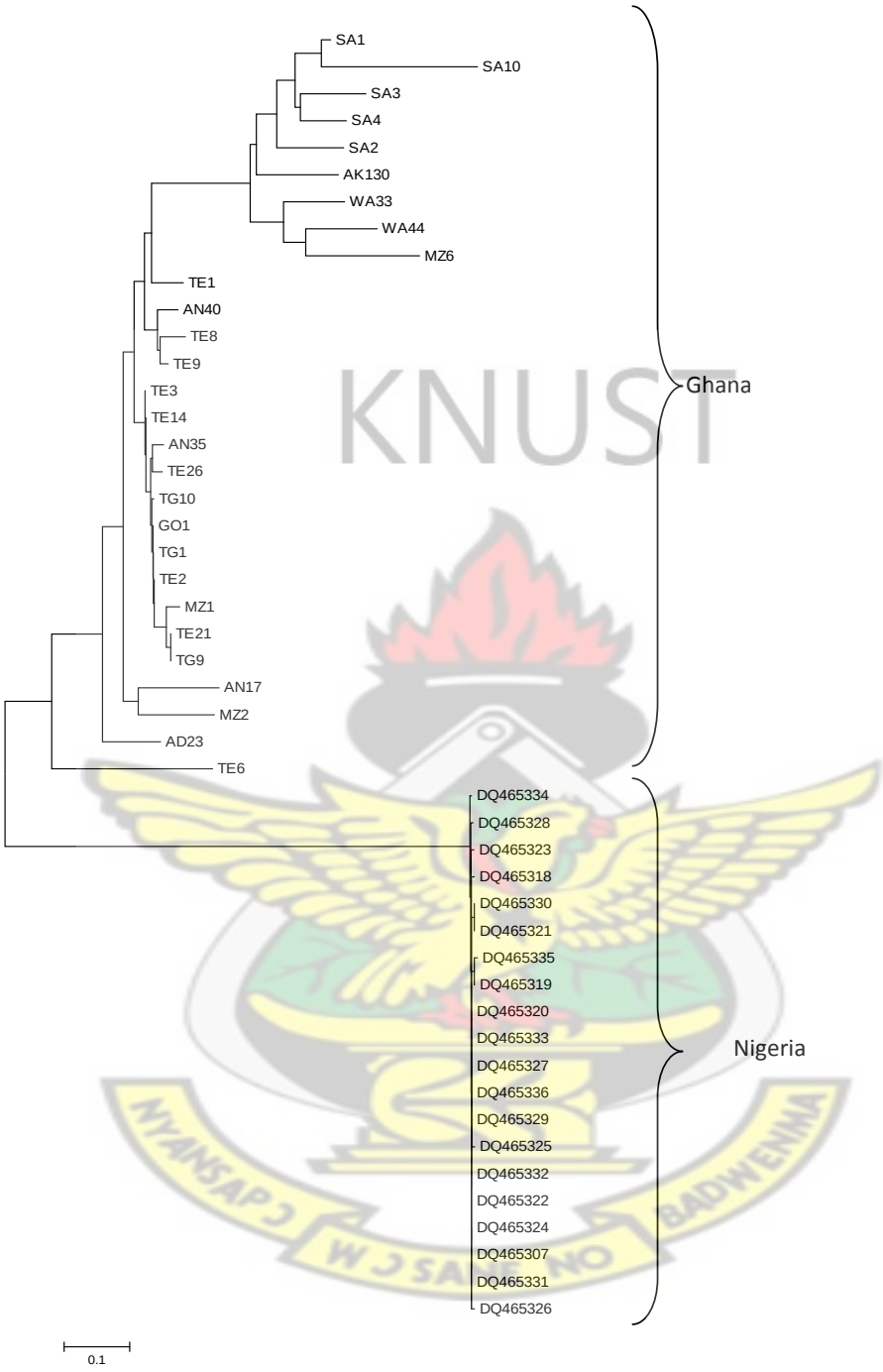


Figure 4.17: Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 *An. gambiae* s.s sequences from Ghana and GenBank sequences from Nigeria. Phylogenetic tree plotted in Mega 4 software.

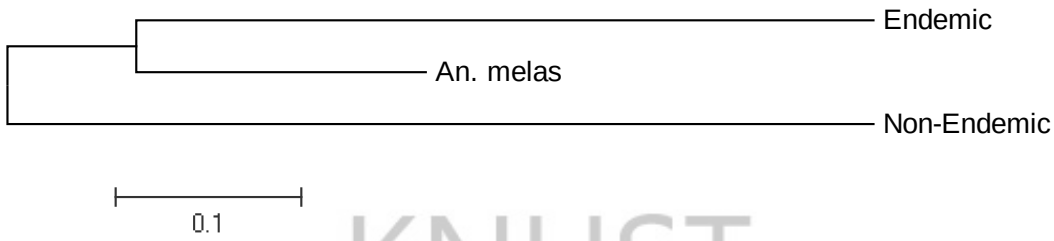


Figure 4.18: Neighbour-joining analysis of Kimura 2-parameter (K2P) distances of CO1 *An. gambiae* s.s sequences from LF endemic and non-endemic areas. Phylogenetic tree plotted in Mega 4 software.

4.5 Analyses of *W. bancrofti* Sequences

Seven specimens from Gomoa District and 15 from Bongo District were sequenced. The obtained sequences have been entered in '*Wuchereria bancrofti* from Ghana' file in the Project section of the Barcode of Life website (http://www.barcodinglife.org) and submitted to GenBank with accession numbers GQ479497-GQ479518.

The similarity index (degree of overlap) between the Northern and Southern consensus sequences was estimated to be 38.3%. This shows that the 2 sequences are considerably different. There is a smaller difference in nucleotide composition between the Northern samples, as shown by the mean nucleotide composition distance of 0.090, compared to that between the Southern samples (2.817). The mean evolutionary diversity between the two groups was 0.314 (SE=0.028) while the mean evolutionary diversity for the entire population was 0.363 (SE=0.038). Analysis for positive selection using the Fisher's Exact Test of Neutrality for Sequence Pairs (Zhang *et al*. 1997) showed no evidence of selection between sequence pairs. However, the Codon-based Test of Positive Selection between sequences, i.e. the probability of $D_N > D_S$, revealed a positive selection between two of the samples (WB 19 and WB 11) in the south (Z=2.073, p=0.02) at a confidence interval of 0.05. The equality of evolutionary rate between the consensus sequences from the North and the South was tested using *F. martis* as an outgroup in Tajima' relative rate test (Tajima, 1993). The χ^2 test statistic

was 7.44 (P = 0.00637) at 0.05 confidence interval, rejecting the null hypothesis of equal rates between lineages.

The phylogenetic analyses revealed samples from the North and South clustering together (Fig. 4.19). However, some samples from the South were closer to the Northern samples, and may have been the progenitors of the Northern population. Analysis using the Maximum Parsimony method revealed similar trends, but with higher bootstrap values (Fig. 4.20). The rooted phylogenetic tree using *F. martis* as an outgroup (Fig. 4.21) reveals the Southern samples to be the most distant population. Fig. 4.22 also shows the evolutionary relationship between the Ghanaian consensus sequences and other sequences from GenBank.

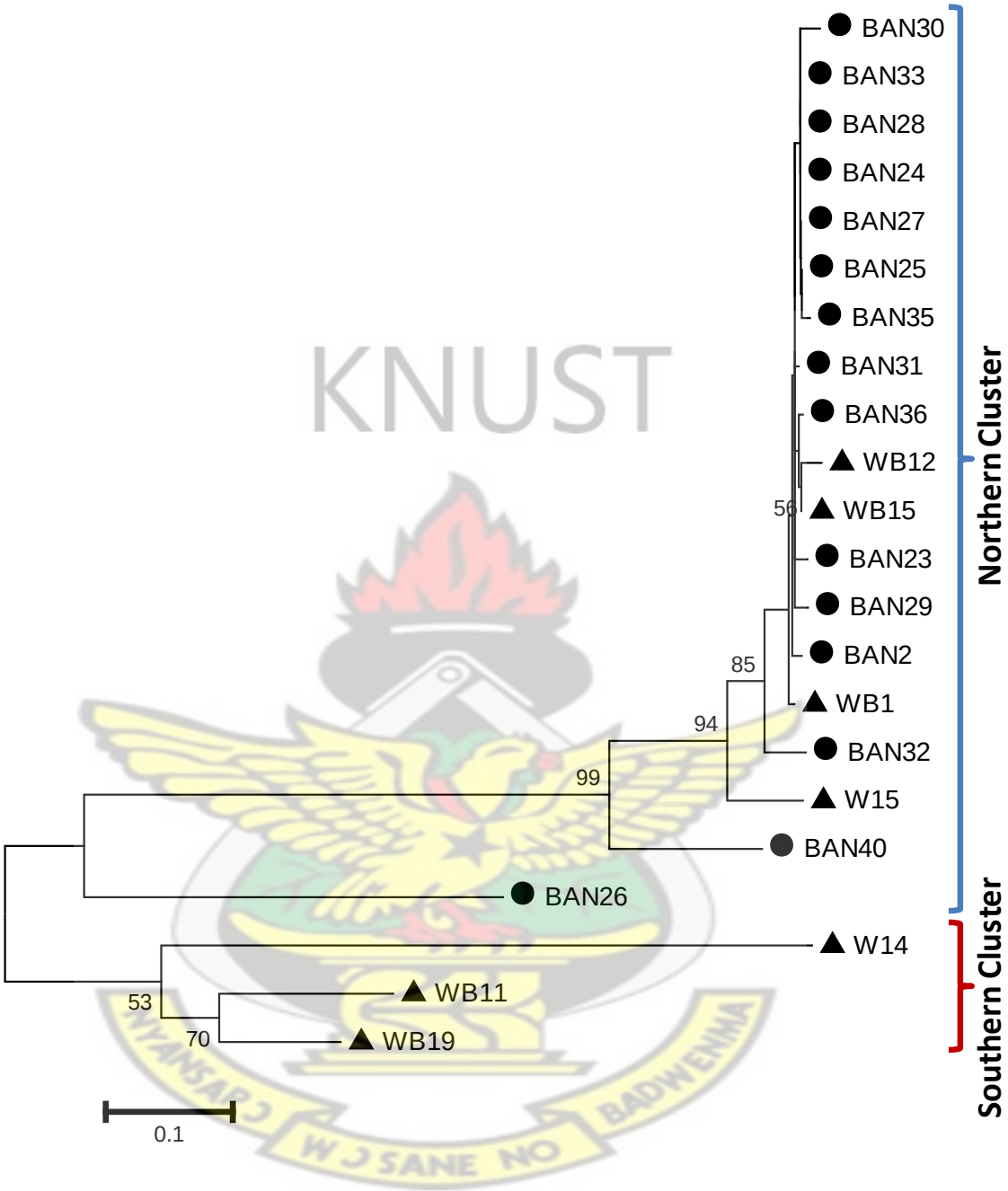


Figure 4.19: Neighbour-Joining tree based on analysis of Kimura 2-parameter (K2P) distances of CO1 *W. bancrofti* sequences from Northern and Southern Ghana. Triangles denote samples from Gomoa in the South and circles denote samples from Bongo in the North

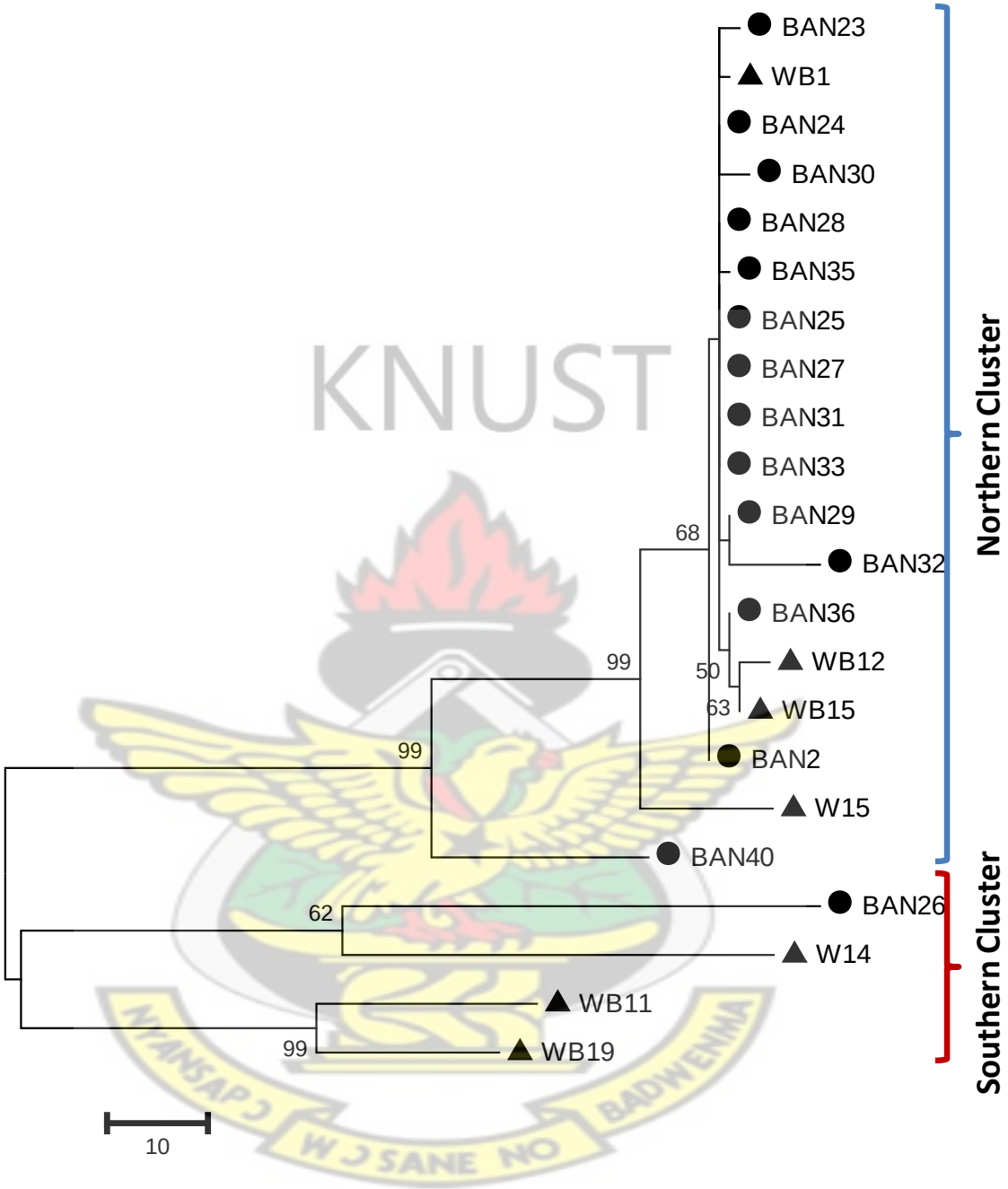


Figure 4.20: Phylogenetic tree inferred using the Maximum Parsimony method. The MP tree was obtained using the Close-Neighbor-Interchange algorithm in which the initial trees were obtained with the random addition of sequences (10 replicates). Triangles denote samples from Gomoa in the South and circles denote samples from Bongo in the North.

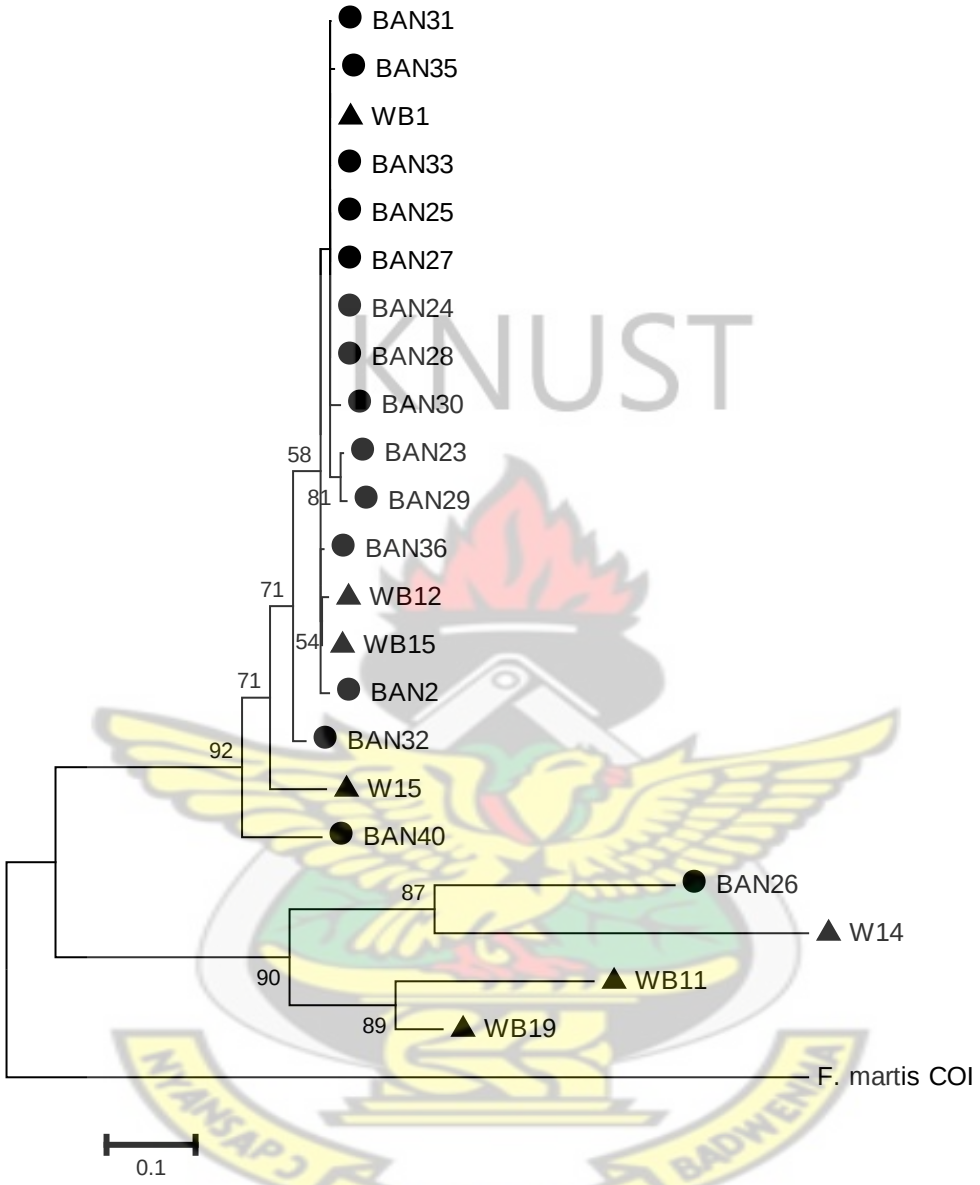


Figure 4.21: Neighbour-Joining tree based on analysis of Kimura 2-parameter (K2P) distances of CO1 *W. bancrofti* sequences from Northern and Southern Ghana, using *F. martis* as an outgroup. Triangles denote samples from Gomoa in the South and circles denote samples from Bongo in the North.

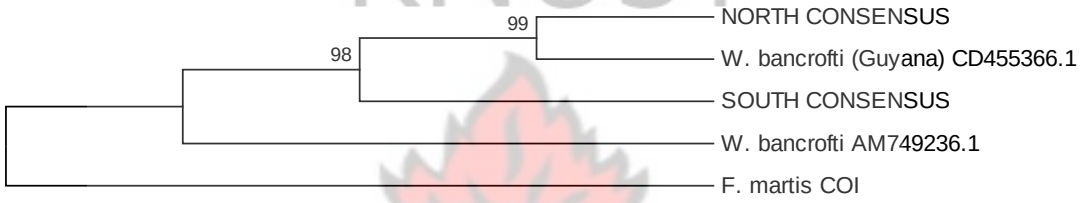


Figure 4.22: Neighbour-Joining tree based on analysis of Kimura 2-parameter (K2P) distances of CO1 *W. bancrofti* consensus sequences from Northern and Southern Ghana and other sequences from GenBank, using *F. martis* as an outgroup.

CHAPTER FIVE: DISCUSSION AND CONCLUSION

5.1 Vector Distribution and Disease Association

5.1.1 Mosquito Species and Biting Patterns of *Anopheles gambiae*

This is probably the first, biggest study to report on the distribution of mosquito species across Ghana. During the study, the predominant mosquito species was *An. gambiae* s.l (64.96%), followed by *Culex*, *Mansonia*, *An. funestus* and *Aedes* species (21.35%, 8.30%, 2.39% and 2.53% respectively). Other minor species such as *An. rufipes*, *An. pharaoensis* and *An. coustani* were also reported. Of the *An. gambiae* s.l, 3 sibling species were observed. These were the *An. gambiae s.s* (67.2%), *An. melas* (27.7%) and *An. arabiensis* (3.1%). While the *Anopheles* species are believed to be the major vectors of LF in West Africa (Appawu *et al*., 2001), all the other species observed are also potential vectors of LF (World Health Organization 2000) and their role in disease transmission in the different ecological areas remains to be determined. The effects of climate change on vectors of diseases may affect their susceptibility to infection. An example can be given of *Culex quinquefasciatus* species which have differed susceptibility to *W. bancrofti* from West Africa and East Africa (Kuhlow and Zielke, 1978, Maxwell *et al*., 1990, Appawu *et al*., 2001). Also, even though *Mansonia* species are vectors of *W. bancrofti* in parts of Asia but have not been identified as vectors in Africa, they are known to transmit the animal filaria *Dirofilaria immitis* and

D. repens in certain parts of Africa (Service, 1990). As such, it is important to constantly determine the susceptibility of these vectors in different areas.

The focus of this study, however, was on the *An. gambiae* and as such, the biting patterns were determined only for this species. The peak biting time for *An. gambiae* was observed to vary between sites. This however was not significant. The biting rates showed an increased night time peak between 11 pm and 5 am. As observed throughout Africa, the peak biting time of *Anopheles* mosquitoes occurred after 10 p.m. when most people are asleep (Taye *et al*., 2006). Ada, Ampain, Ankwanda, Mampong, Santrokofi and Tempane showed a normal biting pattern and peak biting time around 12 a.m. However, Akoraboukrom, Butre, Malzeri, Nabori, Nsokone and Wakasi Damai showed a peak biting time around 3 a.m., while Gowrie and Oseikokokrom had peak biting time at 5 a.m. The late biting behaviors and difference in peak biting time observed could possibly be interpreted as different *An. gambiae* populations exhibiting adaptations to their environment, such as the increased use of insecticide treated nets. A similar observation has been reported in Senegal (Ndiath *et al*., 2008).

5.1.2 General Distribution of *Anopheles gambiae* s.s

Most entomological studies on *An. gambiae* in Ghana, have focused on small area/district based collections (Appawu *et al*., 1994, Midega, 2001, Sibomana, 2002, Appawu, 2006, Tuno *et al*., 2010). The present study represents the first nationwide study aimed at identifying the distribution of the members of the *An. gambiae* complex,

the molecular form of the *An. gambiae* s.s, and finally the genetic variability within the *An. gambiae*. This study found the *An. gambiae* s.s as the predominant *Anopheles* species and confirms the reports from other studies (Appawu *et al*., 1994, Midega, 2001, Sibomana, 2002, Yawson *et al*., 2004, Appawu, 2006).

The distribution of the *An. gambiae* M and S forms varied significantly across the country. The two molecular forms were found sympatrically in most locations, except in some areas in the middle belt of the country where only the S form was observed, and in certain areas in the northern Savannah and coastal Savannah areas where only the M form was observed. A spatial cluster analysis, and resulting high Z score values in *An. gambiae* M and S form dominant areas, also showed that along the coast and in the northern Savannah, the *An. gambiae* M form was predominant and spatially clustered, while the S molecular form was most common and spatially clustered in the middle belt. The clustering remained relatively consistent irrespective of the different collection methods. This positive, spatial autocorrelation of the *An. gambiae* M and S forms indicates that their distribution is influenced by distinct environmental factors such as temperature and humidity, and habitat characteristics.

5.1.3 Effect of Environmental Factors on the Distribution and Density of the *Anopheles gambiae* M and S Forms

The distribution of *Anopheles* mosquito species has been shown to be dependent on environmental factors (Muturi *et al*., 2007, Yawson *et al*., 2007, Sogoba *et al*., 2008, Costantini *et al*., 2009) as well as on human population changes and anthropological effects, which may lead to land-use changes (Molyneux, 2003, Walsh *et al*., 1993). This current study shows the wide variability in distribution and abundance of the *An. gambiae* M and S forms, which appear to be driven by a range of environmental factors.

The *An. gambiae* M form is mostly found in the northern and southern Savannah areas, whereas the *An. gambiae* S form is mostly found in the middle forest belt of the country. The presence of the *An. gambiae* M form in the Savannah areas may be explained by the presence of permanent breeding conditions provided by irrigation facilities (Toure *et al*., 1998) and ponds of water resulting from rivers and run-offs since the M form is known to be associated with flooded areas. The S form on the other hand is heavily dependent on rainfall (Diabate *et al*., 2003, Yawson *et al*., 2004).

From the environmental analyses elevation, precipitation and temperature are important variables driving the spatial distribution of each molecular form, and the differences between them. In particular, temperature appears to be a key factor

determining the distribution of the M and S forms; probably due to influences on their reproduction as reported for *An. arabiensis* and *An. gambiae s.s* (Kirby and Lindsay, 2009). That the *An. gambiae* S form is dominant in the middle belt of the country may be due to the lower mean temperatures, highest recorded rainfall and mountainous topography all of which provide a cooler environment, conducive for the S form, and supported by Costantini and colleagues (Costantini *et al*., 2009). Observations on the predominance of the *An. gambiae* S form in a larger part of the country from this study support the suggestion that the *An. gambiae* S form, has broader environmental ranges and therefore is found in more locations than the M form (Esnault *et al*., 2008). On the other hand, higher temperature may lead to higher developmental rates and better physiological conditions in the M form as they are more prevalent and seemingly better adapted to higher temperatures, than the S form. As such, the *An. gambiae* M form shows a more latitudinal range in West Africa than the S form (Ndiath *et al*., 2008), being the most dominant form encountered in many studies undertaken in hot, arid regions of the Sudan-Savannah or Sahelian zones (Toure *et al*., 1998, Bayoh *et al*., 2001, della Torre *et al*., 2005, Costantini *et al*., 2009).

In this study, the density of the M and S forms was closely linked to their distribution. As such, higher proportions of each form were observed in the areas where they are mostly distributed. Variations in population densities of these two molecular forms have been observed in populations in Mali and Cameroon as well as between the various chromosomal forms in Mali (Slotman *et al*., 2007, Sogoba *et al*., 2008, Costantini *et al*., 2009).

5.1.4 *Anopheles gambiae* s.s and Disease Association

In the current study, malaria prevalence was positively associated with the *An. gambiae* S form, whilst high LF prevalence and high LF transmission zones were associated with the *An. gambiae* M forms. Other studies have suggested that the Mopti form of *An. gambiae s.s* is more associated with *W. bancrofti* than with malaria transmission (Hunter, 1992, Ijumba and Lindsay, 2001) and also that it is a relatively poor vector of malaria compared with other species such as the Savannah form of *An. gambiae* (Coluzzi, 1993, Carnevale *et al*., 1999). The reasons for this association may be due to vector-parasite interactions, vector competence of *Anopheles* species, agricultural practices and insecticide resistance. An analysis of the LF distribution map by Gyapong and colleagues (Gyapong *et al*., 2002), also indicates that the disease distribution in West Africa has the highest prevalence in the hotter Sudan/Sahel Savannah areas, which are also *An. gambiae* Mopti chromosomal form dominant areas (Bayoh *et al*., 2001, Kelly-Hope *et al*., 2006). Hence, the occurrence of high LF prevalence in hot, arid areas may be explained by the dominance of the *An. gambiae* M form, which prefers higher temperature areas.

5.2 *Anopheles gambiae* s.s Diversity

Analysis of nucleotide composition showed higher A+T composition in the *An. gambiae s.s.* The pattern of nucleotide usage suggests that there is an A+T mutational bias within members of the *An. gambiae* s.s, as is observed in other species of mosquitoes (Cywinska *et al*., 2006). Also, the A+T composition is lower in first- and second-position sites than in third-position sites. Furthermore, the asymmetric rates in nucleotide substitutions confirm that the high A+T composition is caused by an underlying mutational bias. 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. As such, this high A+T bias observed in the *An. gambiae* may be responsible for the observed differentiations in terms of chromosomal and molecular forms and reported speciation (Coluzzi *et al*., 1985, della Torre *et al*., 2005).

The observations from the phylogenetic analyses revealed clusters of *An. gambiae* s.s according to ecological zones (Fig. 4.16) and confirm the results of Yawson and colleagues, in Ghana (Yawson *et al*., 2007). This suggests that ecological barriers may act as important restrictions to gene flow. The existence of ecological differentiation may be due to environmental pressures such as the formation of the Dahomey Gap (DG) (Maley, 1999) during the Pleistocene era. During this era, the area

of the Guinea-Congo forest belt greatly reduced, leading to the formation of forest refugia and the formation of the DG. Thus, exposure to changing environmental conditions could predispose *Anopheles species* to selection pressures, and increase in diversity and speciation (Coluzzi *et al*., 1985, Lehmann *et al*., 1997, Kamau *et al*., 1998, della Torre *et al*., 2001, Michel *et al*., 2005) as reported in the *An. gambiae* populations in West Africa.

The observation of Ghanaian and Nigerian clusters from the phylogenetic analysis (Fig. 4.17) and the non-equality of evolutionary rates between the Ghana and the Nigeria populations, separated by at least 300km and the existence of the DG, provide further evidence for genetic differences based on distance and ecological zones. This observation is supported by a study in Nigeria which revealed significant levels of differentiation among *An. gambiae* populations, based on ecological zones (Matthews *et al*., 2007). Also, another study conducted in Mali concluded that the genetic difference at microsatellite loci was consistent with the isolation-by-distance model (Carnahan *et al.,* 2002) –where populations that live near each other are genetically more similar than populations that live further apart. As such, the formation of the DG between Ghana and Nigeria, through climatic events, may have resulted in the selection pressure, establishing the two different populations observed in this study and lead to the suggestion that the DG may be an important barrier to gene flow, establishing a division of the *An. gambiae* population. The present study is an initial step towards a phylogenetic guide for *An. gambiae* in West Africa based on COI sequences and additional sampling within the entire region may clarify the importance of the DG in shaping the evolution of the *An. gambiae* population in West Africa.

The phylogenetic analyses of the M and S forms of the *An. gambiae* s.s revealed that the S form is the most distant (parental) form (Fig. 4.16B). As such, this observation leads us to the hypothesis that '*the M form of the An. gambiae s.s may have evolved from the S form, as a result of environmental changes in West Africa*'. Thus, before the environmental changes that occurred during the late Pleistocene era (about 10,000 years ago) and which led to the reduction of the West African Rainforest, West Africa was densely forested (Maley, 1999), and may have only been populated with what is today called the S form of *An. gambiae* s.s. This may be confirmed by this and other studies (Bockarie *et al*., 1993, Yawson *et al*., 2004, Tuno *et al*., 2010) that reveal the S form to be the only species reported in forest areas. However, the climate effects and the formation of the DG may have led to an increase in temperature, which provided suitable environmental conditions for the evolution of a new form of the *An. gambiae* s.s, named the M form. Eventually, with current deforestation patterns, climate change and increase in temperature, the M form of the *An. gambiae* s.s became distributed throughout Ghana and other West African countries. This hypothesis may also be explained with the suggestion that the *An. gambiae* M form shows a more latitudinal range in West Africa than the S form (Ndiath *et al*., 2008), being the dominant form encountered in many studies undertaken in hot, arid regions of the Sudan-Savannah or Sahelian zones (Bayoh *et al*., 2001, Toure *et al*., 1998, della Torre *et al*., 2005, Costantini *et al*., 2009). As such, the *An. gambiae* M form is more prevalent and seemingly better adapted to higher temperatures, than the S form, as observed from the environmental analyses in the present study.

5.3 *Wuchereria bancrofti* Diversity

The data from this initial study indicates that the Southern samples could be more ancient than the Northern samples. This data provides some relevant information about the possible route of entry of the parasite into Ghana and its subsequent spread. It has been hypothesized that filarial parasite dispersed from somewhere in Southeast Asia about 3000 years ago to Africa, by infected seafarers (Laurence, 1989). Thus, *W. bancrofti* may have probably been introduced into Ghana and other Western African countries, by merchants who came in through the sea for commercial activities. Inland transportation could then have facilitated the spread and mixing up of *W. bancrofti* populations. Unfortunately, *W. bancrofti* COI sequences from Asia are not available, in order to compare and determine the similarity between regions.

The tree topology of the COI sequences analyzed revealed a cluster of the Northern and Southern samples, with few exceptions. This could be attributed to environmental selection pressures, especially when analyzed with respect to the evolutionary relationship between the consensus sequences and sequences from other parts of the world. Although in the present study a small number of samples were analyzed, the results reveal some level of genetic variability in *W. bancrofti* populations in Ghana, thus adding to the very few studies that aimed at understanding the genetic

heterogeneity of *W. bancrofti* (Kumar *et al*., 2002, Thangadurai *et al*., 2006, Nuchprayoon *et al*., 2007).

Various reasons could explain the differences between samples from both areas. The first could be a result of migration of microfilaremic individuals from the South to the North, for commercial activities. As such, migration being an important activity in the Northern Region could have resulted in the transfer of parasites from the South to the North. Another reason based on geoclimatic factors could also be suggested for the observed differences. A study in India reported high genetic divergence and varying gene flow between *W. bancrofti* populations and attributed these differences to geoclimatic factors and 50 years of antifilarial chemotherapeutical pressure on the parasite (Thangadurai *et al*., 2006). While, Ghana has only had about 10 rounds of MDA, the effects of chemotherapeutical pressures on *W. bancrofti* in Ghana may be very limited compared to the study in India. However, the samples from the Northern samples were collected from the Sudan Savannah zone while the Southern samples were collected from the Coastal Savannah zone. As such, differences in geoclimatic zones in Ghana may be responsible for the observed differences between the Northern and Southern samples.

The divergence of the Northern samples from the more distant Southern ones, may imply the development of biological factors (such as the development of tougher cuticles) that could explain the higher prevalences of LF symptoms observed in the

North compared to the South (Gyapong *et al*., 1996a). As observed for the forest and savannah forms of *Onchocerca volvulus* (Zimmerman *et al*., 1992), the geographic strains of parasites may be important in disease pathogenicity. As such, differing vector-parasite interactions based on these differences in parasite populations could be hypothesized, to explain the high LF prevalence in the North. One could also hypothesize that; the difference in LF prevalence between the north and south is due to the virulence of *W. bancrofti* as a result of sequence variations between the north and south populations. In fact, suboptimal treatment responses in *W. bancrofti* (Eberhard *et al*., 1991) and selection for Benzimidazole resistance in Ghana and Burkina Faso (Schwab *et al*., 2005) have been reported. As such, *W. bancrofti* populations with these characteristics will produce higher disease prevalences. There is therefore the need for more detailed studies; comparing sequence diversity in *W. bancrofti* populations to Ivermectin/Albendazole treatment failures and drug resistance markers.

5.4 Combined Influence of Vector and Parasite Diversity on LF Distribution in Ghana

The distribution of LF in Ghana by Gyapong and colleagues (1996a) corresponds to the distribution of the vectors, as observed in this study. The present study revealed a significant genetic difference between endemic and non-endemic groups of *An. gambiae* s.s. These differences in the vectors may predispose them to defects, such as the absence of cibarial teeth (McGreevy *et al*., 1978, Lowrie, 1991). For example, *Aedes* species exhibit the phenomenon of limitation (Pichon, 2002), due to the absence of cibarial teeth (McGreevy *et al*., 1978, McGreevy *et al*., 1982). Recent work, done in the Gomoa district of Ghana has shown that the number of cibarial teeth differed significantly between the M and S forms of the *An. gambiae* s.s., with the M form having a less number of teeth (Amuzu *et al*., 2010). This observation may therefore explain the high prevalence of LF in *An. gambiae* M form dominant areas, since less number of teeth means more parasites could pass through with less damage. As such, the observed distribution pattern of LF in Ghana may be influenced by the vector-parasite interactions. Also, unlike malaria which is transmitted by one vector genus (*Anopheles*), LF is a disease transmitted by many insect genera (World Health Organization 2002). However, these insects though present in all countries, may be refractory to *W. bancrofti*, depending on the different geographical areas. This, thus, raises the question about the diversity of the different species in various geographic locations. As an example, the current distribution of LF also poses questions as to why

Culex species are refractory in West Africa but are the main vectors in East Africa (Appawu *et al*., 2001, Subramanian *et al*., 1998, World Health Organisation, 2002). Thus, this study proposes that divergent vector and vector-parasite evolution due to major environmental changes may be responsible for the observed refractoriness. *Culex* unfortunately is not a forest type mosquito and the history of the West African vegetation, in relation to natural and anthropogenic processes, may play a significant role in the refractoriness of *Culex* in West Africa. Similarly, this "*An. gambiae - W. bancrofti"* evolution/co-adaptation may also explain the observed LF distribution in Ghana.

The observed distribution of LF in Ghana could also be explained in terms of the parasite strain and its compatibility with the vectors. The sequence differences in *W. bancrofti* from this study may suggest that the geographic strains of *W. bancrofti* could enhance or limit their survival in the vectors. In a susceptibility study by Jayasekera and colleagues, *Culex quinquefasciatus* strains from Liberia had a low susceptibility to *W. bancrofti* from Liberia, while the same strain had a high susceptibility to *W. bancrofti* from Sri Lanka (Jayasekera *et al*., 1980). Consequently, the study concluded that the Liberian and Sri Lankan strains of *W. bancrofti* differed in their ability to infect specific mosquito strains. This observation may be explained by genetic differences in *W. bancrofti* which may enhance or limit its survival in the vector, and therefore, affecting the vector-parasite interactions, through various mechanisms. An example can be the possession of tougher cuticles, enhancing their permeability through the stomach walls and passage through the cibarial teeth, without much damage.

5.5 Conclusion and Recommendations

This thesis sets out to explain the distribution of LF in Ghana which is characterized by a pattern of endemicity, separated by a continuous band of non-endemicity. To achieve this objective, the study explored the impacts of diversity in the *An. gambiae* s.s (the major vector of lymphatic filariasis) and *W. bancrofti* on the distribution of the disease.

It was observed that the distribution of the *An. gambiae* M and S forms varied significantly across the country, even though they were sympatric in most locations. Spatial cluster analysis revealed a positive, spatial autocorrelation of the *An. gambiae* M and S forms with their geographic location, and indicated that their distribution is influenced by distinct environmental factors and habitat characteristics. Multiple regression analysis revealed temperature as a key factor influencing the distribution of the two forms, and explained the dominance of the M form in the Savannah zones and the S form in the middle Forest belt.

Analyses of disease prevalence with the molecular forms of the *An. gambiae s.s,* indicated that high LF prevalence and high LF transmission zones were significantly associated with the *An. gambiae* M form. An analysis of the LF distribution with environmental factors also indicated a correlation with temperature. The disease distribution in Ghana and West Africa has the highest prevalence in the hotter Sudan/Sahel Savannah areas, which are also *An. gambiae* M form dominant areas. This

could be due to the influence of temperature on vector species and hence on the disease. Further analysis of the evolutionary relatedness among the *An. gambiae* s.s sequences revealed also clusters along ecological zones. This ecological differentiation may be due to environmental pressures and suggests that ecological barriers may act as important restrictions to gene flow (Yawson *et al*., 2007). Analyses of the M and S forms of the *An. gambiae* s.s also revealed that the S form could be the parental form. Finally, there was also phylogenetic grouping between *An. gambiae* s.s collected in LF endemic and non-endemic areas.

Analysis of *W. bancrofti* diversity populations of the North and the South showed evidence of significance between the two populations. The results also showed that the Southern samples could be the progenitors of the Northern samples and provide relevant information about the possible route of entry of the parasite into Ghana and its subsequent spread. The differences in the two populations could explain the higher prevalence of LF symptoms observed in the North compared to the South (Gyapong *et al*., 1996a), but requires further studies. As observed for the forest and savannah forms of *Onchocerca volvulus* (Zimmerman *et al*., 1992), the geographic strains of parasites may be important in disease pathogenicity.

A limitation to this study is the small number of sites involved in the analysis of the *An. gambiae* M and S distribution. This coupled with their uneven distribution between the southern half and the northern half of the country and the use of previously modeled LF and malaria data could introduce potential bias in the analyses. Undertaking spatial statistical analysis with a larger dataset in the future, and further modeling their distributions through ecological niche modeling (Costantini *et al*., 2009,

Levine *et al*., 2004, Stockwell and Peters, 1999) could further enhance our understanding of the distribution of the disease and its vectors, as well as defining the spatial limits of the vectors' distribution which is crucial for disease control. This would, however, require a constant update of the vector database generated in the present study as well as collection of data from remote areas of the country where no available data exists. Also, further studies on vector-parasite associations are required to determine the importance of the M and S forms of the *An. gambiae* s.s in disease transmission.

In this study, analysis of COI mitochondrial DNA has revealed further phylogenetic differences within *An. gambiae* s.s in different regions of Ghana. Considering that both karyotypes and rDNA alone could not satisfactorily describe the *An. gambiae* population over its entire range, further studies aimed at determining markers of differentiation in the COI that could be used to better study the phylogeny and population structure of *An. gambiae* will be needed in order to understand the mechanisms of reproductive isolation and speciation as well as their consequences for evolution in the *An. gambiae*. A larger sample size from within the West African Region – which has the largest number of ecological zones, will be needed in order to shed light on the genetic relationship and our understanding of the population structure of *An. gambiae* s.s in the region.

Sequencing of *W. bancrofti* samples also provided information about the diversity in the population in Ghana. Though this study cannot conclusively determine whether the Northern and Southern populations are different in terms of physiological responses and the effects on disease epidemiology, the observed differences do provide

a baseline for further investigations. In order to further explore the results observed in this study, it is recommended that further studies with a much larger sample size and more detailed analyses be conducted on the genetic variability of *W. bancrofti* populations. Studies encompassing parasites from different geo-climatic regions will further enhance our understanding of *W. bancrofti* diversity as well as vector-parasite co-adaptations.

In conclusion, this study revealed that the distribution of LF in Ghana is influenced by the diversity in the *An. gambiae* s.s and *W. bancrofti*. The diversity in the *An. gambiae* s.s is affected by the ecological zones and temperature is the major environmental factor affecting the distribution of the M and S forms of the *An. gambiae* s.s. Together, the diversity in *An. gambiae* s.s and *W. bancrofti* may have bearings on the vector-parasite interactions affecting the LF transmission dynamics in the various endemic areas in Ghana.

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APPENDIX I: IRB Approval for the study

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
INSTITUTIONAL REVIEW BOARD

(UNIVERSITY OF GHANA)

Phone: +(233) 21 500374 /501178
Fax: +(233) 21 502182
Email: Director@noguchi.mimcom.net
Telex No. 2556 UGL GH

P.O. Box LG581
Legon
Ghana

My Ref. No: DF.22

4th July, 2007

Your Ref. No:

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824

NMIMR-IRB CPN 046/06-07

On 4th July, 2007 the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting approved the protocol titled.

TITLE OF PROTOCOL : **Species Diversity in Lymphatic Filariasis Vectors and the Potential for Targeted Vector Control in Maintaining Biodiversity.**

INVESTIGATORS : **Professor Daniel Adjei Boakye**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 3rd July, 2008. You are to submit annual reports for continuing review.

Signature of Chairman: .....
Rev. Dr. Samuel Ayete-Nyampong
(NMIMR – IRB, Chairman)

cc: Professor Alexander K. Nyarko, PhD
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon.

APPENDIX II: Information sheet and consent form

INFORMATION SHEET

Information sheet for parents/individuals participating in the "Species diversity in lymphatic filariasis vectors and the potential for targeted vector control in maintaining biodiversity" study.

Introduction: This consent form informs you about the background, aims and the method of this study. In addition it explains the anticipated benefits, potential risk of the study and the discomfort it may entail. Finally it informs you of inclusion criteria and your rights regarding participating in this study.

Purpose of the research: The Global Programme for the Elimination of Lymphatic Filariasis (GPELF) is based on a strategy of MDA with albendazole and DEC or ivermectin. The idea is to reduce the parasite load in the human host thereby preventing transmission. A target of 80% coverage of the population at risk for at least 5 years has been proposed. This ideal is not always achievable because of programmatic issues of drug distribution, and the perpetual threat of drug resistance. Even if this level of coverage is achieved, the biodiversity of vectors and their differing abilities to transmit low level parasitaemia may lead to a failure to stop transmission in some regions, In view of the above, vector control is now considered an important and integral part to achieve elimination in specific areas where MDA alone will not provide the solution.

In the areas where vector control needs to be implemented, an integrated vector management (IVM) strategy targeted at the major vectors may need to be adhered to and coordinated with MDA to give the best results at least cost. To achieve this, it is necessary to recognize the biodiversity in the mosquito vectors, have reliable identification of vector species, insights into vector behaviour and an understanding of the biology and ecology of specific vectors

A database of vector species, including their attributes and distribution, is lacking in most LF endemic areas including Ghana. Some information does exist in various publications and archival systems, such as Ministry of Health reports, but is not in a format that can be easily accessed by those involved in the management of disease control programmes. It is essential to collate this information as well as to collect new information to provide a data package for effective control when needed. The focus of this proposal therefore is to identify and characterise the bio-diversity of LF vectors across Ghana, create a database relative to vector diversity and apply Geographic Information System to vector studies for effective control and monitoring.

The total duration of the study is two years.

Procedure: There will be screening for microfilaraemia in the population. 100µl of finger prick blood per subject will be mixed with 900µl 3% acetic acid so that the number of microfilariae could be determined by microscopy. 20 adults (all of whom will provide their informed consent) will be selected as blood meal sources. Each village will be divided into four sections and a house per section selected randomly for overnight mosquito collection using the human-

landing catch method from 1800 hours till 0600 hours the following morning. A separate house will also be selected from each section for collection of indoor resting mosquitoes by pyrethrum spray catch method during the hours of 0600 hours to 0800 hours. There will be two collections per village every three months during the first year. This is to cover the dry and rainy seasons and thus identify any seasonal variations that may be present. The overnight mosquito catches will be kept in ideal conditions for morphological identification and dissection the next morning. All mosquitoes identified as *An. gambiae* s.l. and *An. funestus* s.l. will be stored dry for later species identification by PCR.

Risks and Discomfort: There is no major risk associated with your participation in this study apart from the bite you may get from the mosquitoes and the discomfort you may experience when you or your ward is pricked for the drawing of blood. The amount of blood to be taken is very small and will not result in any damage to you.

Benefits: Any person with infection will be treated free of charge by the project. There will be no direct benefit for subjects' participation in this study. However, the main benefit of your participation is indirect as you will help us understand the diversity of vectors in the transmission of the parasite. At the end of the study, you will be contributing significantly to the design of programs aimed at eradicating the disease in your community.

Incentives: You will not be provided any incentive other than refreshment if you take part in this study. This will be provided after the exercise.

Confidentiality: Your records will be kept in a secure location at NMIMR. All information collected during the study will be stored in a file which will not have your name on it, but a study number assigned to it. Only the research team will have access to the names associated with the study numbers and for special reasons such as treatment. It is likely that data obtained from tests done on you may be published in medical journals; however, your identity will not be disclosed.

Your Right to refuse or withdraw: You have the right not to take part in the study if you do not want to, and this will not affect you or your ward in any way. During the study you may stop from participating in the study at any time. Refusal to participate in or withdraw from this study will not have any penalties or loss of benefits that you may be entitled to. Your position in the community will also not be affected in any way, even if you decide to stop from participating in the study.

Contact information: You may ask any questions about the study now or later. For any further information you may contact any of the following:

Rev. Dr. Samuel Ayete-Nyampong

(Chairman, Institutional Review Board/Ethics Committee)

C/o NMIMR

P.O.Box LG 581, Legon

Tel: +233 20 8152360

Prof. Daniel Adjei Boakye

(Principal Investigator)

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Prof. Michael David Wilson

Deputy Director

NMIMR

P.O.Box LG 581, Legon

Tel: 021 501178/79

Email: mwilson@noguchi.mimcom.org

Consent Form

I have read this consent form. I have received satisfactory answers to my questions. I understand that my participation is voluntary. I know about the purpose, methods, risks and possible benefits of the research study to judge that I want to participate. I consent voluntarily to allow my ward to participate in this study. I understand that I have the right to withdraw from the study at any time, and I know that I can call on any member of the study team if I have any questions or concerns.

Name of participant_____

Parent's Name _____ Signature of participant/parent _____

Witness Name _____ Signature of witness _____

Primary Investigator Signature _____

Date _____ Place _____

(Study ID number assigned to this participation) _____

APPENDIX III: Mosquito records database

LOCALITY	LAT	LONG	YEAR	CATCH	SPECIES	ID	No.	REFERENCE
MADINA	5.4035600	-0.1020200	2004		GAMBIAE	12	30	ABABIO K. GRACE 2004 MPHIL UNIV. OF GHANA MEDICAL SCHOOL
OKYEREKO	5.2485400	-0.3616700	2003	PSC	GAMBIAE	127	386	ANDERSON BENJAMIN 2003 HND SCIENCE LAB TECH, ACCRA POLY
BEWADZE			2003	PSC	GAMBIAE	32	386	ANDERSON BENJAMIN 2003 HND SCIENCE LAB TECH, ACCRA POLY
KORLE BU	5.3238700	-0.1420300	2005		GAMBIAE	30	60	SEDZIAFA L. MAWULI 2005 HND SCIENCE LAB TECH, ACCRA POLY
AIRPORT	5.59772	-0.18588	2005		GAMBIAE	30	60	SEDZIAFA L. MAWULI 2005 HND SCIENCE LAB TECH, ACCRA POLY
KND	10.89250	-1.14353	2004	PSC	GAMBIAE	304	349	FREMPONG K. KWADWO 2004 BSC ZOOLOGY UNIV. OF GHANA
OKYEREKO	5.2485400	-0.3616700	2003	HLC	FUNESTUS	405	8233	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
BEWADZE			2003	HLC	FUNESTUS	221	660	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
OKYEREKO	5.2485400	-0.3616700	2003	HLC	PHARAONENSIS	415	8233	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
BEWADZE			2003	HLC	PHARAONENSIS	7	660	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
OKYEREKO	5.2485400	-0.3616700	2003	HLC	COUSTANI	2	8233	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
OKYEREKO	5.2485400	-0.3616700	2003	HLC	GAMBIAE	602	626	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
BEWADZE			2003	HLC	GAMBIAE	196	202	OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
OKYEREKO	5.2485400	-0.3616700	2003	HLC	GAMBIAE			OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
BEWADZE			2003	HLC	GAMBIAE			OKOYE P. NKEM 2003 MPHIL UNIV. OF GHANA
CENTRAL REGION			2006	HLC	FUNESTUS	112	1271	CLEMENT ISAAC 2006 MPHIL UNIV. OF GHANA
CENTRAL REGION			2006	HLC	GAMBIAE	3	126	CLEMENT ISAAC 2006 MPHIL UNIV. OF GHANA
CENTRAL REGION			2006	HLC	MELAS	123	126	CLEMENT ISAAC 2006 MPHIL UNIV. OF GHANA
GOMOA DISTRICT			2005	HLC	GAMBIAE	30	32	KWANSA-BENTUM BETHEL 2005 MPHIL UNIV.OF GHANA
GOMOA DISTRICT			2005	HLC	FUNESTUS	267	286	KWANSA-BENTUM BETHEL 2005 MPHIL UNIV.OF GHANA
MADINA	5.4035600	-0.1020200	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4035600	-0.1020200	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4035600	-0.1020200	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4035600	-0.1020200	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
LEGON	5.3575300	-0.1088800	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
NEW ACHIMOTA	5.3714400	-0.1406600	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
WHITE CROSS	5.3865900	-0.1426500	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
HWIDA	5.1545700	-0.4801300	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
OKYEREKO	5.2485400	-0.3616700	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MUUS	5.3904700	-0.1528500	2002	Larval	GAMBIAE	1	1	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
DOME	5.3980500	-0.1426700	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
WEST LEGON	5.3928700	-0.1249900	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4036700	-0.1025400	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4046600	-0.1025100	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
UNIV. OF GHANA	5.3875900	-0.1122600	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4043900	-0.1026400	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA

DZORWULU	5.3704900	-0.1170400	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
KORLE BU	5.3238700	-0.1420300	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
KORLE BU	5.3238700	-0.1420300	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
KORLE BU	5.3238700	-0.1420300	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
KORLE BU	5.3238700	-0.1420300	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
LEGON	5.3573400	-0.1090600	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
ADENTA	5.4242800	-0.0882600	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
ADENTA	5.4243500	-0.0880000	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
ACHIMOTA	5.3697100	-0.0359400	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
DOME	5.3980000	-0.1427300	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
LEGON	5.3575600	-0.1089000	2002	Larval	GAMBIAE	7	7	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
MADINA	5.4015300	-0.0963900	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
GBC	5.3473500	-0.1084000	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
LEGON	5.3575000	-0.1087500	2002	Larval	GAMBIAE	10	10	SIBOMANA ISAIE 2002 MPHIL UNIV. OF GHANA
KPONE	5.68333	0.06667	2006	HLC, PSC	GAMBIAE	480	1209	POUMO TCHOUASSI DAVID 2006 MPHIL UNIV. OF GHANA
KORLE BU	5.53719	-0.22727	2005	Larval	GAMBIAE	90	90	ACHONDUH OLIVIA 2005 MPHIL UNIV. OF GHANA
AIRPORT RES. AREA	5.59772	-0.18588	2005	Larval	GAMBIAE	90	90	ACHONDUH OLIVIA 2005 MPHIL UNIV. OF GHANA
ADABRAKA (HOHOE)	7.0988100	0.2851400	2005	Aspirators/Larval	GAMBIAE	42	50	KAHINDI C. SAMUEL 2005 MPHIL UNIV. OF GHANA
ATABU NEWTOWN	7.0815300	0.2881500	2005	Aspirators/Larval	GAMBIAE	46	50	KAHINDI C. SAMUEL 2005 MPHIL UNIV. OF GHANA
KLEDZO	7.0735300	0.2752300	2005	Aspirators/Larval	GAMBIAE	33	50	KAHINDI C. SAMUEL 2005 MPHIL UNIV. OF GHANA
LIKPE	7.0979900	0.3531300	2005	Aspirators/Larval	GAMBIAE	38	50	KAHINDI C. SAMUEL 2005 MPHIL UNIV. OF GHANA
DODOWA	5.8833333	-0.1166667	2004		GAMBIAE	106	143	OTIENO JOHN OGECHA 2004 MPHIL UNIV. OF GHANA
OKYEREKO	5.4166667	-0.6	2004		GAMBIAE	37	143	OTIENO JOHN OGECHA 2004 MPHIL UNIV. OF GHANA
TONO			2001	PSC	GAMBIAE	402	464	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-516
TONO			2001	PSC	ARABIENSIS	62	464	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-517
VEA			2001	PSC	GAMBIAE	562	618	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-518
VEA			2001	PSC	ARABIENSIS	56	618	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-519
AZOKA			2001	PSC	GAMBIAE	306	356	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-520
AZOKA			2001	PSC	ARABIENSIS	50	356	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-521
TONO			2001	PSC	FUNESTUS	254	1534	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-522
VEA			2001	PSC	FUNESTUS	471	2504	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-523
AZOKA			2001	PSC	FUNESTUS	48	862	APPAWU ET AL., 2001 TROP. MED. INT. HEALTH 6(7): 511-524
KND	10.89250	-1.14353	2002	HLC	GAMBIAE	711	728	APPAWU ET AL., 2004 TROP. MED. INT. HEALTH 9(1):164-169
KND	10.89250	-1.14353	2002	HLC	ARABIENSIS	17	728	APPAWU ET AL., 2004 TROP. MED. INT. HEALTH 9(1):164-170
WEIJA	5.5722500	-0.3368600	2004	Aspirators	GAMBIAE	50	50	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
ADA FOAH	5.7847200	0.6271300	2004	Aspirators	GAMBIAE	100	100	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
SUHUM	5.9100000	-0.4000000	2004	Aspirators	GAMBIAE	50	50	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM

ASUTUARE	5.9808600	-0.0545500	2004	Aspirators	GAMBIAE	50	50	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
TWIFU PRASO	5.6400000	-1.5400000	2004	Aspirators	GAMBIAE	50	50	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
ESIAM	5.3900000	-0.9700000	2004	Aspirators	GAMBIAE	50	50	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
NKWANTA	8.2599400	0.5216900	2004	Aspirators	GAMBIAE	100	100	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
HOHOE	7.3743600	0.5316000	2004	Aspirators	GAMBIAE	114	114	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
AXIM	4.8663000	-2.2387000	2004	Aspirators	GAMBIAE	90	100	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
AXIM	4.8663000	-2.2387000	2004	Aspirators	MELAS	10	100	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
AGONA NKWANTA	4.8881000	-1.9643000	2004	Aspirators	GAMBIAE	100	100	BOAKYE ET AL., 2004 REPORT TO THE GHANA MALARIA CONTROL PROGRAM
KPONE	5.68333	0.06667	2007	HLC/ Larval	GAMBIAE	151	160	FREMPONG K. KWADWO 2007 MPH SCHOOL OF PUBLIC HEALTH, UNIV. OF GHANA
KPONE	5.68333	0.06667	2007	HLC/ Larval	ARABIENSIS	9	160	FREMPONG K. KWADWO 2007 MPH SCHOOL OF PUBLIC HEALTH, UNIV. OF GHANA
HWIDA	5.25000	-0.80000	2007	HLC	GAMBIAE	24	67	AMUZU HILARIA 2008 MPHIL UNIV. OF GHANA
HWIDA	5.25000	-0.80000	2007	HLC	MELAS	40	67	AMUZU HILARIA 2008 MPHIL UNIV. OF GHANA
HWIDA	5.25000	-0.80000	2007	HLC	FUNESTUS	5	72	AMUZU HILARIA 2008 MPHIL UNIV. OF GHANA
MAMPONG	5.4333333	-0.6333333	2007	HLC	GAMBIAE	280	290	AMUZU HILARIA 2008 MPHIL UNIV. OF GHANA
MAMPONG	5.4333333	-0.6333333	2007	HLC	FUNESTUS	42	332	AMUZU HILARIA 2008 MPHIL UNIV. OF GHANA
ADA	5.78472	0.62713	2008	HLC	GAMBIAE	168	195	DE SOUZA ET AL., 2008 UNPUBLISHED
ADA	5.78472	0.62713	2008	HLC	MELAS	25	195	DE SOUZA ET AL., 2008 UNPUBLISHED
ADA	5.78472	0.62713	2008	HLC	ARABIENSIS	2	195	DE SOUZA ET AL., 2008 UNPUBLISHED
AMPAIN	4.95620	-2.40160	2008	HLC	GAMBIAE	3	201	DE SOUZA ET AL., 2008 UNPUBLISHED
AMPAIN	4.95620	-2.40160	2008	HLC	MELAS	193	201	DE SOUZA ET AL., 2008 UNPUBLISHED
ANKWANDA	5.06667	-1.40000	2008	HLC	GAMBIAE	98	194	DE SOUZA ET AL., 2008 UNPUBLISHED
ANKWANDA	5.06667	-1.40000	2008	HLC	MELAS	96	194	DE SOUZA ET AL., 2008 UNPUBLISHED
ANKWANDA	5.06667	-1.40000	2008	HLC	ARABIENSIS	5	201	DE SOUZA ET AL., 2008 UNPUBLISHED
BUTRE	4.82180	-1.91600	2008	HLC	GAMBIAE	0	100	DE SOUZA ET AL., 2008 UNPUBLISHED
BUTRE	4.82180	-1.91600	2008	HLC	MELAS	100	100	DE SOUZA ET AL., 2008 UNPUBLISHED
GOWRIE	10.85956	-0.83927	2008	HLC	GAMBIAE	139	172	DE SOUZA ET AL., 2008 UNPUBLISHED
GOWRIE	10.85956	-0.83927	2008	HLC	ARABIENSIS	33	194	DE SOUZA ET AL., 2008 UNPUBLISHED
HWIDA	5.25	-0.8	2008	HLC	GAMBIAE	13	18	DE SOUZA ET AL., 2008 UNPUBLISHED
HWIDA	5.25	-0.8	2008	HLC	MELAS	5	18	DE SOUZA ET AL., 2008 UNPUBLISHED
KPONE	5.6833333	0.0666667	2008	HLC	GAMBIAE	10	10	DE SOUZA ET AL., 2008 UNPUBLISHED
MALZERI	9.50439	-0.02847	2008	HLC	GAMBIAE	32	32	DE SOUZA ET AL., 2008 UNPUBLISHED
MAMPONG	5.4333333	-0.6333333	2008	HLC	GAMBIAE	61	61	DE SOUZA ET AL., 2008 UNPUBLISHED
NABORI	9.15311	-1.85256	2008	HLC	GAMBIAE	86	87	DE SOUZA ET AL., 2008 UNPUBLISHED
NABORI	9.15311	-1.85256	2008	HLC	ARABIENSIS	1	100	DE SOUZA ET AL., 2008 UNPUBLISHED
NSOKONE	7.4960700	-2.02551	2008	HLC	GAMBIAE	83	83	DE SOUZA ET AL., 2008 UNPUBLISHED
OSEIKOKOKROM	6.13036	-1.82328	2008	HLC	GAMBIAE	166	166	DE SOUZA ET AL., 2008 UNPUBLISHED
SANTROKOFI	7.2102900	0.47448	2008	HLC	GAMBIAE	47	47	DE SOUZA ET AL., 2008 UNPUBLISHED

APPENDIX IV: Publication

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PLoS one

Environmental Factors Associated with the Distribution of *Anopheles gambiae* s.s in Ghana; an Important Vector of Lymphatic Filariasis and Malaria

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Abstract

Anopheles gambiae s.s mosquitoes are important vectors of lymphatic filariasis (LF) and malaria in Ghana. To better understand their ecological aspects and influence on disease transmission, we examined the spatial distribution of the *An. gambiae* (M and S) molecular forms and associated environmental factors, and determined their relationship with disease prevalence. Published and current data available on the *An. gambiae* species in Ghana were collected in a database for analysis, and the study sites were georeferenced and mapped. Using the *An. gambiae s.s* sites, environmental data were derived from climate, vegetation and remote-sensed satellite sources, and disease prevalence data from existing LF and malaria maps in the literature. The data showed that *An. gambiae* M and S forms were sympatric in most locations. However, the S form predominated in the central region, while the M form predominated in the northern and coastal savanna regions. Bivariate and multiple regression analyses identified temperature as a key factor distinguishing their distributions. *An. gambiae* M was significantly correlated with LF, and 2.5 to 3 times more prevalent in the high LF zone than low to medium zones. There were no significant associations between high prevalence *An. gambiae* s.s locations and malaria. The distribution of the *An. gambiae* M and S forms and the diseases they transmit in Ghana appear to be distinct, driven by different environmental factors. This study provides useful baseline information for disease control, and future work on the *An. gambiae* s.s in Ghana.

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Introduction

The mosquito *Anopheles gambiae* sensu lato (s.l.) contains seven species, of which *An. gambiae sensu stricto* (s.s), *An. arabiensis* and *An. melas* are three of the major vectors of lymphatic filariasis (LF) and malaria caused by *Wuchereria bancrofti*, and *Plasmodium falciparum* respectively in West Africa [1,2]. In Ghana, previous studies have found the *An. gambiae* s.l and the *An. funestus* to be the major vectors of LF in the southern coastal zone and in the northern region of the country [3–7].

Vector control is considered an important tool for diseases transmitted by mosquitoes and other insect vectors [8]. The current Ghana policy on vector control against *Anopheles* vectors prioritizes the use of insecticide treated materials and indoor residual spraying. However, the efficiency of these interventions will depend on information on the distribution and abundance of the main vectors, the specific molecular forms of *An. gambiae* s.s and the levels of insecticide resistance within them [9,10].

Studies on the distribution of the *An. gambiae* complex in Africa [9,11], highlighted gaps in our knowledge, and Coetzee and colleagues [9] further recommended an urgent need for baseline surveys on the distribution of these malaria vectors in areas where no reliable information exists. Ghana is one of these countries. The largest and most recent study published on the abundance, distribution and levels of insecticide resistance in *An. gambiae* s.s covered 11 sites across different ecological zones of the country [12]. This study found that the *An. gambiae* S form predominated across the country, except in the arid north where only the M form was found. While this was the first major attempt at presenting the geographical distribution and ecological variations in *An. gambiae* s.s in Ghana, it is not known how these data compare with other *An. gambiae* s.s data, whether there are specific environmental factors driving the distributions of the M and S molecular forms, and if there are any associations with LF and malaria distributions [13,14].

In order to better understand the ecological aspects of this important vector, their influence on the epidemiology of LF and malaria, this study collated data on *An. gambiae* s.s in Ghana, and aimed to i) examine the spatial distribution of the *An. gambiae* M and S forms across the country, ii) identify key environmental factors associated with their distribution, and iii) determine their relationship with LF and malaria prevalence distributions. It is envisaged that this information will help develop a comprehensive profile on the ecology of *An. gambiae* s.s in Ghana, which will assist researchers and the diseases control programs in the country.

Methods

Study site

The Republic of Ghana is a developing West African country bordered to the north by Burkina Faso, to the east by Togo, to the

West by Cote d'Ivoire and to the south by the Gulf of Guinea [15]. It has a total surface area of 239, 460 sq km with a coastline of 539 km, and approximately a population of 23 million inhabitants. The climate is warm and comparatively dry along the southeast coast, hot and humid in the southwest, and hot and dry in north. The ecology can be divided into six zones; the mangrove zones at the coastline, the coastal savanna, the evergreen forest in the south-west, the moist semi-deciduous forest in the central area, the guinea savanna in the north and the sudan savanna in the north-east. The terrain is mostly low plain, with dissected plateau in the south-central area. The elevation ranges from 0 to 880 m above sea level. There are two main seasons; a rainy season from April to October and dry season from November to March.

Entomological data and mapping

Entomological studies on *An. gambiae* s.l in Ghana were identified from various sources including published articles in peer-reviewed journals, unpublished works from MPhil and PhD theses held at the Noguchi Memorial Institute for Medical Research (NMIMR), Accra-Ghana, as well as on-going studies at NMIMR. The collated data spanned from 2001 to 2008. Information on the location, study period, sample size, collection method, mosquito species and molecular forms, from each study were collated into a database. Various collection methods including; human landing catches, pyrethroid spray catches, larval collections and aspirators were used, depending on the location of the study sites and the objectives of the various studies. However, irrespective of the collection method, data collected from the same location in different years were considered separately. With the exception of few sites, most locations (i.e collection site) in the database were geo-referenced using the latitude and longitude coordinates obtained by cross-checking the names with data from the GEOnet Names Server [16], and Directory of Cities and Towns in the World [17] databases.

All data were imported into the geographical information systems software ArcGIS 9.2 (ESRI, Redlands, CA) for mapping and spatial analyses. First, the overall distribution of the *An. gambiae* s.l, and the different prevalence distributions of *An. gambiae* M and S across the country were mapped. Mosquito collection methods were also compared, to highlight differences in sampling. Second, spatial analysis of the *An. gambiae* M and S prevalences were examined using ArcGIS Spatial Analyst and Statistics tools (ESRI, Redland, CA). The Moran's *I* statistic was used to determine spatial autocorrelation patterns i.e. clustered, dispersed, random, and the Getis-Ord Gi* statistic was used to identify the specific locations where high and low prevalences were clustered (Z scores, 95% confidence levels (CI) +1.96 and −1.96 standard deviations). In addition, the kernel density estimation (KDE) method, non-parametric way of estimating the probability density function, was used to create a continuous surface representing the high to low density distributions of each molecular form.

Environmental data and analysis

To examine environmental factors associated with the prevalence distributions of *An. gambiae* M and S in Ghana, specific data on elevation, vegetation, precipitation, temperature and humidity were obtained for each location (i.e. collection site), and compiled into a database, for descriptive and statistical analyses in SPSS 16.0 (SPSS, Inc, Chicago, IL).

Elevation data were derived from the U.S Geological Survey's ETOPO2 Digital Elevation Model available from ESRI (Redlands, CA). The elevation at each collection site was determined by importing the digital elevation map into ArcGIS 9.2 and extracting the underlying value (metres). Vegetation and climate data were based on the mean annual values for the specific year of study, obtained from the best available sources via the Climate Data Library of the International Research Institute for Climate and Society [18]. Vegetation cover was based on Normalized Difference Vegetation Index (NDVI) satellite data extracted from the LandDAAC MODIS version 005 West Africa from USGS [19,20]. Precipitation (mm), temperature (C°) and specific humidity (qa) measures were obtained from satellite data from the National Oceanic and Atmospheric Administration (NOAA) and based on daily mean readings taken 2 meters above the ground [21–25].

First, the relationship between *An. gambiae* M and S, and each environmental variable was examined using bivariate correlations, Pearson's correlation coefficient (2 tailed P values$\leq$0.05 significance). Stepwise multiple linear regression analysis was then used to identify the environmental factor that would best predict the distribution of each molecular form. To account for environmental variables that may be highly correlated with each other, the level of colinearity tolerance in the stepwise regression procedure was set at $\geq$0.8 and only variables above this threshold were accepted in the models. Second, to better understand the environmental parameters associated with the *An. gambiae* M and S forms, mean environmental measures between high and low prevalence sites were compared using the Mann-Whitney *U* test with Bonferroni correction for multiple comparisons.

Relationship with disease prevalence distributions

To examine the relationship between the *An. gambiae* M and S prevalence and the distribution of disease, maps on the LF prevalence [13] and *P. falciparum* prevalence [14] for West Africa were imported into ArcGIS 9.2 and geo-referenced. The LF map was modeled from the *W. bancrofti* seroprevalence data collected in 2000 from 401 villages throughout Benin, Burkina Faso, Ghana and Togo [13]. The *P. falciparum* malaria prevalence map was modelled on extensive data obtained from children aged 2–10 years in non-epidemic periods, using a generalized linear mixed model [14].

The *An. gambiae* s.s collection sites were used as focal points, whereby the underlying disease prevalence data could be compared with the entomological data. The LF and malaria prevalence data, corresponding to the latitude and longitude of each mosquito collection site, were extracted and exported for descriptive and statistical analyses, which included bivariate correlations, and comparison of means between high and low prevalence sites of the *An. gambiae* M and S forms.

Further, it was of particular interest to explore the entomological and environmental characteristics in different LF transmission zones based on the prevalence data map in Gyapong et al. 2002 [13]. The prevalence distributions ranged from 0 to 30%, and were classified into distinct transmission zones, which were digitized in ArcGIS 9.2. The entomological and environmental data from each of collection sites within each zone were summarized, and significant differences identified by comparing the standard errors (+2SE) of the means.

Results

Entomological mapping

The collated *An. gambiae* species complex database contained 143 records with a total of 12,607 mosquitoes, reflecting both larval and adult catches. From this, the distribution of *An. gambiae* s.l was mapped (Figure 1). The most dominant species was *An. gambiae* s.s, which was found at 114 sites (total n = 10,028), followed by *An. melas* (6 sites, total n = 469) and *An. arabiensis* (8 sites, total

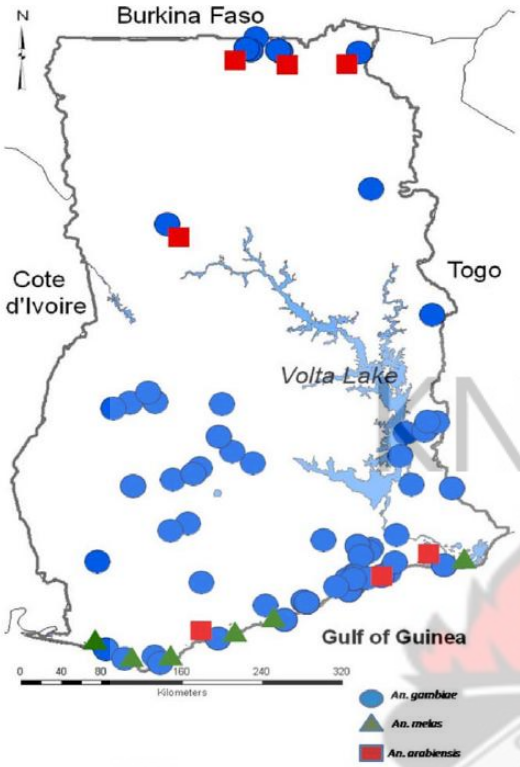

Figure 1. Distribution of *An. gambiae* s.l mosquitoes.
doi:10.1371/journal.pone.0009927.g001

n = 240). The map indicates that the distribution of *An. gambiae* s.s was widespread, while *An. melas* was primarily found along the coastal Savanna zones, a predominantly marshy environment, and *An. arabiensis* mainly in the northern savanna zone. *Anopheles funestus*, the second most important vector of malaria and lymphatic filariasis in Ghana, was also recorded in 9 sites with a total of 1,825 mosquitoes. Refer to supplementary data File S1 "*Anopheles* distribution records in Ghana" for further information on *An. funestus* and other *Anopheles* mosquito species, collection sites and coordinates, year and month of collection (where available), collection methods, numbers collected and identified, and the data sources.

Of the *An. gambiae* s.s data, a total of 70 collection sites had information on the M form (n = 2,826) and S form (n = 4,098). The collection sites were located predominately in three geographical regions i.e. the south eastern, central western and north central. The distribution of *An. gambiae* M and S forms varied across the country in different proportions (Figure 2). Figure 2 indicates the distribution of each species according to the various collection methods. Overall, *An. gambiae* M and S were sympatric in most locations. However, *An. gambiae* M form was more prevalent in the northern savanna, and coastal savanna areas of the country, and in four sites it was the only species observed. In contrast, *An. gambiae* S form was more prevalent in the middle region of the country, and in seven locations it was the only species collected. Interestingly, bivariate correlation analysis between each species indicated that their prevalences were negatively correlated (−0.763).

While, it is possible that the geographical grouping of collection sites may influence trends, the spatial analyses carried out in this study indicated a positive spatial autocorrelation or clustering for both the *An. gambiae* M (MI = 0.19, Z score = 4.2, $P \leq 0.01$) and *An. gambiae* S (MI = 0.19, Z score = 4.2, $P \leq 0.01$) forms. The resultant Z scores of the Getis-Ord Gi* hot spot analyses (using inverse-distance weighting), indicated similar trends with significantly different clustering of high and low prevalences of the *An. gambiae* M and S forms. These spatial trends were overlaid a density

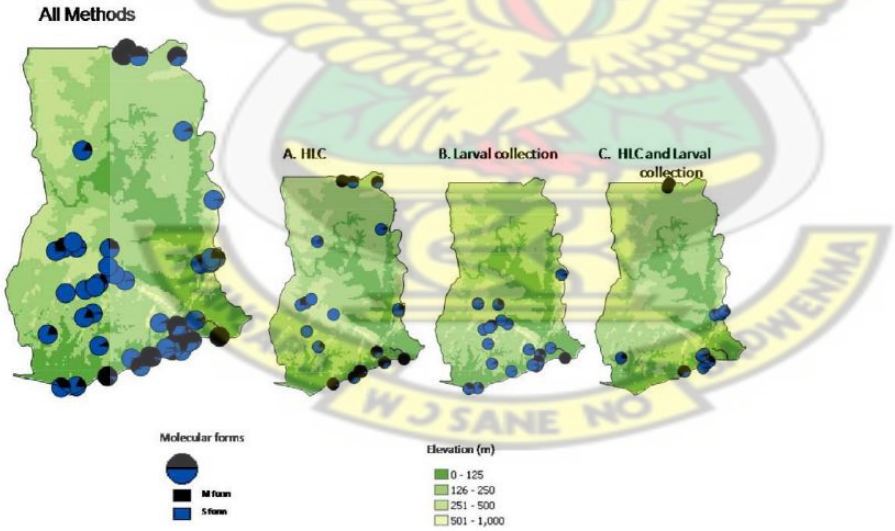

Figure 2. Distribution of *An. gambiae s.s* molecular form by collection methods.
doi:10.1371/journal.pone.0009927.g002

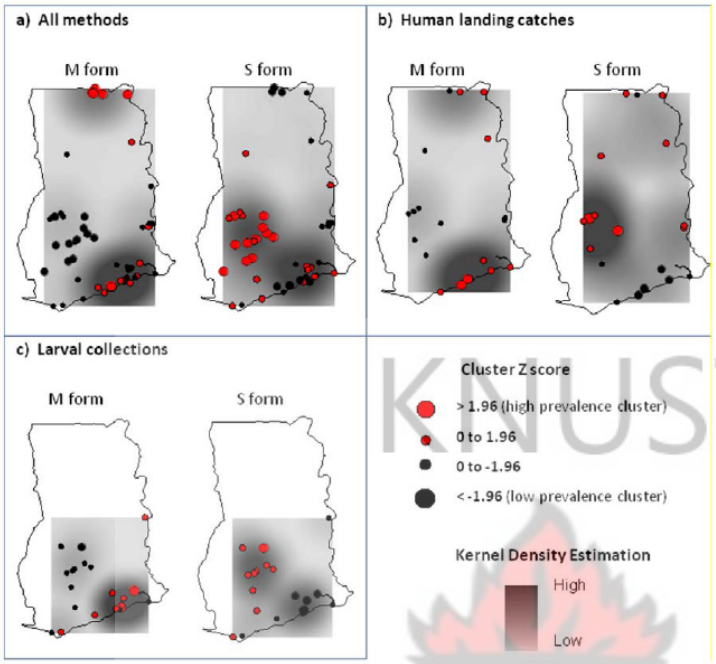

Figure 3. Spatial clustering trends and density distributions of *An. gambiae* s.s molecular forms.
doi:10.1371/journal.pone.0009927.g003

distribution surface map shown in Figure 3, further highlighting the high to low patterns of each species.

Environmental analysis

The relationship between the prevalence of *An. gambiae* M and S forms, and the environmental variables are shown in Table 1. Overall, bivariate correlation analysis indicated that the *An. gambiae* M form was significantly positively associated with temperature ($r = 0.51$), and negatively with elevation ($r = -0.28$), precipitation ($r = -0.33$), and humidity ($r = -0.26$). This contrasts to the *An. gambiae* S form, which was found to be significantly negatively associated with temperature ($r = -0.58$), and positively with elevation ($r = 0.30$) and rainfall ($r = 0.41$). Interestingly, elevation, precipitation and temperature correlations increased when data were stratified by the two main collection methods, HLC and larval collections (Table 1). Multiple regression analyses of all data (n = 70), indicated that temperature was an important variable for both molecular forms, explaining for *An. gambiae* M, 28% ($R_2 = 0.28$, $F = 25.8$, $P \leq 0.001$) and for *An. gambiae* S, 36% ($R_2 = 0.36$, $F = 37.9$, $P \leq 0.001$) of the variance in the model (Table 2).

For each molecular form, comparisons of environmental measures between locations with significantly high and low prevalences, defined by positive Z scores (≥+1.96) and negative

Table 1. Bivariate correlations between *An. gambiae* s.s. molecular forms and environmental and epidemiological variables.

	All Methods n = 70		Human Landing Catch n = 26		Larval Collection n = 28	
	M form	S form	M form	S form	M form	S form
Elevation	−0.28*	0.30*	−0.54**	0.58**	−0.42*	0.51**
NDVI	−0.14	0.22	−0.18	0.19	−0.18	0.25
Rainfall	−0.33**	0.41**	−0.63**	0.75**	−0.39*	0.60**
Temperature	0.51**	−0.58**	0.61**	−0.72**	0.16	−0.42**
Humidity	−0.26*	0.08	−0.07	−0.10	0.19	−0.43
LF	0.46**	−0.48**	-	-	-	-
Malaria	−0.14	0.26*	-	-	-	-

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
doi:10.1371/journal.pone.0009927.t001

Table 2. Multiple regression model for environmental variable predicting the presence of the *An. gambiae* M and S forms.

Species/predictor variable	Standardized Coefficient beta	T statistic	P value
An. gambiae M (Constant)		−4.76	<0.0001
Temperature	0.527	5.08	<0.0001
An. gambiae S (Constant)		6.67	<0.0001
Temperature	−0.601	−6.16	<0.0001

doi:10.1371/journal.pone.0009927.t002

Z scores (≤−1.96) respectively, are shown in Table 3. Overall, locations with high *An. gambiae* M prevalences had higher NDVI and temperatures, but lower elevation, precipitation and humidity measures than those locations with lower prevalences by these species and/or where the prevalence of *An. gambiae* S form was higher. Statistical comparisons indicated significant differences (*P* value<0.004 Bonferroni corrected) between elevation, precipitation and temperature for both *An. gambiae* M and S forms between the high and low prevalence areas.

To further explore the differences in elevation, precipitation and temperature, the mean prevalence of *An. gambiae* M and S forms was plotted across a range of environmental groupings (Figure 4). *An. gambiae* M prevalences were found to be higher at elevations of 0–200 m, and where mean daily precipitation ranged between 1.0–2.5 mm, and mean daily temperatures ranged between 26.1–27.6°C. In contrast, *An. gambiae* S prevalences were found to be higher at elevations >200 m, and where mean daily precipitation ranged between 2.6–3.8 mm, and mean daily temperatures ranged between 24.5–26.0°C.

Disease association

The relationship between the *An. gambiae* M and S, and LF and malaria prevalences were first examined using bivariate correlation analysis. Results shown in Table 1, indicate that *An. gambiae* M was significantly positively associated with LF ($r = 0.46$), while *An. gambiae* S form was significantly negatively associated with LF ($r = -0.48$), but positively with malaria ($r = 0.26$). Interestingly, correlation analysis between each mosquito species, and each disease indicated significant negative associations between *An. gambiae* M and *An. gambiae* S (r = −0.76), and between LF and malaria prevalence (r = −0.41).

Table 3. Comparison of mean environmental and epidemiological measures between high and low prevalence areas of *An. gambiae* s.s molecular forms.

Variable	*An. gambiae* s.s			
	M Form	High Low	S Form	High Low
Elevation	147	276**	272	107**
NDVI	0.42	0.50	0.49	0.44
Precipitation	2.1	3.1**	3.1	2.0**
Temperature	27.0	24.9**	24.9	26.8**
Humidity	0.0161	0.0181	0.0181	0.0174
LF	20.0	2.4**	2.2	17.1**
Malaria	55.9	47.0	55.8	33.9

Note. High = Z score≥+ 1.96, Low = Z score≤−.1.96.
**Significant at the 0.004 level after Bonferroni Correction.
doi:10.1371/journal.pone.0009927.t003

Second, we compared disease prevalences between high and low *An. gambiae* M and S sites (as described above). Overall, locations with high *An. gambiae* M prevalences (Z scores≥+1.96), were found to have significantly higher LF prevalences (20%) than those locations with low prevalences (Z scores≤−1.96), by these species (2.4%) and/or where the prevalence of *An. gambiae* S form was significantly high (2.2%). No significant differences were found between malaria prevalence and each mosquito species (Table 3).

Finally, we examined the LF data in Gyapong et al. 2002 [13], and identified three main transmission zones i.e. zero/low (<1%, n = 19), medium (1–10%, n = 32) and medium/high (10–30%, n = 19). Graphical presentation of these three zones is included in Figure 5, which summarizes mean entomological and environmental measures for the sites within each zone. In the medium/high LF transmission zone, the mean *An. gambiae* M prevalence (53.2%,) and temperatures (26.8°C) were found to be significantly higher (±2SE), and *An. gambiae* S prevalence (25.6%) significantly lower, than those found in medium transmission (21.1%; 25.5°C; 64.7%) and low transmission (21.4; 25.3°C; 64.4%) zones. In the zero/low transmission zone, precipitation measures (2.83 mm) were found to be significantly higher (+2SE), than those in the medium transmission (2.34 mm) to high transmission (1.99 mm) zones (Figure 5). Refer to the supplementary File S2 for further details on Figure 5.

Discussion

Most entomological studies on *An. gambiae* s.l in Ghana [3,4] have focused on small area/district based collections except the study by Yawson and colleagues [12]. Although the study by Yawson *et al.* attempted a broader coverage, this study represents the first nationwide review aimed at identifying the distribution of the various member species of the *An. gambiae* s.l, the molecular forms of the *An. gambiae* s.s, together with key environmental drivers and how they may relate to diseases. This study confirms previous observations that *An. gambiae* s.l is the major human biting mosquito species in Ghana [3,4,12,26] and within the *An. gambiae* s.l, *An. gambiae* s.s is the predominant species [3,4]. Other members of the *An. gambiae* s.l found in Ghana are *An. arabiensis* and *An. melas*.

The distribution of the *An. gambiae* M and S forms varied significantly across the country. The two molecular forms were found sympatrically in most locations, except in some areas in the middle region of the country where only the *An. gambiae* S form was observed, and in certain areas in the northern savanna and coastal savanna areas where only the *An. gambiae* M form was observed. This is confirmed by the spatial analysis and high Z score values in *An. gambiae* M/S form dominant areas. Also, the clustering remained relatively consistent irrespective of the different collection methods. Along the coast and in the northern savanna, the *An. gambiae* M form was predominant and clustered, while the S molecular form was most common and clustered in the middle belt. This positive spatial autocorrelation indicates that *An. gambiae* M/S distributions are geographically defined, and nearby areas are likely to comprise the same or similar species compositions, than those further away. The distribution of each species is also influenced by distinct and geographical related environmental factors and habitat characteristics. For example, the dominance of the *An. gambiae* M form in the northern and coastal savanna areas may be due to the wide presence of permanent breeding conditions provided by irrigation facilities [27] and ponds of water resulting from rivers run-offs since the M form is known to

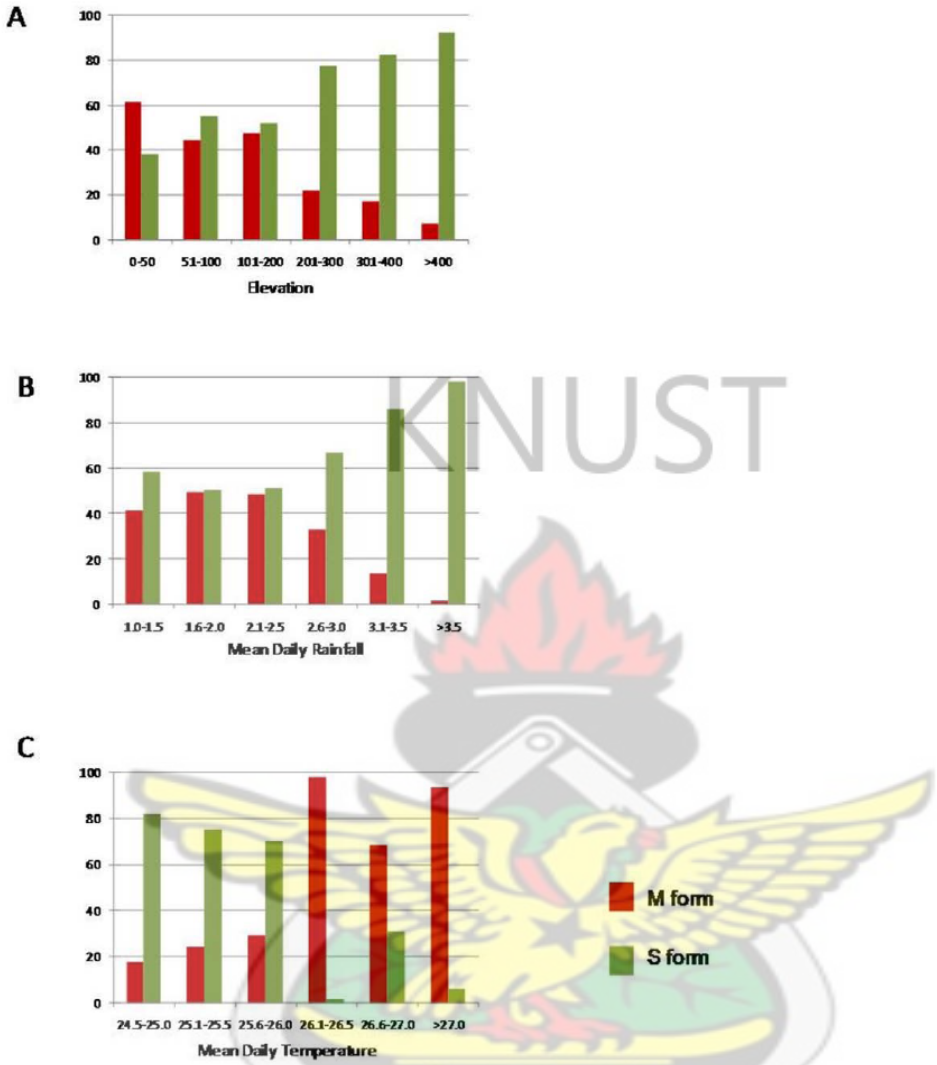

Figure 4. Mean prevalence of *An. gambiae* M and S forms plotted against elevation, precipitation, and temperature groupings.
doi:10.1371/journal.pone.0009927.g004

be associated with flooded areas, while the S form is heavily dependent on rainfall [12,28]. The dominance of the *An. gambiae* S form in the middle region of the country may be explained by the fact that this region is mountainous, forested, with lower mean temperatures and the highest recorded rainfall in Ghana, which supports the findings of our study. In sub-Saharan Africa the abundance and distribution of *Anopheles* mosquito species is dependent on environmental factors and ecological zones [29–32] as well as on human population changes and anthropological effects, which may lead to land-use changes ultimately affecting vector distribution and abundance [33,34] and as shown in a recent paper by Costantini and colleagues [35]. This current study did not focus on human factors but was able to show the wide variability in abundance and distribution of the *An. gambiae* M and S forms, which appear to be driven by a range of environmental factors. The relation between human population density and vector distribution is an indirect one and difficult to measure. However, it may be inferred that the NDVI, which is a measure of vegetation greenness and density, is somehow affected by the degree of land occupancy and exploitation by humans through the action of occupation and clearance for agricultural and developmental purposes.

Variations in the vector population densities of these two molecular forms have been observed in populations in Mali and Cameroon as well as between the various chromosomal forms in Mali [32,35,36]. Observations on the distributions and the predominance of the *An. gambiae* S form in a larger part of the country from this study confirm suggestions that the *An. gambiae* S

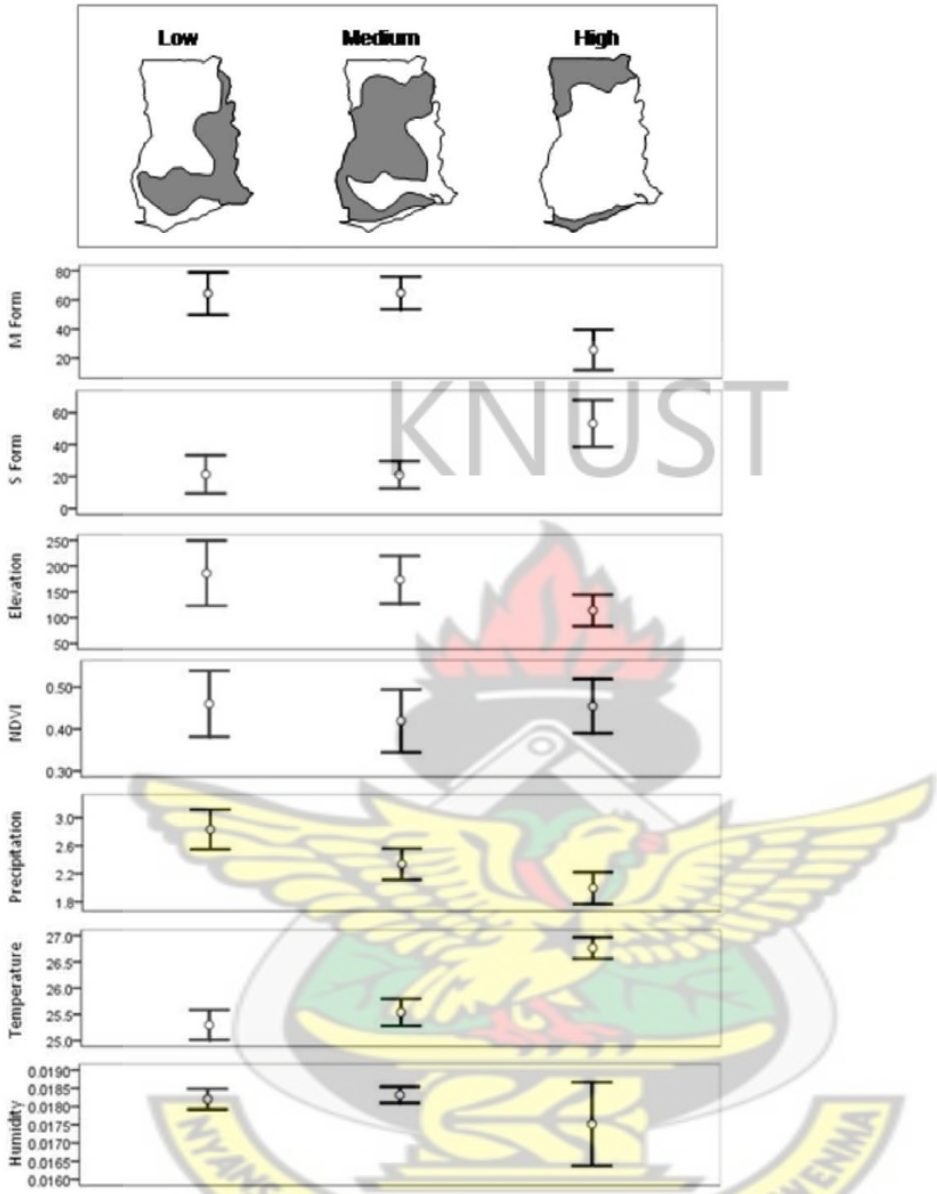

Figure 5. Summary of entomological and environmental variables in different LF transmission zones. Note: The maps represent the LF transmission zones in Ghana. The graphs below summarize the mean entomological and environmental measures for the sites within each zone. NDVI stands for Normalized Difference Vegetation Index.
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form, has broader environmental ranges, and therefore is found in more locations than the M form [37]. Our environmental analyses suggest that elevation, precipitation and temperature are important variables driving the spatial distribution of each mosquito species, and the differences between them. In particular, temperature appears to be a key factor distinguishing the two species; probably due to influences on their production as reported for *An. arabiensis* and *An. gambiae s.s* [38]. The *An. gambiae* M form was more prevalent and seemingly better adapted to higher temperatures, than the S form. This is in agreement with the suggestion that the *An. gambiae* M form shows a more latitudinal range in West Africa than the S form [39], being the most dominant form encountered in hot, arid regions of the Sudan-savanna or Sahelian zones [27,35,40–42].

The mosquito vectors' association with each disease might explain the negative association between LF and malaria described

by Kelly-Hope and colleagues [43]. In this current study, malaria prevalence was positively associated with the *An. gambiae* S form, whilst high LF prevalence and high transmission zones were associated with high temperatures and significantly high *An. gambiae* M prevalences. This relationship between *An. gambiae* M and S forms with LF and malaria supports previous studies that suggest the Mopti form of *An. gambiae s.s* is more associated with *W. bancrofti* than malaria transmission [44,45] and also that it is a relatively poor vector of malaria compared with other species such as the Savanna form of *An. gambiae* [46,47]. The LF distribution map by Gyapong and colleagues [13], indicates that the disease distribution in West Africa has the highest prevalence in the hotter Sudan/Sahel savanna areas, which are also *An. gambiae* Mopti chromosomal form dominant areas [41,43].

The results from this study provide useful information on the distribution of the *An. gambiae* M and S forms in Ghana, highlighting the environmental factors that may play a role in determining their distributions. The information provided also marks a beginning in understanding the LF disease distribution pattern in Ghana relative to the forms of the *An. gambiae* s.s. Despite the limitations of this study in using previously modeled LF and malaria data, these results are very useful for disease control and allocation of resources, especially for LF, which together with its vectors appear to be restricted to hotter, less elevated regions of the country. Another limitation to this study is the small number of sites involved in the analysis of the *An. gambiae* M and S distribution. This coupled with their uneven distribution between the southern half and the northern half of the country could introduce potential bias in the analyses. Undertaking spatial statistical analysis with a larger dataset in the future, and further modeling their distributions through ecologic niche modeling [11,35,48], could further enhance our understanding of the distribution of the disease and its vectors, as well as defining the spatial limits of the vectors' distribution which is crucial for disease control. This would, however, require a constant update of the vector database generated in this study as well as collection of data from remote areas of the country where no available data exists.

Supporting Information

File S1 Data file for Anopheles mosquito species, collection sites and coordinates, year and month of collection (where available), collection methods, numbers collected and identified, and the data sources.

Found at: doi:10.1371/journal.pone.0009927.s001 (0.04 MB XLS)

File S2 Summary of entomological and environmental variables in different LF transmission zones.

Found at: doi:10.1371/journal.pone.0009927.s002 (0.08 MB DOC)

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Author Contributions

Conceived and designed the experiments: DKdS LAKH MDW BL DAB. Analyzed the data: DKdS LAKH. Contributed reagents/materials/analysis tools: DAB. Wrote the paper: DKdS LAKH MDW BL DAB.

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