Systematic study of the new *Anopheles funestus*-like species from Malawi

Vezenegho Samuel Bumuh

‘A thesis submitted to the Faculty of Health Science, University of the Witwatersrand Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy’

Johannesburg, 2012

DECLARATION

I, Samuel Bumuh Vezenegho declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

…… [Signature of candidate]

……23rd …….Day of……April….2012

DEDICATION

I dedicate this thesis to our God and Lord. To my wife, Confidence and Son, Valery; parents, Clement and Regina Bumuh, brothers and sister for their love and encouragement

PUBLICATION AND PRESENTATIONS:

Publication:

Vezenegho, S. B., Bass, C., Puinean, M., Williamson, M. S Field, L. M., Coetzee, M and Koekemoer, L. L (2009). Development of multiplex real-time PCR assays for identification of members of the *Anopheles funestus* species group. *Malaria Journal* 2009, **8:**282 (Appendix VII)

Presentations:

Vezenegho S. B., Coetzee, M., and Koekemoer, L. L. Biological and behavioral characterization of *Anopheles funestus-like* from Malawi. Parasitological Society of Southern Africa Conference (PARSA) 10-13 July 2011

Vezenegho S. B., Bass. C., Williamson. M., Puinean. M., Koekemoer. L. L., Coetzee M. and Field, L. Development of *Anopheles funestus* species identification. Rothamsted Research UK. 14 July 2009.

Vezenegho S. B., Bass. C., Williamson. M., Puinean. M., Koekemoer. L. L., Coetzee M. and Field, L. Development of multiplex real -time PCR assays for identification of *Anopheles funestus* species group. Faculty of health science research day 22 September 2010

Vezenegho S. B., Bass. C., Williamson. M., Puinean. M., Koekemoer. L. L., Coetzee M. and Field, L. Vector population monitoring tools and development of *Anopheles funestus* species identification. Liverpool school of Tropical Medicine, Liverpool, UK. 10 July 2009.

ABSTRACT

Morphological similarity between malaria vectors and non-vectors occurring in sympatry has serious consequences if the killer diseases have to be controlled. Malaria in Malawi is transmitted by *Anopheles gambiae, An. arabiensis* and *An. funestus*. This vector diversity is further complicated by the recently discovered *An. funestus*-like species which is morphologically similar to *An. funestus,* and found in association with humans. Currently there is no single assay available that differentiates *An. funestus*-like from the other African members in the *An. funestus* group.

The objective of this study was to investigate the biology and behavior of the newly discovered *An. funestus*-like species and its possible role in malaria transmission. This information will assist in the implementation of vector control programs. In addition to this, the study investigated the development of a DNA based assay to differentiate between the members of the *An. funestus* group and to morphologically described *An. funestus*-like species.

Anopheles mosquitoes were collected resting indoors and outdoors from Karonga in Malawi. Specimens were identified morphologically and molecularly using chain reaction PCR. Identified samples were analyzed by ELISA for blood meal source and *Plasmodium* sporozoite infection. *Anopheles funestus*-like was morphologically compared with *An. funestus*. Real time based PCR was developed and compared to the current multiplex or allele-specific PCR (AS-PCR) assay for sensitivity and performance. The IGS region of the rDNA gene was investigated for development of AS-PCR. Phylogenetic relationship of mosquitoes was constructed from ITS2 and D3 sequences.

Adult *An. funestus* mosquitoes (n = 391) were collected during April and September, 2010. Karonga contributed 63.9% and Likoma Island 36.1%. Of the identified specimens (n = 347) *An. funestus*-like comprised 10.4%, *An. rivulorum* 31.7%, *An. funestus* 57.3% and *An. parensis* 0.6%. Most of the *An. funestus*-like species were collected resting indoors 91.7% (33/36) compared to outdoors 8.3% (3/36). The species was predominant during the dry season 63.9% (23/36) compared to the wet season. A total of 19 *An. funestus*-like females were analyzed for blood meal source. Mixed blood meal from goat and bovine was found in 7 specimens and a single blood meal from goat in 3 specimens.. The rest of the *An. funestus*-like was negative for blood meal. An overall dry season infection rate of *An. funestus*-like species by *Plasmodium vivax* was 5% (1/20) in this study and 3.1% (2/64) from samples collected in 2009 was found. However, the possibility of false positivity could not be excluded and further study is urgently needed to investigate this. Real-time PCR for the identification of members of the *An. funestus* group was found to be more sensitive (0.02ng/µl) than AS-PCR (0.04ng/µl) and had performance comparable to AS-PCR. AS-PCR developed from the intergenic spacer region of rDNA discriminates *An. funestus*, *An. rivulorum*, *An. vaneedeni* and *An. parensis*.

Of all assays developed in this study, the hydrolysis probe assay is the most reliable assay for identifying members in the *An. funestus* group. This study confirmed the existence of *An. funestus*-like species in sympatry with *An. funestus* group members. *An. funestus*-like was predominantly found resting indoors (endophilic) but preferring animal over human blood (zoophilic). No consistent morphological characters were found to discriminate between *An. funestus* and *An. funestus*-like based on morphological data, *An. funestus*-like is very similar and closely related to *An. funestus* which is supported by phylogenetic analysis. However, Restriction Fragment Length Polymorphism separates the two species.

ACKNOWLEDGEMENTS

First and foremost I am indebted to my supervisors, professors Maureen Coetzee and Lizette Koekemoer who perceived the project. I am grateful for your expertise, kindness, patience and thoughtful guidance throughout my PhD program. You gave me the confidence to explore my research interest and taught me both consciously and unconsciously how to become a good researcher. I am also thankful for the excellent examples you both provided as successful women in science. Professors, without your financial support and constant supervision, this project wouldn't have been a success story. Dr. Chris Bass was so instrumental making me felt at home in Rothamsted Research, United Kingdom. He didn't only take time to train me with real-time PCR technique but could come to the lab during his sick leave just to give me more guidance. Doctor, I am grateful and say thank you.

I want express my gratitude to Professor Richard Hunt for his patience in teaching me the ecological aspects of medical entomology during our field trip to Karonga in Malawi and laboratory mosquito rearing skills. The Karonga health department staffs are thanked for their assistance during our stay in Karonga. The Karonga inhabitants were friendly and allowed us to collect mosquitoes from their houses without which mosquito collections would have been unsuccessful.

I need to thank my readers Dr Basil Brooke, Dr Riann Christian, Dr. Givemore Munhenga, Micheal Osae and Oliver Wood, for their helpful comments and for taking off time from your busy schedules to make sure this thesis is worth reading. I will like to convey my gratitude to the following; Dr Gerald Kimbi, Dr. Givemore Munhenga, Dr. Alphonse

Neba, Dr. Joel Mouatcho, Dr. Godlove Enongene, Dr. Sylvester Matambo and Ms Hiba Abdalla for their sharp sense of humour and encouragement which was always appreciated. The rest of the staff and students of VCRU and Malaria Entomology Research Unit are thanked for their invaluable support.

This work wouldn't have been possible without financial support. Thanks to Professor Maureen Coetzee for funding from the National Research Foundation/Department of Science and Technology (NRF/DST) Research Chair Initiative awarded to her. Thanks to the University Witwatersrand for the Carnegie PhD bursary awarded to me.

At this stage I want to say thanks to my dad Toh Clement Bumuh, my mom Vuke Regina Bumuh, my sister, brothers, my wife and son and Kedjom family in South Africa. You all provided me with continuous encouragement and particularly my parents who instilled in me the philosophy that education is the best tool to survive and that were there is a will there is a way. These had driven me through from primary school (CBC Nkwen, Bamenda, Cameroon) to University of Witwatersrand, Johannesburg, South Africa. Lastly, I thank GOD for giving me a chance in life and making my dream come true.

TABLE OF CONTENT

CHAPTER TWO

Biological and behavioural characterization of *Anopheles funestus* group, including *Anopheles funestus*-like species from Northern Malawi

CHAPTER THREE

Morphological description of *Anopheles funestus*-like from Malawi

CHAPTER FOUR

Development of DNA-based molecular assays for the identification of *Anopheles funestus* group members including *An. funestus*-like species

CHAPTER FIVE

General Discussion and Conclusions

APPENDICES

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

LIST OF TABLES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

ABBREVIATIONS AND SYMBOLS

Amp	ampicillin
ATP	adenosine triphosphate
ANOVA	analysis of variance
AS-PCR	allele specific polymerase chain reaction
BSA	bovine serum albumin
BLAST	basic logic alignment search tool
bp	base pair
°C	centigrade
CSP	circumsporozoite protein
cm	centimetre
CDC	center for disease control
CV	coefficient of variation
CI	confidence interval
DDT	diethyl diphenyl trichloroethane
ddH_2O	distilled deionized water
dH_2O	deionized water
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
dsDNA	double stranded deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
DNase	deoxyribonuclease
E. coli	*Esherichia coli*
EDTA	ethylenediamine tetraacetate
ELISA	enzyme limked immunosorbent assay
EST	external transcribe spacer
et al	and others
FANG	*Anopheles funestus* from Angola
F_1	first filial generation

hr	hour
HBI	human blood index
IPTG	isopropyl-β-D-thiogalactopyranoside
IGS	intergenic spacer
IRS	indoor residual spraying
ITN	insecticide treated net
ITS	internal transcribe spacer
IUPAC	international union of pure and applied chemistry
Ig G	immunoglobulin G
kdr	knockdown resistance
kb	kilo bas
LB	luria-broth
mA	milliampere
µM	micro molar concentration
µl	microlitre
min	minutes
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
ng	nanogram
nM	nanomole
NICD	national institude for communicable diseases
NHLS	national health laboratory service
NCBI	national centre for biotechnology information
NTS	non-transcribe spacer
OD	optical density
%	percentage
PBS	phosphate buffered saline
PCR	polymerase chain reaction

p	probability level
pf	*Plasmodium falciparum*
pH	potential of hydrogen
pg	picogram
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
RFLP	Restriction fragment length polymorphism
SDS	sodium-dodecyl-sulfate
s	seconds
S	svedberg sedimentation unit
s.l	sensu lato
s.s	sensu stricto
SNP	single nucleotide polymorphism
SD	standard deviation
Taq	*Thermus aquaticus*
Tris	tris (hydroxymethyl) aminomethane
TW	tween
USA	United State of America
V	volt
VCRU	vector control reference unit
WHO	World Health Organisation

CHAPTER ONE

General Introduction

1.1 Global malaria perspective

Malaria is a life-threatening infectious disease in many tropical and subtropical areas and is endemic in over 106 countries according to the World Health Organization (WHO, 2010) (Figure 1.1). Globally, malaria accounts for 245 million clinical cases and 781,000 deaths per year (WHO, 2010), 90% of these occurring in Africa. Children under the ages of five and pregnant women are the most vulnerable risk group (WHO, 2005). Africa is most affected by malaria and this can be attributed to numerous factors. Some of these include civil unrest which interrupts control programs, resistance of malaria parasites to anti-malaria drugs (WHO, 2004), resistance of mosquito vectors to chemical insecticides (Hargreaves *et al*. 2000), vector abundance due to climate change (Mouchet *et al*. 1998), migration of people and water management projects including dams for electricity, agriculture and irrigation (Service, 1984).

Charles Laveran demonstrated that malaria is caused by a protozoan parasite belonging to the phylum Apicomplexa and genus *Plasmodium* (Garnham, 1966). Five species of plasmodia (*P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*) causes malaria in human. Of these, *P. falciparum* causes the most severe infections and the highest mortality in Africa (WHO, 2005). *Plasmodium knowlesi* has recently been found to infect both humans and monkeys in Malaysia (Cox-singh *et al*., 2007). Certain anopheline mosquitoes play an important role in vectoring malaria to humans and animals. One of the

three principal African vectors of human malaria is *Anopheles funestus* (Gillies and De Meillon, 1968). The other two are members the *An. gambiae* species complex, namely *An. arabiensis* and *An. gambiae sensu stricto*. In addition to these, at least seven other minor vectors are also involved in malaria transmission (Gillies and De Meillon, 1968).

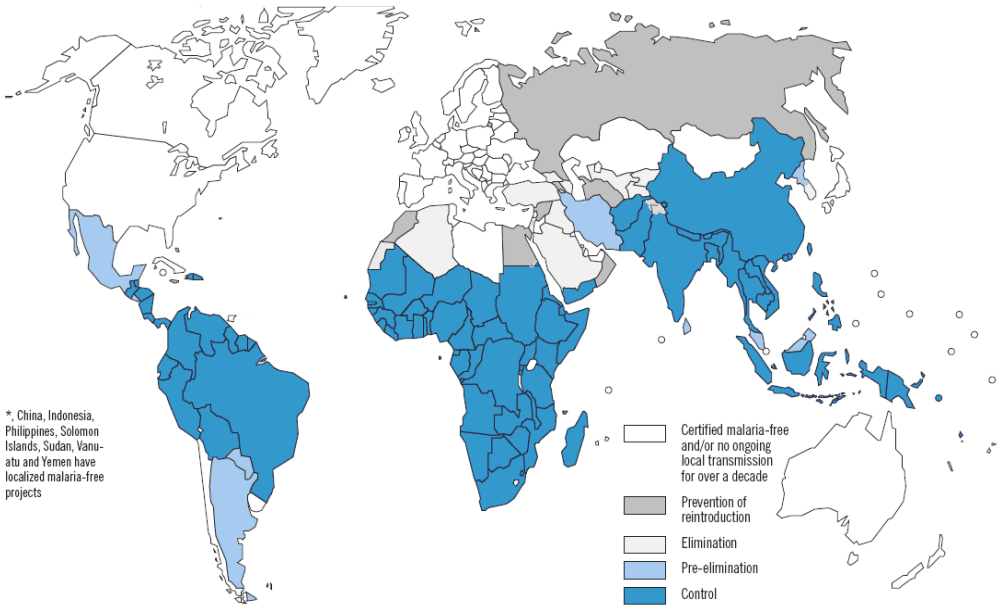


Figure 1.1 Malaria-free countries and Malaria endemic countries in phases of control*, pre-elimination, elimination and prevention (WHO 2008).

1.2 Malaria control

Early diagnosis and treatment of patients as well as controlling malaria vectors constitute key measures in mitigation of the diseases and reducing both illness and death as well as the socio-economic burden caused by malaria.

1.2.1 Malaria vector control using insecticides

According to the WHO, malaria vector control forms an important part of the global malaria strategy and remains the most effective approach for the prevention of malaria transmission (WHO, 2006). Chemical controls are most often used for vector control and

include approximately 12 insecticides from four classes (organochlorines, organophosphates, carbamates and pyrethroids) (WHO, 2001). The major insecticide based strategies are indoor residual spraying (IRS) of dwellings and associated structures, insecticide treated nets (ITNs) and larviciding. IRS has successfully reduced malaria cases in many areas in Africa such as the recent control campaign on Bioko Island, Equatorial Guinea (Kleinschmidt *et al*., 2006), and the decades long campaign in South Africa (Hargreaves *et al*., 2000). Insecticide treated nets (ITNs) repel or kill mosquitoes (Lines, 1996). Categories of ITN include conventionally treated nets, (treated by dipping the net in WHO recommended insecticides) and Long Lasting Insecticide Nets (LLINs) made in factories using specially formulated netting with insecticide embedded within the fibres. Only pyrethroids are recommended for treating nets (Zaim *et al*., 2000). Unfortunately, wide spread resistance to pyrethroids threatens IRS and ITN applications (N'Guessan *et al*., 2007).

All of the recommended insecticides used for vector control target the central nervous system of insects (Nauen, 2006). However, each insecticide class targets a different site as demonstrated in Figure 1.2. Insecticides belonging to the organophosphate and carbamate classes principally target the enzyme acetylcholinesterase (AChE), preventing normal hydrolysis of the neurotransmitter acetylcholine in synaptic clefts (Hemingway *et al*., 2004). Pyrethroids target the voltage gated sodium channel and disrupt the regulation of sodium ion flux across neuron membranes (Brengues *et al*., 2003). DDT acts on the same target site as pyrethroids (Brengues *et al*., 2003). The mode of action of DDT has not been clearly established but it also disrupts the balance of sodium and potassium ions within the nerve axon, such that normal nerve impulses are prevented (Whiteacre and Ware, 2004).

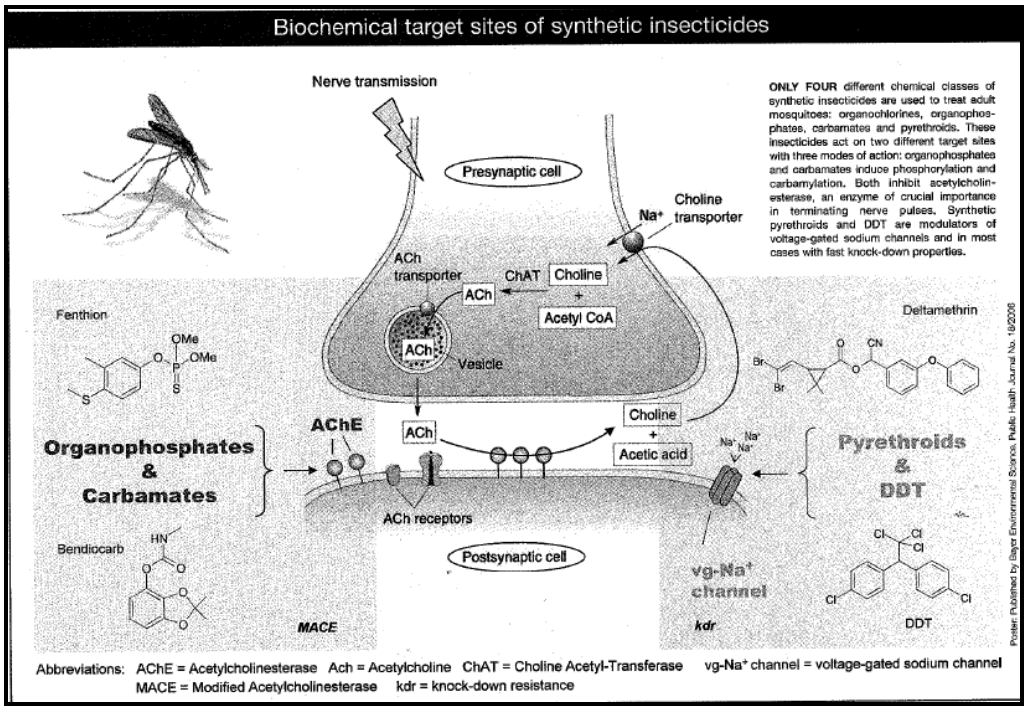


Figure 1.2 Action sites of insecticides used for vector control. (Nauen, 2006)

1.2.2 Biological control of malaria vectors

Biological control encompasses the introduction of mosquito larval and pupal predators (e.g. mosquito fish *Gambusia affinis* (Rose, 2001)), or entomopathogenic organisms such as *Bacillus thurigiensis* var *israelensis* serotype H-14 (*Bti*) (Service, 1983) and *Metarhizium anisopliae* and *Beauveria bassiana* (Chapman, 1974; Roberts and Hajek, 1992) into the breeding sites. Biological control is generally species specific and is more environmentally friendly than chemical insecticide use (Mulla *et al*., 2003). It has been used in African countries such as Burkina Faso (Skovmand and Sanogo, 1999) and the Gambia (Majambere *et al*., 2006). Biological control methods, however, suffer from difficulties associated with the colonization and management of the predators as well as cost incurred with the frequent application of entomopathogenic organisms such as *Bti*.

1.2.3 Other control methods

Genetic control of mosquitoes has garnered tremendous interest in the past two decades. This has been triggered by the development of resistance to insecticides, environmental concerns as well as the development of new technologies. One application of genetic engineering is the transformation of a *Plasmodium* susceptible strain of mosquito, to one refractory to *Plasmodium* infection (Beerntsen *et al*., 2000; Ito *et al*., 2002). This idea was first employed when *Aedes aegypti* was genetically engineered to be refractory to dengue virus. This was achieved using the Sindbis virus to express an antisense RNA molecule, complimentary to a segment of the dengue virus genome (Olsson *et al*., 1996). However, the observed expression was only transient and not heritable and the desired refractoriness could not be driven into the wild population. Transformation was accomplished by using transposable elements such as Minos and Mariner isolated from *Drosophila hydei* and *D. mauritiana* respectively (Franz *et al*., 1991; Madhora *et al*., 1991). Refractory genes can also be driven into the wild populations by using symbionts. This approach has been achieved by incorporating anti pathogen genes into the genome or plasmids of symbionts e.g. bacteria (Durvasula *et al*., 1997).

Currently, malaria vector control using the sterile insect technique (SIT) (Pates and Curtis, 2005) is being investigated. In this technique laboratory reared male mosquitoes, conspecific and compatible with the target population, are sterilised with gamma-irradiation or chemosterilants, e.g. cobalt-60 (Coleman and Alphey, 2004; Davidson, 1969) and are then mass-released into the wild (Hassan *et al*., 2010; Helinski *et al*., 2008). There they hopefully mate with wild females causing them to lay sterile eggs, eventually suppressing the target population. However, this technique has not been used to effectively control malaria to date (Hassan *et al*., 2010).

Other control methods include source reduction which aims at eliminating mosquito vector breeding sites. The disadvantage of source reduction is that the breeding sites of some vector species are usually small, dispersed and transient, making complete coverage almost impossible (Pates and Curtis, 2005).

1.3 African malaria vectors

Sir Ronald Ross, a British scientist working in India, found out that some species of mosquitoes act as intermediate hosts for both human and bird malaria parasites (Garnham, 1966; Harrison, 1978). Further research revealed that the malaria parasite is transmitted from human to human by various species of *Anopheles* mosquitoes which mainly feed between sunset and sunrise (Gillies and De Meillon, 1968; WHO, 2005). In sub-Saharan Africa, the major vectors are members of the *Anopheles gambiae* species complex and the *An. funestus* species group.

1.3.1 The *Anopheles gambiae* complex

This complex consists of seven recognized sibling species which are morphologically identical: *An. gambiae* Giles, *An. arabiensis* Patton, *An. quadriannulatus* Theobald species A and B, *An. merus* Dönitz, *An. melas* Theobald and *An. bwambae* White (Gillies and Coetzee 1987; Hunt *et al.*, 1998). *Anopheles gambiae s.s* and *An. arabiensis* are the most efficient vectors in the complex. Their efficiency is partly a result of their preference for human blood meals and their ability to quickly adapt to environmental changes initiated by human habitation and agriculture (Collins and Paskewitz, 1995). *Anopheles merus*, *An. melas* and *An. bwambae* tend to be localised vectors. *Anopheles merus* and *An. melas* in particular are not always implicated in malaria transmission where they occur. Both species of *An. quadriannulatus* are non-vectors.

1.3.2 The *Anopheles funestus* group

This group traditionally consisted of nine morphologically similar species (*An. funestus s.s* Giles, *An. rivulorum* Leeson, *An. leesoni* Evans, *An. vaneedeni* Gillies and Coetzee, *An. parensis* Gillies, *An. confusus* Evans and Leeson, *An. aruni* Sobti, *An. fuscivenosus* Leeson and *An. brucei* Service) with some members distinguishable only at the early developmental stages (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Cohuet *et al.* (2003) described a taxon based on biological, morphological and genetic characteristics which are related to *An. rivulorum* and provisionally named it *An. rivlorum*-like. This species is a non-vector and has been recorded in Cameroon and Burkina Faso. Spillings *et al*. (2009) discovered yet another species in Malawi which is morphologically similar to *An. funestus* and named it *An. funestus*-like. A detailed taxonomic description of each new species and their phylogenetic relationship within the *An. funestus* group will be important in terms of understanding their biology and could have implications for the design of vector control strategies. The most efficient vector in the *An. funestus* group is *An. funestus s.s*. (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

1.4 Systematics of the *Anopheles funestus* group

Classification of this group was re-evaluated using morphological, chromosomal and molecular data by Harbach (2004) due to similarities with the South-east Asian *An. minimus* group. This resulted in a composite group referred to as the *An. funestus* group. According to this classification the *An. funestus* group consists of five subgroups (*An. funestus* and *An. rivulorum* from sub-Saharan Africa, *An. minimus* (including one African species), *An. aconitus* and *An. culicifacies*) all from Southeast Asia. The *An. funestus* subgroup consists of five species (*An. aruni, An. confusus, An. funestus, An. parensis* and *An. vaneedeni*). The *An. minimus* subgroup consists of *An. flavirostris*, *An. leesoni*

(African), *An. fluviatilis* complex and the *An. minimus* complex. The *An. aconitus* subgroup consists of *An. aconitus*, *An. filipinae*, *An. mangyanus*, *An. pampanai* and *An. varuna*. The *An. culicifacies* subgroup consists of the *culicifacies* complex, and lastly the *An. rivulorum* subgroup consists of *An. brucei*, *An. fuscivenosus*, *An. rivulorum* and *An. rivulorum*-like species (Harbach, 2004). Only the common species found in sub-Saharan Africa will be briefly discussed below.

The development of cyptic species complexes amongst anopheline taxa has added a level complexity to the understanding of malaria transmission patterns and the design of effective vector control strategies. Despite years of research, the systematics of anophelines has not being fully elucidated (Spillings *et al*., 2009). Whereas the population genetics and systematics of the *An. gambiae* complex has received considerable attention, the *An. funestus* group has received less attention in this regard despite its epidemiological importance. This is primarily attributable to the technical difficulties associated with identifying member species within the group, the general assumption that indoor resting *An. funestus* sensu lato are inevitably *An. funestus* sensu stricto without the need for deeper investigation, and technical difficulties with laboratory colonization of *An. funestus* group members. Cytogenetic analyses and the later development of molecular systematics have provided important insights into the species diversity of these mosquitoes.

1.4.1 *Anopheles funestus s.s.*

Anopheles funestus s.s., henceforth referred to as *An. funestus,* is one of the most efficient human malaria vectors. It transmits *P. falciparum* with infection rates usually in the range of 2-5% (Gillies and De Meillon, 1968). However, higher infection rates in *An. funestus* have been recorded i.e. 11% (Temu *et al*. 2007); 22% (Gillies and De Meillon 1968); 5.4%

(Hargreaves *et al*., 2000). A *P. falciparum* infection rate of 4.9% was recorded by in *An. funestus* collected on Likoma Island, Malawi (Hunt *et al*. 2010). *Anopheles funestus* also plays a role in transmitting pathogens such as bancroftian filariasis and O'nyong-nyong fever (Gillies and De Meillon, 1968).

Amongst the members of this group, *An. funestus* has the widest geographic distribution in sub-Saharan Africa (Gillies and De Meillon 1968). *Anopheles funestus* prefers to breed in bodies of clear water that are large and more or less permanent, containing vegetation in the form of short grasses with little shading of the water surface. Rice growing areas have been associated with *An. funestus*. Unusual breeding sites include wells, domestic water containers and deeply sunken limestone streams all devoid of vegetation (Gillies and De Meillon, 1968). *Anopheles funestus* larvae can stay submerged under water for long periods (Gillies and De Meillon 1968).

Anopheles funestus adult females are highly anthropophilic (human feeding) and prefer to blood-feed indoors (Gillies and De Meillon 1968). They mainly feed during the second half of the night until early morning but will also feed during the day inside dark houses (Gillies and De Meillon, 1968). After feeding they rest indoors rendering them especially susceptible to control by IRS (Gillies and De Meillon, 1968). Little information is available on the swarming activities of male *An. funestus*. However, Charlword *et al*. (2003) observed swarms of male *An. funestus* within sandy clearings surrounding houses in Mozambique.

Cytogenetic investigations have revealed a series of chromosomal inversion polymorphisms in *An. funestus s.s*. populations in West, Central, South and East Africa.

Based on the assortment, chromosomal linkages and relative frequencies of each alternative inversion arrangement, five chromosomal forms of *An. funestus s.s*. have been described in West Africa. In Burkina Faso, the Kiribina and Folonzo chromosomal forms were described (Costantini *et al*., 1999). Three forms were identified in Senegal namely, Skouvar, Wassadou and Dielmo (Lochouarn *et al*., 1998). The inversion polymorphism arrangements in East and Southern African populations are similar to the Folonzo form. However, mitochondrial polymorphisms using microsatellite DNA markers show three distinct clusters of *An. funestus* in Southern Africa (Michel *et al*. 2005a,b). These clades can be extrapolated to populations east of the Great Rift Valley (Tanzania, Malawi, Mozambique and Madagascar), populations west of the Rift Valley (Nigeria, Mali, Burkina Faso and Kenya) and populations in central Africa (Gabon and coastal Angola). Garros *et al*. (2004) showed, by means of restriction fragment length polymorphism (RFLP), intragenomic variation within *An. funestus* populations from sub-Saharan Africa. PCR amplified D3 fragments of rDNA digested with the enzyme *Msp* I gave either an M, W or MW type designation to each sample tested. Further genotyping by Koekemoer *et al*. (2006) using RFLP showed that *An. funestus* specimens from the west and east sides of the Rift Valley could be classified as either W-type or M-type respectively with the MW type associating with the west side. Two additional types were also recorded: the Y-type from Malawi and the Z-type from Angola, Ghana Malawi, and Zambia.

1.4.2 *Anopheles rivulorum*

Anopheles rivulorum is the second most widespread member in the group and is found mainly in eastern and southern Africa. Its distribution ranges from Botswana to Ethiopia, Mozambique, South Africa and Zimbabwe (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Localised occurrences in West Africa include Côte d'Ivoire Mali, and

northern Nigeria (Gillies and De Meillon, 1968). They feed mainly on animals (zoophilic) although they will bite humans outdoors in the absence of animals. After feeding *An. rivulorum* rest outdoors (exophilic) (Gillies and De Meillon, 1968). They have only once been implicated in malaria transmission in Tanzania where they were found infected with *Plasmodium* sporozoites (Wilkes *et al.,* 1996).

1.4.3 *Anopheles vaneedeni*

Anopheles vaneedeni is known only from South Africa (Gillies and Coetzee, 1987). This species was shown to be able to transmit *P. falciparum* in the laboratory when females experimentally infected were found to be positive for oocysts in the stomach walls as well as sporozoites in the salivary glands (De Meillon *et al.*, 1977). No record is available for *Plasmodium* infection of this species in nature. They are found breeding in habitats similar to those of *An. funestus.* Female mosquitoes of this species bite humans outside houses in the early hours of the night but they predominantly feed on cattle (Gillies and Coetzee, 1987). They rest in pit-shelters along with other species such as *An. leesoni* (De Meillon *et al.*, 1977).

1.4.4 *Anopheles parensis*

Anopheles parensis is mainly distributed in Eastern Africa from Kenya and Tanzania, south to Swaziland and northern KwaZulu-Natal, South Africa. The larvae are found breeding in permanent swamps and ponds among reeds and emerging vegetation. Unlike its sibling species, it is scarce or absent in streams and moving water (Gillies and De Meillon, 1968). *Anopheles parensi*s has previously been collected resting indoors in Kenya but was not infected with human malaria parasites (Gillies and Furlong, 1964). Kamau *et al*. (2003) also collected them resting indoors and a small proportion (1.44%) was found to

have taken human blood meals. This may imply that *An. parensis* has the potential to be a minor vector even though there is no record of it been infected with *P. falciparum* (Gillies and De Meillon, 1968).

1.4.5 *Anopheles leesoni*

Anopheles leesoni has been recorded from savanna regions of Eastern and Western Africa. Its distribution ranges from Ethiopia, Kenya and Uganda to Mozambique and South Africa. In West Africa, they have been found in Mali, Ivory Coast and northern Nigeria (Gillies and De Meillon, 1968). Their larval habitat is similar to that of *An. funestus* but are usually found at the edges of slow flowing streams (Gillies and De Meillon, 1968). The adults are frequently collected in natural resting sites and are sometimes found inside houses. They are presumed to be zoophilic and are not implicated in malaria transmission.

1.5 *Anopheles funestus*-like

The newly discovered *An. funestus*-like species from Malawi was found resting indoors and has not to date been implicated in malaria transmission. By analysing molecular, cytogentic and cross-mating data, Spillings *et al.* (2009) proved that *An. funestus*-like is a distinct species. Its chromosomal banding patterns show a homosequential banding arrangement with *An. funestus* but, interestingly, are fixed for the inverted arrangements of the paracentric chromosomal inversions 3a, 3b, and 5a which are commonly polymorphic in *An. funestus*. Hybrid progeny produced from crosses between *An. funestus* and *An. funestus*-like showed consistent asynapsis between homologous chromosomes together with mutation at the *An. funestus* primer binding site (3 base pair deletion and a T to C transition) are both suggesting a genetic discontinuity between the parents (Spillings *et al*., 2009). To date, very little information is known about the biology of this new species.

1.6 Identification of members of the *Anopheles funestus* group

The members of this group are morphologically similar as adults but many can be identified using characters from the aquatic stages. Cytogenetic methods have historically been employed, but molecular methods are now commonly used.

1.6.1 *Morphological identification*

Morphological identification of members of the *An. funestus* group depends on analysis at different life stages according to the taxonomic keys in Gillies and De Meillon (1968) and Gillies and Coetzee (1987). It involves collecting wild adult females and inducing them to lay eggs which are reared through to adults. This method is time consuming because the early life stages of the life cycle can take 4-6 weeks for complete development. Rearing larvae to adults is extremely difficult and if larvae die early, accurate identification is not possible. The following summary of the morphological differences between members of the *An. funestus* group is adapted from Gillies and De Meillon (1968) and Gillies and Coetzee (1987).

A) Adults:

Male *An. parensis* differ from *An. funestus* in that it has small pale scales at the base of its club in 90% of specimens. The presence of small patches of pale scale found at the apex of the sixth vein of the females makes it different from the other members of the *An. funestus* group except *An. brucei*. *Anopheles vaneedeni* tends to have very narrow pale bands at the joints of the tarsomeres.

B) Eggs:

Anopheles rivulorum eggs have a chorion with smaller size bosses than *An. funestus*. *Anopheles leesoni* differs from the rest of the *An. funestus* group members in that the egg chorion is unornamented with small and uniform size bosses. *An. leesoni* eggs have a frill which occupies the full length of the egg. *Anopheles confusus* differs from *An. funestus* by the presence of smaller sized bosses on the chorion. The eggs of *An. aruni*, *An. parensis*, *An. funestus* and *An. vanedeeni* are morphologically indistinguishable while those of *An. brucei* and *An. fuscivenosus* are undescribed.

C) Larvae:

Anopheles leesoni larvae differ from those of the rest of the *An. funestus* group by the presence of paired metathoracic plates. The abdominal plate is similar to that of *An. confusus*. Larvae of *Anopheles aruni*, *An. parensis* and *An. vanedeeni* are morphologically indistinguishable. *Anopheles rivulorum* larvae differ from *An. funestus* in that: they have sutural hairs which are branched from the base; the main abdominal plates are shallow and are three to four and a half times deep as wide; the accessory plates range in number from 1-3; sternites are without belts of spicules.

D) Pupae:

The pupae of *An. parensis* and *An. vaneedeni* are indistinguishable from those of *An. funestus*. *Anopheles rivulorum* pupae differ from those of the other members in that their pupal paddle fringe is not extended along the posterior border.

1.6.2 *Cytogenetic analysis for species identification*

Cytogenetic analysis as a method of species identification can be made by examination of the giant polytene chromosomes found in the ovarian nurse cells of half-gravid adult females (Coluzzi and Sabatini, 1967; Hunt, 1973). This method is based on the identification of species specific chromosomal banding sequences. These differences arise as a consequence of the divergent evolution of fixed chromosomal rearrangements resulting from chromosomal inversions and translocations.

Green and Hunt (1980) and Green (1982) published polytene chromosome maps detailing fixed inversion differences evident in the chromosomal banding patterns that allowed for the species identification of some members of the *An. funestus* group. Two fixed inversions on the X chromosome differentiate *An. parensis* from *An. funestus* but the banding patterns of *An. vaneedeni* and *An. funestus* are homosequential (Green and Hunt, 1980). The chromosomes of *An. parensis* differ from those of *An. funestus* and *An. vaneedeni* by a single fixed polymorphic inversion on arm 3 (Green and Hunt, 1980). The chromosomal map for *An. leesoni* is genetically distinct from that of *An. rivulorum*, *An. confusus* and *An. fuscivenosus* and is closely related to the oriental *An. minimus* group (Green, 1982; reviewed in Coetzee and Fontenille, 2004). Some disadvantages of this technique are that only half gravid adult females can be used, a high level of expertise is required for chromosomal banding pattern interpretation and the technique is labour intensive.

1.6.3 *Molecular species identification*

The DNA based Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method was first used by Koekemoer *et al*. (1998) to distinguish *An. funestus* from *An. vaneedeni*. A year later PCR-single strand conformation polymorphism (PCR-

SSCP) was used to distinguish *An. funestus*, *An. rivulorum*, *An. leesoni* and *An. vaneedeni* but unfortunately did not distinguish between *An. vaneedeni* and *An. parensis* (Koekemoer *et al*., 1999).

A multiplex-PCR assay is currently used to identify five members of the *An. funestus* group: *An. funestus*, *An. rivulorum*, *An. leesoni*, *An. vaneedeni* and *An. parensis* (Koekemoer *et al.*, 2002). Briefly, this assay employs a universal forward primer and species-specific reverse primers designed from the internal transcribed spacer region 2 (ITS2) of ribosomal DNA (rDNA). The PCR products produced are species-specific when visualized on ethidium bromide stained agarose gels. Using this technique requires electrophoresis of PCR products which is time consuming and involves using carcinogenic ethidium bromide. This method, however, is relatively inexpensive.

1.7 New techniques for the identification of species-complexes

Real time PCR assays such as the TaqMan assay, henceforth referred to as the hydrolysis probe assay, high resolution melts and melt curves are currently being used for identifying species or for detecting particular mutations. Details on the molecular chemistry of each of these techniques will be discussed briefly in Chapter 4.

1.8 Speciation concepts

The widely used hierarchical systematic scheme for classifying living organisms by Carolus Linneaus was necessitated by an expansive biodiversity and a belief that the scheme was immutable (Claridge *et al*., 1997). Although the scheme was seen to work well for plants and animals, Linneaus did not explicitly define what constitutes a species. A

number of different species concepts exist and continue to grow. The most widely used species concepts are discussed below.

1.8.1 *Biological or reproductive species concept*

Early work of Dobzhansky (1935) and Mayr (1942) made them well known researchers in the field of biological species concepts, emphasizing reproductive isolation. A species here is a group of interbreeding natural populations that are reproductively isolated from other groups (Mayr, 1996). The populations have acquired genetic changes, or ‘’isolating mechanisms’’ that prevent them from breeding and producing fertile offspring. The isolating mechanisms can be morphological, genetic or behavioral features and are categorized as either premating (prezygotic i.e**.** geographical, habitat, behaviour, temporal, mechanical and gametic isolations) or postmating (postzygotic i.e. reduced hybrid viability, reduced hybrid fertility and hybrid breakdown). This concept faced various forms of criticism because of its severe limitations. It lacks universality as it is not applicable to asexual organisms (Wheeler and Platnick, 2000) and to populations of organisms living in allopatry (Mallet, 1995). However, this concept has some clear advantages. The status of a species has very little to do with the observable differences that exist between populations. It also presents biological and genetic meaning.

1.8.2 The recognition species concept

This concept was introduced well after the widely accepted biological species concept (BSC). It was prompted by the difficulties in reconciling characteristics of mosquito species evolution within the constraints of the BSC (Paterson, 1985). By this concept, species are populations of individual biparental organisms which share a common fertilization system. This model is based on the specific mate recognition system (SMRS)

which are invariant suites of traits for efficient finding and recognition of mates (Paterson, 1993). A new species arises when a fertilization system adapts to a new habitat so as to make sure effective fertilization is possible. SMRS ensures mating as well as preventing hybridization with other species. The biological species concept was misleading according to Paterson. He claimed firstly, that the term isolating mechanism used by Dobzhansky/Mayr indicated that reproductive isolation was adaptive which is unlikely. Secondly, that prezygotic compatibility, comprised of mating and fertilization signals, is the reality underlying species. The advantages of the recognition species concepts include the fact that SMRS is more easily observed than interbreeding in nature. Also, it deals with what happens after speciation.

1.8.3 *Phylogenetic species concept*

Revolutionary developments in molecular biology techniques such as DNA sequencing allowed for a novel definition of species. Classification is based on sequence characterization. Various phylogenetic species concepts exist, some of which include those of Simpson and Cracraft. According to Simpson (1951), a species is defined as a lineage evolving separately from other lineages with its own unilateral evolutionary role and tendency. Cracraft (1983) defined a species by this concept as the smallest diagnosable cluster of individual organisms within which there is a pattern of ancestry and descent. The advantage of this concept is that it is applicable to both sexually and asexually reproducing organisms. The short-comings faced by this concept are: the characters to be used, the level of divergence that will constitute a species and the fact that the concept does not address the mechanism of speciation.

1.8.4 *Similarity species concept / Morphological species concept*

This concept defined species based on overall similarity in anatomical characteristics as the determining factor in discriminating species. In brief, species constitute a group of individual organisms that are morphologically similar and are distinguishable from individuals of another group (Claridge *et al*., 1997) as cited by (Rossello-Mora and Amann, 2001). By this model, morphology is an expression of genetics. The advantage of this concept is that morphological characters can be observed readily without handling or harming the organism. The pitfalls of this concept are i) it relies on expert interpretation and cannot explain the existence of sympatric species which are morphologically similar and occur in the same habitat but are reproductively isolated, ii) it fails to address sexual dimorphism and iii) cannot account for genetic polymorphisms not expressed as distinct morphology. The concept is therefore prone to misidentification.

1.9 Research rationale

Socio-economic development in many African countries is hampered by the burden of malaria. The effective control of malaria vectors requires accurate information in terms of species vector incrimination and the management of insecticide resistance where it occurs in malaria transmitting populations. Because the major African malaria vector species are members of either the *An. gambiae* complex or the *An. funestus* group which include non-vector species, accurate species identification is necessary for vector incrimination and for the assignment to species of insecticide resistance phenotypes where detected. Species identification relies on the application of appropriate species concepts and the development of methodologies that allow for unambiguous species identification of unknown samples or populations.

The malaria vector and non-vector members of the *An. funestus* group are morphologically similar to each other. Currently, there is no single molecular assay that can be used to distinguish between the *An. funestus* group members including *An. funestus*-like in a single reaction. The assay of Koekemoer *et al.* (2002) failed to identify the new *An. funestus*-like species because of the presence, in *An. funestus*-like, of a three base pair deletion and a T to C transition in the *An. funestus* specific primer site (Spillings *et al.*, 2009). As a result, the *An. funestus* specific primers cannot anneal to *An. funestus*-like DNA during amplification.

1.10 Aims

The aim of this project was to develop a molecular assay that can be used to identify all members of the *An. funestus* group, including *An. funestus*-like, in one reaction as well as to provide additional information on the malaria vector status and general biology of *An. funestus*-like.

1.11 Specific objectives:

i) To develop, test and evaluate new assays for species identification of members of the *An. funestus* group (from field and insectary samples) including the new *An. funestus*-like species from Malawi.

ii) Determine the malaria vector status, host preference, biting and resting behaviour of *An. funestus*-like.

iii) Investigate the phylogenetic relationship between the *An. funestus* group and *An. funestus*-like.

iv) To morphologically describe *An. funestus*-like.

CHAPTER TWO

Biological and behavioural characterization of the *Anopheles funestus* group, including *Anopheles funestus*-like, from northern Malawi

2.1 Introduction

Malawi (94,079 sq km) is a landlocked country in southeast Africa bordering on Mozambique, Tanzania and Zambia. The eastern border is mostly occupied by Lake Malawi and the country has a north-south rift valley which is flanked by mountains and plateaus. The climate is sub-tropical with two distinct seasons: the rainy season from November to May and a dry season from May to November (www.infoplease.com/ipa/A0107747.html accessed 20 March 2011).

Like many other African countries, malaria remains the leading cause of mortality and morbidity in Malawi where it is endemic. The Presidents Malaria Initiative: Malaria Operational Plan-Malawi 2010 and 2011 best describe the impact and transmission of malaria in Malawi. Below is a summary from this source. Of a population of 13.1 million, 97% are at risk of exposure to malaria. An estimated six million malaria cases were reported in Malawi in 2006, accounting for 33% of outpatients. The major causative agent is *Plasmodium falciparum* which accounts for approximately 98% of malaria infections with the remaining 2% caused by *P. malariae* and *P. ovale*.

The main vectors of malaria in Malawi are *Anopheles gambiae s.s*, *An. funestus s.s* and *An. arabiensis*. Although malaria is endemic and perennial in this country, higher transmission

occurs along the shores of Lake Malawi and the lowlands of the Shire Valley. The peak period for transmission follows immediately after the rainy season from October to April. In terms of economic loss to the country, the disease accounts for a huge workforce deficit of about 15-25 days per year. Also, 28% of annual income is spent on treating the disease.

Malaria prevention and treatment in Malawi includes case management and vector control. Management of the disease depends on early diagnose and treatment. Malaria is diagnosed by either microscopic or rapid diagnostic tests. As part of the global initiative to eradicate malaria, chloroquine was widely used as the front-line therapy for *P. falciparum* in sub-Saharan Africa. It was recommended for treating uncomplicated malaria in children below five years in Malawi due to its availability, low cost and efficacy. Unfortunately its usage was hampered by the development of resistance in *P. falciparum*. This resulted in a switch in treatment in 2006. Artemisinin-based combined therapies (ACT) are currently the recommended front-line treatment for uncomplicated malaria caused by *P. falciparum*. Intermittent preventive treatment for pregnant women consists of the administration of sulfadoxine-pyrimethamine (SP) which reduces anaemia in the mother and the unborn child as well as the incidence of consequent low birth weight.

Malaria vectors are controlled mainly by indoor residual spraying and the use of insecticide treated nets (Presidents' Malaria Initiative Operational Plan-Malawi 2010). In Malawi, the National Malaria Control Programme (NMCP) recommended the pyrethroid ICON-CS (Lambda-Cyhalothrin capsule suspension) for indoor residual spraying (IRS). Currently, selected pilot areas including the Nkhotakota district are part of a feasibility study to ascertain the operational, logistical and human resources requirements for malaria vector control. Sixty percent of households in Malawi own one or more insecticide treated

nets (ITNs), particularly long lasting insecticide nets (LLINs), and 55% of children under the age of five as well as 49% of pregnant women utilize these nets which are distributed for free. Pyrethroid resistance in *An. funestus* and *An. arabiensis* has recently been reported from Malawi (Rehman *et al*. 2011).

Study rationale

Despite important advances in the use of chemotherapy to target malaria parasites, knowledge of the behaviour and ecology of malaria vectors in Malawi is lacking. During 2007, the Vector Control Reference Unit (VCRU/NICD), Johannesburg, South Africa, conducted a malaria survey on behalf of the Malawian Department of Health, to determine the insecticide susceptibility status of malaria vectors in Karonga. Indoor resting mosquitoes were collected and a proportion of these were morphologically identified as members of the *An. funestus* group. They were preserved for further laboratory processing at the VCRU. However, the routine molecular species identification assay of Koekemoer *et al.* (2002) failed to identify many of these specimens to species level. It was subsequently established that the bulk of the unidentified *An. funestus* group specimens represented a thus far undescribed species within this group provisionally named *An. funestus*-like (Spillings *et al.* 2009). Fifty four *An. funestus*-like specimens (collected during the dry season) were tested for *P. falciparum* infection using an Enzyme Link Immunosorbent Assay (ELISA) (Wirtz *et al*., 1998). None showed positive and their blood meal sources were not determined (Spillings *et al.* 2009). *Anopheles funestus*-like thus currently carries the status of a relatively rare non-vector species.

2.2 Aim and objectives

The aim of this chapter was to further investigate the role, if any, of *An. funestus*-like in malaria transmission in Karonga, Malawi. Specific objectives were:

2.2.1 To establish the host preference of *An. funestus*-like

2.2.2 To determine its vectorial capacity for other *Plasmodium* species

2.2.3 To determine its resting behaviour

2.3 Materials and Methods

2.3.1 Study area

The field component of this study was carried out in Karonga - a town in northern Malawi - and Likoma Island on Lake Malawi. There are distinct wet and dry seasons at these localities. Karonga has a mean annual rainfall of 1025mm (Kwapata *et al*., 2007). Six sites in five villages were selected for specimen collection based on availability of breeding sites. The villages in the North of Karonga include Mwenetete (two sites (9°48.257S; 53°52.161E and 9°47.635S; 33°52.342E)), Mwakabighili (9°49.65S; 33°52.33E), Kwambwe (9°54.032S; 33°55.792E) and Mwampaghatwa, about 2 km North of Mwenetete. In the South of Karonga, specimens were collected from Wovwe (10°18.627S; 34°07.901E) Figure 2.1. Specimens from Likoma Island ((12º04S, 34º44E) Hunt *et al*., 2010) were provided by Professor Richard Hunt.

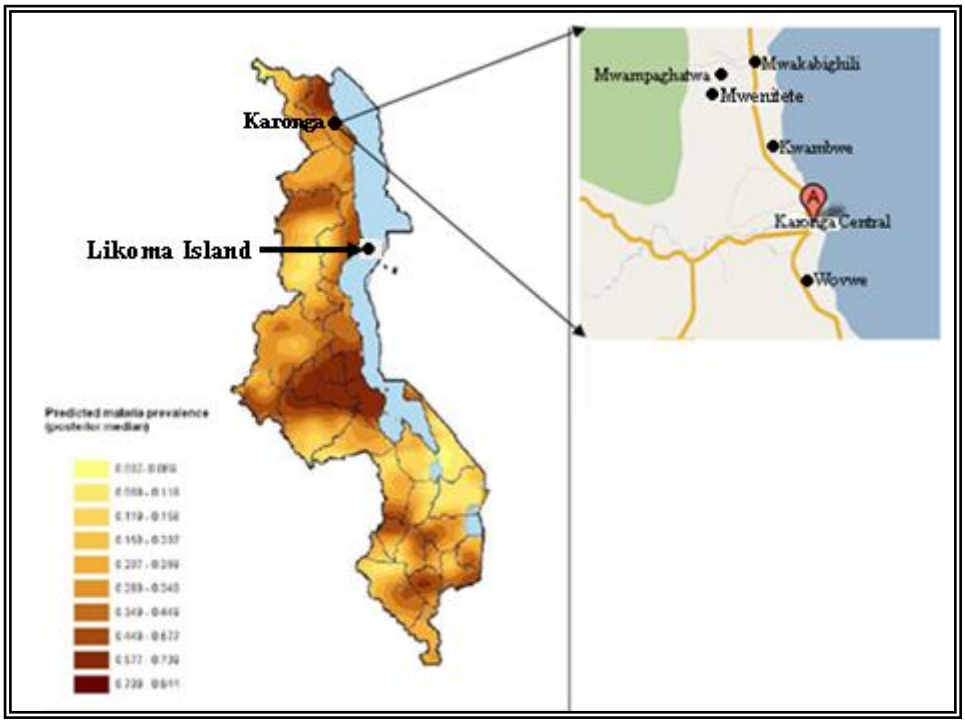


Figure 2.1 Map of Malawi adopted from Kazembe *et al*. (2006) showing Karonga town and Likoma Island. The side map showing sample collection sites was obtained from Google earth map (http://maps.google.co.za, accessed on 20 May 2011).

The primary economic activities in all villages where specimens were collected include farming, fishing and general trading. Rice is mostly cultivated in the north, whilst maize and tobacco farming is predominant in the south. Human dwellings were mainly constructed using either brick or wood and mud (wattle and daub) with thatched roofs without ceilings. Houses in the study area were unsprayed and there was an average of two bed nets per house most of which were insecticide treated. Some inhabitants slept in sheds as a consequence of an earth quake in the region. Domestic animals and livestock including chickens, dogs and cattle are kept in close proximity to dwellings. Piped water, health facilities and good drainage were absent in all villages. Home owners were approached through regional malaria control leaders and an informed consent obtained before the commencement of collections.

2.3.2 Mosquito collections

Mosquitoes resting either inside houses or outdoors were collected using an aspirator. Collections were done from 21 - 29 April and from 24 September - 8 October, 2010 in the mornings (8:00 am to 12:00 pm) and afternoons (15:00 pm to 17:00 pm). Houses without nets or those with untreated nets that were close to breeding sites were preferentially sampled to maximize the collection. Outdoor sites searched included tree holes, empty containers and animal shelters. Human landing catches and cattle kraal collections (Appendix III) were performed in Kwambwe village late in the evenings. Collected mosquitoes were immobilised using ethyl acetate and the gonotrophic state of each female was recorded by observing their abdomens with a hand lens. Each mosquito was then individually desiccated in a 0.2ml eppendorf tube containing silica gel (Fisher Scientific, Fair Lawn, NJ) and kept under ambient conditions. A sub-set of the collected females were stored alive, maintained on a 10% sugar solution and were transported to the Botha De Meillon insectary, Vector Control Reference Unit (VCRU), Johannesburg, South Africa for further analysis.

2.3.3 Mosquito rearing

The insectary was maintained at temperature of 25 ± 2°C and relative humidity of 75 ± 5%. A day/night cycle of 12 hours each including a dusk/dawn gradient of 45 minutes was mimicked. Live wild-caught females from Karonga and Likoma Island were individually placed in glass vials lined with moistened filter paper and were induced to lay eggs. The eggs from each female were transferred to polythene plastic bowls (27cm x 16cm x 6.5cm) 1/3 filled with distilled water. Each family was reared separately. Emerging F_1 larvae were fed with a mixture of powdered dog biscuits (West's traditional crunching biscuit treats, Martin and Martin, South Africa) and yeast (Vital Health Foods, South Africa) prepared at

a ratio of 3:1. F_1 adults were maintained on 10% sugar water for insecticide susceptibility bioassays.

2.3.4 Insecticide susceptibility tests

Insecticide susceptibility tests were performed using the standard WHO protocol (WHO, 1998). Before test exposures were conducted, the efficacy of the insecticide papers was tested by exposing samples of the insecticide susceptible *An. funestus* laboratory strain, FANG. This colony originates from Angola and has been in colony since 2002. All papers used in subsequent tests induced 100% mortality in the exposed FANG samples. Samples of two to three day old non-blood fed F_1 adults (mixture of males and females) were then tested. For all tests, samples of 3-25 F_1 progeny by family were transferred into a holding tube. They were then transferred to an exposure tube lined with an insecticide impregnated filter paper treated with a resistance diagnostic dose (4% DDT, 0.05% deltamethrin and 0.01% bendiocarb). The number of tubes used for exposure was DDT (2), deltamethrin (9) and bendiocarb (7). Adjacent to each test, a control tube containing 25 mosquitoes (FANG) were exposed to papers without insecticide. Following a one hour exposure, the number of mosquitoes knocked down in each tube was recorded and all mosquitoes were transferred back to their respective holding tubes and provided with a cotton pad soaked in a 10% sucrose solution. Final mortality was recorded twenty four hours post-exposure. In instances where the mortalities of the controls were between 5% and 20%, the mortalities of the corresponding test samples were corrected using Abbot's formula (Abbott, 1925):

$$[(E-C) / (100-C)] \times 100$$

E and C represent percentage mortality of mosquitoes in the exposure and control tests respectively. If no mortality was observed in the control, the mortality data for the test

samples were pooled. All susceptibility tests were performed at a temperature of 25 ± 2°C and relative humidity of 75 ± 5%.

2.3.5 Mosquito laboratory processing

2.3.5.1 DNA extraction

The protocol described by Collins *et al*. (1987) detailed in Appendix IIA was used for extracting DNA from the abdomens of single mosquitoes. Preparation of DNA extraction solutions are given in Appendix IA.

2.3.5.2 Mosquito identification

Species-specific identification of all *An. funestus* group samples, including the mothers of each family described above, was performed according to the cocktail PCR assay of Koekemoer *et al*. (2002). Unidentified samples from this assay were subjected to PCR identification according to Spillings *et al*. (2009) which is used for identification of *An. funestus*-like. Those samples that did not produce PCR products in either assay were sequenced and analysed for identification. Protocols are detailed in Appendix IIB and IIC.

2.3.5.3 Sporozoite infectivity rate detection by ELISA

To establish the vector status of the populations represented by the wild caught female specimens, a sporozoite Enzyme-Linked Immunosorbent Assay (ELISA) that detects circumsporozoite proteins (CSP) in the head and thorax of each specimen was performed (Wirtz *et al.* 1992). The head and thorax of each *An. funestus* group female was separated from the abdomen with the aid of a sterile blade and forceps and analysed. Positive controls consisting of synthetic peptides for *P. falciparum* and *P. vivax* (Cat. No. Pf-PC

and Pv-PC Washington DC, USA), supplied by Dr. R. Wirtz of the CDC Atlanta, USA, were freshly prepared for each ELISA. Antibodies of the other *Plasmodium* species were not available during this study and were therefore not used. Negative *An. funestus* controls, consisting of seven insectary-reared unfed females (FANG), were processed in the same manner as the test samples. To quantify the degree of infection in *An. funestus*-like ELISA-positive specimens, a *P. vivax* positive control was serially diluted to 0pg/50µl (100, 50, 25, 12, 6, 3, 1.5 and 0) from 100pg/50µl in triplicate. The CSP-ELISA procedure was then conducted after which a standard curve was plotted against which ELISA positives could be compared and quantified. The prepared homogenates, if not used on the same day, were stored at -70ºC until analysis. The preparation of the chemicals and solutions are given in Appendix IC while the protocol for this assay is detailed in Appendix IID.

2.3.5.4 Blood meal identification

Blood meal identification was determined by the direct enzyme-linked immunosorbent assay (ELISA) method of Beier *et al.* (1988) using field collected females identified as 'fed' following examination of the abdomen using a hand lens. The chemicals and solutions for this assay are given in Appendix I D and a detailed protocol is provided in Appendix II E. This assay employs anti-host immunoglobulin G (IgG) conjugates directed against human, pig, dog, chicken, bovine and goat blood. The presence of all these animal hosts was confirmed during the field collections. The abdomen of each female was separated from the thorax using a sterile scalpel blade. Each abdomen was individually placed in a labelled 1.5ml eppendorf microcentrifuge tube and homogenized. Negative controls consisted of four unfed female *An. funestus* mosquitoes (FANG) which were processed in the same way as the test samples. Positive controls included 1µl of host blood diluted in 50µl PBS and were freshly prepared as needed. Blood for the positive controls

was collected by trained staff at the Department of Agriculture, University of Pretoria, South Africa.

2.3.5.5 Data analysis:

Resting behaviour:

Mosquitoes were regarded as "indoor resting" when caught resting inside houses and "outdoors resting" when caught from cattle kraals.

Seasonality:

Anopheles funestus-like mean abundances for the wet and dry seasons were calculated as a ratio of the total specimens collected per season to the total number of *An. funestus*-like identified in the study.

Sporozoite infectivity rate:

Samples with optical densities greater than twice the average of the optical density of the negative controls were considered positive for *Plasmodium* infection and were repeated for confirmation (Wirtz *et al.*, 1992). The percentage infectivity rate was then calculated by mosquito species and *Plasmodium* species. The equation for the standard curve was used to calculate the concentration of *Plasmodium* in infected *An. funestus*-like specimens.

Blood meal identification:

Samples were considered positive for a particular blood source if their absorbance values exceeded the mean plus three times the standard deviation of the four negative controls (Beier *et al.*, 1988). The percentages of mosquitoes with human and animal (pig, dog,

chicken, bovine and goat) blood were calculated. The blood source with the greatest percentage was considered the main blood source.

2.4 Results

Owing to financial constraints only two field trips were conducted in the course of this study. Specimens collected from 21- 29 April 2009 were termed wet season collections because they were collected during the rainy season which was characterised by an abundance of breeding sites. Those collected from 24 September - 8 October 2010 were regarded as dry season collections.

2.4.1 Mosquito collections

A total of 391 adult *Anopheles* mosquitoes were collected from indoor and cattle kraal collections from both localities. The bulk of the samples analysed were from Karonga (63.9%, n = 250) and these comprised of wet season (48.4% n = 121) as well as dry season (51.6% n = 129) collections (Table 2.1). In the Karonga wet season collections, indoor-resting samples accounted for 87.6% of the sample while a small number (12.4%) were collected outdoors. Dry season samples were predominantly comprised of indoor resting mosquitoes (99.2%) whilst the rest were collected outdoors.

Table 2.1 Distribution of *Anopheles funestus* group collections from Kironga and Likoma Island.

Seasons	Karonga (n = 250)					Likoma Island (n = 141)	TOTAL
	Collection sites						
	Mwakabighili n (%)	Mwenetete n (%)	Wovwe n (%)	Kwambwe* n (%)	Mwampaghatwa n (%)		
Wet	21(8)	20(7.6)	65(24.8)	15(5.7)	-	141(53.8)	262 (67)
Dry	25(19.4)	88(68)	1(0.8)	1(0.8)	14(10.9)	-	129 (33)
	46(11.8)	108(27.6)	66(16.9)	16(4)	14(3.6)	141(36)	**391**

* Specimens from this site were all cattle kraal collections, n = total sample collected, % = percentage calculated from grand total

The Likoma Island samples were all collected during the wet season (n = 141) and were mainly collected indoors. This area was not originally part of the study. However, with an opportunity to visit this site, these collections were included to broaden the search for *An. funestus*-like.

2.4.2 *Anopheles funestus* group species identification

Figure 2.2 shows species-specific PCR results for the identification of the *An. funestus* group members including *An. funestus*-like.

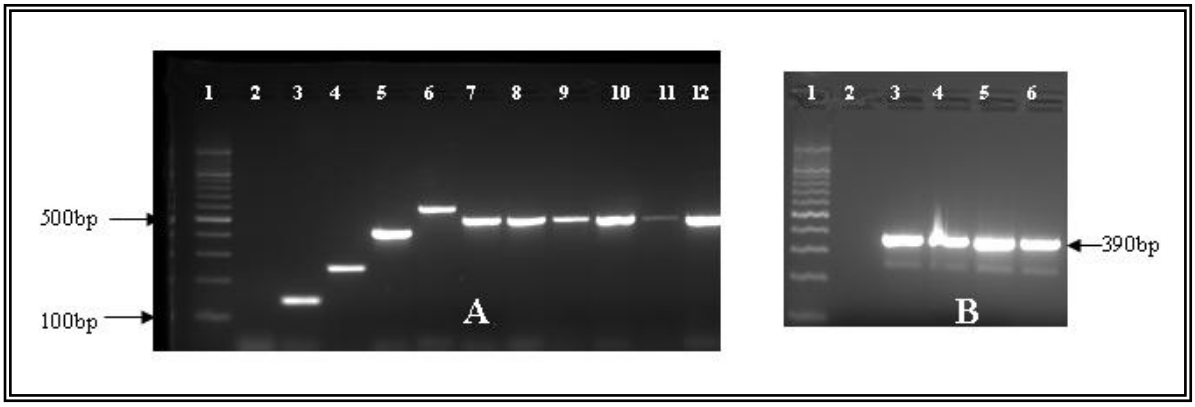


Figure 2.2 2.5% agarose gels stained with ethidium bromide showing PCR products amplified using species-specific primers for *Anopheles funestus* group identification. **A:** Lane 1: Molecular marker; Lanes 2 to 7: Controls, Lane 2: Negative control; Lane 3: *An. leesoni*; Lane 4: *An. parensis*, Lane 5: *An. rivulorum*, Lane 6: *An. vaneedeni*, Lane 7: *An. funestus*; Lanes 8-12: Test samples; **B:** Lane 1: Molecular marker; Lane 2: Negative control; Lane 3: *An. funestus*-like positive control; Lanes 4 to 6: Test samples.

The ITS2-region of samples unidentified using either assay of Koekemoer *et al*. (2002) or Spillings *et al*. (2009) were amplified and subsequently sequenced to allow for species identification by analysing the sequences of the species specific primer sites. Figure 2.3 shows PCR products generated from the rDNA ITS2-region. Results obtained from analysis of the ITS2-region of rDNA were included in Table 2.2.

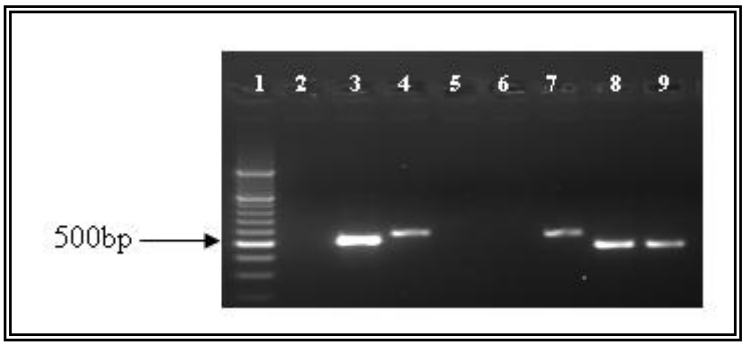


Figure 2.3 A 2.5% agarose gel stained with ethidium bromide showing PCR amplification of the ITS2-region of the rDNA gene. Lane 1: DNA marker; Lane 2: Negative control; Lane 3: *An. rivulorum* (control), Lanes 4 to 9: test samples.

Tables 2.2 and 2.3 show the distribution of *An. funestus* group members identified from the wet and dry season collections respectively. A total of 349 (89.3%) of the collected *Anopheles* mosquitoes were identified and 42 (10.7%) could not be identified. *Anopheles funestus* was the predominant species collected indoors during the wet season and *An. rivulorum* predominated indoors during the dry season. Within the *An. funestus* group, *An. rivulorum* and *An. funestus* were the only species identified from the outdoor collections (Kwambwe.

Table 2.2 *Anopheles funestus* group species identified from wet season indoor and outdoor collections.

Collection site	***Anopheles funestus* group species**			**Total**
	An. funestus-like **n (%)**	***An. rivulorum*** **n (%)**	***An. funestus*** **n (%)**	**n =224**
Mwakabighili	3 (20)	12 (80)	-	15
Mwenetete	8 (42.1)	10 (52.6)	1 (5.3)	19
Wovwe	-	3 (4.8)	60 (95.2)	63
*Kwambwe	2 (16.7)	9 (75)	1 (8.3)	12
Likoma Island	-	-	135 (100)	135
Total	**13**	**34**	**197**	**224**

* Specimens from this site were all cattle kraal collections. n = number of samples identified, % = percentage of identified sample

Table 2.3 *Anopheles funestus* group species identified from dry season indoor and outdoor* collections.

Collection site	***Anopheles funestus* group species**				**Total n = 103**
	funestus-like n (%)	***rivulorum*** n (%)	***funestus*** n (%)	***parensis*** n (%)	
Mwakabighili	3 (15)	17 (85)	-	-	20
Mwenetete	13 (19.1)	52 (76.5)	1 (1.5)	2 (2.9)	68
Wovwe	-	-	1 (100)	-	1
*Kwambwe	1 (100)	-	-	-	1
Mwampaghatwa	6 (46.2)	7 (53.8)	-	-	13
Total	23	76	2	2	**103**

* Specimens from this site were all cattle kraal collections. -: implies no sample and n: number of sample. n = number of samples identified, % = percentage of sample.

Figure 2.4 shows the variation in the number of *An. funestus*-like collected during the wet and dry seasons per collection site. *Anopheles funestus*-like was consistently collected in both seasons from Mwakabighili, Mwenetete and Kwambwe. Mwenetete was the most productive site (n = 12) during the dry season. A larger proportion of *An. funestus*-like (68.6%, n = 35) was collected during the dry season than the wet season (31.4%, n = 35). For both seasons, the majority of mosquitoes were collected indoors except in Kwambwe where all the mosquitoes were collected from a cattle kraal.

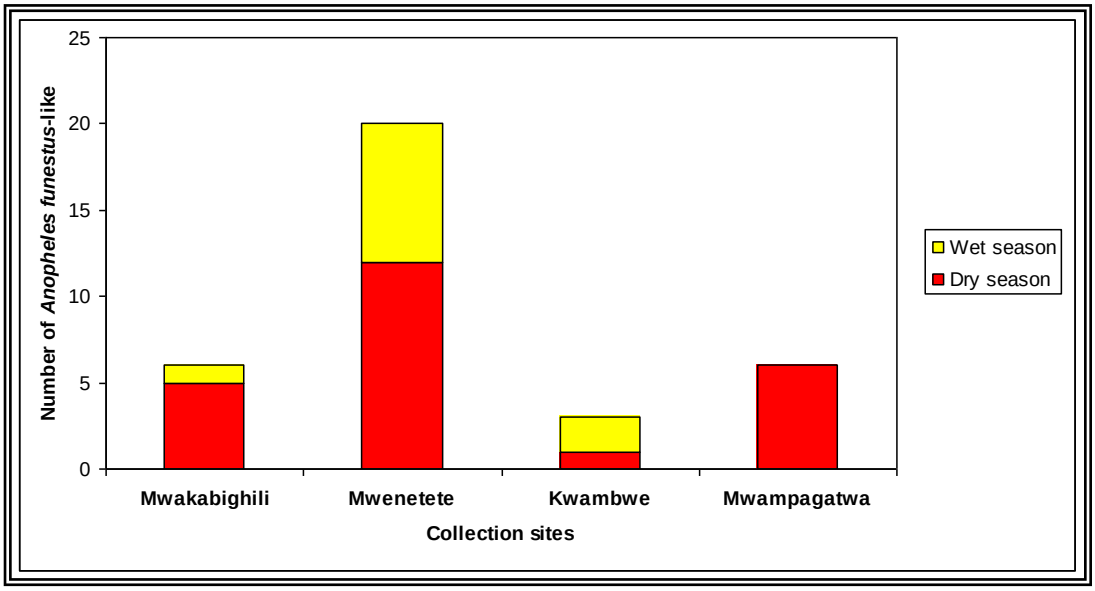


Figure 2.4 Comparative analysis of seasonal predominance of *An. funestus*-like in Karonga. In Mwampaghatwa village, specimen collection was done only during the dry season.

2.4.3 *Insecticide susceptibility tests*

Only the collections from Wovwe successfully produced sufficient F_1 adult progeny (n = 272) for insecticide susceptibility tests. The progeny from females collected from the other sites either died during the aquatic stages or the eggs failed to hatch. According to WHO recommendations, 24 hour post exposure mortality of 98 – 100% indicates full susceptibility, 80 - 97% suggests a possibility of resistance that needs to be confirmed and

less than 80% suggests resistance to the insecticide tested. Analysis based on these indicators shows that *An. funestus* s.s from Wovwe is resistant to deltamethrin (72.2% mortality) and bendiocarb (61.9% mortality), and is susceptible to DDT (98.1% mortality) (Figure 2.5). None of the tested F_1 specimens were *An. funestus*-like.

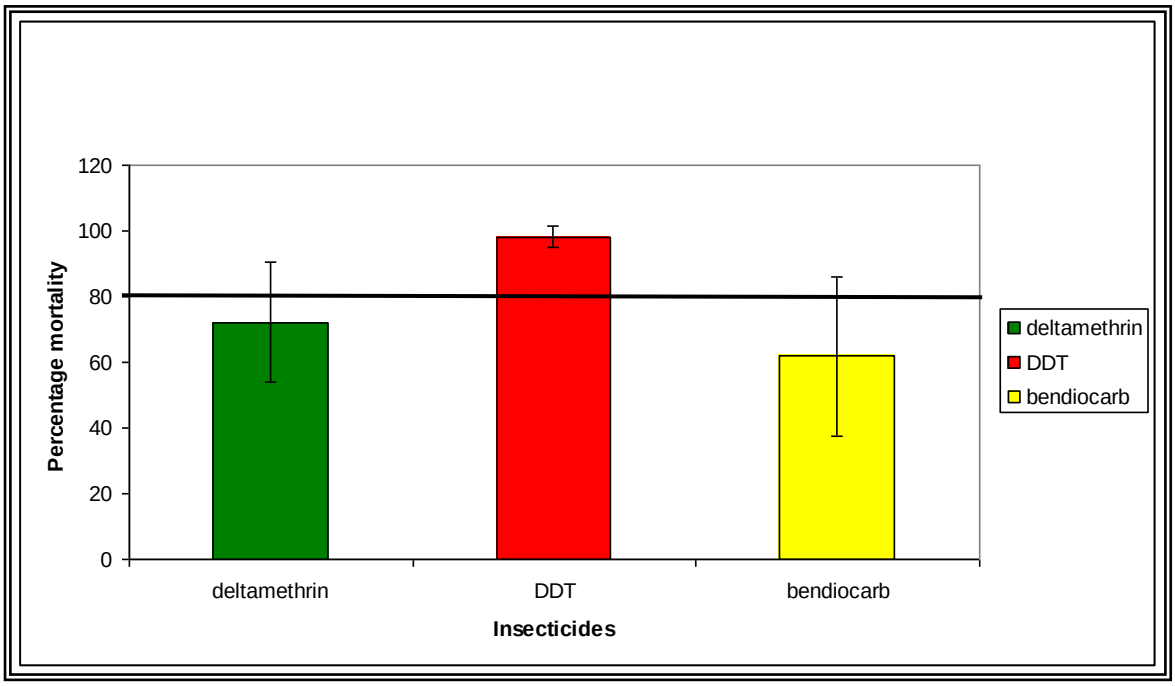


Figure 2.5 Mean proportional insecticide susceptibilities of *Anopheles funestus* from Wovwe Karonga Malawi to deltamethrin, DDT and bendiocarb. The horizontal line at 80% mortality indicates the cut off point below which the percentage mortality indicates resistance. Error bars represent standard deviation

2.4.4 *Blood meal source identification*

A total of 117 blood engorged female *Anopheles* were analysed for their blood source. Of these, only 54 samples (46.2%) tested positive for host blood. The detailed results of appendix IV are summarised in Table 2.4. The combined species data show that single human or animal blood meals constituted 3.7% (2/54) and 88.9% (48/54) respectively. Mixed blood meals represented 7.4% (4/54) of the sample.

Table 2.4 Blood source preferences of members of the *An. funestus* group.

Anopheles species	Total (n = 54)	Blood meal source		Mixed blood (%)
		Human (%)	Animal (%)	
An. funestus-like	10	-	10 (100)	-
An. rivulorum	34	-	31 (91.2)	3 (8.8)
An. parensis	1	-	1 (100)	-
An. funestus	9	2 (22.2)	6 (66.7)	1 (11.1)

Different combinations of mixed blood meals (MBM) were observed for each mosquito species - Appendix IV. The number of MBM combinations by species was *An. funestus* 4, *An. rivulorum* 5, and *An. parensis* 1. The dominant combination for most species is bovine and goat.

A total of 21 female *An. funestus*-like mosquitoes collected during the dry and wet seasons were analysed for their blood meal source (Figure 2.6). Ten of twenty one (47.6%) were positive for animal blood and none for human blood. Seven specimens (70%) tested positive for mixed blood from bovine and goat and only 3 (30%) for single blood meals from goat.

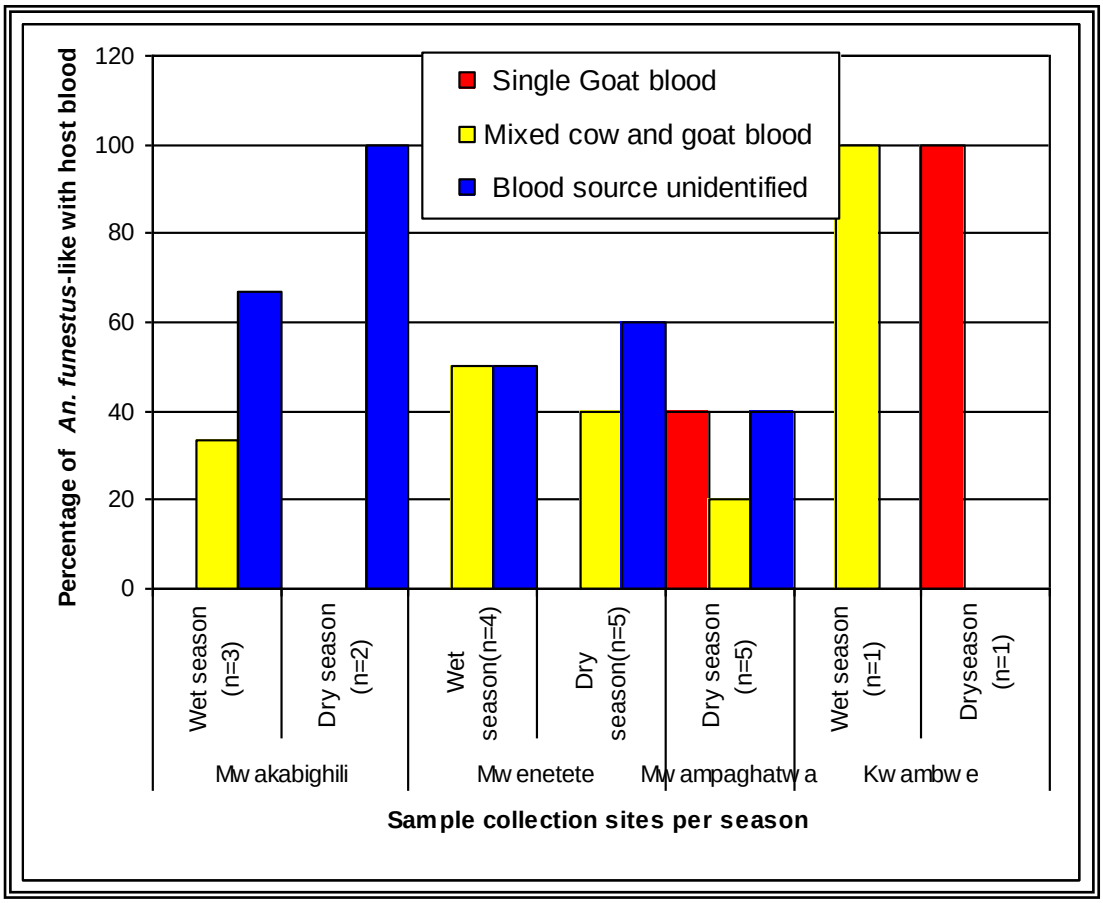


Figure 2.6 Results of ELISA against bovine and goat antibodies for blood meal identification of blood-fed female *An. funestus*-like collected during the dry and wet seasons from different sites in Karonga. 'n' indicates number of samples analysed. Collections were not performed during the wet season in Mwampaghatwa.

Human blood index (HBI)

Human blood indices detected from these collections were as follows. *An. rivulorum* collected from Mwakabighili was 12.5%, *An. rivulorum* and *An. funestus* from Mwenetete was (10% and 8.3%) and 100% respectively. For *An. funestus* from Wovwe, the HBI was 25. The HBI for *An. funestus*-like was zero - Appendix IV.

2.4.5 *Plasmodium* sporozoite infectivity rate

Of the five species of *Anopheles* screened for *P. vivax* and *P. falciparum* using Circumsporozoite Protein (CSP)-ELISA, only *An. funestus* tested positive for *P. falciparum* at a proportion of 5.9% (n = 134). *Anopheles funestus*-like was found to be infected with *P. vivax* with an infection rate of 6.7% (n = 15) (Table 2.5). The infected *An. funestus*-like samples were all from Mwenetete giving an infection rate for this species at that locality of 20% (n = 5). All the other anopheline species were CSP negative (Table 2.5). Irrespective of seasons, an overall infection rate for *An. funestus* with *P. falciparum* in this study was 4.4% (n = 181) and 4% (n = 25) for *An. funestus*-like infected with *P. vivax*. As a consequence of the infection of *An. funestus*-like with *P. vivax*, 210 specimens collected in 2008 from Karonga which showed negative for *P. falciparum* were subsequently screened for *P. vivax* and an infection rate of 3.1% (n = 64) was obtained. Confirmation of the positive infection of *An. funestus*-like with *P. vivax* using PCR according to Snounou *et al*., (1993) proved unsuccessful.

Table 2.5 Detection of *Plasmodium* species infection by ELISA

Sites	Season	*Anopheles* species	n	*P. falciparum*		*P. vivax*	
				Positive (n)	**Infectivity rate (%)**	**positive**	**Infectivity rate (%)**
Likoma Island	Wet	*An. funestus*	134	8	5.9	0	0
Karonga	Wet	*An. funestus*	45	0	0	0	0
		An. rivulorum	29	0	0	0	0
		An. funestus-like	10	0	0	0	0
	Dry	*An. funestus*-like	15	0	0	1	6.7
		An. rivulorum	50	0	0	0	0
		An. funestus	2	0	0	0	0
		An. parensis	1	0	0	0	0

n = Total samples tested.

2.4.5.1 *Plasmodium vivax* Quantification

To determine the parasite load in infected *Anopheles funestus*-like, *P. vivax* infection was quantified. The average of the absorbance values at each concentration of the serial dilution was determined and a standard curve was constructed (Figure 2.7).

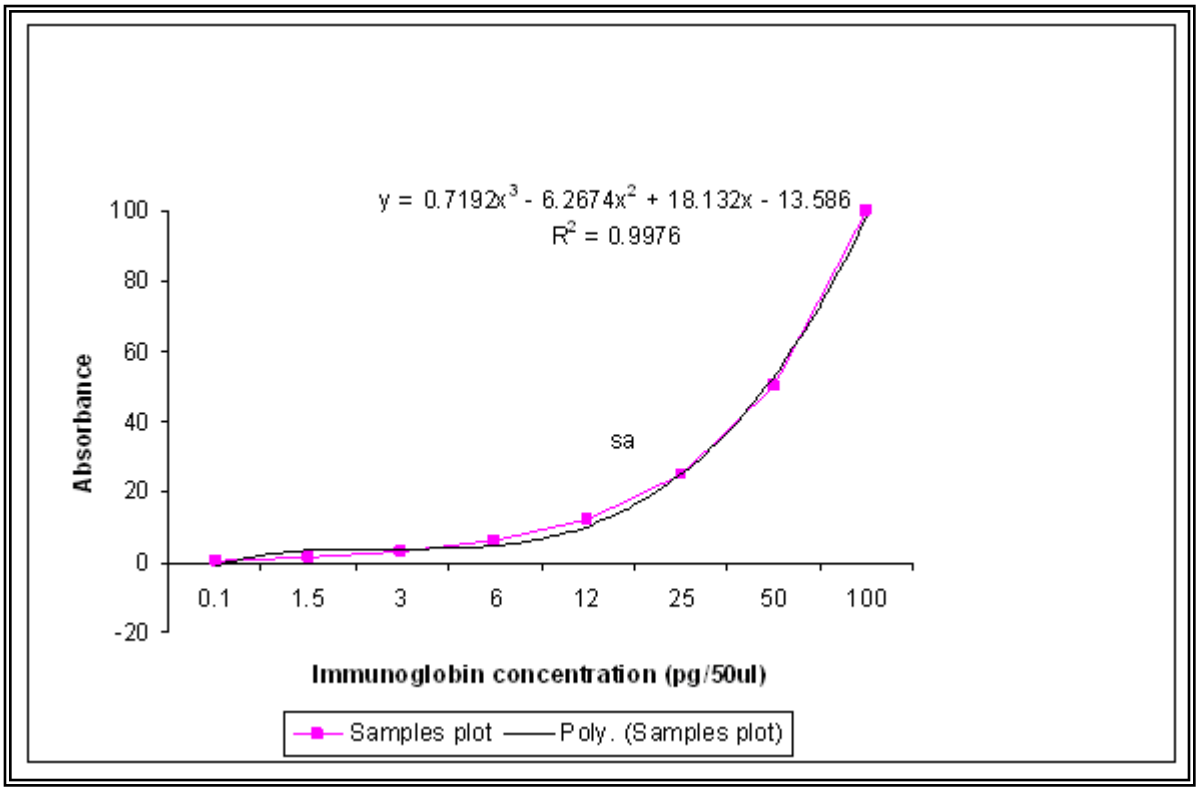


Figure 2.7 *Plasmodium vivax* ELISA assay standard curve. The equation for the best fit polynomial that matches the graph for the test samples was obtained using Microsoft Excel 2003.

Three *An. funestus*-like specimens (one from this study (Table 2.5) and two from Spillings *et al*., 2009 collections) were found to be positive for *P. vivax* using ELISA with absorbance values of 0.182, 0.121, and 0.122. Because of the low values, extrapolation of the corresponding *P. vivax* concentration from the standard curve proved difficult. However, an equation for the standard curve was determined from which the corresponding *P. vivax* concentrations of 2.468, 2.464 and 2.464 pg/50µl were obtained.

2.5 Discussion

2.5.1 Collection methods

Anopheles funestus group mosquitoes were successfully collected indoors and from cattle kraals in northern Malawi. Identification of such samples to species is critical because it enables differentiation between those species that transmit malaria and those that do not. Apart from *An. funestus* which is highly endophilic, *An. rivulorum*, *An. leesoni* and *An. parensis* can also be found resting indoors (Gillies and De Meillon, 1968; Kamau *et al.*, 2003-; Mouatcho *et al*., 2007), negating the assumption that indoor-resting *An. funestus* group samples are most likely *An. funestus* s.s. *Anopheles funestus*-like has previously been collected indoors from Karonga (Spillings *et al*., 2009; Hunt personal communication), although attempts to collect this species using the human landing catch method failed, suggesting that it is essentially exophilic.

Based on morphological and molecular data, this study highlights the presence of four sibling species in the *An. funestus* group in Karonga i.e. *An. funestus*, *An. rivulorum*, *An. parensis* and *An. funestus*-like, while *An. funestus* was the only species collected on Likoma Island. Previous studies conducted in the Karonga region reported the presence of *An. rivulorum*, *An. funestus*-like (Spillings *et al*., 2009) and *An. funestus* (Chiphwanya J personal communication). The additional presence of *An. parensis* is reported here, although in low numbers. *Anopheles rivulorum* was predominant in Mwakabighili, Mwenetete and Kwambwe whilst *An. funestus* predominated in Wovwe and Likoma Island based on data from the wet and/or dry season collections. The predominance of *An. rivulorum* over *An. funestus* is surprising as *An. funestus* has previously been associated with rice agro-ecology (Gillies and

De Meillon, 1968). The predominance of *An. funestus* in Wovwe was expected as the breeding sites there are conducive for this species.

Only 10.7% (n = 391) of collected *An. funestus*. s.l could not be identified to species using the species-specific PCR of Koekemoer *et al.* (2002) and Spillings *et al.* (2009), even after two attempts. This may be attributed to misidentification by human error, failure of the DNA extraction process, the presence of inhibitors after DNA extraction, the presence of other member species of the *An. funestus* group not detected by these assays or the degradation of DNA due to storage problems.

2.5.2 *Insecticide susceptibility test*

Results from this study shows that *An. funestus* population from Wovwe are resistant to deltamethrin and bendiocarb but fully susceptible to DDT. Although the underlying resistance mechanisms in this population have yet to be evaluated, it is unlikely that knock-down resistance (*kdr*) mutations are present because they normally confer resistance to pyrethroids and DDT (Martinez-Torres *et al*., 1998; Dabire *et al*., 2009).

The detection of deltamethrin and bendiocarb resistance in *An. funestus* observed in Wovwe tallies with previous reports from southern Africa. In Mozambique, Abilio *et al*. (2010) reported resistance to the pyrethroid lambda cyhalothrin and bendiocarb in a population of *An. funestus* from Mugeba and Majaua in Mozambique. Similarily, Hunt *et al*. (2010) recently observed the same resistance profile in *An. funestus* from Likoma Island. The above resistance scenario in southern Africa differs from that recorded in east Africa. In Uganda, Morgan *et al*. (2010) reported susceptibility to bendiocarb and deltamethrin and suspected resistance to DDT

in *An. funestus*. Results from this study, highlight the possible spread of resistant genes in *An. funestus* populations from southern Africa. This emphasises the need to collaborate and incorporate insecticide resistance management strategies in the various malaria control programs within the region.

2.5.3 *Blood meal analysis*

The interpretation of the blood meal results obtained during this study should be handled with caution. Relatively few samples from each species per season were analysed for their blood meal sources, precluding a definitive conclusion on the feeding behaviours of each species. Furthermore, a comparison of HBI between species cannot be reliably made.

All four *An. funestus* group members analysed for their blood sources showed a distinct preference for animal blood over human blood. *Anopheles funestus*-like were positive for bovine and goat blood, even though the majority of them were collected indoors. Similarly, the other members of the *An. funestus* group fed predominantly on bovine and goat as well as dog, chicken and pig, suggesting that these mosquitoes obtained their blood meals outdoors from domestic animals and livestock before entering houses to rest. These feeding and resting behaviours amongst member species of the *An. funestus* group have previously been recorded (Kent and Norris, 2005), although this study describes for the first time the zoophilic and endophilic behaviours of *An. funestus*-like. The HBI for indoor and outdoor collected *An. funestus*-like were zero, further supporting evidence that this species is zoophilic and therefore a non-vector of human malaria.

A low median HBI of 10% was observed for all other member species of the *An. funestus* group. This may be attributable to bed net usage, forcing mosquitoes to obtain blood from domestic animals and livestock such as goats and cattle. The unidentified blood sources may have been obtained from other animals such as horses, donkeys, birds and rats. This highlights the need to include a wider variety of hosts for blood meal source determination as well as a need for more sensitive assays such as the PCR methods described by Kent *et al*. (2007).

2.5.4 *Plasmodium sporozoite infection*

The most recent *Plasmodium* infectivity study in Karonga is described in Spillings *et al*. (2009) from wet season collections. In their study, anopheline mosquitoes were screened only for *P. falciparum* infection and all specimens tested negative. In this study, wet and dry season collections together with homogenates from the 2008 collections were screened for *P. falciparum* and *P. vivax* infection. The absence of *P. falciparum* infection in all tested specimens from Karonga does not necessarily imply a total absence of malaria transmission because of the presence of the major malaria vector *An. funestus*.

An important result from this study was the data showing infection of *An. funestus*-like with *P. vivax* during the dry season. This observation appears to contradict other evidence showing that *An. funestus*-like is entirely zoophilic (*P. vivax* is a human malaria). However, it is most likely that the CSP-ELISA produced false positives as previously reported for other anopheline species (Lochouarn and Fontenille, 1999; Koekemoer *et al*., 2001; Mouatcho *et al*. 2007). Alternatively, these data may imply that one or more of the domestic animal species from which the *An. funestus*-like specimens were acquiring blood are reservoirs of *P. vivax*.

This scenario is highly unlikely although it has previously been shown that squirrel monkeys can incubate this parasite (Young *et al*., 1971). Repeated attempts using PCR to confirm the sporozoite ELISA results were unsuccessful. This could be attributed not only to the limited amount of CSP-positive homogenate available, but also to sample degeneration as a consequence of repeated freezing and thawing. Further investigation into the infection of *An. funestus*-like by *P. vivax* using a more sensitive technique such as real time PCR (Bass *et al*., 2008), which employs *Plasmodium* parasite specific probes, is recommended.

2.6 Conclusion

The dynamics of malaria transmission in the Karonga region of northern Malawi are poorly understood. Currently, the only formal vector control intervention there is the use of mosquito nets most of which are untreated. Informative baseline surveys are therefore needed to facilitate the design of a co-ordinated malaria control programme. In order to implement an integrated vector management programme centred on IRS, a survey of the vector species composition, the pattern of malaria transmission, the prevalence of *Plasmodium* infection in implicated vector populations and the insecticide susceptibility status of target vector populations is required.

Much of this information is provided in this study in which the presence of four species of the *An. funestus* group (*An. funestus*, *An. rivulorum*, *An. parensis* and *An. funestus*-like) is described. Evidence of insecticide resistance is presented for the major malaria vector *An. funestus* in Karonga. Although *An. funestus*-like is shown to be zoophilic and mainly endophilic, its vector status in terms of the transmission of *P. vivax* needs to be clarified.

CHAPTER THREE

Morphological description of *Anopheles funestus*-like from Malawi

3.1 Introduction

Morphological identification is the first step in any field study and remains vital to any vector control program. Since morphological keys developed by Gillies and De Meillon (1968) and later updated by Gillies and Coetzee (1987), very few additional studies on the morphology of malaria vectors has been done. The importance of correct morphological identification can never be over-emphasized and incorrect identification can result in loss of valuable resources both in the field and in the laboratory. Furthermore, this will impact on the recommendations made for implementation of a control program or the maintenance of an existing vector control program.

In 2007, mosquitoes were collected from Karonga in Malawi and morphologically identified as belonging to the *Anopheles funestus* group. However, these specimens were subsequently found to be a new species, named provisionally *An. funestus*-like (Spillings *et al*., 2009). It is against this background that the morphological characterization of *An. funestus*-like was undertaken to investigate if any morphological characteristics could be used to differentiate between *An. funestus*-like and *An. funestus s.s.*

3.2 Materials and Methods

Mosquitoes morphologically studied

Mosquitoes examined in this study were laboratory F_1 progeny reared from wild caught gravid females of *An. funestus*-like from Karonga in Malawi. Comparative specimens were *An. funestus s.s* from Malawi and Madagascar. All the specimens were pinned and originated from the VCRU Medical Entomology museum collection. The *An. funestus*-like specimens were reared from 2007 collections while the *An. funestus s.s* samples from Malawi and Madagascar were reared in 2010 and 1996 respectively. All specimens were reared in the Botha De Meillon insectary of the NICD/NHLS Johannesburg, South Africa prior to pinning.

Anopheles species identification

The wild caught females from which the F_1 progeny were obtained had previously been identified morphologically as members of the *An. funestus* group using the keys of Gillies and De Meillon (1968) and Gillies and Coetzee (1987). Further identification to species level was performed according to the protocols of Koekemoer *et al*. (2002) for *An. funestus* and Spillings *et al*. (2009) for *An. funestus*-like.

3.2.1 Morphological measurements

All measurements were done using a Wild Heerbrugg M5-71661 microscope containing a micrometer eye piece and the following characteristics were examined and recorded in mm: Wing length, wing spot ratio, palpal ratio and leg morphology.

3.2.1.1 Wing length measurement

Ten families consisting of four to five females F_1 progeny per family (n = 49) of wild caught *An. funestus*-like and wild *An. funestus s.s* (n = 42) were used for wing measurements (Figure 3.1). The left forewing was measured from the base of the arculus to the apical margin at X200 magnification. In cases where the left wing was damaged, it was replaced by the right wing.

3.2.1.2 Wing morphology and wing spot ratio

Wing spot names and numbering were according to De Meillon *et al*. (1977). Wings for *An. funestus* and *An. funestus*-like species were observed for the presence or absence of costal spots, vein spots and fringe spots. Wing spots 8, 9 and 10 (Figure 3.5) were measured in both species at X200 magnification. The wing spot ratio was calculated as:

$$\frac{\text{Length of costa spot 8 + Length of costa spot 10}}{\text{Length of costa spot 9}}$$

3.2.1.3 Palpal ratio

The palp of each female mosquito was mounted on a microscope and length of palp segments III, IV (subapical dark band) and V (apical pale band) were measured at X200 magnification (Figure 3.8 and 3.9). The palp ratio was determined according to De Meillon *et al.* (1977) using the formula:

$$\text{Palp ratio} = \frac{\text{Length of palp segment III + Length of palp segment V}}{\text{Length of palp segment IV}}$$

3.2.1.4 Leg morphology

The fore-, mid- and hind legs were mounted under an Olympus SZ2-ILST microscope. The joints of the tarsomeres were observed for pale bands while the complete leg was observed for any diagnostic character.

3.2.2 Data analysis

One way analysis of variance (ANOVA) was used to check for variance in morphological character within and between members of each species. Pair wise comparison of means was done using student-t- test. All statistical analysis was done using statistic 7 package.

3.3 Results:

3.3.1 Wing Length measurements

Figure 3.1 shows a representative wing of female *Anopheles funestus*-like indicating points from which wing-length measurements were made.

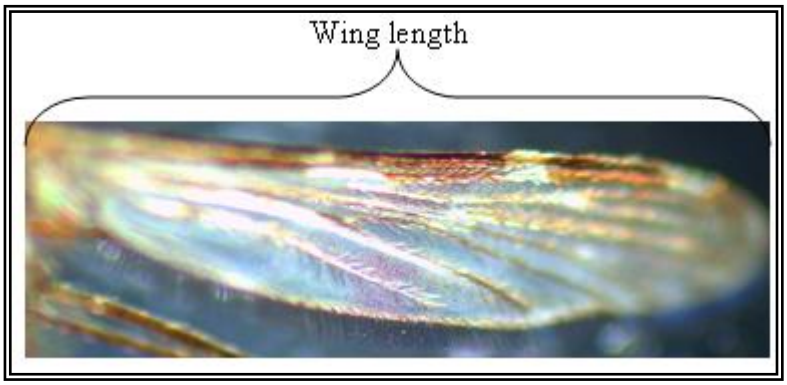


Figure 3.1 The wing of *Anopheles funestus*-like species

Results for mean wing length measurement are summarized in Figure 3.2.

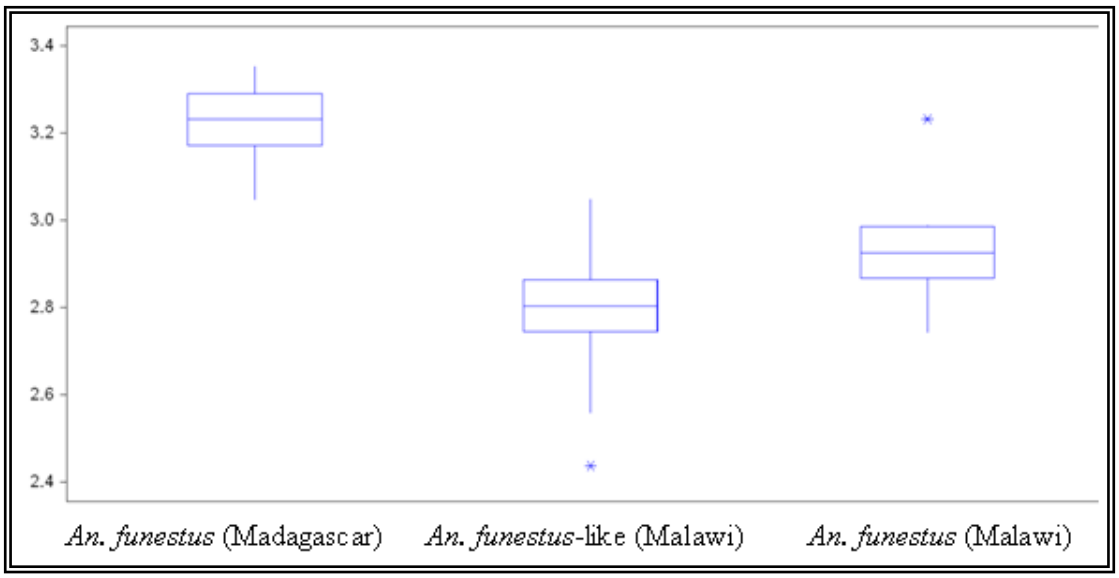


Figure 3.2 Distribution of wing length of *Anopheles funestus* and *An. funestus*-like. Box and whisker plot showing wing length mean in mm (bar in box), interquartile range (box), wing length range (whiskers) for female *An. funestus* and *An. funestus*-like species.

Mosquito's wing length raw data were summarised using statistic 7 parkage (Appendix V) and wa found to be normally distributed. Statistical analysis using ANOVA showed significant difference in mean wing length between *An. funestus*-like, *An. funestus* from Madagascar and Malawi (P = 0.0000, F = 80.55). After pair wise comparisons using student *t*- test, significant difference in mean wing length was recorded for *An. funestus s.s* species from both countries (p = 0.0000) and between each of the *An. funestus* species and *An. funestus*-like (p = 0.0000).

3.3.2 Wing morphology and wing spot ratio

3.3.2.1 Wing morphology

Figure 3.3 shows wing morphological diagnostic characters that were investigated in *An. funestus* and *An. funestus*-like species.

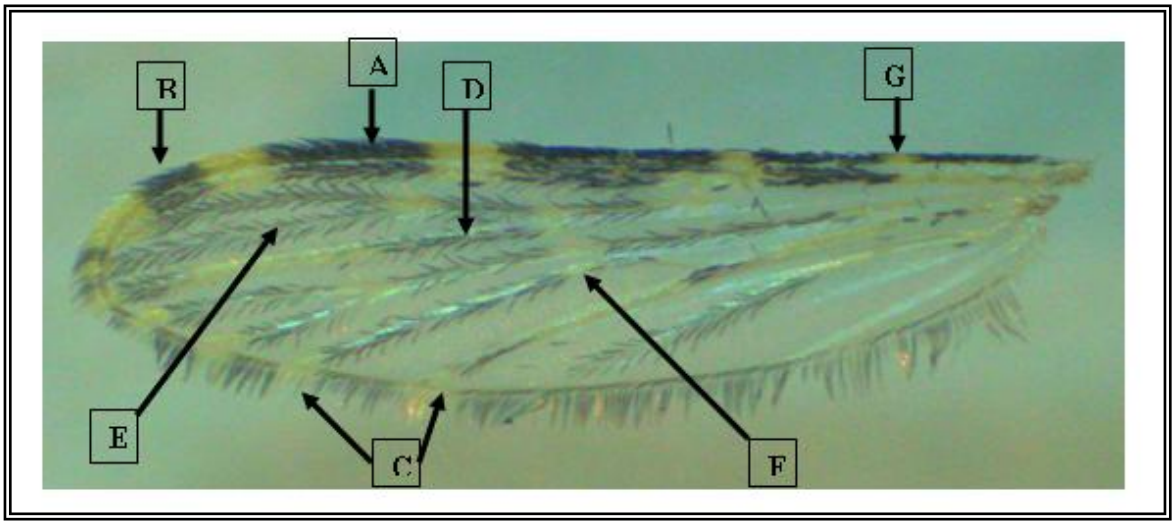


Figure 3.3 Female *An. funestus* left wing showing distinctive morphological characters. A and B: costa black spots 3 and 4 respectively, C, fringe spots, D: Vein 3, E: Vein 2.2, F: Pale spot on vein 5.1, G: Pre-sector pale spot (Gillies and Coetzee, 1987)

Table 3.1 shows wing morphological characters investigated for discrimination of *An. funestus* and *An. funestus*-likes species.

Table 3.1 Comparative morphological characters for *An. funestus* and *An. funestus*-like species

Morphological character	***An. funestus s.s* (n = 22)**	***An. funestus*-like (n = 45)**
Wing field	Predominantly dark	Predominantly pale
Costal black spot 3 and 4	Equally shaded	3 more shaded than 4
	Spot 3 is broader	Spot 3 is Narrower
Wing fringe spots	6 fringe spots	5 fringe spots
	Distinct	Not distinct
Vein 5	Bifurcate ends on fringe spots 5 and 6	Bifurcate ends on fringe spots 4 and 5
Pre-sector pale spot	Present in majority of samples	Absent in all samples

Individual characters investigated within and between families were quantitatively analyzed. The number of members in each family ranged from 3 to 5.

For wing field colour, all specimens of *An. funestus* were predominantly dark while *An. funestus*-like was pale brownish in colour (Fig 3.4). For colour intensity of costal black spots 3 and 4, 95.4% (n = 22) of *An. funestus* analyzed had both spot equally dark and in one sample (4.6%) spot 4 was darker. In all specimens of *An. funestus*-like analyzed for this character,

costal black spot 3 was darker than spot 4. Spot 3 was 3 times broader than spot 4 in *An. funestus* while it was 2 times broader in *An. funestus*-like.

Five distinct pale fringe spots were observed in 9% (n = 22) and six in 45.5% (n = 22) *An. funestus* which also had fringe spots 1 to 3 merged in 13.6% (n = 22) specimens (Figure 3.4). For *An. funestus*-like, 26.8 % (n = 45) individuals had 5 distinct fringe spots and in 6.7% (n = 45) specimens, fringe spots 4 and 5 were merged. The rest of the specimens for both species had damaged fringes or were difficult to score.

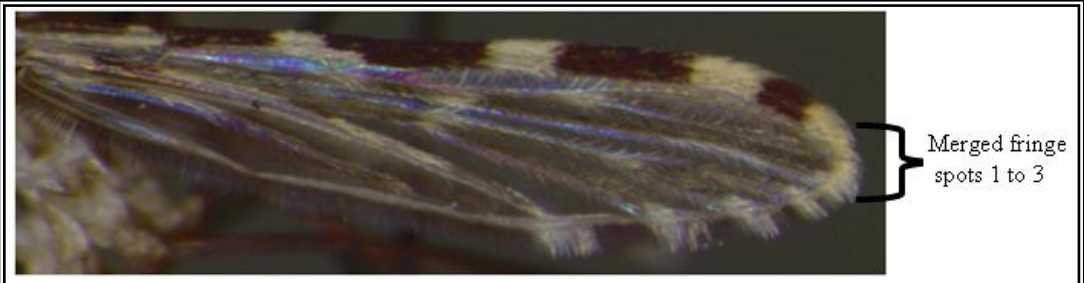


Figure 3.4 Dorsal view of *An. funestus* right wing showing merged fringe pale spots 1 to 3

Analysis of the pale spot on wing vein 5.1 showed that 90.9% (n = 22) and 97.8% (n = 45) were positive for this character in *An. funestus* and *An. funestus*-like species respectively. Bifurcation of wing vein 5 terminated in fringe pale spots 4 and 5 in 4.5% (n = 22) and in fringe spots 5 and 6 in 50% (n = 22) of *An. funestus*. In *An. funestus*-like, 33.3% (n = 45) had this bifurcation terminating on fringe spots 5 and 6.

The pre-sector pale spot was present in 77.2% (n = 22) *An. funestus* and absent in the rest of the samples. The same character was absent in all specimens of *An. funestus*-like.

3.3.2.2 Wing spot ratio

Female wing costal spots used for the determination of wing spot ratio are represented Figure 3.5

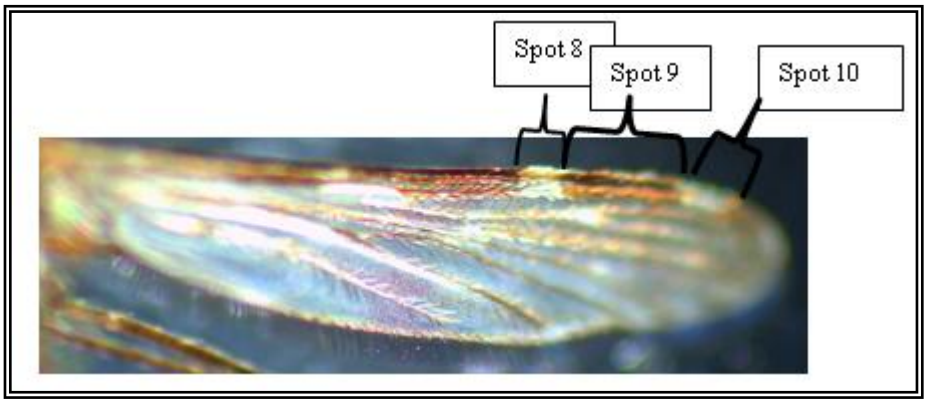


Figure 3.5 Dorsal view of female *An. funestus*-like right wing showing wing spots 8 (pale), 9 (brown) and 10 (pale) measured at 40X magnification for calculation of wing spot ratio.

Table 3.2 show results for biometrical data recorded for adult female wing spot ratios of species investigated.

Table 3.2 Quantitative taxonomic character detailing the ratio between combined lengths of 8th and 10th wing spots and length 9th wing spot in adult females of *Anopheles funestus*-like and *Anopheles funestus* species

An. species /Country	Length	No	Observed range	Mean	95%CI	SD	CV
An. funestus-like/Malawi	Wing segment 9	44	3.658-4.2670	3.9902	3.8969-4.0834	0.3067	7.6875
	wing segments 8+ 10	44	2.72-4.3500	3.3293	3.2046-3.4540	0.4101	12.319
	Wing spot ratio	44	0.638-1.1900	0.8402	0.7997-0.8807	0.1333	15.867
An. funestus /Madagascar	Wing segment 9	22	4.267-5.4860	4.7659	4.5679-4.9639	0.4466	9.3711
	wing segments 8+ 10	22	3.048- 5.4860	4.3225	4.0229-4.6222	0.6758	15.634
	Wing spot ratio	22	0.556-1.2860	0.9212	0.8312- 1.0112	0.2029	22.029
An. funestus /Malawi	Wing segment 9	20	2.4380-3.6580	3.5055	3.3232-3.6878	0.3896	11.114
	wing segments 8+ 10	20	2.7210-3.8100	3.2931	3.1630-3.4232	0.2779	8.4384
	Wing ratio	20	0.7440-1.3390	0.9555	0.8768-1.0341	0.168	17.581

Comparison of the mean wing spot ratio for *An. funestus*-like and *An. funestus* from both countries using ANOVA, showed that there is significant difference between the three (p = 0.0194, F = 4.13). Pair wise comparison of mean wing spot ratio for *An. funestus*-like and *An. funestus* from Malawi showed significant difference (p = 0.0045). There was no significant difference between the mean spot ratio of *An. funestus*-like and *An. funestus* from Madagascar (p = 0.0998) as well as between *An. funestus* samples from both countries (p = 0.5567).

Since there was no statistical difference (p = 0.0150) in mean wing spot ratio data for *An. funestus* from the two countries, the data were pooled for comparison with that of *An. funestus*-like (Figure 3.6). Results obtained indicated a significant statistical difference for both species (p = 0.0069).

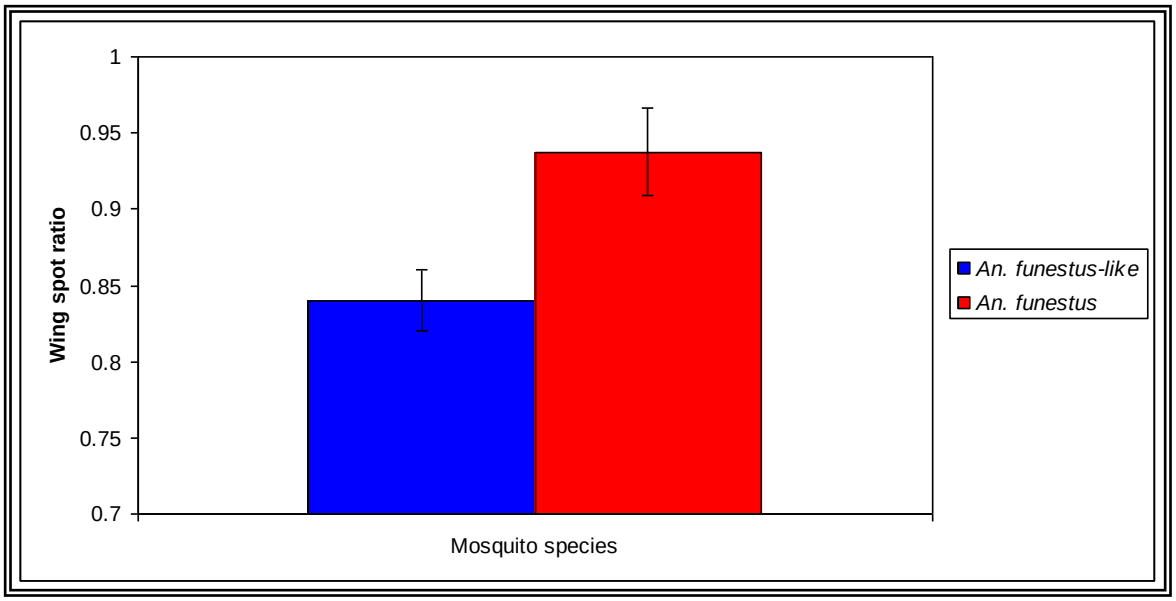


Figure 3.6 Comparison of mean wing spot ratio for *An. funestus s.s* from Malawi and Madagascar with that of *An. funestus*-like species. The bar in the column represents standard error.

Further analysis of distribution of the data shows that, 14.28% of the pooled *An. funestus* could be separated from *An. funestus*-like. The percentage overlap using this character was 93% (Figure 3.7).

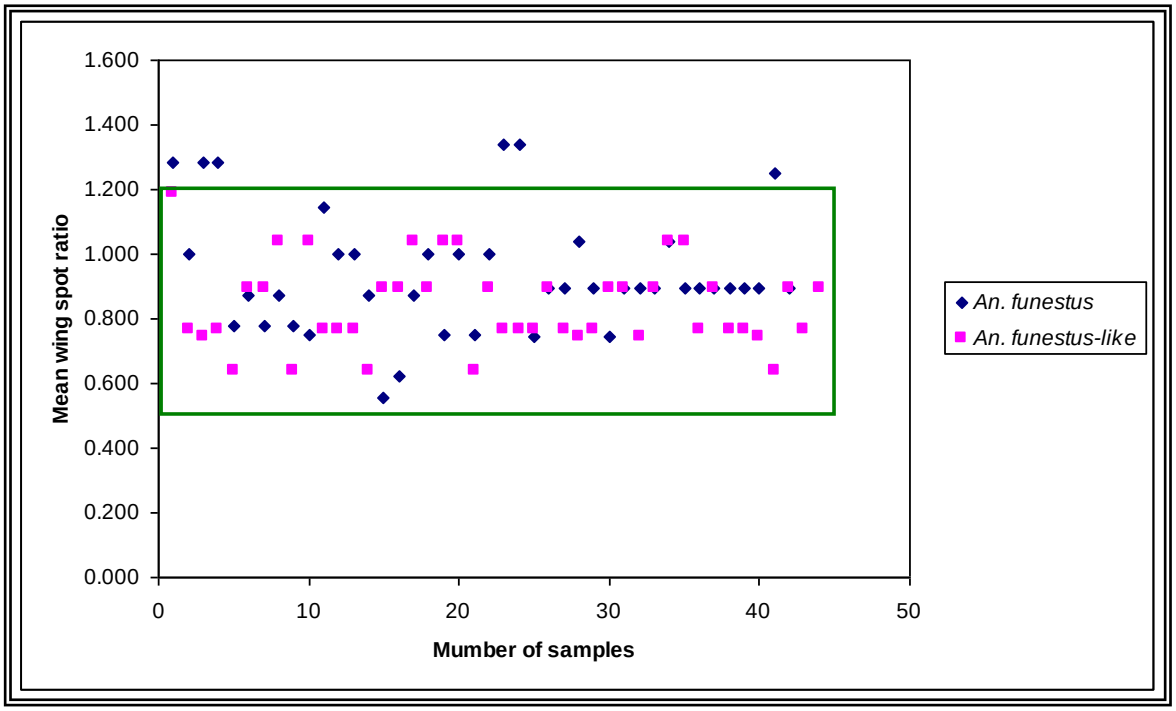


Figure 3.7 Scatter plot analysis of mean wing spot ratio for *Anopheles funestus s.s* and *An. funestus*-like species.

3.3.3 Palp measurement and palp ratio determination

The female palp of *An. funestus* and *An. funestus*-like analyzed in this study is presented in Figure 3.8 and 3.9 which also show palpal bands measured.

Figure 3.8 *Anopheles funestus* palp showing A: segment 3; B: segment 4 and C: segment 5

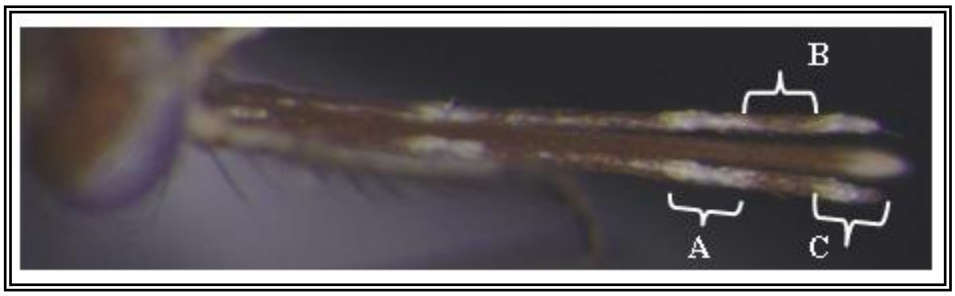


Figure 3.9 *Anopheles funestus*-like species palp

Biometrical data recorded for adult female palp ratios for species under investigation are presented in Table 3.1. Comparing the mean palpal ratios of *An. funestus*-like species and *An. funestus* from Malawi and Madagascar using ANOVA, showed that there was a significant difference between the three groups ($p = 0.0000$, $F = 12.76$). Pair wise comparisons using two sample t test, showed a significant difference between *An. funestus*-like and *An. funestus* from the two countries ($p = 0.0000$). However, no significant difference between *An. funestus* samples from both countries ($p = 0.2595$) was recorded.

Table 3.3 Quantitative taxonomic character detailing ratios between combined lengths of 3rd and 4th palpal segment and length of 4th segment in adult females of *Anopeheles funestus*-like and *Anopheles funestus* species

An.species /Country	Length of segment	No	Observed range	Mean	95%CI	SD	CV
An. funestus-like/Malawi	Length of palp segment 4	44	1.2190 - 3.0480	2.1197	2.0104 - 2.2290	0.3595	16.962
	Length of palp segments 3+ 5	44	1.829 - 4.2670	3.0618	2.8853 - 3.2383	0.5805	18.96
	Palp ratio	44	1.0000 - 2.3330	1.475	1.3712 - 1.5788	0.3413	23.143
An. funestus /Madagascar	Length of palp segment 4	22	1.829 - 3.6580	2.743	2.5429 - 2.9431	0.4513	16.453
	Length of palp segments 3+ 5	22	2.438 - 3.6580	2.9648	2.7727 - 3.1569	0.4332	14.611
	Palp ratio	22	0.667 - 1.6670	1.1129	0.9998 - 1.2261	0.2552	22.932
An. funestus /Malawi	Length of palp segment 4	20	1.8290 - 2.4380	2.2858	2.1591 - 2.4124	0.2706	11.837
	Length of palp segments 3+ 5	20	1.8290 - 3.0480	2.7127	2.4961 - 2.9292	0.4628	17.06
	Palp ratio	20	0.7500 - 1.6670	1.2	1.0894 - 1.3106	0.2364	19.689

Data for mean palp ratios for *An. funestus s.s* species from both countries, were pooled for comparison with the mean palp ratio for *An. funestus*-like (Figure 3.10). Statistical analysis using the two sample *t*-test revealed a significant difference between the mean palp ratio for *An. funestus*-like and the combined mean palp ratio for the *An. funestus* species (p = 0.0000).

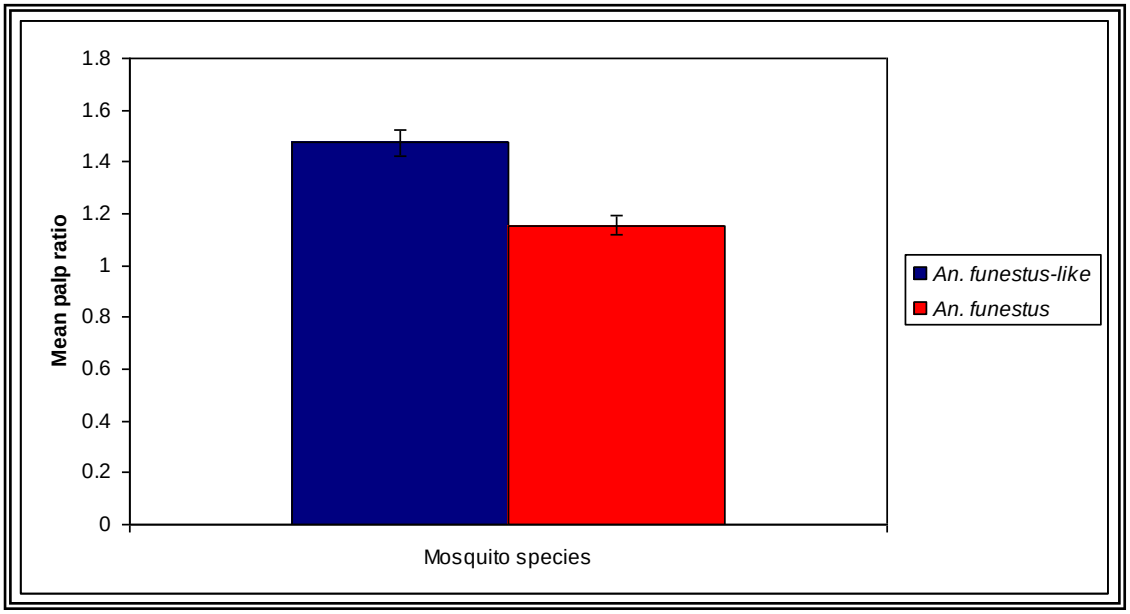


Figure 3.10 Comparison of mean palp spot ratio for *An. funestus s.s* from Malawi and Madagascar with that of *An. funestus*-like species. The bar in the column represents standard error.

There was an overlap in the ranges of the palp index for *An. funestus* and *An. funestus*-like (Figure 3.9). A palp index of less than 1 indicated *An. funestus* and an index greater than 1.7 indicated *An. funestus*-like. Combining the female *An. funestus* from Malawi and Mozambique resulted in 19% (n = 42) distinctively identified. For *An. funestus*-like (n = 44) 13.6% could be distinctively identified using palp index. Percentage overlap for both species was 83.7%.

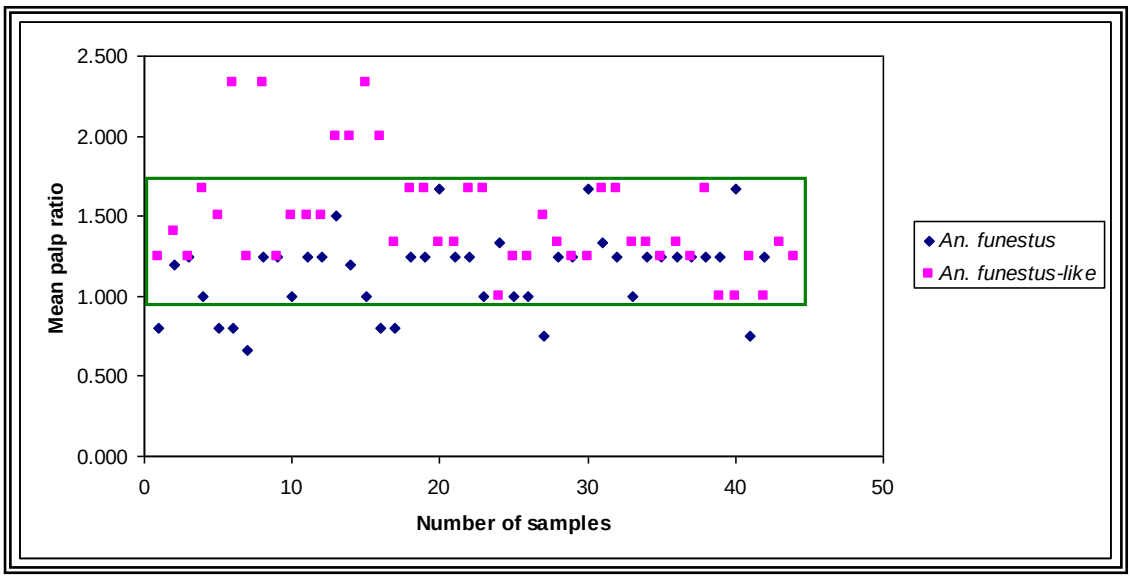


Figure 3.11 Scatter plot analysis of mean palp ratio for *Anopheles funestus s.s* and *An. funestus*-like species.

3.3.4 Scatter plot analysis of wing spot against palp spot ratio

Scatter plot analysis to segregate *An. funestus* families from *An. funestus*-like is presented in Figure 3.12. Results showed that 70% (n = 10) of *An. funestus* segregated from *An. funestus*-like which showed a 66.7% (n = 9) segregation. A percentage overlap of 26% was obtained when both species were considered.

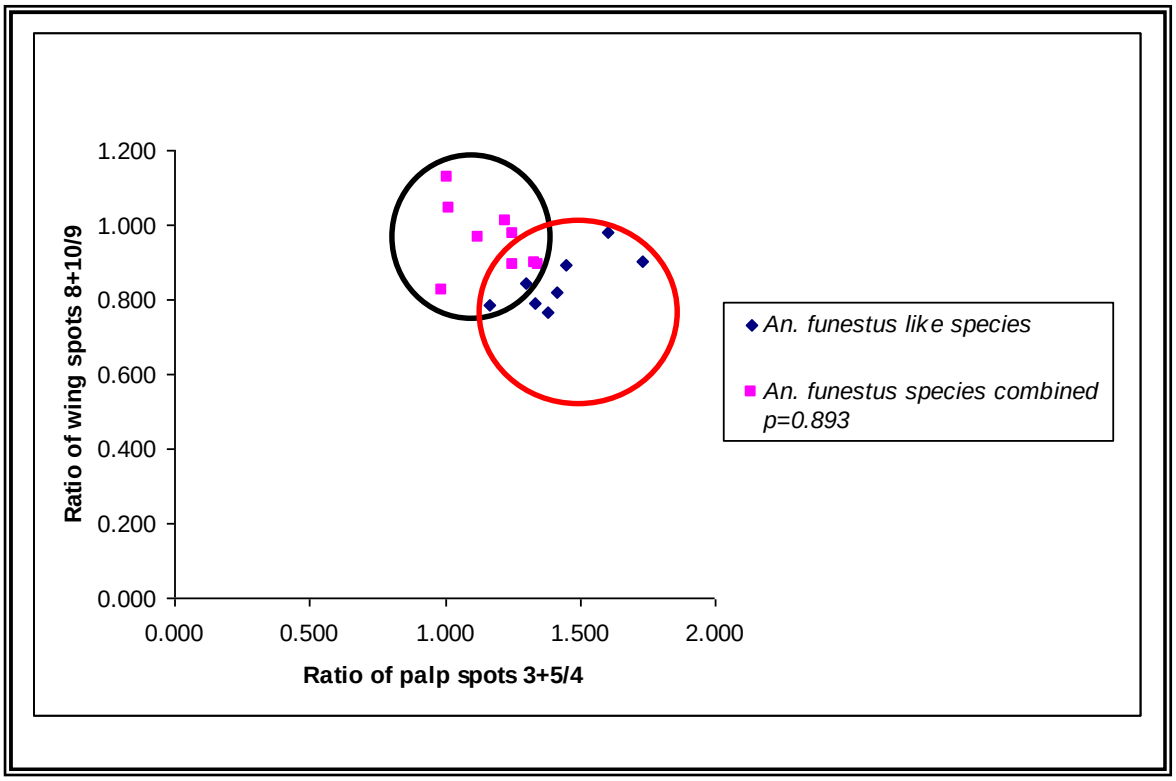


Figure 3.12 Segregation of adult female *Anopheles funestus s.s* and *An. funestus*-like families by means of ratios of wing spots 8 + 10/9 and palp spots 3 + 5/4.

3.3.5 Female leg morphology

Female fore-, mid-, and hind tarsomeres for *An. funestus* and *An. funestus*-like analyzed for leg appearance are shown in Figure 3.13 and Figure 3.14 respectively. Results of the comparative analysis obtained using individuals of distinct progenies showed that in *An. funestus*, the tarsomere bands are apical and pale in colour (Figure 3.13 A, B and C) where as in *An. funestus*-like, the band are at the articulations of the tarsomeres and more whitish (Figure 3.14 A, C and C). No variation was observed within progenies and between families using this morphological character.

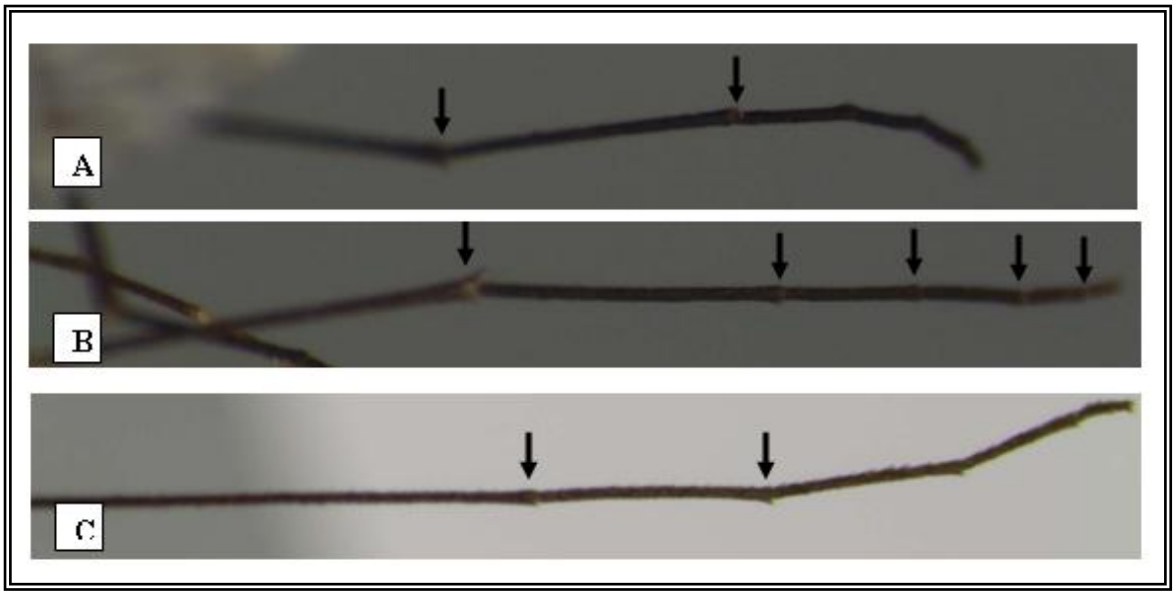


Figure 3.13 Female *Anopheles funestus* tarsomeres fore (A), mid (B) and hind (C) legs with apical pale bands on joints

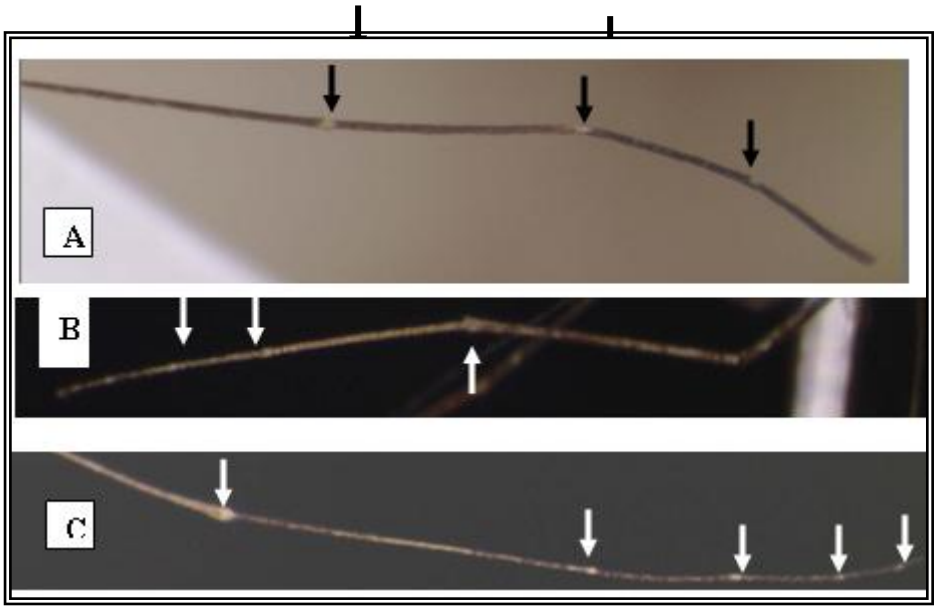


Figure 3.14 Female *Anopheles funestus*-like tarsomeres fore (A), mid (B) and hind (C) legs with whitish spots on joints

3.4 Discussion

Female characters deemed to be of greater taxonomic significance by De Meillon *et al.* (1977) were investigated in this study in an attempt to morphologically discriminate *An. funestus*-like from *An. funestus*. The specimens examined were all reared in the same insectary under standard insectary conditions. Irrespective of the 11 year difference between the F_1 progeny at time of analysis, both species show similarity in the morphological characters studied.

3.4.1 Wing length measurement

Statistically significant differences in mean wing lengths observed for each population of mosquito facilitated their discrimination using this character. Wing length measurements can be used to separate *Anopheles funestus*-like from *An. funestus* originating from Madagascar. However, this character could only partially separate *An. funestus*-like and *An. funestus* from Malawi or the two *An. funestus* populations. The wild females for *An. funestus*-like and *An. funestus* from Malawi examined in this study were sympatric at Wowve and were collected during similar seasons but at different times (2 year interval). The natural environmental conditions for the parents and the insectary conditions for the progeny were similar. This probably allowed both species of mosquitoes to be of the same body size which has been shown by other investigators to correlate to wing length (Lyimo and Takken, 1993). The fact that *An. funestus* from Madagascar could be completely separated from *An. funestus*-like and only partially separated from *An. funestus* from Malawi, may suggest that geographic location contributes to the difference observed or might be due to larval diet when F_1 progeny were reared.

3.4.2 Wing morphology

Female *Anopheles funestus* wing colour has been described as predominantly dark by Gillies and De Meillon (1968). In this study the wing colour for *An. funestus*-like was more brownish and served as an identification character. Though the costal black spot 3 and 4 colour intensity has not been reported, this study showed that an over whelming majority of the *An. funestus* females had both spots equally shaded. This feature was however different in all specimens of *An. funestus*-like analyzed as the costal black spot 3 was darker than costal spot 4. The wing fringe pale spots in *An. funestus*-like were indistinct compared with the distinct spots in *An. funestus*.

3.4.3 Wing spot and palpal ratios

Separation of *Anopheles funestus*-like from *An. funestus* using wing spot ratio was unsuccessful. All wing spot ratio values for *An. funestus*-like fell within the range of the *An. funestus* wing spot ratio of. However, any *An. funestus* specimens with wing spot ratio above 1.2 can be discriminated from *An. funestus*-like which had this value as it highest wing spot ratio.

Palpal index remains a vital morphological character for discriminating species of anophelines. It was employed by De Meillon and Evans (1935) to describe two anophelines, *An. cameroni* and *An. walravensi* from South Africa. In this study, there was no significant difference in the palp ratio between *An. funestus* from Malawi and Madagascar. However, mean palp ratio was significantly different for *An. funestus*-like and *An. funestus* but unfortunately produced a huge overlap of 83.5% for the values. This leaves the use of palpa ratio unsuitable for discriminating these species.

The simultaneous use of two characters to separate insect species is not new. Palp band ratio and wing spot ratio have been shown to be highly discriminatory. It has been used to separate *Anopheles funestus* from *An. aruni*? (now referred to as *An. vaneedeni*) without an overlap between the two species (De Meillon *et al*., 1977). Though in this study such an attempt failed to separate *An. funestus*-like from *An. funestus*, it did produce a more reliable separation tool than using each of these ratios individually. The percentage overlap resulting from this application was much lower compared to that obtained using other characters. Overlap using this character was also observed on separating *An. vaneedeni* and *An. funestus* by Gillies and Coetzee. (1987).

3.4.4 Leg morphology

The black leg colour for *An. funestus* and the brown colour for *An. funestus*-like were discriminatory at a high degree and proved to be useful. A few samples could not be analyzed as the legs were damaged. The pale spots on the joints of the legs looked to be a promising character for separating these species.

The use of coloration of wing spots and legs is subjective and may not be useful in the field where reference specimens would not be available for comparison of wild specimens. Unfortunately, there was insufficient time to look for discriminating characters at larvae stage for *An. funestus*-like.

3.5 Conclusion

Morphological discrimination of members of the *Anopheles funestus* group has been difficult. This is attributed to the degree of overlap in morphological characters exhibited within this group. The present study showed that a combination of characters is required to

separate *An. funestus* from *An. funestus*-like. Scatter plot analysis of wing spot and palpal ratios proved to be the most discriminatory method for these species. Because it was sometimes difficult to ascertain observed differences in some samples, this study will recommend the use of mosquitoes that has only been dead for an hour for morphological studies on both species in combination with molecular identification tools developed by Spillings *et al.* (2009).

CHAPTER FOUR

Development of DNA-based molecular assays for the identification of *Anopheles funestus* group members including *An. funestus*-like ♣

4.1 Introduction

Members of the *Anopheles funestus* group are morphologically similar in their adult stage (Gillies and De Meillion, 1968; Gillies and Coetzee, 1987), making identification of species problematic. The difficulty in discriminating malaria vectors from non-vectors can undermine vector control efforts. Historically, identification was performed by morphological (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987) and cytogenetic methods (Green, 1982; Green and Hunt, 1980). Various constraints associated with these techniques led to the search for novel identification methods. DNA-based molecular approaches based on PCR have superseded the older identification methods over the last decade (Koekemoer *et al*.,2002 and Bass *et al*., 2007b).

A 'gold standard' Allele specific-Polymerase Chain Reaction (AS-PCR) protocol by Koekemoer *et al*. (2002), which can be used to identify all five species in the *An. funestus* group, failed to identify samples morphologically identified as *An. funestus* s.l, collected resting indoors in Malawi. Following sequencing of the internal transcribed spacer 2 (ITS2) region of rDNA, cytogenetic evaluation of polytene chromosomes and crossbreeding experiments, Spillings *et al*. (2009) showed that the unidentified specimens are a new species which is provisionally named *An. funestus*-like. Efforts detailed in

♣ Work has been published in Malaria Journal. See Appendix VII, page 167

Spillings *et al*. (2009) to develop a multiplex PCR assay to discriminate *An. funestus* group members from *An. funestus*-like based on the ITS2 regions proved difficult due to overlap in diagnostic fragment (PCR product) size. These findings highlight the need for a better identification assay which can identify all members of the *An. funestus* group including *An. funestus*-like. High throughput real-time PCR techniques have recently been used for species genotyping and brief descriptions of some of the commonly used methods are presented below.

4.1.1 Melt Curve Analysis (MCA)

This technology uses SYBR Green I dye which intercalates into double stranded DNA (dsDNA) produced after PCR (Lekanne *et al*., 2002). The resulting effect is 100 fold increase in fluorescence when bound to the DNA than when it is unbound. Subjecting the PCR products to increasing temperature results in denaturisation and hence a decrease in fluorescence as the dye is released. Plotting a negative first derivative of the collected fluorescence against temperature results in melt peaks with characteristic melting temperatures (TM). The TM is highly dependent on factors such as GC content, length and sequence of the amplicon. SYBR Green popularly used in MCA has some limitations. These include its ability to bind to non-specific products, redistributing during melting, inhibiting amplification at high concentration (Monis *et al*., 2005) and a preference for DNA with high TM for binding (Giglio *et al*., 2003). In addition, it is also unable to detect a difference in TM of 0.2 degrees between samples.

4.1.2 High Resolution Melt analysis (HRMA)

High resolution melting analysis can be used to discriminate between DNA fragments that differ at sequence level. Firstly, PCR is used to amplify a region of interest in the presence

of fluorescence dyes such as SYTO9. The dye intercalates in the PCR products and produces fluorescence. A HRM analysis is then performed. HRM involves a stepwise increase in temperature resulting in dissociation of dsDNA into single strands with a decrease in fluorescence as the dye is released. The shape of the melt curve of fluorescence against temperature is characteristic for a particular sample (Liew *et al*., 2004). This technique has been used by Bass *et al.* (2007a) to detect both the West (L1014F) and East (L1014S) African *kdr* mutations in *An. gambiae*.

4.1.3 TaqMan PCR (Hydrolysis probe assay)

This assay relies on the 5'- 3' exonuclease activity of *Taq* DNA polymerase, which degrades species-specific probes labelled with a reporter and a quencher dye during PCR polymerization. The released reporter dye, when free from the quenching effect of the quencher dye, produces fluorescence which is monitored in real-time (Lyamichev *et al*., 1993). This technique has been successfully used to distinguish between the main malaria vectors *An. arabiensis* and *An. gambiae s.s* from closely related non-vectors or minor vectors (*An. quadriannulatus, An. melas* and *An. merus*) (Bass *et al*., 2007b). The Hydrolysis probe assay is advantageous over the conventional PCR technique in that it is fast, more efficient and safe. The disadvantages of this technique are that the probes and the real-time equipment are very expensive. The rDNA gene has been of interest in molecular diagnostics partly because it is present in hundreds of tandem repeats per cell nucleus in multicellular organisms as well as bacteria.

4.2 Structure of ribosomal DNA (rDNA) gene

The ribosomal deoxyribonucleic acid (rDNA) genes in eukaryotes are transcribed to ribosomal ribonucleic acid (rRNA). Ribosomal RNA constitutes the greatest portion of

cellular RNA. To produce this large amount of rRNA, the rDNA gene is present in multiple transcriptional units or tandem repeats separated from each other by non-transcribed intergenic spacers (NTS) (Beckingham, 1982). Each repeat consists of 18S, 28S and 5.8S (S = Svedberg sedimentation value) and is separated from the next repeat by two external transcribed spacers (ETS) and a non-transcribed spacer (NTS) Figure 4.1 (Hwang and Kim, 1999). Both the ETS and the NTS form the intergenic spacer (IGS) and are highly variable. This makes the IGS a suitable candidate for designing species-specific assays even though it is of great size compared to the smaller internal transcribed spacer (ITS) region. The functions of the ETS and NTS regions are unclear (Van der Sande *et al*., 1992). The 18S and 5.8S genes are separated by the ITS1 and the 5.8 and 28S by ITS2. The ITS region has a high mutation rate compared to the coding regions and is therefore useful for distinguishing closely related species. The 28S subunit varies in length depending on organism due to contraction or expansion of variable joining sequences located at 10-12 specific points. These sequences have been referred to as variable divergent or D domains (Gonzalez *et al*., 1985).

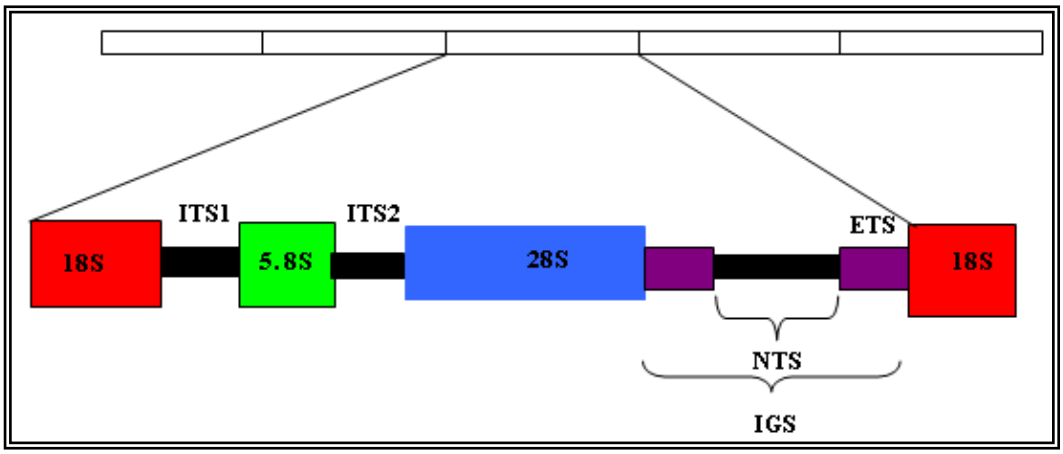


Figure 4.1 Schematic representation of a transcription unit of eukaryotic rDNA with tandem repeats of 18S, ITS1, 5.8S, ITS2, and 28S. (Modified from Hwang and Kim, 1999; Hunter *et al*., 2007)

4.3 Aim and objectives

Accurate identification of disease transmitting mosquitoes remains an integral part of any effective vector control program. The aim of this study was to investigate the use of multiplex PCR assays (real-time PCR-based assays and conventional PCR) for the identification of members of the *An. funestus* group. The specific objectives were:

4.2.1 To design new species diagnostic assays including real-time PCR and Allele Specific PCR (AS-PCR) for *An. funestus* group identification. The following real-time PCR assays were investigated: Melt Curve Analysis, High Resolution Melt, TaqMan single nucleotide polymorphism (SNP) genotyping (hydrolysis probe assay).

4.2.1 To investigate the development of an allele specific (AS)-PCR from the larger more variable IGS region.

4.4 Materials and Methods

Mosquito samples, DNA extraction and quantification

Mosquito specimens collected from various localities in Ghana, Mozambique and South Africa were used in this study. DNA for the initial optimization of each real-time assay was extracted using either the Livak *et al*. (1984) or Collins *et al*. (1987) methods. For AS-PCR based on the IGS sequence, DNA was extracted from mosquitoes collected from Malawi using a DNA extraction *prep*GEM^{TM} insect kit (ZyGEM, New Zealand, PIN0050). Detailed DNA extraction methods are provided in Appendix II A. All DNA concentrations and qualities were determined by absorption at 260nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies-Wilmington, USA). DNA samples with a 260/280 ratio greater than 1.7 were classified as pure DNA and those with a lower ratio

were considered contaminated with proteins. Only pure DNA samples were used for analysis.

Mosquito identification

Control mosquitoes were identified as belonging to the *An. funestus* group by using the morphological keys of Gillies and Coetzee (1987). The mosquitoes were further identified to species level using the assay of Koekemoer *et al*. (2002). *Anopheles funestus*-like were identified using the protocol of Spillings *et al*. (2009). The methodologies for both molecular identification methods are provided in Appendix II B and C. The species-specific primers used in these assays produce diagnostic amplicons of distinct sizes Table 4.1.

Table 4.1 Polymerase chain reaction (PCR) primers and sizes of the amplified products for a species-specific diagnostic test for the *An. funestus* group

Species	Primers	Primer sequence (5'- 3')	Product size
An. funestus group	UV	TCT GAA CTG CAG GAC ACA T	-
An. funestus	FUN	GCA TCG ATG GGT TAA TCA TG	500 bp
An. vaneedeni	VAN	TGT CGA CTT GGT AGC CGA AC	550 bp
An. funestus-like	FUN-LIKE	GTT TTC AAT TGA ATT CAC CAT T	440 bp
An. rivulorum	RIV	CAA GCC GTT CGA CCC TGA TT	400 bp
An. parensis	PAR	TGC GGT CCC AAG CTA GGT TC	250 bp
An. leesoni	LEES	TAC ACG GGC GCC ATG TAG TT	153 bp

Real-time PCR assays were designed based on the ITS2 regions of ribosomal DNA gene sequences. Sequences for different species of the *An. funestus* group, available from the National Centre for Biotechnology Information (NCBI) data base, were obtained and aligned using DNASTAR lasergene 7 MegAlign in order to design species-specific primers and probes.

4.4.1 Development of real-time based PCR

Costing for real time assays consumables were calculated in 2009 and are given in US dollar. Each of the assays requires 1 hour 30 minutes to perform.

4.4.1.1 Melt Curve Analysis assay

For this assay, the original primers of Koekemoer *et al*. (2002) were used except for the VAN and FUN primers, specific for *An. vaneedeni* and *An. funestus* respectively, which were replaced by VAN3 and FUN1 primers. Redesigning of the primers was necessary to ensure that amplicons with distinct optimal melting temperatures were generated during PCR. A 20µl PCR reaction mixture consisting of 1µl genomic DNA, 10µl SensiMix™ (Quantace), 0.4µl SYBR Green 1 (Quantace) and 250nM of each primer (UV, FUN1, LEES, VAN3, PAR and RIV) was prepared. PCR was performed using a Rotor-Gene 6000 (Corbett Research) thermal-cycler under the following cycling conditions: One cycle of 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds. The PCR products were immediately subjected to a melting step of 72-95ºC rising by 1ºC and holding for 90 seconds for pre-melting for the first step and 5 seconds for each of the subsequent steps. Changes in the fluorescence of SYBR Green 1 during PCR and melt steps were acquired on the green channel of the PCR machine.

4.4.1.2 High Resolution Melt (HRM) assay

HRM was performed using the primers of Koekemoer *et al*. (2002). The PCR reaction (25µl) consisted of 12.5µl SensiMix HRM kit, 200nM of each primer, 1µl Eva Green dye and 1µl genomic DNA. PCR was run in a Rotor-Gene 6000 under the following cycling conditions: A cycle of 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds, 56ºC for 30 seconds and 72ºC for 30 seconds. After PCR, the products formed were subjected to a melting step, consisting of a 77-94ºC rise in 0.1ºC increments followed by holding for 90 seconds for pre-melting and a subsequent 2 seconds for each step. Changes in fluorescence of Eva Green dye were monitored on the green channel during the PCR and melting phase.

4.4.1.3 Hydrolysis probe assay

Ribosomal DNA gene alignment produced species-specific sequences for the design of species-specific probes. The alignment unfortunately failed to produce common regions for the design of a universal forward and reverse primer. As a consequence, the universal and species-specific primers of Koekemoer *et al*. (2002) were used except for the primers specific for *An. parensis* and *An. vaneedeni.* The sequence over which both primers annealed were between their corresponding species-specific probe annealing sites and the universal primer's binding site. Conventionally, hydrolysis probe binding sites should be located between the forward and reverse primers needed for amplification of a target sequence. These primers were replaced by designing two new primers (new PAR and VAN1 diagnostic for *An. parensis* and *An. vaneedeni* respectively) (Table 4.2).

Each species-specific probe was designed to anneal to a sequence between the universal forward primer and a corresponding species-specific primer (Figure 4.2).

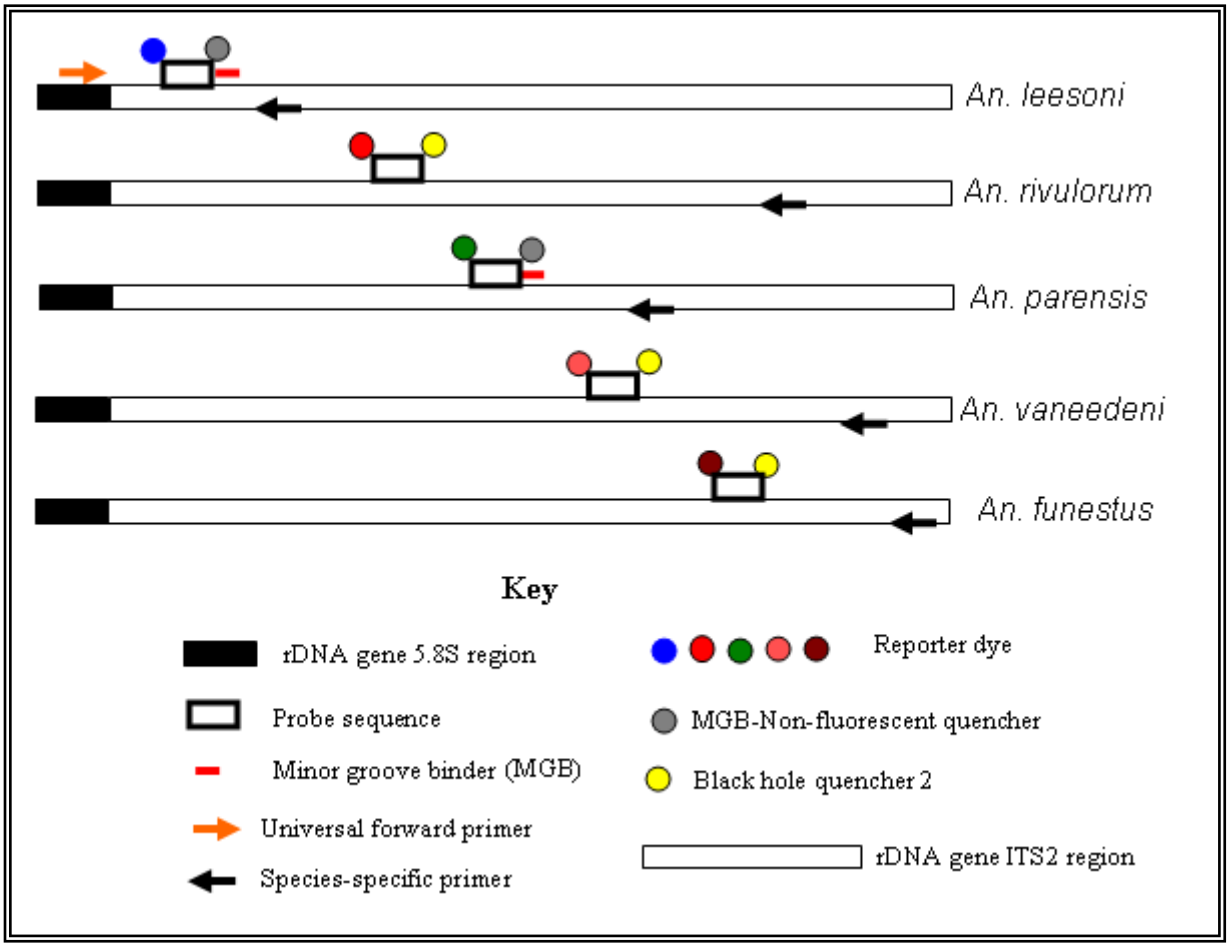


Figure 4.2 Design of the hydrolysis probe assay for the identification of members of the *An. funestus* group

Probes designed to anneal over sequences with two or less species-specific single nucleotide polymorphisms (SNPs) were designed as minor groove binder (MGB) probes (Applied Biosystems). MGB's increase the melting temperature between matched and mis-matched probes (Afonina *et al*., 1997) and were designed using primer Express™ Software Version 2.0 (Applied Biosystems). Probes annealing over sequences with more than two species-specific SNPs were manually designed and synthesised as dual-labelled probes by Thermo Fisher Scientific or Biosearch Technology. Each probe was labelled at its 5' end with a reporter dye and at the 3' end with a quencher dye (Table 4.2).

Table 4.2 Primers and probes used in the identification of *An. funestus* group species using the hydrolysis probe assay

Name	Primer/ Probe	Reporter dye	Sequence (5'-3')	Quencher
VAN1	Primer	_	AAACCCCAAGATGTGCTCC	_
New PAR	Primer	_	ATACTTGTGTGTGTGTGTATTTG	_
RIV TM	Probe	Cy5	CTATGGCGAGACCCCGTCTAGTG	BHQ2a
FUN TM	Probe	ROX	CATGGGGAAATTCAATCGAAAACCTCT	BHQ2a
PAR TM	Probe	VIC	CGGAACCTAGCTTGG	MGBNFQ[b]
VAN TM	Probe	Quasar 706	CGTTGTGAAAAATGGAGATTCATTTGAAAA CC	BHQ2[a]
LEES TM	Probe	6-FARM	CCGACCGATGTACA	MGBNFQ[b]

4.4.2 Analytical sensitivity of developed the hydrolysis probe assay compared to allele specific PCR

The DNA concentrations of five species of mosquitoes (*An. funestus, An. rivulorum, An. parensis, An. leesoni* and *An. vaneedeni*) were measured on a NanoDrop ND-1000 spectrophotometer and diluted to 20ng/µl. These were then serially diluted down to a 1 in 1 X 10^6 dilution. DNA (1µl) of the representative dilution was used as a template in AS-PCR, MCA, HRM and hydrolysis probe assays. AS-PCR was performed according Koekemoer *et al*. (2002) with minor modifications. The final reaction volume was changed from 13.5µl to 14µl and ready made PCR master mix was used. The 14µl reaction volume contained 6µl of ReddyMix PCR master mix (Thermo fisher scientific, UK) and 0.24µM of each primer. The thermal cycling conditions were unchanged. For real-time PCR, the reaction volumes and cycling conditions were as optimised. Analytical sensitivity was defined as the lowest DNA concentration that displayed a detectable fluorescence which is above the cycle threshold (Ct) for the hydrolysis probe assay. For AS-PCR, analytical sensitivity was considered as the lowest DNA concentration with a diagnostic band observable on an agarose gel.

4.4.3 Determination of assay performances in a blind trial

The performance of the real-time assay and AS-PCR of Koekemoer *et al*. (2002) was assessed in a blind trial by identifying samples whose identities were kept blind prior to the test. Samples tested (n = 96) consisted of DNA from *An. funestus* (n = 26), *An. parensis* (n = 23), *An. rivulorum* (n = 13), *An. leesoni* (n = 19), *An. vaneedeni* (n = 10), water (n = 1) and *Plasmodium* species (n = 4). DNA samples were of variable quality and quantity. The numbers of correct scores, failed reactions and miscored samples were recorded and compared for each assay.

4.4.4 Robustness and validation of the hydrolysis probe assay

Genomic DNA samples extracted between the years 2001-2010 from mosquitoes from different regions across Africa (Zambia, Malawi, South Africa, Congo and Mali) were obtained from collections stored at -80ºC. These specimens, which had previously been identified using the morphological keys of Gillies and De Meillon (1968) and the AS-PCR protocol of Koekemoer *et al*. (2002) as belonging to the *An. funestus* group, were re-identified using the hydrolysis probe assay. The percentage of samples with the same identity from both assays was calculated.

4.4.5 Development of New Allele Specific-PCR assays

4.4.5.1 Primer design for the amplification of the rDNA IGS region

To obtain sequence information of the IGS region for members of the *An. funestus* group, known sequences of the 18S and 28S subunits of rDNA genes from insects closely related to the *An. funestus* group were obtained from the NCBI website. The 18S sequences of rRNA genes for *Drosophila melanogaster* (GI: 158246), *An. gambiae* (GI: 103421630), *Aedes aegypti* (GI: 169930869) and *Culex quinquefasciatus* (GI: 66269563) were obtained and aligned using DNASTAR lasergene 7 MegAlign. Primers were preferably designed from regions with greatest sequence homology between two or more species. A 23-mer reverse primer designated IGS Rev was designed from *An. gambiae* and *C. quinquefasciatus* sequences and was positioned 80bp from the 5' end of the 18S gene (Figure 4.3). The forward primer, IGS Forward 1, was designed 145bp downstream of the 3' end of the 28S gene of *D. Melanogaster*.

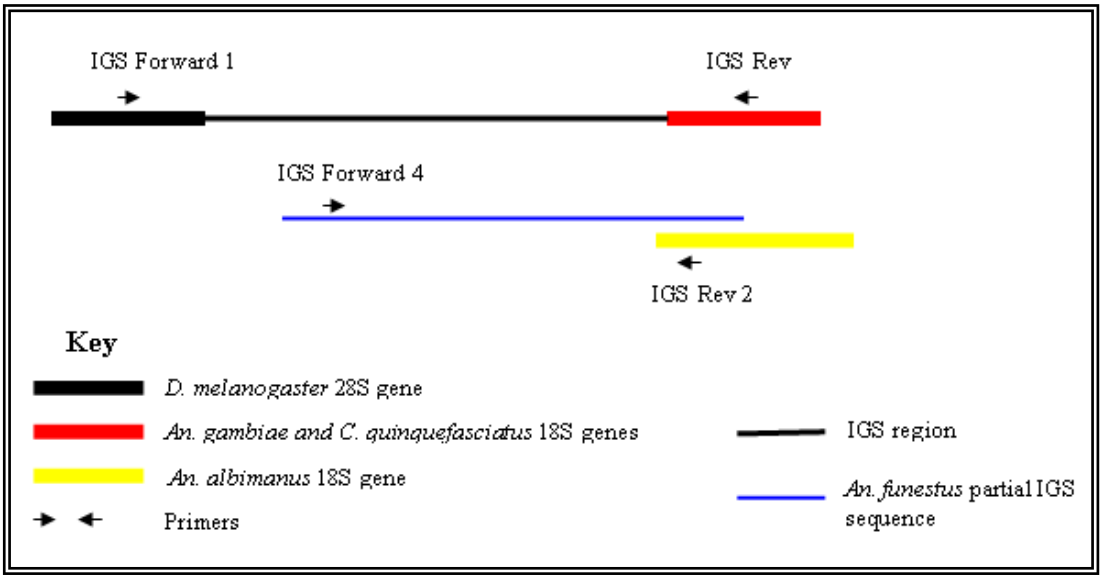


Figure 4.3 Schematic diagrams showing a partial rDNA gene with positions of primers and alignment of the *An. albimanus* 18S gene with partial IGS sequence.

The IGS sequence for *An. funestus* obtained after sequencing PCR products, generated using the IGS Forward 1 and IGS-Rev primers, was aligned with the *An. albimanus* 18S gene sequence. The 5' end of the *An. albimanus* 18S gene sequence overlapped by 102bp with the *An. funestus* 18S sequence. A second reverse primer IGS Rev 2 was then designed 48bp from the *An. albimanus* 18S gene sequence. The last primer, IGS forward 4, was designed from the *An. fuenstus* IGS region. Apart from the *An. gambiae* universal primer (UN) of Scott *et al*. (1993), all other primers were designed with the aid of fermentas REviewer™ and synthesized by Inqaba biotec, South Africa. Details of primers are found in Table 4.3.

Table 4.3 Primers used in the amplification of the partial IGS region of the *An. funestus* group.

Species	Name of primer	5'-3'primer sequence	TM (ºC)
An. funestus	IGS Forward 1	TTC GAT CAC CTG ATG CCG C	**62.00**
	IGS Rev	TAC TGA GCC TTA TGC GGT TTC AC	**62.77**
An. vaneedeni	IGS Forward 4	CAC CTG ATG CCG CAG GTT G	**64.48**
An. parensis	IGS Rev 2	CTA CTT AGA CAT GCA TGG C	**58.01**
An. rivulorum	UN*	GTG TGC CCC TTC CTC GAT GT	**58.3**
	IGS Rev	TAC TGA GCC TTA TGC GGT TTC AC	**62.77**

***** Universal primer (UN), originally designed by Scott *et al*. (1993).

4.4.5.2 Optimization of PCR for the amplification of the rDNA IGS region

A 50µl PCR reaction consisted of the following: 1 X LA buffer (Takara biomedical group Shiga Japan, Cat. No. KA3701DA), 0.4mM dNTPs, 0.5µM of each primer pair, 2.5U of LA *Taq* DNA polymerase and 50-100ng genomic DNA. The $MgCl_2$ concentration for reactions in which DNA from *An. funestus*, *An. leesoni*, *An. rivulorum* and *An. funestus*-

like was used as the template was 2mM while for *An. parensis* and *An. vaneedeni*, the $MgCl_2$ concentration was 3mM and 2.5mM respectively. The PCR cycling conditions were as follows: 1 cycle initial denaturation at 94°C for 1 minute, 30 cycles of denaturation at 98°C for 5 seconds, annealing temperature (56°C for 30 seconds for *An. funestus* and *An. funestus*-like, 38°C for 30 seconds for *An. vaneedeni*, and *An. rivulorum*; 46°C for 30 seconds for *An. parensis*), extension at 68°C for 15 seconds. This was followed by a final extension at 72°C for 10 minutes. A no template control consisting of only PCR mix was included for each reaction. The reverse and forward primer pair used in this assay to amplify products in each species are provided next to the species in Table 4.3.

A volume of 10µl of PCR products was mixed with 3µl ficoll loading dye and loaded into the wells of a 1% (W/V) SeaKom® LE agarose (Lonza, Rockland USA, 50004) 1 X TAE (Tris acetic acid EDTA) gel pre-stained with 0.5µg/ml of ethidium bromide. Electrophoresis of products was performed in a 1 X TAE buffer (Appendix I B) using a Bio-Rad PowerPac 300 power supply at 90V, 400mA for 50 minutes. Amplification products were visualised with an ultraviolet (UV) transilluminator system (Vacutec, G-BOX from Syngene) and the image captured. The amplicon sizes were estimated by comparison to the standard band size of a 10Kb HyperLadder ™ I (Bioline, USA, BIO 33053).

4.4.5.3 Cloning, screening and sequencing of IGS products

The IGS PCR products generated were either gel purified using QIAquick gel extraction kit (QIAGEN, South Africa, 28707) (Appendix II F) or directly used in a ligation reaction (Appendix IIH). Ligation is the process whereby an insert (PCR product) is annealed into a vector by phosphodiester bonds. Forming of the bond by joining the 3’ hydroxyl and 5’

end of nucleic acids is controlled by the enzyme DNA ligase. The recombinant DNA formed during ligation was used to transform JM109 chemical competent cells (Appendix III).

Transformation is the introduction of plasmids into chemically competent cells. These cells have been treated such that their membrane becomes permeable to the plasmids (recombinant DNA). They were obtained from Lucigen, Middleton Cat. No 60107-2. Ligation and transformation were performed using the pGEM-T Easy vector kit (Promega, USA, A1360) following the manufacturer's instructions. The preparation of transformation solutions are given in Appendix I E. Transformed competent cells were plated on luria broth (LB) agar/ampicillin/IPTG/ X-gal plates (Appendix II I). The plates were incubated at 37ºC overnight and white colonies were screened for inserts. Three controls (two positive and one negative) were included during cloning. The first positive control, a ligation control consisting of a known insert together with all ligation reagents and competent cells was provided in the kit. The control template ligates into the pGEM-T Easy vector and produces white colonies after transformation and incubation. The second positive control was a transformation positive control for the determination of transformation efficiency. It consisted of a closed super-coiled plasmid (pUC1**9** DNA) and JM109 *E. coli* competent cells. This control should produce many white colonies after plating. The negative control consisted of all ligation reagents except an insert and was expected not to produce any colonies. It was used to identify contamination in the cloning process.

i) Screening of clones with insert by colony PCR

Screening was performed to determine whether the recombinant plasmids contained the correct insert. Some 350 white clones were selected for screening from LB agar plates (Appendix II G). Using a sterile yellow tip, colonies were scraped from the agar plate and the tip stirred in 25µl PCR mix. The tip was also stirred in 500µl 50µg/ml AMP/LB solutions in a 1.5ml eppendorf tube and incubated overnight at room temperature in a Labcon shaking incubator (Labcon, South Africa, Model FSIE 5P08). Glycerol solution was added to the culture to a final concentration of 30%, properly mixed and stored at -70ºC for future analysis. The PCR mixture consisted of all reagents used to generate the insert except for the primers which were replaced with 0.24µM each of SP6 and T7 primers specific for pGEM-T Easy vector.

The colony PCR cycling conditions consisted of an initial denaturation of 94°C for 2 minutes followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 56.5ºC for 30 seconds and 72°C extension for 30 seconds. A final extension was performed at 72°C for 10 minutes and the reaction held at 8°C until they were removed from the thermo-cycler. A total volume of 3µl of the colony PCR product was mixed with 2µl ficoll loading dye (Appendix IB) and analysed on a 1% TAE agarose gel as described previously (section 4.4.5.2). The colony PCR product (10µl) with the correct insert was sent for sequencing to Macrogene, (South Korea). Sequencing was done in both directions using T7 and SP6 primers. At least two PCR products of the same size from different clones originating from the same ligation and transformation reactions were sequenced to confirm the identity of the insert.

4.4.5.4 Analysis of IGS sequences for species-specific PCR design

Electropherograms and sequences for PCR products were obtained on the Macrogene website (http://dna.macrogene.com) and manually edited using Chromas lite version 2.0. PCR products in most cases were between 1500 and 3000bp, and only a read length of 900bp of sequencing data can reliably be obtained. The forward and reverse primer sequence for each product was therefore analysed separately. The cloning vector sequence was removed from the sequences and the portion of sequence belonging to the PCR product was blasted in the NCBI website (http://blast.ncbi.nlm.nih.gov). Only reverse and forward sequences that aligned with rDNA 18S or 28S genes respectively for anophelines, or drosophila species and gave the highest percentage similarity were selected for analysis. For each species, selected forward or reverse sequences were aligned together using DNASTAR lasergene SeqMan 2007 version and a consensus sequenced generated. The consensus sequences for reverse and forward sequences were aligned with *An. albimanus* rDNA 18S and 28S gene sequences respectively. This allowed determination of the IGS region that has been sequenced for each species. The IGS sequences obtained for different species were aligned for species-specific primer design Table 4.4.

Table 4.4 Species-specific primers for the identification of members of the *Anopheles funestus* group.

Species	Primers	Primer sequence (5'- 3')	Product size
An. funestus group	UN18S-IGS-Rev	TTACTGGTAGGATCAACCAG	-
An. funestus	FUNIGS	GCTTGAGCCCCTGAATAAGGG	630bp
An. vaneedeni	VANIGS	CATAATACGAAGGCAGCGCTGTC	554bp
An. parensis	PARIGS	CAAGTGGAACCAATTGATAG	363bp
An. rivulorum	RIVIGS	GATAATAGGGACCCGGGAGC	213bp

4.4.5.5 Optimisation of Allele Specific PCR based on the IGS region of an rDNA gene

Each species-specific primer was used together with the universal primers (UN18S-IGS-Rev) in a PCR and the reaction optimised for the formation of single PCR products. The size of each product was confirmed by comparison with a standard DNA molecular ladder. A multiplex PCR containing all the individual primers in a single reaction was optimised. A 13.5µl volume of this reaction mixture consisted of 1 X Buffer, 0.23mM of each dNTP, 1.2mM $MgCl_2$, 0.17µM of primer RIVIGS, 0.24µM each of primer UN18S-IGS-Rev, FUNIGS, VANIGS and PARIGS, 0.5U *Taq* DNA polymerase and 1 µl DNA. The PCR cycling conditions consisted of an initial denaturation of 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55ºC for 30 seconds and a 72°C extension for 40 seconds. A final extension was performed at 72°C for 10 minutes. PCR products (10µl) were mixed with 3µl ficoll loading dye and loaded into the wells of a 2.5% ethidium bromide stained agarose gel submerged in a 1 X TAE buffer. The first well of the gel was loaded with GeneRular^{TM} 100bp DNA ladder (Fermentas, USA, SM0242)

from which PCR product sizes were determined. The second well was used as a negative control and the rest of the wells were used for test samples. Electrophoresis of products was performed using a Bio-Rad PowerPac 300 power supply at 110V, 400mA for 1hr: 30 minutes. Amplification products were visualised and images captured as previously described (section 4.4.5.2).

The optimised AS-PCR was validated by re-identifying 114 DNA samples from Zambia, Malawi, Mali, Mozambique, DRC, South Arica, Ghana and Uganda belonging to the *An. funestus* group. The samples were previously identified as *An. funestus* (n = 97), *An. rivulorum* (n = 39), *An. parensis*, *An. vaneedeni* and *An. leeseoni* using the protocol of Koekemoer *et al*. (2001) and the hydrolysis probe assay developed in this study. The percentage match in identity was calculated in order to validate the performance and accuracy of this assay.

4.4.6 Restriction fragment length polymorphism of the *Anopheles funestus*-like and *An. funestus* rDNA D3 region

The 28S domain 3 (D3) of rDNA for *An. funestus* and *An. funestus*-like was amplified and analysed according to the protocol of Garros *et al*. (2004) with minor modifications. Briefly, a 25µl reaction volume consisting of: 1 X reaction buffer, 1.5mM $MgCl_2$, 1µM each of D3A and D3B primers (Koekemoer *et al*., 1998), 0.2µM dNTPs, 2U *Taq* DNA polymerase and 1µl DNA template. The PCR cycling conditions consisted of: 1 cycle initial denaturation at 94ºC for 3 minutes; 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 45ºC for 40 seconds, extension at 72ºC for 40 seconds and a final extension of 72ºC for 10 minutes. A negative control to rule out experimental errors consisted of all reagents except DNA. PCR product (5µl) was subjected to electrophoresis

(100V, 400mA, for 1hr) on a 2.5% TAE agarose gel stained with ethidium bromide. The gel was visualized and images captured as described in section 4.4.5.2. PCR product sizes were determined by comparison to standard band sizes of a GeneRular^{TM} 100bp DNA ladder (Fermentas, USA, SM0242).

Digestion of PCR products was carried out in a 20µl reaction volume consisting of: 12.3µl of sterilised distilled water, 2µl Buffer A, 0.2µl BSA, 5µl PCR amplified product and 0.25µl *Hpa* II restriction endonuclease. The buffer, BSA and enzyme were supplied by Promega, USA (Cat. No. R6315). The digestion mixture was incubated at 37ºC for 2 hours using a thermo-cycler. The digest (10µl) was mixed with 4µl ficoll loading dye and loaded into the wells of a 3% TBA agarose gel stained with 0.5µg/ml ethidium bromide. The loaded gel was then subjected to electrophoresis using a Bio-Rad Powerpac power supply at 110V, 400mA for 1hour. Post electrophoresis, the remainder of the D3 PCR products (10µl), representative of the PCR-RFLP patterns observed, were sequenced by Macrogene (South Korea). In instances where the D3 PCR gave multiple products, the products were cloned into pGEM-T Easy vectors (Appendix II H and I). Colony PCR was performed and the products analysed on agarose gels as previously described in section 4.4.5.3 to confirm the presence of the inserts. The colony PCR products for each fragment were then sequenced. The *Hpa* II restriction sites on the sequences were determined and a restriction map constructed for each RFLP type.

4.4.7 Phylogenetic relationship of *Anopheles funestus*-like and other members of the *An. funestus* group

The rDNA ITS2 region of *An. funestus*-like (n = 12), *An. funestus* (n = 14) and *An. parensis* (n = 4) was amplified according to the protocol of Koekemoer *et al.* (2002) using genomic DNA. The protocol was slightly modified. A 25µl volume of the PCR mixture consisted of 1 X reaction buffer, 3mM $MgCl_2$, 0.5µM each of ITS2A and ITS2B primers, 0.205mM of dNTPs, 1U *Taq* DNA polymerase and 50-100ng DNA template. The PCR cycling conditions consisted of: 1 cycle initial denaturation at 94ºC for 2 minutes; 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds, extension at 72ºC for 40 seconds and a final extension of 72ºC for 10 minutes. PCR product (5µl) was analysed on a 2.5% TAE ethidium bromide stained agarose gel as described in section 4.3.2.4 (i). PCR products were sequenced in both directions using the ITS2A and ITS2B primers by Macrogene (South Korea). The ITS2 and D3 sequences were aligned separately using BioEdit and saved as a fasta file. The sequences were imported into MEGA 5 for phylogenic analysis. To assess intraspecific variation within the *An. funestus*-like population, the ITS2 sequences were compared with one another.

4.5 Results

Mosquito identification

Species re-identifications of each sample matched their corresponding previous identifications. Figure 2.1 in chapter 2 shows diagnostic PCR products for the identification of a set of *An. funestus* group samples. *Anopheles funestus*-like was not successfully discriminated from the other members of the *An. funestus* group using real-time based PCR and was therefore not included in results.

4.5.1 Development of real-time based PCR

4.5.1.1 Melt Curve Analysis

The species characteristic melt curves produced by *An. leesoni*, *An. vaneedeni*, *An. rivulorun*, *An. parensis* and *An. funestus s.s.* are shown in Figure 4.4 which was obtained by plotting a negative first derivative of fluorescence against temperature. The negative control produced a slight increase in fluorescence at late cycles during PCR optimisation which was reduced by decreasing cycling number.

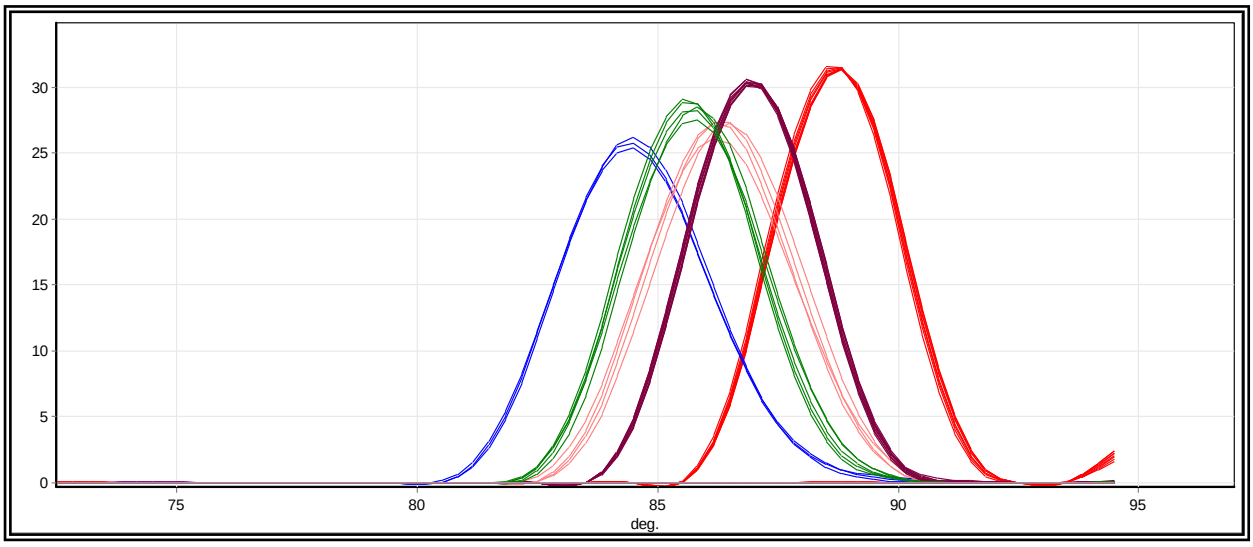


Figure 4.4 Identification of members of the *An. funestus* group by melt-curve real-time PCR analysis. In this example five to seven specimens of *An. rivulorum* (red trace), *An. leesoni* (blue trace), *An. funestus* (brown trace), *An. parensis* (green trace) and *An. vaneedeni* (pink trace) were tested. A plot of negative first derivative of the collected fluorescence against temperature results in melt peaks with characteristic melting temperatures.

Attempts to include *An. funestus*-like in the MCA assay were unsuccessful. The melt curve obtained for this species was indistinguishable from that of *An. funestus*. The redesigning of primers did not produce a distinct melt curve for *An. funestus*-like, and this species was excluded from the assay.

Variability of melting temperature in the Melt Curve assay

The average melting temperatures of 10 replicate PCR samples for each species subjected to MCA are shown in Figure 4.5. *Anopheles leesoni* gave the lowest melting temperature and *An. rivulorum* the highest. Melting temperature variation was observed within replicates for all species except *An. funestus* where it was constant. *Anopheles vaneedeni* showed the greatest variation with a minimum and maximum melting temperature of 84.5°C and 86.5°C respectively.

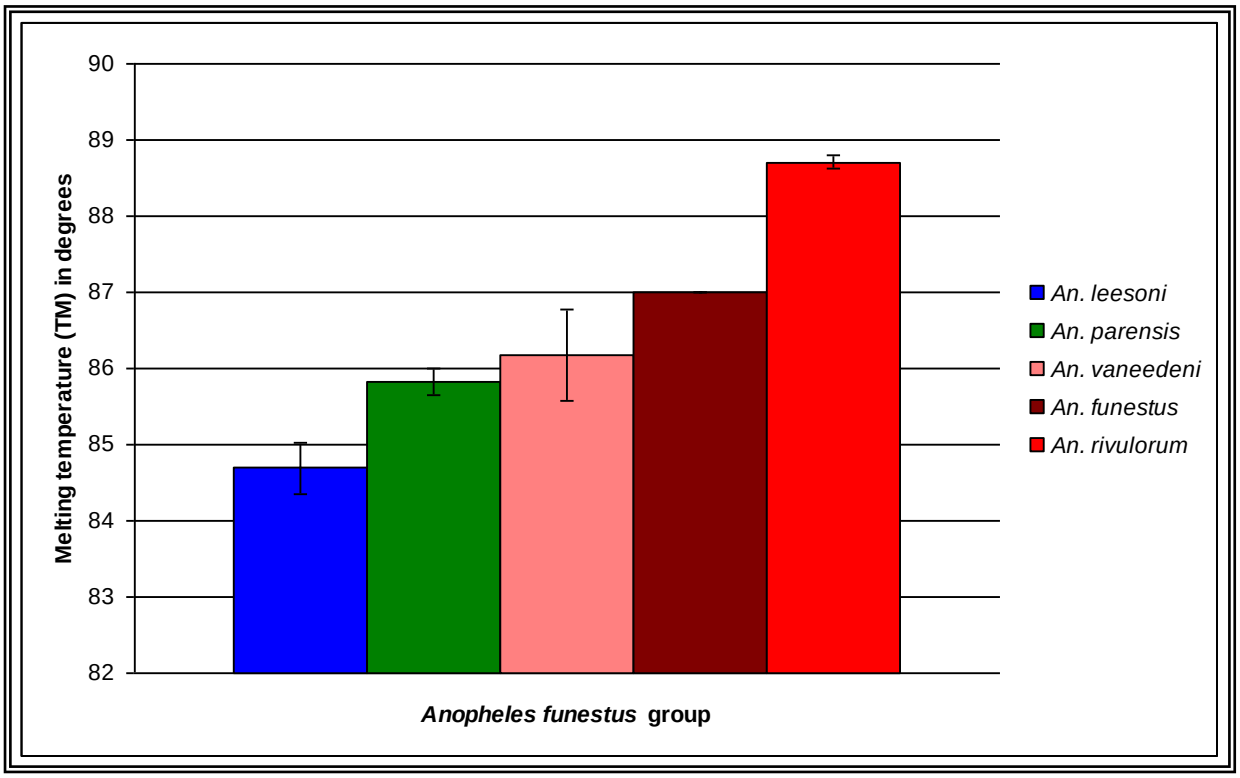


Figure 4.5 Average melting temperatures and standard deviations for fives species of the *An. funestus* group.

Owing to difficulties encountered in differentiating *An. vaneedeni* from *An. parensis* and *An. funestus*, this assay was not pursued further. Instead, high resolution melt was investigated.

4.5.1.2 High Resolution Melt

Figure 4.6 shows a normalized plot as well as a difference plot generated from the associated Rotor-Gene Software (version 1.7) for several representative specimens of each species. The normalized plots (Figure 4.6 A) initial fluorescence signal for the pre-melt stage was fixed at 100% and the final fluorescence at the post–melt stage at 0%. The species specific curves of the normalized plot are not vastly distinct from each other. Figure 4.6 (B) shows the normalized data using a difference plot. This alternative analysis highlights the deviations between the selected genotype (*An. vaneedeni*) as the standard and the other samples.

Anopheles funestus-like PCR products produced similar curves to that of *An. vaneedeni* based on HRM analysis and was therefore excluded from the assay design. Similar to MCA, the HRM method, in a blind trial, misidentified some samples including the misidentification of water as *An. funestus* and *An. rivulorum*. The constraints imposed by MCA and HRM motivated for the investigation of the hydrolysis probe assay which, though expensive, is well known for its high specificity.

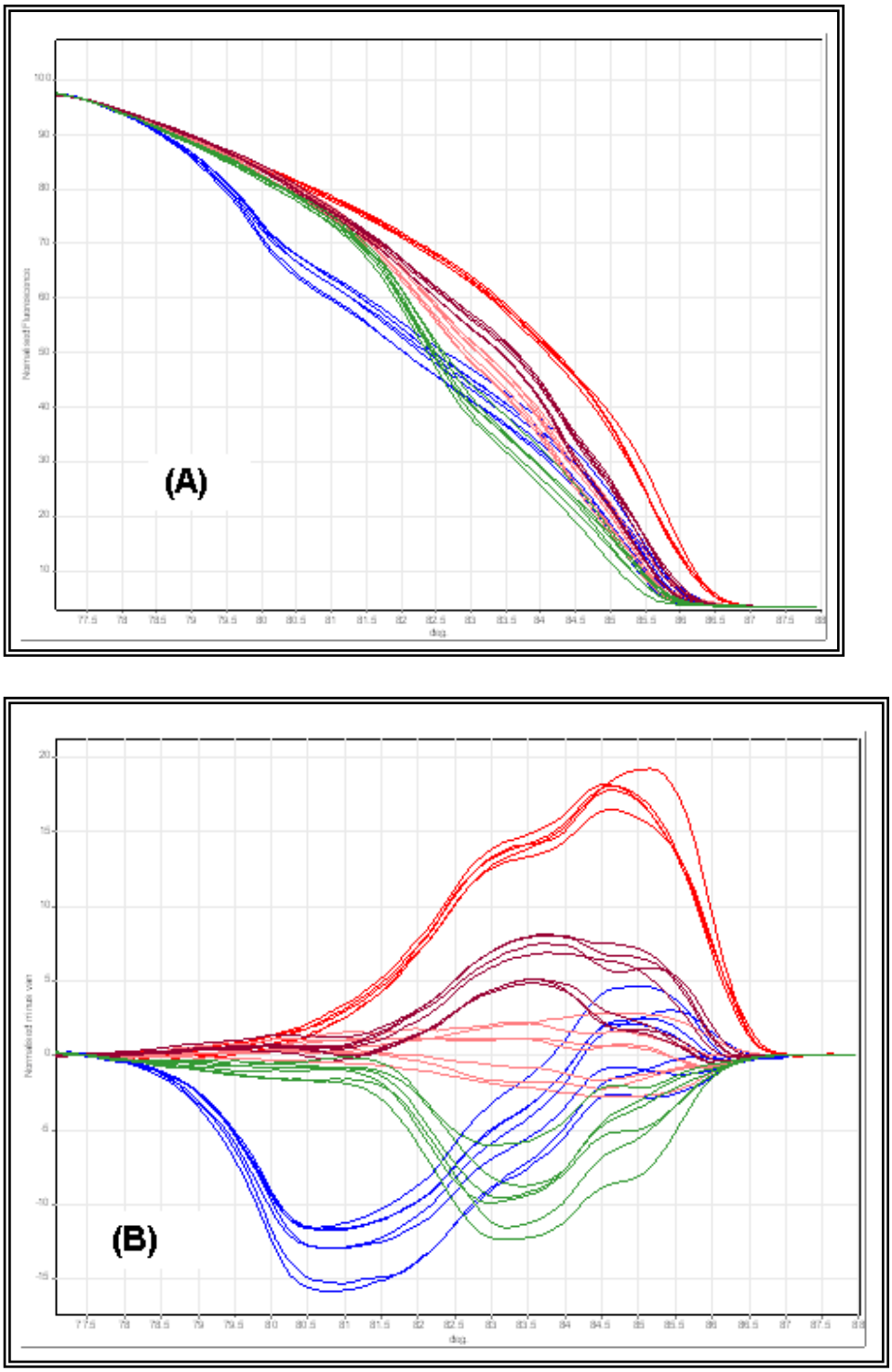
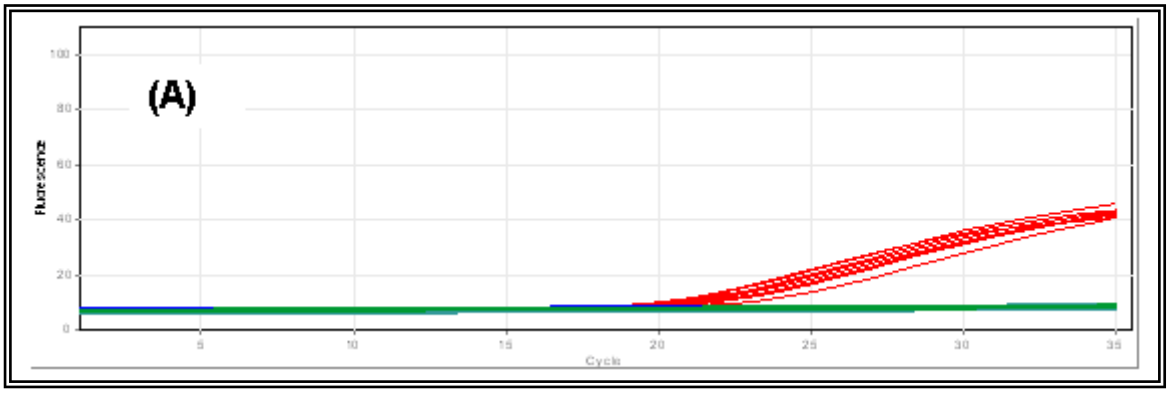
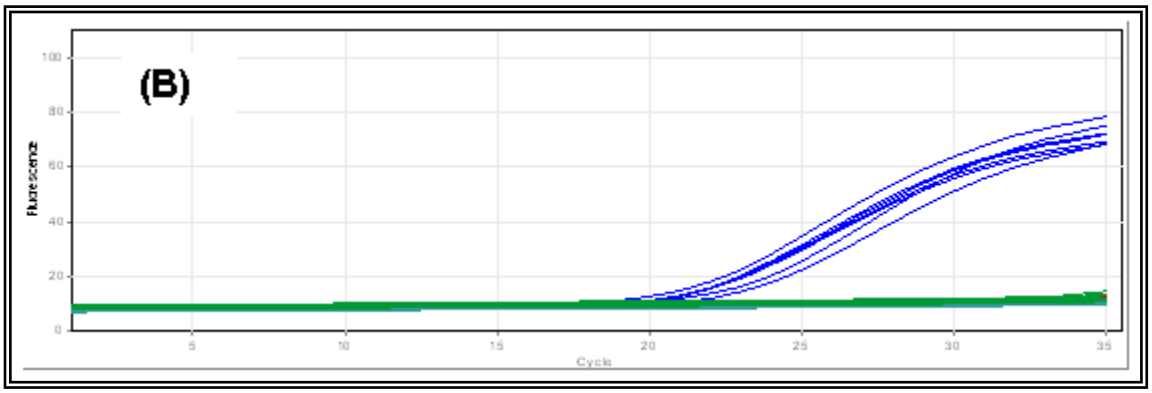


Figure 4.6 Identification of members of the *An. funestus* group by High Resolution Melt analysis. In this example, five to seven specimens of *An. rivulorum* (red trace), *An. leesoni* (blue trace), *An. funestus* (brown trace), *An. parensis* (green trace) and *An. vaneedeni* (pink trace) were tested. **A**) Normalised melt curve for different *An. funestus* group species. **B**) Difference plot for samples as in (A) in which *An. vaneedeni* was selected as the reference standard.

4.5.1.3 Hydrolysis probe assay

The hydrolysis probes and primers for the multiplex assay designed in this study effectively identified all the reference control templates of the five members of the *An. funestus* group. A series of preliminary tests were performed for different concentrations of primers (125nM to 250nM) and probes (100nM to 200nM). That combination of primer and probe concentration giving optimal fluorescence and the smallest Ct value was selected for the assay. Figure 4.7 shows an example of using this assay for the identification of seven to ten samples of each species. Measured increases in the fluorescence of Cy5 (probe RIV), 6FAM (LEES probe), ROX (FUN probe), VIC (PAR probe) and Quasar 705 (VAN probe) respectively identified *An. rivulorum*, *An. leesoni*, *An. funestus s.s.*, *An. parensis* and *An. vaneedeni* specimens. Though no hybrid was included here, an increase in two or more of the dyes would indicate a hybrid or a contaminated sample.





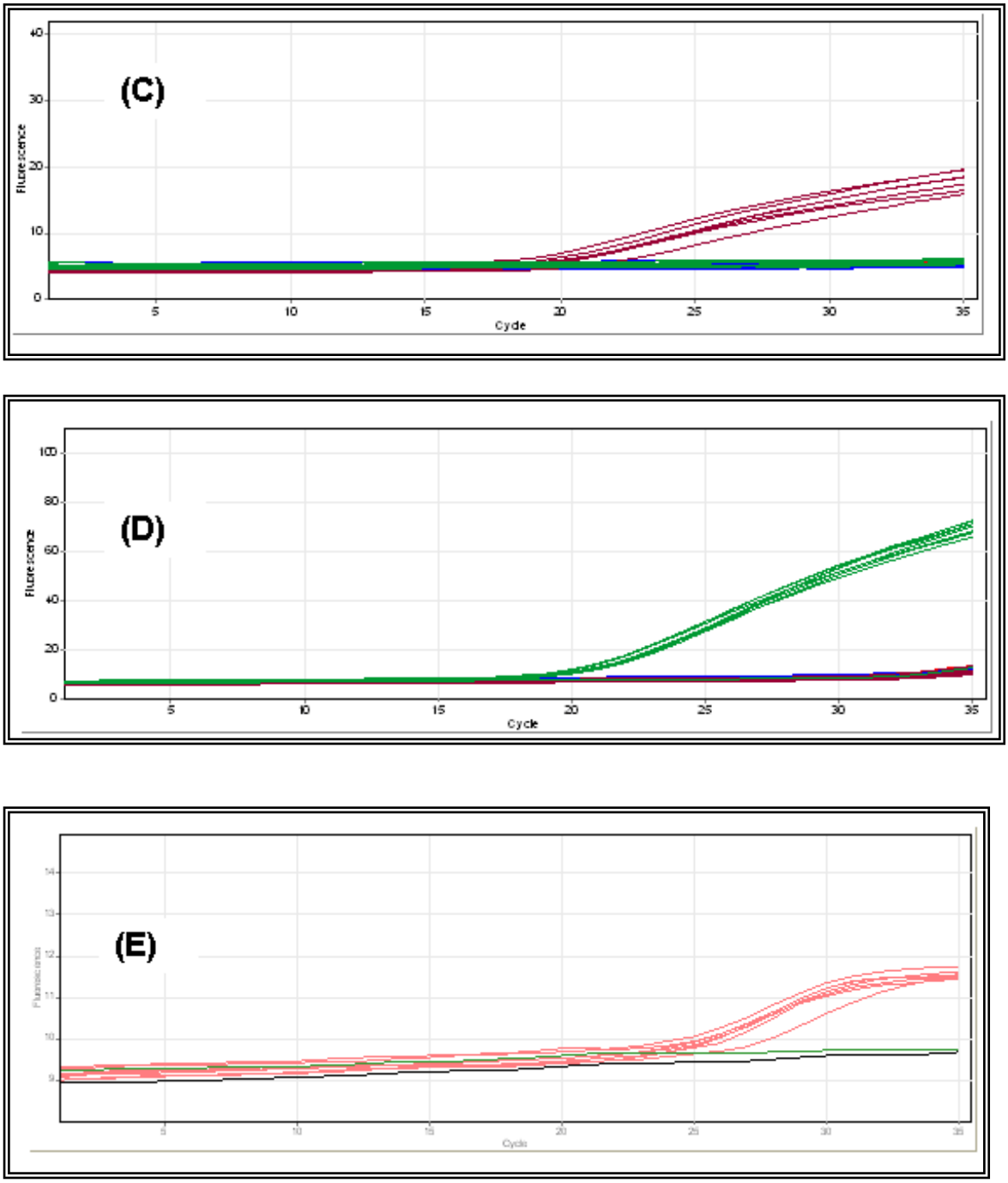


Figure 4.7 Identification of members of the *An. funestus* group using the multiplex hydrolysis probe assay. Seven to ten specimens of *An. rivulorun* (red trace), *An. leesoni* (blue trace), *An. funestus* (brown trace), *An. parensis* (green trace) and *An. vaneedeni* (pink trace) were tested. (A) Cycling of the RIV probe (Cy5 labelled), (B) cycling of the LEES probe (6FAM labelled), (C) cycling of the FUN probe (ROX labelled), (D) cycling of the PAR probe (VIC labelled) and (E) cycling of the VAN probe (Quasar 705 labelled)

4.5.1.4 Analytical sensitivity of real-time assays and AS-PCR

The sensitivity of real-time PCR and AS-PCR was evaluated using diluted samples from each of the five species. Only the sensitivity of the hydrolysis probe assay was investigated because it was the only assay that reliably discriminated between members of the *An. funestus* group. The hydrolysis probe had template detection limits of 1 in 1000 dilution representing 0.02ng DNA. The AS-PCR assay could only detect template up to a limit of 1 in 5 000 dilution, equivalent to 0.04ng of DNA (Figure 4.8).

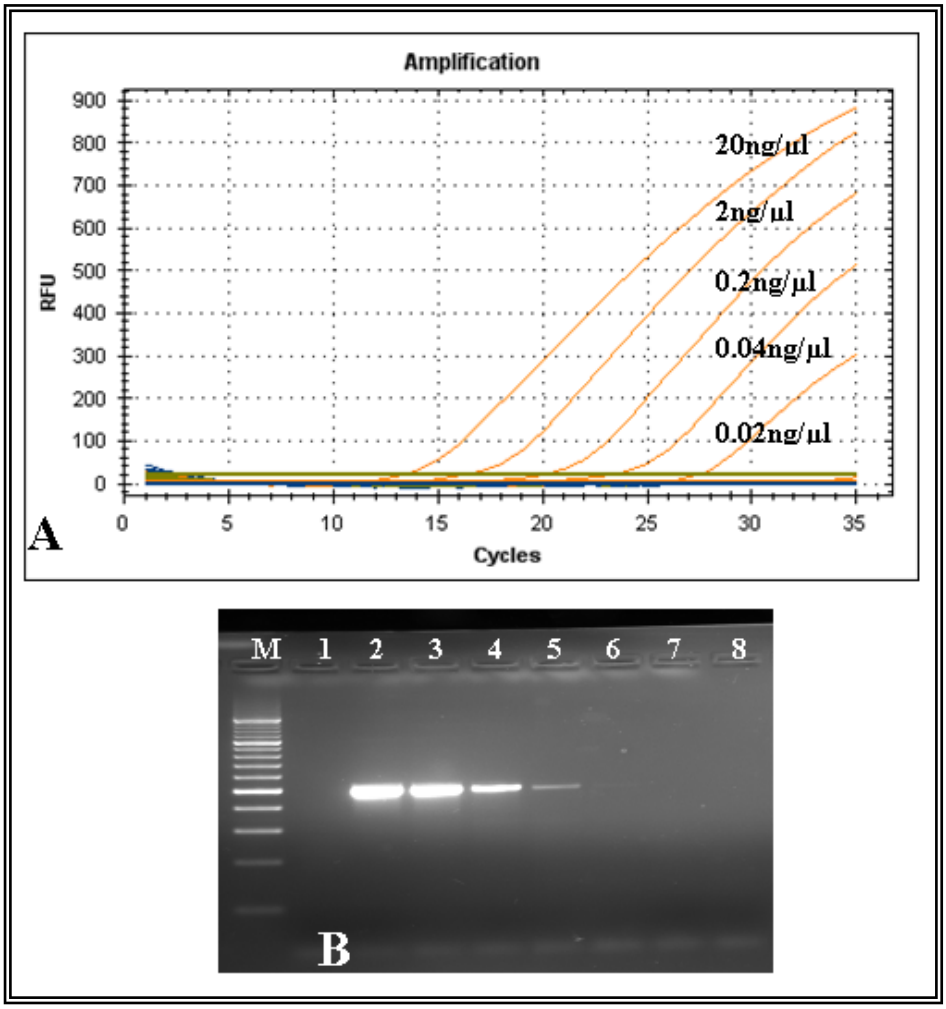


Figure 4.8 Comparison of analytical sensitivity of real-time PCR and AS-PCR assays for the detection of *An. funestus* 20ng/µl stock DNA serially diluted down to a 1 in 1 x 10^6 dilution. **A:** Hydrolysis probe assay amplification plot showing concentration of detected DNA. **B:** A 2.5% agarose electrophoretic gel stained with ethidium bromide showing *An. funestus* AS-PCR single specific bands of 500bp detected DNA. Lane M: Marker; Lane 1: Negative control (no template); Lane 2: 20 ng/µl; Lane 3: 2 ng/µl; Lane 4: 0.2 ng/µl; Lane 5: 0.04 ng/µl; Lane 6: 0.02 ng/µl; Lane 7: 0.004 ng/µl; Lane 8: 0.002 ng/µl.

4.5.1.5 Blind species identification trial

Results obtained for four assays in the species identification blind trial are presented in Table 4.5. These data indicate that AS-PCR followed by the hydrolysis probe assay produced the highest scores. Misidentification of samples was associated with MCA and HRM. All four assays failed to identify some samples.

Table 4.5 Performance of four assays in the *Anopheles funestus* group species identification blind trial.

	AS-PCR	**MCA**	**HRM**	**Hyrolysis probe assay**
Correct scores	90	84	88	89
Failed reactions	6	4	2	7
Misidentifications	0	8	6	0

4.5.1.6 Validation of the hydrolysis probe assay

The hydrolysis probe assay was the most reliable of the real-time assays for *An. funestus* group species identifications and was therefore further investigated. The efficacy of this assay for rapid screening of large numbers of field collected mosquitoes was assessed by testing 427 stored DNA samples extracted from field collected mosquitoes. Comparative results for specimens positively identified by the AS-PCR and hydrolysis probe assays are presented in Table 4.6. These data indicate 90.4% (n = 427) concordance between the hydrolysis probe assay and AS-PCR. A total of 9.6% specimens could not be identified using the hydrolysis probe assay.

Table 4.6 Detailed results of re-identification of AS-PCR identified *An. funestus* group samples using the hydrolysis probe assay.

An. funestus species	Number of samples	Correct score	Failed reaction
An. parensis	14	14	0
An. funestus	321	285	36
An. leesoni	13	8	5
An. vaneedeni	8	8	0
An. rivulorum	71	71	0
	427	**386**	**41**

None of the real-time PCR assays investigated in this study successfully identified *An. funestus*-like together with the other members of the *An. funestus* group in a single assay based on the ITS region of rDNA. The IGS region was therefore investigated as an alternative region from which a cost effective assay could be designed.

4.5.2 Development of Allele-specific PCR assay on the rDNA IGS region

4.5.2.1 Amplification and sequence analyses of *Anopheles* species rDNA IGS regions

Following PCR amplification of the IGS region, products by species of the *An. funestus* group were resolved on a 1% agarose gel. *Anopheles rivulorum* gave a single product of 1000bp while the other species gave multiple products ranging from 1000 to above 10Kbp (Figure 4.9).

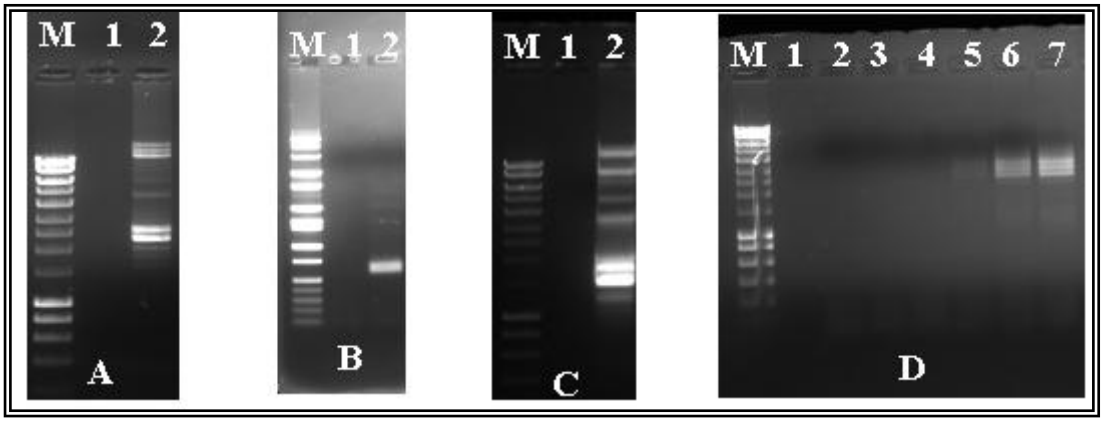


Figure 4.9 1% agarose gels stained with ethidium bromide showing rDNA IGS generated PCR products. Lane M: DNA marker; Lane 1: No template negative control. **A:** (*An. funestus*), **B** (*An. rivulorum*) and **C** (*An. parensis*): Lane 2 (A, B and C): PCR products, **D** (*An. vaneedeni*) Lanes 2, 3 and 4: No amplification, lanes 5-7: PCR products.

i) Cloning and screening of clones

The transformation efficiency obtained during cloning ranged from 2.3 x 10^7 cfu/µg to 2.9 x 10^8cfu/µg. A total of 350 colonies were screen for inserts by the colony PCR method. Of these, 104 colonies had the inserts of interest based on the expected size of PCR product.

ii) IGS sequence analysis

Table 4.7 shows statistical data obtained after blast searches of IGS sequences from various species of the *An. funestus* group in the NCBI data base. The IGS sequences aligned continuously along the length of the sequence for *An. funestus* clone AF4 microsatellite (AF171034.1) and *Aedes aegypti* rDNA gene (gb/U65375.1). Due to high variability in the IGS region, the query (sequence blasted in the data base) coverage by the hit sequence (sequences submitted and available on the data base) was low, ranging from 6% to 13%. However, the 18S gene sequence produced a high match. The percentage similarity between the query and the hits ranged from 98% to 100%. The second highest hit was with *An. albimanus* (gb/L78065.1) for all sequence blasts. The E-values in Table 4.7 describe the likelihood of a sequence occurring in the data base by chance.

Table 4.7 Statistical data obtained after blast searches of *An. funestus* group partial IGS sequences in the NCBI data base.

Query sequence	**Highest Hit**	**QC**	**E-value**	**Max ID**	Max scores
An. funestus (1331bp)	*An. funestus*	6%	$4e^{-39}$	100%	172
An. parensis (545bp)	*Ae. aegypti*	12%	$2e^{-24}$	100%	122
An. rivulorum (686bp)	*An. funestus*	13%	$1e^{-37}$	98%	167
An. vaneedeni (588bp)	*Ae. aegypti*	11%	$2e^{-24}$	100%	122

A representative partial IGS sequence for *An. vaneedeni* is presented in Figure 4.10.

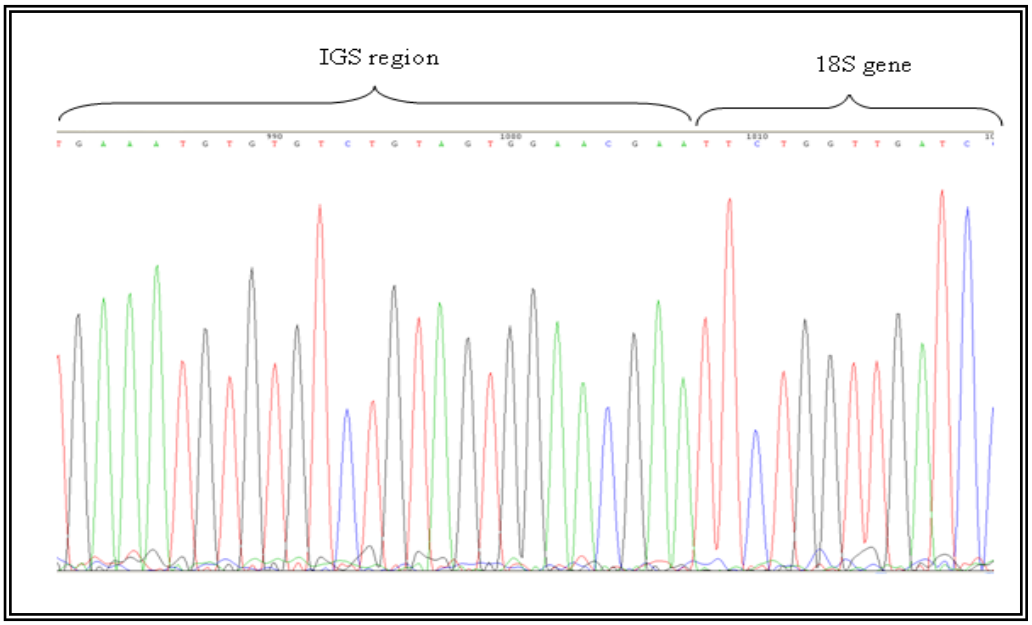


Figure 4.10 Partial rDNA gene sequence (IGS region and I8S gene) for *An. vaneedeni* obtained from sequencing PCR products with reverse primers.

Alignment of the IGS consensus sequences for four species of the *An. funestus* group using DNASTAR-MegAlign is shown in Figure 4.11. The consensus sequences were obtained from reverse sequences. The portion of the alignment in the blue rectangular box is homologous to the 5' end of the *An. albimanus* 18S gene sequence.

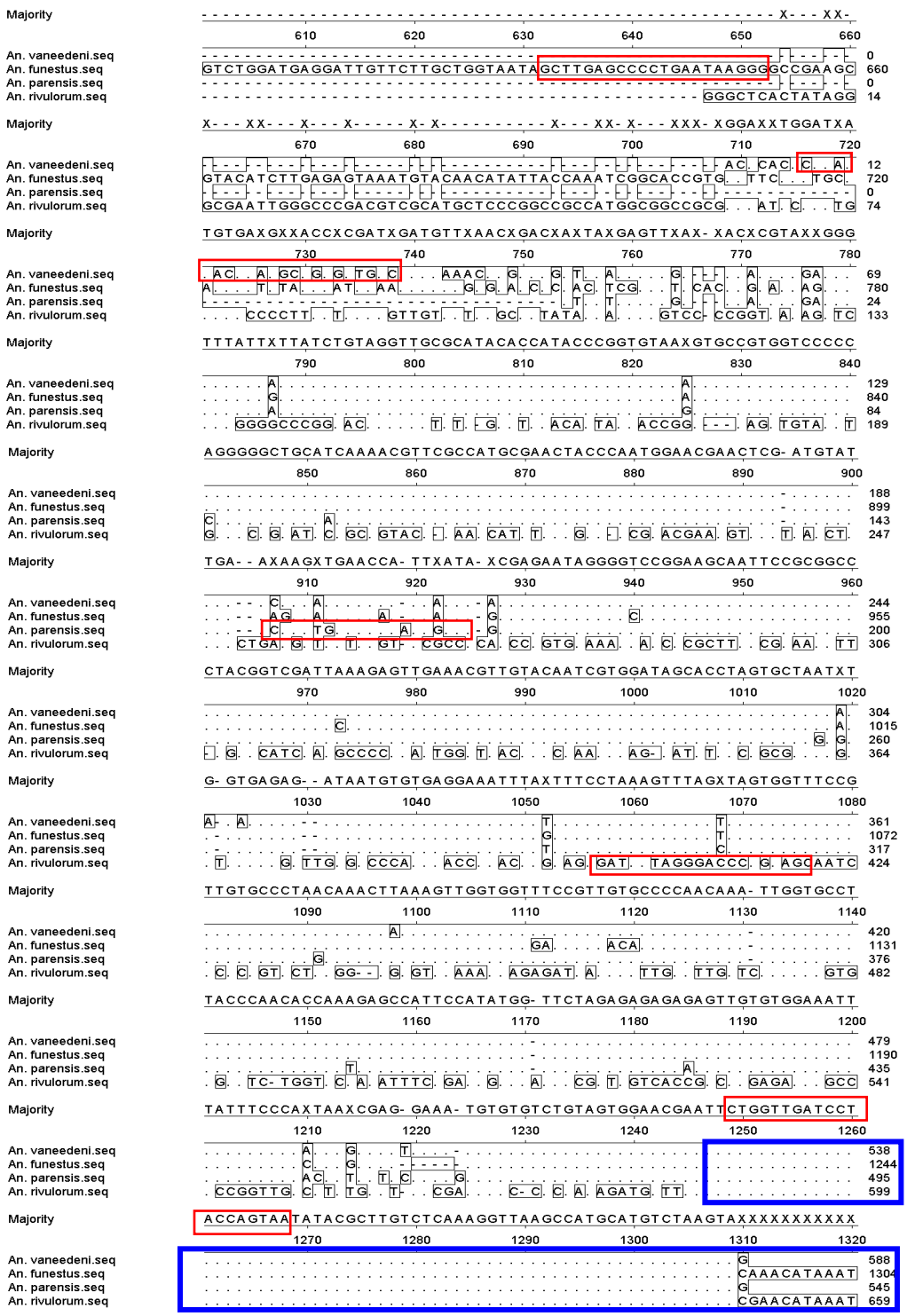


Figure 4.11 Alignment of *An. funestus* group partial IGS sequences. Sequences in the blue box are identical to the sequence at the 5' end of the *An. albimanus* 18S rDNA gene. Sequences in the red boxes indicate possible diagnostic species-specific primer sites for each species. The universal primer is indicated on the consensus sequence.

PCR products in clones sent for sequencing had sizes ranging from 1Kb to 4Kb. Sequencing data obtained were between 545bp to 1300bp from reverse sequences. Analyses of the forward sequences showed that not more than 200bp of sequence data could be obtained from the IGS region for *An. funestus*-like. For the other species investigated, even though sequencing data with read lengths ranging from 500bp to 700bp were obtained, analyses indicated that the IGS region had not been sequenced.

4.5.2.2 Multiplex AS-PCR based on the IGS region of rDNA

The conserved 18S gene sequence (Figure 4.11) was used for designing a universal reverse primer. Diagnostic species-specific primers are shown in red rectangle boxes (Figure 4.11) and were designed from sequences with high variability compared with the other species. The positions of primers were also chosen such that PCR products with discriminating sizes are produced.

The optimisation of single AS-PCR for each species resulted in the production a single diagnostic amplicon each for *An. funestus* (620bp), *An. vaneedeni* (550bp), *An. parensis* (360bp) and *An. rivulorum* (200bp) as estimated from agarose gel migration. In the multiplex PCR, *An. funestus*, *An. rivulorum* and *An. vaneedeni* produced the expected products. *Anopheles parensis* however produced the diagnostic 360bp product in addition to the *An. vaneedeni* 550bp product (Figure 4.12). A series of Multiplex AS-PCRs, in which one of the species-specific primers was omitted from each, was set up to determine the origin of the *An. vaneedeni* 550bp product. Data showed that in the absence of the *An. vaneedeni* specific primer, the *An. vaneedeni* 550bp product is not formed (Figure 4.12).

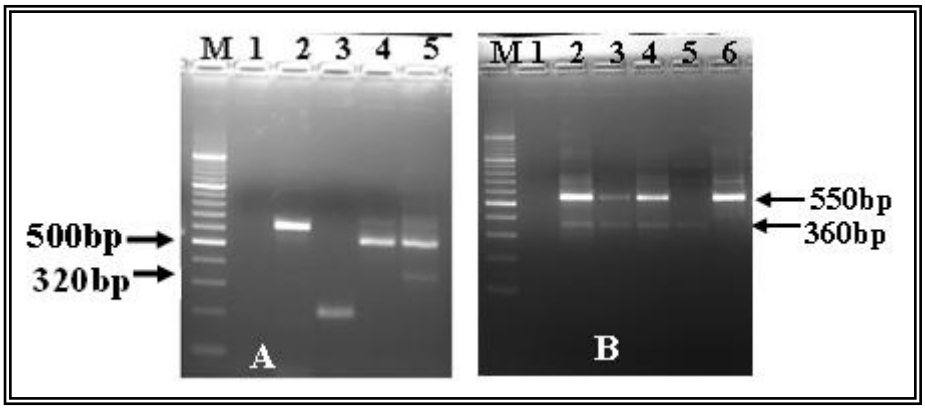


Figure 4.12 1% agarose gel stained with ethidium bromide showing AS-PCR products. **A:** Multiplex AS-PCR for identification of *An. funestus* group species. Lane M: DNA marker; Lane 1: No template negative control, Lane 2: *An. funestus*(620bp); Lane 3: *An. rivulorum* (200bp), Lanes 4: *An. vaneedeni* (550bp), Lane 5: *An. parensis* (320bp diagnostic band and 550bp). **B:** Multiplex AS-PCR in which each species-specific primer has been omitted. Lane M: DNA marker; Lane 1 No template negative control; Lane 2: All primers present; Species-specific primers were absent for Lane 3: FUNIGS primer (*An. funestus*); Lane 4: RIVIG (*An. rivulorum*); Lane 5: VANIGS (*An. vaneedeni*) and Lane 6: PARIGS (*An. parensis*).

iii) Validation of the new AS-PCR

A total of 139 DNA samples belonging to the *An. funestus* group that had previously been identified by the AS-PCR of Koekemoer *et al*. (2002) as well as the hydrolysis probe assay developed in this study were assayed again using the new AS-PCR. The species identities of 89.9% (n = 139) of the samples matched those previously obtained. The remainder (13 *An. funestus* and 1 *An. leesoni*) could not be identified using the new AS-PCR.

4.5.3 Amplification and sequencing of ITS2 and D3 regions of rDNA

i) *ITS2 region of rDNA*

Figure 4.13 shows ITS2 amplified products for *An. funestus* group species. The ITS2 products ranged in size from ≈500 to 850bp and single products were obtained for all species investigated.

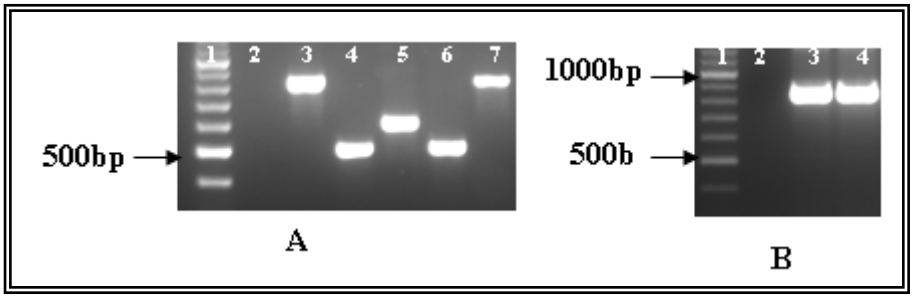


Figure 4.13: 2.5% agarose gel stained with ethidium bromide for visualising *An. funestus* group PCR products. For each gel, lanes 1 and 2 are molecular markers and no template controls respectively. **A:** ITS2 products; lane 3: *An. vaneedeni* (850bp), lane 4: *An. leesoni* (500bp), lane 5: *An. pareensis* (600bp), lane 6: *An. rivulorum* (500bp), lane 7: *An. funestus* (850bp). **B:** Lanes 3 and 4 *An. funestus*-like ITS2 products (850bp)

4.5.3.1 Phylogenetic relationship of *An. funestus*-like with other members of the *An. funestus* group based on ITS2 sequences

The phylogentic tree constructed using ITS2 sequences showed a clear clustering of the individual species in the *An. funestus* group into six groups (Figure 4.14). *Anopheles funestus*-like is more closely related to *An. funestus s.s*, than to the other members of the *An. funestus* group.

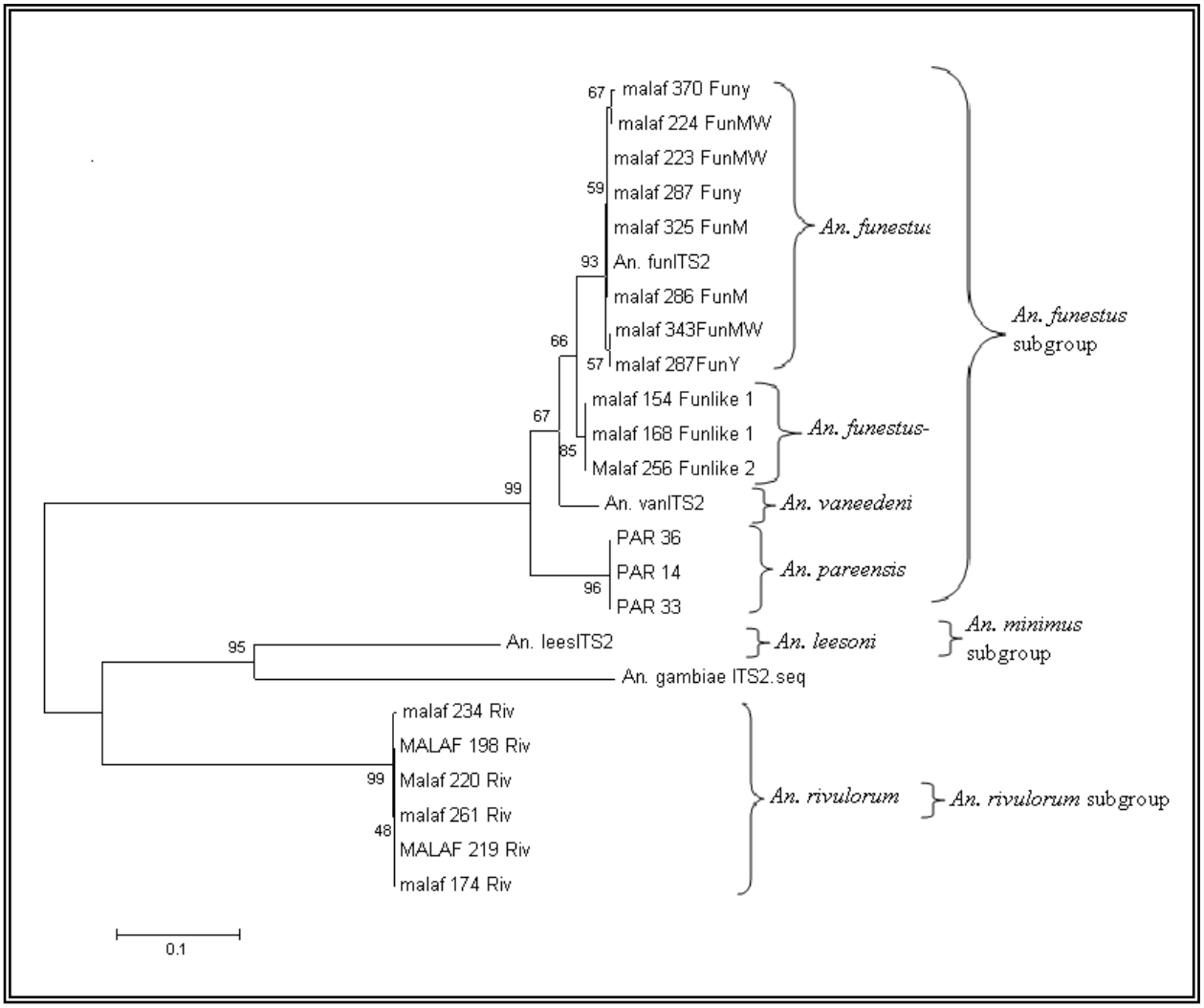


Figure 4.14 Phylogenetic tree of the *An. funestus* group based on nucleotide sequences of the ITS2 region of rDNA (Neighbour joining construction). Funy or FunY= *An. funestus* Type Y, FunMW = *An. funestus* Type MW, FunM = *An. funestus* Type M, Funlike 1 = *An. funestus*-like Type 1, Funlike 2 = *An. funestus*-like Type 2, An. funITS2 = *An. funestus* ITS2, An. vanITS2 = *An. vaneedeni* ITS2, PAR = *An. parensis* (36, 14 and 33 are sample numbers), An. leesITS2 = *An. leesoni* ITS2, Riv = *An. rivulorum* (malaf or MALAF plus number are species labels). *An. gambiae* ITS2 is used as an outgroup

Alignment of the ITS2 sequences of the *An. funestus*-like samples for intra-specific variation investigation suggests that this population is divided into two groups based on single nucleotide polymorphism (Figure 4.15) with a T/Y, A/T or G/A substitution. The IUPAC nucleotide code Y (Figure 4.15) represents either a C (cytosine) or T (thymine).

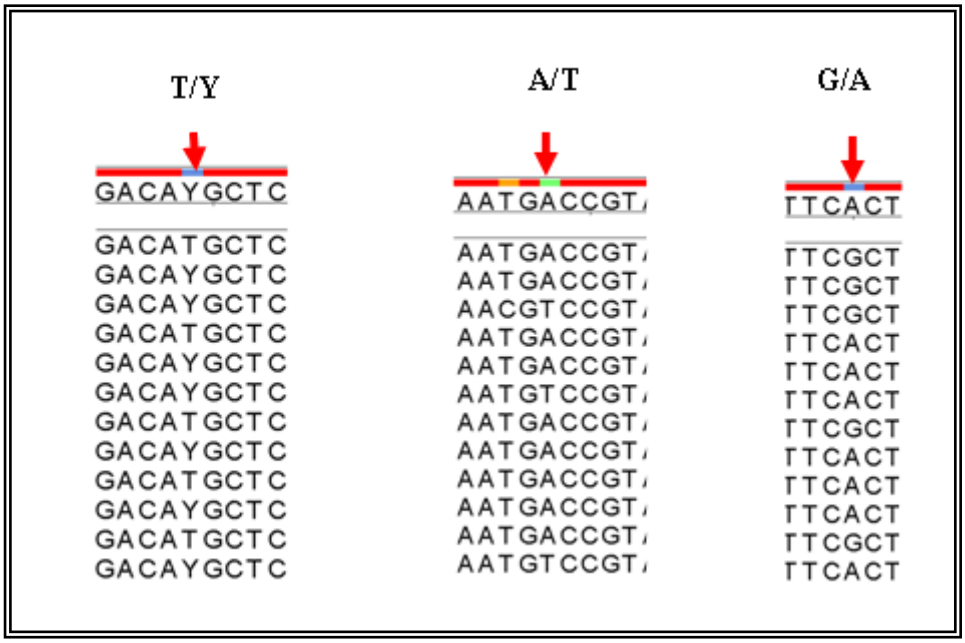


Figure 4.15 ClustalW alignment of *An. funestus*-like ITS2 sequences of rDNA using the DNASTAR MegAlign method. Only portions with a SNP are shown.

ii) *D3 region of rDNA*

Amplification of the D3 region produced two PCR products for *An. vaneedeni* (≈400 and 350bp) and *An. parensis* (≈400 and 330bp) (Figure 4.16A). *Anopheles funestus*-like also gave products of ≈400 and 350bp (Figure 4.16B).

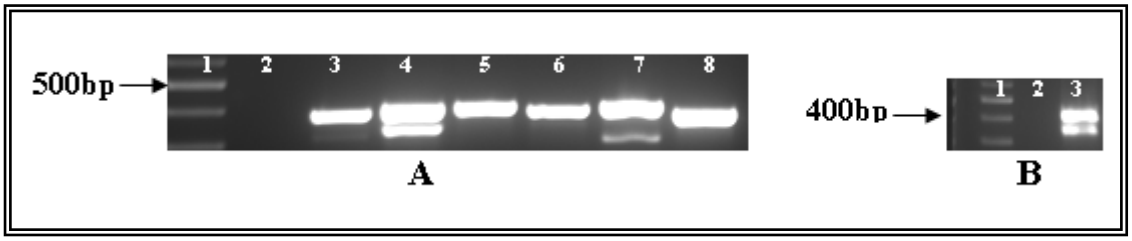


Figure 4.16: 2.5% agarose gel stained with ethidium bromide for visualising *An. funestus* group 28S ribosomal DNA D3 PCR products; Lanes 1 and 2 are for DNA molecular markers and negative controls respectively. **A**: lane 3: *An. funestus* (≈400bp), lane 4: *An. vaneedeni* (≈400bp and 350bp), lane 5: *An. funestus*-like (≈400bp), lane 6: *An. rivulorum* (≈400bp), lane 7: *An. parensis* (≈400bp and 350bp), lane 8: *An. leesoni* (≈400bp). **B:** Lane 3 *An. funestus*-like D3 product (≈400bp and 350bp).

Anopheles funestus-like D3 PCR products with double amplicons were cloned and sequenced. Following blasting of the sequences for these fragments on the NCBI data base, the sequence for the 350bp fragment failed to align with any anopheline sequences. However, its complete length aligned with the 28S rRNA gene of *Leishmania donovani* producing a 100% similarity (E-value = $7e^{171}$). The 400bp fragment sequence aligned with the rRNA 28S gene sequences of various anophelines including *An. funestus*.

4.5.3.2 Restriction fragment length polymorphism of D3 PCR products

A total of 225 specimens were analysed for RFLP genotypes. Data indicated two types of digest pattern for the 30 *An. funestus*-likes samples investigated, called Type1 and Type 2. The Type 1 pattern, which constituted 96.7% (n = 30) of the specimens, gave three products with approximate sizes of 70, 150 and 160bp. Type 2 constituted 3.3% (n = 30) of the specimens and gave a 110bp product in addition to the Type 1 multiple products (Figure 4.17A).

Of the 196 *An. funestus* samples analysed, three Types of digest pattern were obtained (Figure 4.17B). Their proportions were 53.06% (n = 196) Type Y (70bp, 150bp, 150bp and 310bp), 20.41% (n = 196) Type M (70bp and 310bp), and 26.02% (n = 196) Type MW (70bp, 150bp, 160bp and 310bp).

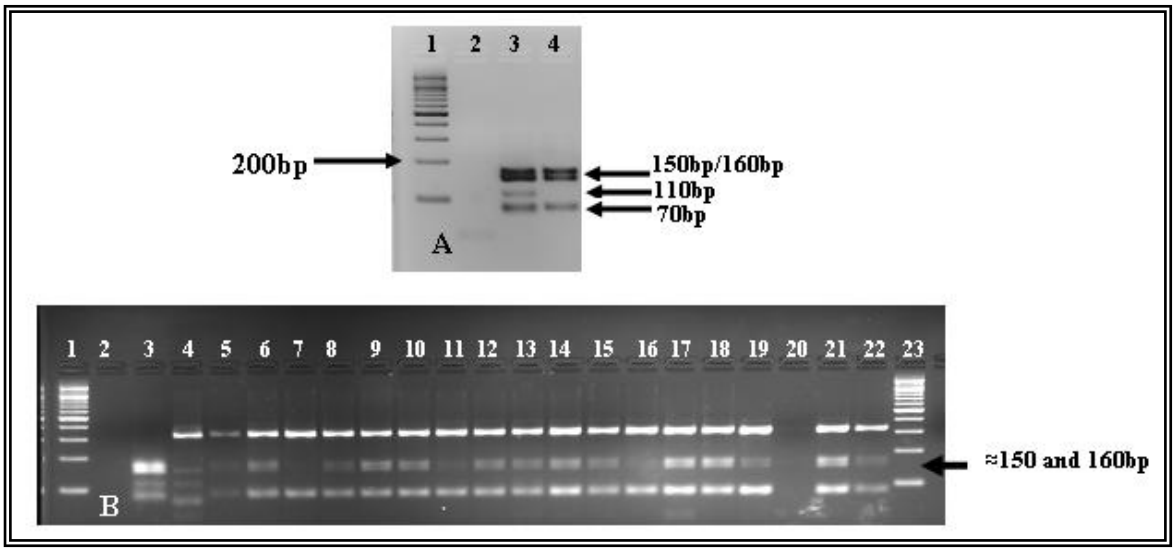


Figure 4.17 A 3% TAB ethidium bromide stained agarose gel showing polymerase chain reaction-restriction fragment length polymorphism patterns for domain 3 (D3) fragments. Lanes 1 and 2 in both gels are for DNA molecular markers and no template negative controls respectively**. A:** *Anopheles funestus*-like *Hpa* II digestion product. Lane 3, Type 2 and lane 4, Type 1. **B:** *Anopheles funestus* s.s *Hpa* II digestion product. Lane 7, Type M; Lane 19, Type Y; and Lane 21, Type MW.

The physical restriction map for the various RFLP-types is showed in Figure 4.18.

Analysis of the D3 sequences showed that *Hpa* II restriction sites (CCGG) ranged from 1 to 4. The first restriction site is 70bp from the 5' end for D3 products generated from all mosquitos except in *An. funestus*-like Type 2 where it is 83bp. The sequences used for the construction of the restriction map excluded the 40bp sequences for D3A (20bp) and D3B (20bp) primers.

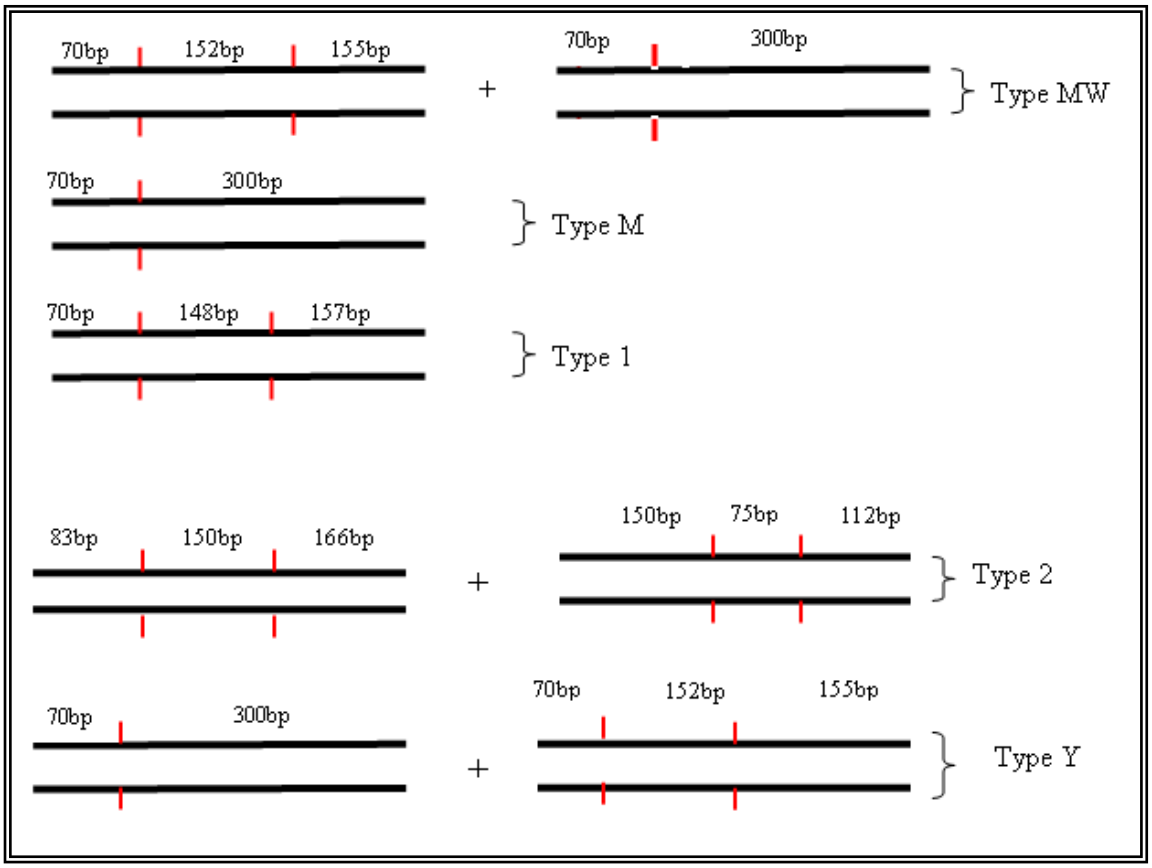


Figure 4.18 Schematic representation of rDNA D3 *Hpa* II restriction mapping for different restriction fragment length polymorphism types. The black bold lines represent DNA strands with approximate sizes in base pair (bp) and the red vertical lines indicate the *Hpa* II enzyme restriction sites. Each restriction site is 4bp and, together with the primer length, was not considered when constructing the restriction map.

4.5.3.3 Phylogenetic relationship of *An. funestus*-like with other members of the *An. funestus* group based on D3 sequences

The phylogenetic tree generated from D3 molecular data clusters the seven species represented, into three groups (Figure 4.19). No difference was observed between *An. parensis*, *An. funestus*-like and *An. funestus* which are all grouped in the same clade.

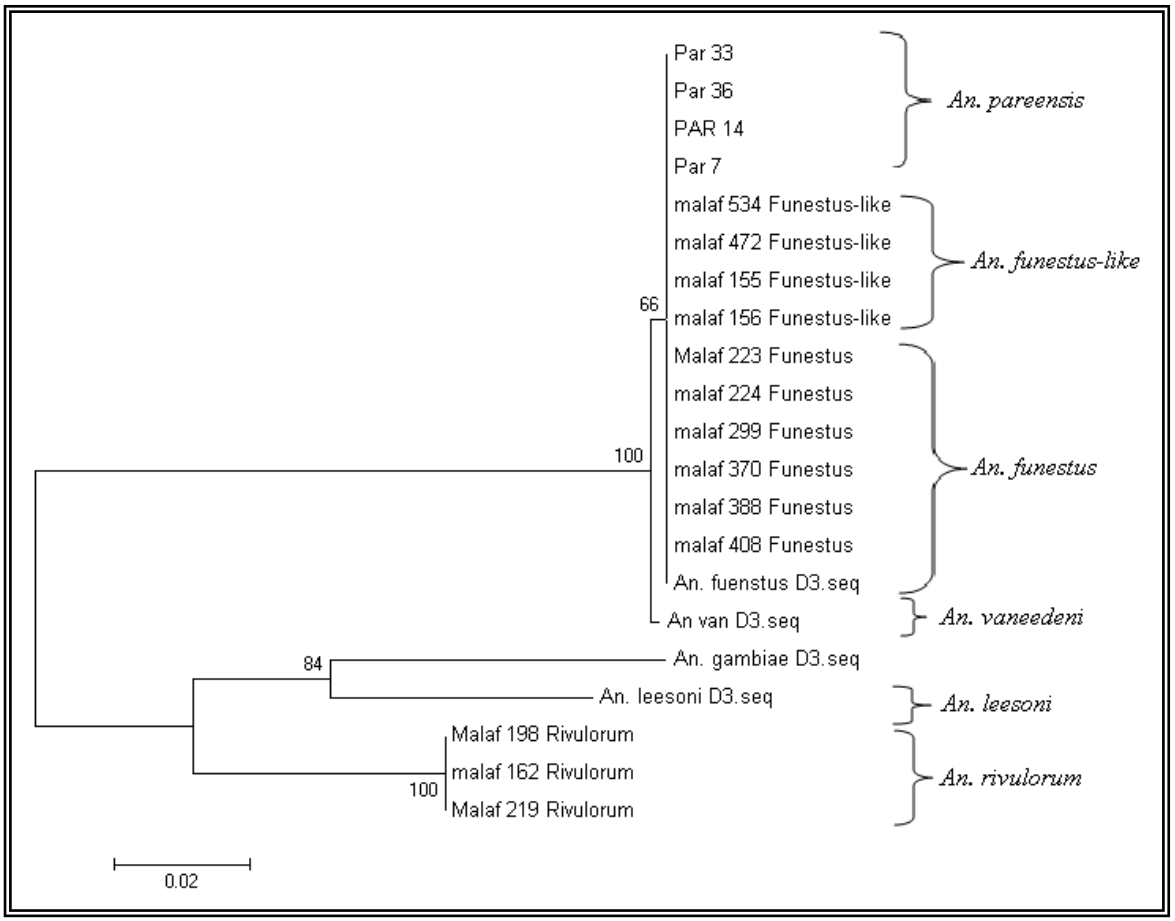


Figure 4.19 Phylogenetic tree of the *An. funestus* group based on nucleotide sequences of domain 3 (D3) of the 28S unit of rDNA (Neighbour joining construction). Par or PAR = *An. parensis*, Funestus-like = *An. funestus*-like, Funestus = *An. funestus*, An. van D.seq = *An. vaneedeni* D3 sequence, Rivulorum = *An. rivulorum*, Malaf/malaf plus numbers are sample codes. *An. gambiae* D3 sequence represent outgroup.

4.6 Discussion

Anopheles funestus group species are morphologically similar. Therefore, unambiguous discrimination between member species should be based on molecular PCR based techniques, enabling differentiation between vector and non-vector species.

4.6.1 Development and optimization of real time PCR assays

Cost effectiveness of assays was the main criterion that was used to decide the sequence in which assays were investigated. Melt curve analysis was first investigated because this

assay employs only standard oligonucleotide primers, and no additional fluorescence labelled probes were required. Furthermore, this assay only requires a single channel real-time PCR machine which is less costly than a multichannel machine. The running cost per sample using this assay was approximately US $0.65. The MCA was initially optimised using the original primers of Koekemoer *et al*. (2002). It became immediately evident that the assay could not accurately discriminate between *An. vaneedeni*, *An. parensis* and *An. funestus*. This was because the amplicons from these species had similar GC content causing a difference of only 0.5ºC in melting temperature between *An. funestus* and *An. vaneedeni* and a 1ºC difference between *An. vaneedeni* and *An. parensis*. Redesigning the species-specific primers for *An. vaneedeni* and *An. funestus* to generate amplicons with different melting temperatures did not sufficiently resolve unambiguous differentiation between these species. This assay could prove useful in Sub-Saharan Africa where *An. vaneedeni* is absent in field collections which results in a clear gap in melting temperature from amplicons generated from *An. funestus* and *An. parensis*. To date, *An. vaneedeni* has only been described from Southern Africa Gillies and Coetzee, (1987). However, if *An. vaneedeni* is unexpectedly present, this assay will lead to misidentification of this species.

Since MCA could not distinctly identify the small differences in melting temperature between the amplicons for the three species, an assay based on HRM was developed. The running cost for this assay is also low (US $0.65) as it only requires standard oligonucleotide primers and a cheap intercalating dye. Unfortunately the cost of acquiring a multichannel real-time machine is expensive. During optimisation, it was noted that there was slight increase in the fluorescence of the dye in the negative controls during late cycles. This was greatly reduced by decreasing the number of PCR cycles but ensuring that

amplification of species diagnostic product reached the full plateau phase. HRM successfully identified all five targeted species with some discrepancies.

To overcome the shortcomings of misidentification associated with both the MCA and HRM assays, an assay based on hydrolysis probe genotyping was developed. The major problems with this assay include the high initial cost of a real-time PCR machine (US$ 35452 and above compared to US$7334 for a standard thermo-cycler at time of writing) and expensive probes. Fortunately, the price of this machine is decreasing.

The hydrolysis probe assay was initially run for 40 cycles which unfortunately resulted in low levels of non-specific amplification during the cycling of LEES (*An. leesoni*) and PAR (*An. parensis*) specific probes. To eliminate this, the number of cycles was dropped to 35. However, the fluorescent signal resulting from the binding of the VAN probe (*An. vaneedeni*) was significantly lower compared to the relative fluorescence from the other probes. Analysing the PCR product on agarose gel electrophoresis confirmed the presence of a single amplicon diagnostic for *An. vaneedeni*. Replacing the fluorescent dye and/or redesigning the probe failed to improve the fluorescent signal generated. However, even with this low fluorescent signal, *An. vaneedeni* specimens were positively identified when the auto-scale function of the Rotor-Gene software was used. In regions where *An. vaneedeni* is absent in its distribution, the species-specific probe can be exempted from the reaction mixture thereby reducing the running cost further. The problem is, in the event that *An. vaneedeni* is present, it will not be identified and therefore a second assay will be needed to screen for this species from the unidentified samples.

The overlap observed in curves from *An. funestus*-like, *An. funestus* and *An. vaneedeni* during optimisation of the MCA and HRM assays could be a consequence of PCR products of similar size and GC content. *Anopheles funestus*-like was therefore excluded from both assays. During the design of a hydrolysis probe assay to also indentify *An. funestus*-like, it was observed that the ITS2 sequence for this species is highly similar to that of *An. funestus* leaving no option for designing an *An. funestus*-like specific probe. *Anopheles funestus*-like was excluded from this assay design as well.

4.6.1.1 Analytical sensitivity of assays

The hydrolysis probe assay developed in this study was more sensitive compared to the conventional AS-PCR. This is similar to the results obtained by other researchers. Bass *et al*. (2008) compared the sensitivity of a single PCR assay and a TaqMan (hydrolysis probe) assay for detecting diluted DNA from four *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*). They found that the hydrolysis probe assay had a detection limit of 0.0002ng DNA for all four *Plasmodium* species compared to a 2ng - 0.004ng range for the single PCR method. The high sensitivity associated with the hydrolysis probe assay can be attributed to the species-specific probes and primers. As the hydrolysis probe assay is expensive to run, the possibility of halving the reagent volumes was investigated. It was found that the sensitivity remained comparable. Due to the high sensitivity of this method it can be used in situations where DNA has degraded as a consequence of poor storage.

4.6.1.2 Blind trial

The performances of the three real-time PCR assays developed in this study together with the AS-PCR of Koekemoer *et al*. (2002) were assessed. The hydrolysis probe assay was the only assay amongst the real-time assays developed with a performance comparable to

the gold standard AS-PCR which had a low failure rate compared to MCA and HRM. This could be attributed to the double specificity of the assay as a consequence of the species-specific probes and primers used. MCA had the highest number of misidentified samples with misidentifications being recorded in *An. funestus*, *An. vaneedeni* and *An. parensis* specimens. The PCR products generated from these species during MCA were of different sizes but had similar melting temperatures. It is known that the melting temperature of an amplicon depends on its GC content, sequence length as well as compositional variation in the nucleotide bases. The GC content and the nucleotide composition for these species are likely to be similar. This molecular observation highlights the close morphological similarity between these species. It is recommended that DNA concentrations between samples for MCA are standardised as variation in the starting templates affects melting temperature. Standardization would require DNA quantification by gel electrophoresis or spectrophotometer. Although this is an additional step and adds to the running cost of the assay, it is likely to reduce misidentification. As with MCA, HRM misidentified a number samples. This included misidentification of two water negative controls as *An. funestus* and *An. rivulorum*, resulting in a potential background problem for the assay.

4.6.1.3 Validation of the hydrolysis probe assay

The high performance of the hydrolysis probe assay in this test confirmed its robustness and sensitivity. It however failed to identify some samples. This could be due to degradation of the template DNA.

4.6.2 AS-PCR development and validation

Partial IGS sequence was obtained from the 3’ direction of the rDNA IGS region for four species of the *An. funestus* group from which AS-PCR has been developed. The protocol

allows for the identification of *An. funestus*, *An. rivulorum*, *An. vaneedeni* and *An. parensis*. Sequence data for *An. funestus*-like obtained during sequencing was approximately 200bp and was part of the 18S gene and not the targeted IGS region. There was not enough time to sequence the IGS region of *An. leesoni*. Difficulties in obtaining IGS sequence for *An. funestus*-like could be due to the presence of secondary structures in the rDNA. As a consequence, both species could not be included in the multiplex PCR. Although this study recommend the inclusion of *An. funestus*-like and *An. leesoni*, the current protocol can be useful for species identification in areas where both species are limited in their distribution. The 550bp product in the multiplex PCR for *An. parensis* is a non-specific product generated by the VARIGS primer specific for *An. vaneedeni*. The rDNA IGS region was not successfully sequenced from the 5' direction for the species investigated. This is most likely due to the presence of secondary structures inhibiting sequencing reaction.

The performance of the AS-PCR assay in identifying previously identified samples was encouraging and indicates its potential for use in *An. funestus* group species identification. Samples not identified by this assay could be as a result of degradation of the DNA.

4.6.3 RFLP genotyping of *An. funestus* and *An. funestus*-like populations

The use of PCR-RFLP for genotyping populations of anophelines is not new. This technique was first developed by van Bortel *et al*. (2000) for genotyping Asian anophelines of the *An. minimus* group and has subsequently been used by other researchers. In this study, amplification of domain 3 (D3) of the 28S rDNA region gave two PCR products. This is similar to results obtained by Garros *et al*. (2004) and Koekemoer *et al*. (1998). The generated D3 products in this study digested with *Hpa* II endonuclease and resulted in the

differentiation of *An. funestus* populations into three RFLP types (Y, M, MW). Similarly, two RFLP Types (1 and 2) have been identified in *An. funestus*-like from Malawi. The RFLP types M and MW found in this study correlate with previous reports of Type M in Kenya and Madagascar as well as Type MW in Mozambique (Garros *et al*. 2004). Koekemoer *et al*. (2006) also reported the presence of Type M in Kenya, Malawi and Tanzania as well as Type MW in Mozambique, South Africa, Tanzania, Uganda and Zambia. The RFLP type W of Garros *et al*. (2004) and Koekemoer *et al*. (2006) is similar to *An. funestus*-like RFLP Type 2. The unique occurrence of RFLP type 2 in the *An. funestus*-like Malawi population probably confirms the high degree of variation in the D3 region of 28S rDNA. The 350bp D3 products for *An. funestus*-like are most likely the products of non-specific amplification.

The RFLP patterns observed in this study can be used to differentiate *An. funestus* from *An. funestus*-like. However, RFLP may not be useful for the identification of *An. parensis*, *An. vaneedeni* and *An. funestus*-like. In this study, the D3 products for *An. vaneedeni* and *An. parensis* were not digested. However, after digesting similar products from both species with MSp I, an isoschizomer to *Hpa* II, similar patterns were obtained to that of Type 2 *An. funestus*-like (Garros *et al*. 2004). Employing this technique in regions where all the species occur will probably lead to misidentification.

The restriction map obtained after analysing the D3 sequence of *An. funestus* with PCR-RFLP Type Y pattern for the *Hpa* II restriction site was similar to that of Type M and not Type MW as expected based on the RFLP pattern observed by agarsoe gel electrophoresis. The likely explanation is that Type Y is a combination of the types M and MW with more repeats of Type M than Type MW. Because the PCR products of Type M outnumber those

of the Type MW, they are inadvertently preferentially sequenced instead of Type MW and therefore produce a Type Y restriction map. However, on analysing the digestion products, considering that more products are analysed on gel than used for sequencing, the resulting restriction map is that of the PCR-RFLP Type MW with faint 152 and 155bp products. Further research is recommended to establish the ratio of these two types (M and MW) in the RFLP Type Y.

4.6.4 Phylogenetic analysis

The phylogenetic tree constructed using ITS2 data clearly discriminated *An. funestus*-like from the other species of the *An. funestus* group. This was expected as the ITS region is more variable than the D3 region. It is not surprising that *An. funestus*-like is most closely related to *An. funestus* as the field collections of *An. funestus*-like were misidentified as *An. funestus*. An attempt to root the ITS2 and D3 phylogenetic trees with the respective sequences for *An. gambiae* failed to produce similar results to those obtained by Garros *et al.* (2004). Although the exact reason is unknown, it is likely that the exclusion of a 15bp fragment at the 5' and 3' ends of the ITS2 and D3 sequences for *An. gambiae* together with those of the other species are responsible for the difference observed. This exclusion was done to ensure that all sequences analysed were of the same length.

4.7 Conclusions

Of the three assays tested against the gold standard AS-PCR of (Koekemoer *et al*., 2002), the hydrolysis probe assay was the most efficient for identification of members of the *An. funestus* species group. Its performance was comparable to that of the AS-PCR and, like the other real time assays, was more sensitive than the AS-PCR. Although this technique

may not currently be affordable for resource-poor laboratories, the initial costs of the required equipment are decreasing.

IGS sequence data for *An. funestus*-like and *An. leesoni* needs to be obtained so that probes for these species can be included in the newly developed AS-PCR for identifying members in the *An. funestus* group. The IGS region is much larger (± 4Kb) than the ITS2 (± 800bp) (Collins *et al*. 1990) and will most likely provide the size variation needed to include *An. funestus*-like in one multiplex assay.

The RFLP results obtained in this study show that this technique can be used to discriminate *An. funestus*-like from *An. funestus*. Both species showed distinct digestion patterns on an agarose gel. ITS2 sequence analyses together with RFLP results for *An. funestus*-like suggest intraspecific variation within this population.

Phylogenetic analysis of the ITS2 region sequences for different species in the *An. funestus* group showed that *An. funestus*-like is closely related to *An. funestus* and should clearly be listed as a member of the *An. funestus* group. Phylogenetically, there is no difference between the different *An. funestus* RFLP types as they were all in the same clade. Of the two data sets investigated, the ITS2 region indicated a distinct clade which links *An. funestus*-like with the other *An. funestus* group species and can also be used to distinguish between *An. funestus*-like and *An. funestus* s.s.

CHAPTER FIVE

General Discussion and Conclusion

5.1 Discussion

In Africa, malaria remains the number one killer disease (WHO, 2010) and the principal vectors are mosquitoes belonging to the *Anopheles gambiae* complex and *An. funestus* group (Gillies and De Meillon, 1968). The malaria vector and non-vector members of the *An. funestus* group are highly similar morphologically (Gillies and De Meillon, 1968; Gillies and Coetzee 1987) which is problematic because efficient identification of anopheline species is of fundamental importance to sustainable malaria vector control. In Malawi, malaria vector control has principally been based on the use of bed nets (Presidents' Malaria Initiative Operational Plan-Malawi 2011). Prior to this study, the Department of Health in Karonga, northern Malawi, was preparing for malaria vector control using indoor residual spraying of insecticides. Success of these interventions relies heavily on baseline information which encompasses mosquito species composition, malaria vector incrimination, behaviour and susceptibility to insecticides of target populations. Spillings *et al*. (2009) describe a new member of the *An. funestus* group provisionally designated *An. funestus*-like from Karonga, expanding our knowledge of the *An. funestus* group. The aim of this study was to systematically describe *An. funestus*-like from the same location. To achieve this aim, specimens were collected from the field and brought to the laboratory for molecular analysis. Museum specimens were used for morphological studies.

5.1.1 Vector composition, distribution and insecticide susceptibility status

Mosquitoes were collected from the field, morphologically sorted and molecularly identified to species where necessary. The anopheline species composition was analysed per collection site. F_1 progeny successfully reared from field-caught adults were exposed to a range of insecticides to determine their susceptibility status.

This study showed that four species of the *An. funestus* group (*An. funestus*, *An. funestus*-like, *An. rivulorum* and *An. parensis*) occur in Karonga. These findings correlate with previous studies. Spillings *et al*. (2009) recorded *An. rivulorum* and *An. funestus*-like in the same area and the occurrence of *An. funestus s.s* in Malawi has previously been recorded (Weeto *et al*., 2004; Chiphwanya, 2004). The collections described here include the first record of *An. parensis* in Karonga. The distribution of these species varied between seasons. *Anopheles funestus s.s* predominated over other species during the wet season while *An. rivulorum* outnumbered the other species during the dry season. *Anopheles funestus*-like were found to be more abundant in the dry season collections compared to the wet season.

The sympatric occurrence of these morphologically similar species in Karonga and the differences in their seasonal occurrence has implications for malaria vector control there. Efficient and unambiguous identification tools are therefore needed to discriminate the vector from the non-vector species before the implementation of a vector control program. Accurate species identification under these circumstances allows for an appraisal of insecticide susceptibility status by species, which in turn allows for the design of a suitable insecticide based vector control intervention. This will avoid the waste of limited resources by targeting non malaria transmitting mosquitoes. Data presented in this study suggest that

vector control at Karonga should be intensified at the beginning of the wet season when *An. funestus* are found in high numbers. Based on the insecticide susceptibility studies conducted there to date, and from previous records from southern Africa (Hunt *et al*., 2010; Abilio *et al*., 2010), pyrethroid and carbamate resistance seems to be spreading across the region. There is need for detailed investigation on the underlying resistance mechanisms in the populations from each country. This information will assist in coming up with appropriate resistance management strategies. This study also recommend, the use of DDT since results from this region have shown that *An. funestus* is still susceptible to this insecticide.

5.1.2 Feeding and resting behaviours

Mosquito blood meal identification indicates the association between species and host (blood source). To understand the ecology of *An. funestus*-like, identification of blood sources was determined from field collected specimens using ELISA (Beier *et al.* 1988). The percentages of mosquitoes with human or animal blood were determined. *Anopheles funestus*-like were positive for animal blood only, indicating no tendency to feed on humans.

An indication of the resting behaviour of *An. funestus*-like was obtained by comparing the relative occurrence of this species in the indoor and outdoor collections. *Anopheles funestus*-like tend to rest inside human dwellings after taking animal blood meal. Should females of this species be incriminated as vectors of malaria, their resting behaviour will render them vulnerable to indoor residual spraying.

5.1.3 *Plasmodium* species infection

Field collected female mosquitoes were analysed for *Plasmodium* infection by means of ELISA (Wirtz *et al.,* 1992). Two species of the *An. funestus* group were infected with *Plasmodium*. *Anopheles funestus s.s* from Likoma Island was infected with *P. falciparum* at a rate of 5.9% In Karonga, *P. vivax* infection was only detected in *An. funestus*-like. These infections were not confirmed using the more sensitive PCR technique (Tassanakajon *et al*., 1993) so it is highly likely that these data are false. False positive *Plasmodium* species infection has previously been reported in zoophilic mosquito species across Africa (Lochouarn and Fontenille, 1999; Sylla *et al*., 2000; Koekemoer *et al.,* 2001; Bigoga *et al*., 2007; Mouatcho *et al.,* 2007*)* which can have serious implications for vector control. False sporozoite positives can cause an overestimation of the entomological inoculation rate and incorrect vector incrimination, leading to a poorly informed vector control strategy (Durnez *et al*., 2011).

5.1.4 Morphological description of *An. funestus*-like

Morphological identification is the first step in mosquito identification. Since *An. funestus*-like was discovered resting indoors with *An. funestus*, it was suspected of being a vector. There was therefore a need to morphologically describe *An. funestus*-like. Members of the *An. funestus* group are morphologically similar to each other. In order to assess morphological variation between *An. funestus*-like and its closest relative *An. funestus*, the progeny of wild-caught females were reared under standard conditions for subsequent morphometric measurements. In particular, wing length, wing morphology, wing spot ratio, palp band ratio and leg morphology were investigated. From these measurements, a plot of palp band ratio against wing spot ratio can tentatively be used to separate these species. However, these ratio's do not unambiguously differentiate *An. funestus*-like from

An. funestus although they can be used to completely differentiate *An. funestus* from *An. vaneedeni* (*An. aruni* of De Meillon *et al.*, 1977). The extent of morphological similarity between *An. funestus*-like from *An. funestus* is therefore highlighted by the fact that none of the characters investigated produced a clear separation between the two. Molecular identification has frequently been used to supplement morphological methods to discriminate members of the *An. funestus* group. This study therefore investigated different molecular assays that can be used for discriminating members of the *An. funestus* group.

5.1.5 Novel DNA based assays for *Anopheles funestus* group identification

Member species of the *An. funestus* group are routinely identified using dichotomous morphological identification keys and the AS-PCR method. Morphological identification to species level within this group requires a high level of expertise which is not readily available in most countries affected by malaria, added to this the mosquitoes species are morphologically very similar. There is therefore a need to supplement morphological identification of mosquito species with molecular methods. Currently, molecular methods available could not distinguish all members of the group in a single assay (Spillings *et al*., 2009). It was therefore decided to investigate several molecular methods in the hope of designing a single assay for the routine identification of all six common *An. funestus* group members (*An. funestus*, *An. rivulorum*, *An. parensis*, *An. vaneedeni*, *An. leesoni* and *An. funestus*-like).

Three multiplex real-time PCR assays (MCA, HRM, and hydrolysis probe assays) based on ITS2 sequences of rDNA that can be used to identify members in the *An. funestus* group were designed and evaluated. For MCA and HRM, species-specific diagnostic PCR products were melted in the presence of a dye. These resulted in species diagnostic melt

curves. For the hydrolysis probe assay, species-specific probes were designed between a universal forward primer and species-specific reverse primers. Hydrolysis of the probes during PCR result in an increase in fluorescent unit of the probe. Of these real-time assays, the hydrolysis probe assay showed a specificity comparable to that of AS-PCR and increased sensitivity to low DNA template concentrations. This assay can therefore be used to identify samples with low DNA concentrations thereby alleviating the extra cost incurred when repeating samples as often happens with the AS-PCR method. Although a separate assay is still required to differentiate *An. funestus*-like, the hydrolysis probe assay is easy to perform, has a high throughput, is closed tube and requires no post PCR processing which not only adds to cost but also requires the use of toxic ethidium bromide.

Also investigated was the design of a conventional PCR based on the IGS region of rDNA as this region is larger and more variable. This region was partially sequenced for four species (*An. funestus*, *An. rivulorum*, *An. parensis*, and *An. vaneedeni*). Diagnostic species-specific primers (forward primers) and a universal reverse primer were designed from the partial IGS sequences to produce a new AS-PCR assay. This new assay successfully discriminates the four species. Unfortunately, PCR amplification and sequencing of the IGS region for *An. funestus*-like and *An. leesoni* was not successful in this study due to time constraints. However, it is recommended that these species are included in the IGS AS-PCR for the identification of all six *An. funestus* group members in a single assay.

5.2 Recommendations for further research

In the course of interpreting the data collected in this study, a number of questions were raised. These were not answered here because they are not directly linked to the original

objectives of this study. Further investigations are recommended to fully characterize *An. funestus*-like.

During this study only a few specimens of *An. funestus*-like were analyzed for blood meal sources which were found to be of cow and/or goat. The majority of these specimens were collected indoors in human dwellings. It will therefore be interesting to know if the tendency toward complete zoophily will be supported by analysis of a larger sample. To achieve this, a large sample of this species needs to be collected indoors and outdoors using different collection methods across seasons. Future collected specimens should be analyzed for host blood source using a more sensitive method such as PCR (Kent and Norris, 2006).

This study showed that *An. funestus* was predominant during the wet season and *An. rivulorum* during the dry season. Although *P falciparum* infected *An. funestus* were detected only in samples from Likoma Island, malaria transmission is likely ongoing in Karonga, as suggested by Chiphwanya (2004) who reported *P. falciparum* infection in *An. funestus* with infection rates of 2.3% and 8.5% in Malawi. Follow-up investigations at Karonga should help resolve the pattern of malaria transmission there.

Resistance to pyrethroid and bendiocarb insecticides was detected in *An. funestus* from Wowve. Insecticide susceptibility data should also be collected from the other collection sites.

5.3 Conclusion

Spillings *et al*. (2009) identified *An. funestus*-like resting indoors together with *An. rivulorum* in Karonga, Malawi, where little information is available concerning mosquito species composition and the pattern of malaria transmission. Data presented here show that four member species of the *An. funestus* group, including *An. funestus*-like, co-exist in Karonga. *Anopheles funestus*-like is zoophilic, endophilic and its infectivity with *P. vivax* requires confirmation. Species identification protocols for real-time and AS-PCR assays have been developed. Of these, the hydrolysis probe assay shares a similar specificity to that of the currently used AS-PCR assay but with a greater sensitivity. This study has advanced existing knowledge for *An. funestus*-like in the field of vector control and has equally provided an alternative and sensitive molecular identification method for discriminating members in the *An. funestus* group.

APPENDIX I

PREPARATION OF CHENICALS AND SOLUTIONS

A) **DNA extraction solution** (Collins *et al*., 1987)

- **8M KAc**
- **Grinding buffer**

  0.08M NaCl

  0.16M Sucrose

  0.06M EDTA

  0.5% SDS

  0.1M Tris-HCl
- **TA (Tris EDTA) Buffer** (Sambrook *et al.*,1989)

  100ml 1M Tris (pH)

  20ml 0.5M EDTA

  Make up volume to 1L.

B) Preparation of PCR solutions Sambrook *et al.* (1989)

- **TAE (Tris Acetic EDTA) Buffer 50X (pH 8)**

  242g Tris base

  37.2 g Na_2 EDTA.$2H_2O$

  57.1M glycial acetic acid

  Make up to 1 L
- **TBE (Tris-Borate EDTA) Buffer 5X (pH 8)**

  54g Tris base

  27.5g boric acid

3.722 g Na_2 EDTA.2H_2O

Make up to 1 L

- **Agarose gel**

  For a 2.5% agarose gel, dissolve 10g agarose in 400ml 1X TAE

  For a 1% agarose gel, dissolve 1g agarose in 100ml 1 X TAE

  The mixture was heated in a microwave oven until all the agarose had melted and the solution had started to boil. The gel solution was allowed to cool to approximately 55ºC. Four µl of a 0.5mg/ml ethidium bromide was added and gently mixed into the gel by swirling. The gel was poured slowly into a gel rack containing combs. Any air bubbles were removed immediately and the gel allowed to solidify for 30 minutes. After the gel had completely solidified, the combs were removed.

- **Ethidium bromide (EtBr)**

  Dissolve 10mg EtBr crystals in 1ml distilled H_2O

- **Ficoll dye**

  50% sucrose

  1ml 0.05M EDTA (pH7.0)

  0.1% Bromophenol blue

  10% ficoll

C) Preparation of sporozoite ELISA solutions (Wirtz *et al*., 1987)

Phosphate Buffer Saline (PBS) 10X

Blocking Buffer (BB) 1L

2.5g Casein

50ml 0.1N NaOH

450ml 10X PBS

0.1ml Phenol red

Suspend casein in NaOH and boil. After casein has dissolved slowly add the PBS and cool. Adjust the pH to 7-7.4 using HCl. Add phenol Red and store for one week in fridge.

Grinding Buffer (BB-NP40)

50ml BB plus 250 µl NP-40. Five ml is enough for 100 samples. Store for one week in fridge

Washing Buffer

Add 500µl Tween-20 to 1L 1X PBS. Mix well and store at 4ºC for one week

D) Preparation of blood meal ELISA solutions (Beir *et al*., 1988)

Phosphate buffered saline, p H 7.4. Store solution at 4 ºC

Boiled Casein, 0.5% (BC)

2.5g Casein

50ml 0.1 N NaOH

450ml PBS

0.05g Thimerosol

0.1ml Phenol red

Suspend the casein in the NaOH and bring it to boil. After the casein is dissolved, slowly add PBS and allow the mixture to cool. Adjust the p H to 7.4 with HCl. Added thimerosol and phenol red then mix well and store on shelf for a week.

Wash Solution (PBS containing 0.5% Tween 20)

For a 500ml washing buffer, add 2.5ml Tween-20 to 497.5ml PBS. Mix well and use the same day.

Enzyme Diluent (BC-Tween 20) 100ml

Add 0.025ml Tween 20 to 99.975ml 0.5%BC

E) Preparation of cloning solutions

- **IPTG stock solution 0.1M**:

  1.2g IPTG was weighted and water added to a final volume of 50ml. The solution was mixed and filtered through a filter paper and stored at 4ºC.

- **X-gal** 50mg/ml (Promega Madison WI USA)

- **LB medium:**

  10g Bacto® trypto, 5g Bacto® yeast extract and 5g NaCl was weighed out and water added to a final volume of one liter. The solution was properly mixed and pH adjusted to 7 with NaOH.

APPENDIX II

STANDARD LABORATORY METHODS

A) DNA extraction protocols

- Lavik *et al*., (1984)

The whole mosquito was homogenized in 0.1ml of 0.5% SDS/ 0.08M NaCl / 0.16M sucrose/ 0.06M EDTA/ 0.I2 M Tris-HCl, pH 9, and incubated at 65ºC for 30 minutes. To the homogenate 14ul 8M stock of potassium acetate was added and incubated on ice for 30 minutes. Debris and precipitated SDS and protein were removed by 10-min centrifugation in a microcentrifuge. Nucleic acid from the supernatant was collected by adding 0.2ml of ethanol, incubated for 2 minutes at room temperature and centrifuged for 5 minutes. The pellets were washed twice with cold 70% ethanol, dried and dissolved in 40ul distilled water.

- Collins *et al* (1987)

The abdomen of a single mosquito was placed in a separate 1.5 ml microcentrifuge tubeand homogenized in 100µl of grinding buffer (0.08M NaCl, 0.16M sucrose, 0.06M EDTA, 0.5 % SDS, 0.1M Tris-Cl, pH 8.6). The homogenate was incubated at 70°C for 30 minutes. Potassium acetate (0.98 M) was added to the heated homogenate, mixed and incubated on ice for 30 minutes. After incubation, room temperature centrifugation was carried out for 15 minutes at 16060 x g. The supernatant was removed and pipetted in to a clean microcentrifuge tube and the pellets discarded.

Two volumes of 99.9% Ethanol compared to the volume of homogenate was added and the solution gently mixed gently by inversion. In order to facilitate DNA precipitation, the tube and contents were incubated overnight at -20°C prior centrifugation at 16060 x g for 35 minutes. The supernatant was removed and the DNA pellet was washed with one volume (100µl) 70% ethanol prior to centrifugation for 10 minutes at 16060 x g. The pellets were then air dried after removing the ethanol. The DNA pellets were resuspended in 100µl of 1 X TE buffer (0.1M Tris and 0.01M EDTA) and stored at -20°C.

- DNA Extraction using *prep*GEM™ Insect kit

A sterile forcep and scalpel was used to dissect insect body part (leg or wing). The body part was placed inside a thin-welled PCR tube for extraction. Into the tube was added 35µl of PCR grade water, 4µl 0f 10X buffer BLACK and 1µl *prep*GEM™. The sample was crushed with a sterile pestle and incubated in a thermal cycler at 75ºC for 15 minutes and 95ºC for 5 minutes. The tube was briefly centrifuged at 16060x g and the supernatant used directly for PCR.

B) *Anopheles funestus* group PCR protocol (Koekemoer *et al*., 2002)

A 13.5µl PCR reaction mixture 0.2ml microcentrifuge tube consisted of the following: 1 X PCR reaction buffer (100mM Tris-HCl pH 8.3, 500mM KCl), 0.23mM dNTPs, 1.38mM $MgCl_2$, 0.24µM FUN, LEES, RIV, VAN, PAR and UV primers, 0.02U of *Thermus aquaticus* (*Taq*) DNA polymerase enzyme (5U/µl) and 1µl DNA template. The buffer, dNTP mix, $MgCl_2$ and *Taq* supplied by Takara biomedicals group Shiga Japan Cat. No. R001AM. The microcentrifuge tube was briefly vortexed for 2 seconds and the tube was centrifuged for 10 seconds at 13,000 rpm. PCR was performed using a thermal cycler (Primus 96, MWG Biotech) using the following cycling conditions: 94°C for 2 minutes

initial denaturation, 35 cycles of 94°C for 30 seconds denaturation for melting of double-stranded DNA, 50°C for 30 seconds annealing of specific primers, 72°C for 40 seconds extension (polymerization) and a final auto extension at 72°C for 10 minutes. Ten microliters of amplicons were mixed with 3µ ficoll loading dye and laoded into the wells of a 2.5% ethidium bromide (10mg/ml) (Cat. No. 15585-011, GibcoBRL, UK) stained agarose gel submerged in a 1X TAE buffer. The PCR products were subjected to electrophoresis at 110V, 400m A for 1 hour 20 minutes or until proper separation of the smaller fragments of the molecular weight marker had been achieved. Each gel contained five positive controls drawn from known laboratory strain of *Anopheles funestus*, and positively identified field specimens of *An. leesoni*, *An. rivulorum*, *An. vaneedeni*, and *An. pareensis*. The negative control consisted of the PCR mix without DNA template. The amplicons were visualized under ultra violet light of a Syngene G-box sydr4/115, gel documentation system and image captured. Mosquitoes were identified by comparing the sizes of their amplicons, to 4µl of a 1kilo base (kb) standard molecular weight GeneRuler^{TM} DNA ladder (Fermentas, Canada Cat. No. SM0331) loaded on the first lane of the gel. Positive controls were loaded after the marker, followed by the negative control and then the samples.

C) *Anopheles funestus* species protocol (Spilling *et al*., 2009)

The PCR mixture was prepared the same as in Koekemoer *et al*. (2002) except that only two primers (UV and a Malaf B) were used. The PCR mixture was carried out in the Primus 96 thermal cycle under the following cycling conditions: 94°C for 2 minutes initial denaturation, 35 cycles of 94°C for 30 seconds denaturation for melting of double-stranded DNA, 45°C for 30 seconds annealing of specific primers, 72°C for 30 seconds extension and a final auto extension at 72°C for 10 minutes. The positive control consisted of a

positively identified field *An. funestus*-like specimen and the negative control was made up of only the PCR mix without template. PCR products were analysed as described in section (Appendix IIB). Positive samples gave bands of approximately 440bp.

D) *Plasmodium* sporozoite ELISA Test Protocol Wirtz *et al*. (1992)

Mosquito preparation

The head and thorax of individual *Anopheles* female mosquito was separated from the abdomen with the aid of a blade and forceps and placed in a sterile 1.5 ml microcentifuge tube. The blade and forceps were rinsed twice in methylated spirit solution and wiped dry. The sample was homogenised in 50µl grinding buffer with a sterile pestle and the pestle washed with 150µl blocking buffer to give a total volume of 200µl. A positive control, consisted of a synthetic peptide standardised against *Plasmodium* species and was prepared fresh on the day of the ELISA. Negative controls, consisted of seven unfed female *An. funestus s.s.* (FANG) prepared as described above and samples were stored in -70 ºC until use (less than two months).

Each well of a 96 well microplate was coated with a solution of 0.200µg/50 µl monoclonal antibody, and the plate was wrapped with aluminium foil before incubating overnight at 4 ºC. The microtitreplate was aspirated and filled with blocking buffer followed by one hour incubation at room temperature. During this incubation, a positive control was prepared by mixing 100pg of with 50µl Blocking buffer.

The wells were aspirated and 50µl of each mosquito homogenate loaded per well. Well A1 of the plate, was designated for the positive control and the last seven wells (H6 to H12) for negative controls. This was followed by 2 hour incubation and washing of wells twice

with PBS-Tween 20. Fifty µl of a 9mg/µl Peroxidase labelled monoclonal antibody (Cat. No. 37.00-24-4, Kirkegaard and Perry Laboratories, Maryland, USA) was added into each well and the plate incubated for one hour at room temperature. The wells were washed 3-4 times with PBS-Tween 20 and 100µl of freshly prepared ABTS peroxidase substrate (2,2'-azino-di-3 ethyl-benzthiazoline) (Cat. No.50-60-18, Gaithersburg, Maryland USA) was added to each well. The plate was incubated in the dark at room temperature for 30-60 minutes to allow the peroxidase reaction to occur. Optical density was measured using a plate reader at a wavelength of 405 nm.

Positive controls used

Plasmodium falciparum: Pf 2+ (Cat. No. Pf-PC Washington DC, USA)

Plasmodium vivax: Pv210-PC (Cat. No. Pv-PC Washington DC, USA)

Monoclonal antibody used

Plasmodium falciparum: Pf 2A10-CDC O1 (Cat. No. 37-00-24-2, Kirkegaard and Perry Laboratories, Maryland, USA)

Plasmodium vivax: Pv 2A10-CDC O1 (Cat. No. 37-00-24-2, Kirkegaard and Perry Laboratories, Maryland, USA)

E) Mosquito blood meal identification using direct ELISA (Beir *et al*., 1988)

Sample preparation

The abdomen of individual mosquito was separated from the head and thorax with the aid of a sterile blade and forceps and placed into a 1.5ml microcentrifuge eppendorf. The blade and forceps were rinsed twice in methylated spirit solution and wiped dry. The specimen

was ground in 50µl 0.01M PBS. This was followed by a 1:50 dilution of the mosquito triturate using PBS.

Blood meal ELISA procedure

Each well of a 96 well microplate was coated with 50µl of diluted mosquito triturate and the plate was wrapped with aluminium foil before incubating overnight at room temperature for 3hours. The microtiter plate wells were aspirated and washed twice with PBS Tween-20. Host specific conjugate (antihost IgG conjugate in either peroxidase for human or phosphatase for bovine) was diluted in 0.5% boiled casein containing 0.025% Tween-20. The dilutions were 1:2000 for antihost IgG conjugated with peroxidase and 1:250 dilutions for antihost IgG conjugated with phosphatase. On to each well was added 50µl host specific conjugate and plate wrapped with aluminium foil before incubated for one hour at room temperature. The wells of the microtiter plate were aspirated and washed thrice with PBS-Tween-20. One hundred µl ABTS peroxidase substrate (22-azino-di 3-ethylbenzthiazoline sulfonate) and phosphatase substrate (p-Nitrophenyl phosphatase) (Cat. No.50-80-00, Gaithersburg, Maryland USA) was respectively added to plate containing antihost IgG conjugated and antihost IgG conjugated to phosphatase. Optical density was measured using a plate reader at wavelength of 405 nm after 30 minutes.

F) QIAquick Gel Extraction kit Protocol

DNA fragment of interest was excised from the gel matrix using a clean sharp scalpel and transferred into a 1.5ml eppendorf tube. The size of the gel slice was determined by weight and three volumes of buffer QG was added to one volume of gel (100mg ≈ 100µl). The tube was incubated at 50ºC until the gel slice had dissolved completely. To help dissolve gel, the tube was mixed by vortexing every 2-3 minutes during the incubation. After the

gel slice had dissolved and the mixture was yellow in colour, one volume of isopropanol was added and mixed. A QIAquick spin column was placed in a 2ml collection tube labelled with the name of the sample. To bind DNA, the sample was applied to the QIAquick column and centrifuged for a minute. The flow-through was discarded and QIAquick column returned in the same collection tube. To remove traces of agarose, 0.5ml of buffer QG was added to the QIAquick column and centrifuged for one minute. The flow-through was discarded and column returned in the same collection tube. The column was washed by adding 0.75ml of buffer PE and centrifuged for one minute. The flow-through was discarded and the QIAquick column was centrifuged for one minute to remove residual ethanol from Buffer PE. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube and the DNA was eluted by adding 50 µl of double distilled water to the center of the QIAquick membrane and centrifuged for one minute. All centrifugation were performed at 13,000 rpm.

G) LB plate with ampicilin/IPTG/X-gal

To a 15g agar was added 1L LB medium mixed and autoclaved. The medium was allowed to cool to about 50ºC (just enough to hold container with the hand). This was followed by the addition to final concentration, ampicillin 100µg/ml, X-gal 80µg/ml and IPTG 0.5m M. The mixture was mixed by swirling and 30-35ml of the medium was poured into petri dishes. The agar was allowed to harden, wrapped in plastic and was stored at 4ºC for a month.

H) DNA ligation

Ligation was carried out using pGEMT-Easy kit (Promega, USA) and the reaction set up in a 0.2ml microcentrifuge PCR tube. The ligation mixtures for test and control reactions are showed in Table V.

Table x Reagents and reaction volume for the preparation of ligation reactions for the cloning of PCR products

Reagents	Reaction volumes (µl)		
	Test sample	+ control	- control
2x Rapid ligation buffer, T4 DNA ligase	5	5	5
P GEM-T Easy vector (50ng)	1	1	1
PCR product	3	-	-
Control insert DNA	-	2	-
T4 DNA Ligase	1	1	1
Double distilled waster	1	1	3
Total volume	10	10	10

+ Control = positive control, - control = negative control, TC= transformation control and - =
no reagent. The ligation reactions were incubated overnight at 4ºC.

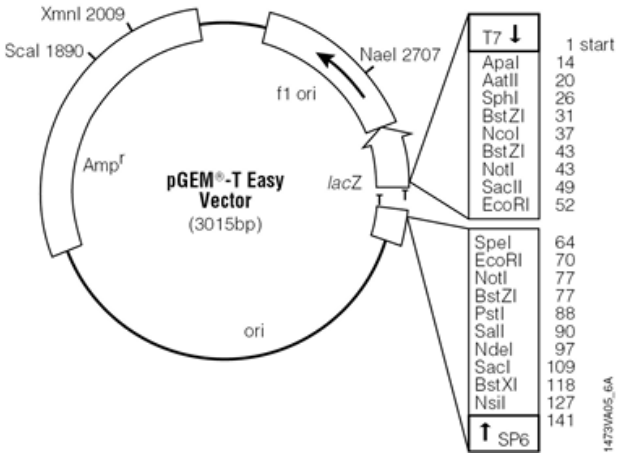


Figure X pGEM®-T Easy Vector circle map and sequence reference point (promega.com)

I) Transformation of *E. coli* JM109 chemical competent cells

The cloning area was wiped properly with 70% ethanol to ensure sterility. 1.5ml eppendorf tubes were labelled as per name of ligation reaction and left on ice to pre-chill before use. Competent cells from -80ºC freezer were allowed to thaw completely on ice for 5-10 minutes. Forty µl of the competent cells was placed into each eppendorf tube. This was followed by the addition of 4 µl of ligation reaction to the 40µl cells on ice. For a transformation control 1ul (1:100 dilution of a 1ng/µl P UC18 DNA) template was added to 9µl water. The content of each tube was briefly stirred with a pipette tip before incubating on ice for 30 minutes. The competent cells were heat shocked by placing the transformation reaction in a 42ºC water bath for 45 seconds. The tube and content were returned immediate to ice for two minutes. A recovery medium (960µl) was added to the cells and the tubes were placed in a shaking incubator at 250 rpm for 1.5 hours at 37ºC. One hundred µl of transformed cells was transferred to the centre of an LB agar plate containing ampicillin (AMP)/IPTG/X-gal. A sterile spreader was used to spread the transformed cells over the entire surface of the plate. The plates were labelled with the name of the ligation reaction, dated and incubated overnight at 37ºC.

APPENDIX III

Cattle kraal collection

Cattle Kraal collection in Kwambwe

APPENDIX IV

Seasonal blood meal source for three species of mosquitoes in the *Anopheles funestus* group across five collection sites

Collection site	Season	*An.* species	Total number of samples tested	Samples with identified Host blood												HBI
				Single blood meal				Multiple blood meal								
				H	D	C	G	BG	BGH	HPDckG	PDG	DchG	HPchB	PckG	HPB	
Mwakabighili	Wet	*An. riv*	**3**	**-**	**-**	**-**	**-**	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0**
	Dry	*An. riv*	**15**	**-**	**-**	**3**	**-**	**3**	**-**	**-**	**-**	**-**	**-**	**1**	**1**	**12.5**
Mwenetete	Wet	*An. riv*	**10**	**-**	**-**	**-**	**-**	**7**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**12.5**
		An. par	**1**	**-**	**-**	**-**	**-**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0**
	Dry	*An. riv*	**25**	**-**	**-**	**2**	**1**	**8**	**-**	**-**	**-**	**-**	**1**	**-**	**-**	**8.3**
		An. fun	**1**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**100**
Mwampaghatwa	Dry	*An. riv*	**5**	**-**	**-**	**-**	**-**	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0**
Wovwe	Wet	*An. fun*	**19**	**1**	**1**	**-**	**-**	**1**	**-**	**1**	**3**	**1**	**-**	**-**	**-**	**25**
	Dry	*An .fun*	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0**
Kwambwe	Wet	*An. riv*	**2**	**-**	**-**	**-**	**-**	**2**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0**

An. riv: *An. rivulorum*, *An. par*: *An. parensis*, *An. fun*: *An. funestus*

H: Human, D: Dog, B: Bovine, G: Goat, ck: Chicken - : no sample

APPENDIX V

Quantitative taxonomic wing length raw data for adult females of *Anopheles funestus*-like and *Anopheles funestus* species

An. species /Country	No	Observed range	Mean	95%CI	SD	SE Mean
An. funestus-like/Malawi	49	2.4380-3.0480	2.7953	2.7569-2.8336	0.1335	0.0191
An. funestus /Madagascar	18	3.0480-3.3530	3.2208	3.1789-3.2628	0.0843	0.0199
An. funestus /Malawi	18	2.7430-3.2320	2.9192	2.8605-2.9780	0.1182	0.278

APPENDIX VI

Ethical clearance waiver

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-100510-1

10/05/2010

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigators: **Professor Maureen Coetzee**

Project title: **Research on mosquitos.**

Reason: This waiver covers all research using mosquitos and mosquito parasites by Professor Coetzee, her staff and students as long as no humans or human tissues are involved. The waiver lasts 5 years and may be renewed.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2010 -05- 10
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

APPENDIX VII

Published manuscript I

Vezenegho, S. B., Bass, C., Puinean, M., Williamson, M. S Field, L. M., Coetzee, M. and Koekemoer, L. L (2009). Development of multiplex real-time PCR assays for identification of members of the *Anopheles funestus* species group. *Malaria Journal*, **8:**282.

Contribution:

I carried out the laboratory work, interpretation of all results and wrote the first and subsequent drafts on the manuscript.

See attachment Appendix VII

REFERENCES

Abbott W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, **18:**265-266.

Abilio, A. P., Kleinschmidt, I., Rehman, A. M., Cuamba1, N., Ramdeen, V., Mthembu, D. S., Coetzer, S., Maharaj, R., Wilding, C. S., Steven, A., Coleman, M., Hemingway, J. and Coleman, M. (2010). The emergence of insecticide resistance in central Mozambique and potential threat to the successful indoor residual spraying malaria control programme. *Malaria Journal*, **10:**110.

Afonina, I., Zivarts, M., Kutyavin, I., Lukhtanov, E., Gamper, H. and Meyer, R. B. (1997). Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Research*, **25:**2657-2660.

Bass, C., Nikou, D., Donnelly, M. J., Williamson, M. S., Ranson, H., Ball, A., Vontas, J. and Field, L. M. (2007a). Detection of knockdown resistance (*kdr*) mutations in *Anopheles gambiae*: a comparison of two new high-throughput assays with existing methods. *Malaria Journal* 2007, **6:**111.

Bass, C., Williamson, M. S., Wilding, C. S., Donnelly, M. and Field, L. M. (2007b). Identification of the main malaria vectors in the *Anopheles gambiae* species complex using a TaqMan real-time PCR assay. *Malaria Journal*, **6:**155.

Bass, C., Nikou, D., Blagborough, A. M., Vontas, J., Sinden, R. E, Williamson, M. S. and Field, L. M. (2008). PCR-based detection of *Plasmodium* in *Anopheles* mosquitoes: a comparison of a new high-throughput assay with existing methods. *Malaria Journal*, **7:**177

Beier, J. C., Perkins, P. V., Wirtz, R. A., Koros, J., Diggs, D., Gargan, T. PII. and Koech, L. K. (1988). Blood-meal identification by direct enzyme-linked immunosorbent assay (ELISA) tested on *Anopheles* (Diptera: Culicidae) in Kenya. *Journal of Medical Entomology*, **25:**9-16.

Beerntsen, B. T., James, A. A. and Christensen, B. M. (2000). Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews*, **64:**15-137.

Bigoga, J. D., Manga, L., Titanji, V. P., Coetzee, M. and Leke, R. G. (2007). Malaria vectors and transmission dynamics in coastal south-western Cameroon. *Malaria Journal*, **6:**5.

Brengues, C., Hawkes, N. J., Chandre, F., McCarroll, L., Dunchon, S., Guillet, P., Manguin, S., Morgan, J. C. and Hemingway, J. (2003). Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gate sodium channel gene. *Medical and Veterinary Entomology*, **17:**87-94.

Chapman, H. C. (1974). Biological control of mosquito larvae. *Annual Review of Entomology*, **19:**33-59.

Charlwood, J. D., Thompson, R. and Madsen, H. (2003). Observations on the swarming and mating behaviour of *Anopheles funestus* from southern Mozambique. *Malaria Journal*, **2:** 2.

Chiphwanya, J. A. (2004). Evaluation of insecticide susceptibility in malaria vector mosquitoes and their role in malaria transmission in central Malawi. M.Sc Thesis, University of Witswatersrand, South Africa.

Claridge, M. F., Dawah, H. A. and Wilson, M. R. (1997). Practical approaches to species concepts for living organisms. Species: the units of biodiversity. Chapman and Hall, London.

Coetzee, M. and Fontenille, D. (2004). Advances in the study of *Anopheles funestus*, a major vector of malaria in Africa. *Insect Biochemistry and Molecular Biology*, **34:** 599-605.

Cohuet, A., Simard, F., Toto, J., Kengne, P., Coetzee, M. and Fontenille, D. (2003). Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for new species. *American Journal of Tropical Medicine and Hygiene*, **62:**200-205.

Coleman, P. G. and Alphey, L. (2004). Editorial: Genetic control of vector populations: an imminent prospect. *Tropical Medicine and International Health*, **9:** 433-437.

Collins, F., Mendez, A. M., Rasmussen, M. O., Mehaffey, C. F., Besansky, N. J. and Finnerty, V. (1987). A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene*, **37:**37-41.

Collins, F. H., Porter, C. H. and Cope, S. E. (1990). Comparison of rDNA and mtDNA in sibling species *Anopheles freeborni* and *Anopheles hermsi*. *American Journal of Tropical Medicine and Hygiene*, **42:**417-423**.**

Collins, F. H. and Paskewitz, S. M. (1995). Malaria: current and future prospects for control. *Annual Review of Entomology*, **40:**195-219.

Coluzzi, M. and Sabatini, A. (1967). Cytogenetic observations on species A and B of the *Anopheles gambiae* complex. *Parassitologia*, **9:**73-88.

Costantini, C, M., Sagnon, N. F., Ilboundo-Sanogo, E., Coluzzi, M. and Bocolini, D. (1999). Chromosomal and bionomic heterogeneities suggest incipient speciation in *Anopheles funestus* from Burkina Faso. *Parassitologia*, **41:**595-611.

"**Countries of the world. Country profile**: Malawi" <http://www.infoplease.com/ipa/A0107747>[accessed 20 March 2011].

Cox-Singh, J., Davis, T. M., Lee, K., Shamsul, S. S., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J. and Singh, B. (2007). *Plasmodium knowlesi* malaria in humans is

widely distributed and potentially life threatening. *Infectious Disease Society of America*, **46:** 165-171.

Cracraft, J. (1983). Species concepts and speciation analysis. *Current Ornithology*, **1:**159-187.

Dabire, K. R., Diabaté, A., Namountougou, M., Toé, K. H., Ouari, A., Kengne, P., Bass, C. and Baldet, T. (2009). Distribution of pyrethroid and DDT resistance and the L1014F *kdr* mutation in *Anopheles gambiae* s.l. from Burkina Faso (West Africa). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **103**:1113-1120.

Davidson, G. (1969). The potential use of sterile hybrid males for the eradication of member species of *the Anopheles gambiae* complex. *Bulletin of the World Health Organization*, **40:** 211-228.

De Meillon, B. and Evans, A. M. (1935). Two new anophelines from South Africa. *Annals of Tropical. Medicine and Parasitology*, **29:**91.

De Meillon, B., Van Eeden. G, J., Coetzee, L., Coetzee, M., Meiswinkel, R. Du Toit, C. L. N. and Hansford, C. F. (1977). Observations on a species of the *Anopheles funestus* subgroup, a suspected exophilic vector of malaria parasites in the north-eastern Transvaal, South Africa. *Mosquito News*, **37:**657-661.

Dobzhansky, T. (1935). A critique of the species concept in biology. *Philosophy of Science*, **2:**344-355.

Durnez, L., Van Bortel, W., Denis, L., Roelants, P., Veracx, A., Trung, H., Sochantha, T. and Marc Coosemans. (2011). False positive circumsporozoite protein ELISA: a challenge for the estimation of the entomological inoculation rate of malaria and for vector incrimination. *Malaria Journal*, **10:**195.

Durvasula, R. V., Gumbs. A., Panackal. A., Kruglov. O., Askoy. S., Merrifield. R. B., Richards, F. F. and Beard. C. B. (1997). Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, **94:**3274-3278.

Franz, G. and Savakis, C. (1991). *Minos*, a new transposable element from *Drosophila hydei*, is a member of the Tc1-like family of transposons. *Nucleic Acids Research*, **19(23):** 6646.

Garnham, P. C. C. (1966). Malaria parasite and other haemosporidia. Blackwell Scientific Publications, Oxford.

Garros. C., Koekemoer, L. L., Kamau., Awolola, T. S., Van Bortel. W., Coetzee, M., Coosemans, M. and Manguin. S. (2004). Restriction fragment length polymorphism method for the identification of major African and Asian malaria vectors within the *Anopheles*

funestus and *An. minimus* groups. *American Society of Tropical Medicine and Hygiene*, **70(3):** 260-265.

Giglio, S., Monis, P. T. and Saint, C. P. (2003). Demonstration of preferential binding of SYBR Green I to specific DNA fragments in realtime multiplex PCR**.** *Nucleic Acids Research*, **31(22):**e136.

Gillies, M. T. and Furlong, M. (1964). An investigation into the behaviour of *Anopheles parensis* Gillies at Malindi on the Kenya coast. *Bulletin of Entomological Research*, **55:**1-16.

Gillies, M. T. and De Meillon, B. (1968). *The Anophelinae of Africa South of the Sahara*. **Publications of the South African Institute for Medical Research, Johannesburg. No**, **54.**

Gillies, M. T. and Coetzee, M. (1987). *A supplement to the Anophelinae of Africa South of the Sahara*. **Publications of the South African Institute for Medical Research, Johannesburg. No. 55.**

Gonzalez, I. L., Gorski, J. L., Campen, T. J., Dorney, D. J., Erickson, J. M., Sylvester, J. E. and Schmickel, R. D. (1985). Variation among human 28S ribosomal RNA genes P*roceedings of the Natational Academy of Science of the United State of America*, 82:7666-7670.

Google earth map (http://maps.google.co.za, accessed on 20 May 2011).

Green, C. A. and Hunt, R. H. (1980). Interpretation of variation in ovarian polytene chromosomes of *Anopheles funestus* Giles, *A. parensis* Gilies and *A. aruni*. *Genetica*, **51:**187-198.

Green, C. A. (1982). Clasdistic analysis of mosquito chromosome data (*Anopheles* (Cellia) Myzomyia). *Journal of Heredity*, **73:**2-11.

Harbach, R. E. (2004). The classification of the genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bulletin of Entomological Research*, **94:** 537-553.

Hargreaves, K., Koekemoer, L. L., Brooke, B. D., Hunt, R. H., Mthembu, J. and Coetzee, M. (2000). *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. *Medical and Veterinary Entomology*, **14:**181-189.

Hassan, M. M., El-Motasim, W. M., Ahmed, R. T. and El-Sayed, B. B. (2010). Prolonged colonisation, irradiation, and transportation do not impede mating vigour and competitiveness of male *Anopheles arabiensis* mosquitoes under semi-field conditions in Northern Sudan. *Malaria World Journal*, **1:**2.

Helinski. M. E. H., Hassan, M. M., El-Motasim, W. M., Malcolm, C. A., Knols, B. G. J. and El-Sayed, B. (2008). Towards a sterile insect technique field release of *Anopheles arabiensis* mosquitoes in Sudan: Irradiation, transportation, and field cage experimentation. *Malaria Journal*, **7:**65.

Hemingway, J., Hawkes, N. J., McCarroll, L. and Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular biology*, **43:**653-665.

Hunt, R. H. (1973). A cytological technique for the study of the *Anopheles gambiae* complex. *Parassitologia*, **15:**137-139.

Hunt, R. H., Coetzee, M. and Fettene, M. **(1998).** The *Anopheles gambiae* complex: a new species from Ethopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **92:**231-235.

Hunt, R. H., Edwardes, M. and Coetzee, M. (2010). Pyrethroid resistance in southern African *Anopheles funestus* extends to Likoma Island in Lake Malawi. *Parasites and Vectors*, **3:**122.

Hunter R, L., LaJeunesse, T. C. and Santos, S. R. (2007). Structure and evolution of the rDNA internal transcribed spacer (ITS) region 2 in the symbiotic dinoflagellates (*Symbiodinium*, Dinophyta). *Journal of Phycology*, **43**:120-128.

Hwang Ui-Wook. and Kim Won (1999). General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics. *The Korean Journal of Parasitology*, **37:**215-224.

Ito, J., Ghosh, A., Moreira, L.A., Wimmer, E. A. and Jacobs-lorena, M. (2002). Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature,* **417:** 452-455.

Kamau, L., Koekemoer, L. L., Hunt, R. H. and Coetzee, M. (2003). *Anopheles parensis*: the main member of the *Anopheles funestus* species group found resting inside human dwellings in Mwea area of central Kenya towards the end of the rainy season. *Journal of the American Mosquito Control Association*, **19:**130-133.

Kazembe, L. N., Kleinschmidt, I., Holtz, T. H. and Sharp, B. L. (2006). Spatial analysis and mapping of malaria risk in Malawi using point-referenced prevalence of infection data**.** *International Journal of Health Geographics***, 5:**41.

Kent, R. J. and Norris, D. E. (2005). Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome b. *American Journal of Tropical Medicine and Hygiene*, **73:**336-342.

Kent, R. J., Thuma, P. E., Mharakurwa, S. and Norris, D. E. (2007). Seasonality, blood feeding behaviour, and transmission of *Plasmodium falciparum* by *Anopheles arabiensis* after an extended drought in southern Zambia. *Journal of Tropical Medicine and Hygiene,* **76:**267-274.

Kleinschmidt, I., Sharp, B., Benavente, L. E., Schwabe, C., Torrez, M., Kuklinski, J., Morris, N., Raman, J. and Carter, J. (2006). Reduction in infection with *Pasmodium*

falciparum one year after the introduction of malaria control interventions on Bioko Island, Equatorial Guinea. *American Journal of Tropical Medicine and Hygiene*, **74:**972-978.

Koekemoer, L. L., Coetzee, M. and Hunt, R. H. (1998). *Hpa* II endonuclease distinguishes between two species in the *Anopheles funestus* group. *Insect Molecular Biology*, **7:**273-277.

Koekemoer, L. L., Lochouarn, L. L., Hunt, R. H. and Coetzee, M. (1999). Single-strand conformation polymorphism analysis for identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group. *Journal of Medical Entomology*, **36:**125-130.

Koekemoer, L. L., Rankoe, E. M., La Grange, J. P., Govere, J. and Coetzee, M., (2001). False detection of *Plasmodium falciparum* sporozoite in *Anopheles marshallii* group of mosquitoes. *Journal of the American Mosquito Control Association*, **17:**160-165.

Koekemoer, L. L., Kamau, L., Hunt, R. H. and Coetzee, M. (2002). A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *American journal of Tropical Medicine and Hygiene*, **66:**804-811.

Koekemoer, L. L., Kamau, L., Garros, C., Manguin, S., Hunt, R. H. and Coetzee, M. (2006). Impact of the rift valley on restriction fragment length polymorphism typing of the African malaria vector *Anopheles funestus* (Diptera: Culicidae). *Journal of Medical Entomology*, **43:**1178-1184.

Kwapata, K., Mwase, W. F., Bokosi, J. M., Kwapata, M. B. and Munyenyembe, P. (2007). Genetic diversity of *Annona senegalensis* Pers. Populations as revealed by simple sequence repeats (SSRs). *African Journal of Biotechnology*, **6(10):**1239-1247.

Lekanne, D., Fijnvandraat, A. C., Ruijter, J. M. and Moorman, A. F. (2002). Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depend on c DNA synthesis conditions. *Analytical biochemistry*, **307:**63-69.

Liew, M., Pryor, R., Palais, R., Meadows, C., Erali, M., Lyon, E. and Wittwer, C. **(2004)** Genotyping of single-nucleotide polymorphisms by high resolution melting of small amplicons. *Clinical chemistry*, **50:**1156-1164.

Lines, J. D. (1996). The Technical Issues. *Net gain, a new method for preventing malaria deaths* (C, Lengeler, J Cattani and D, de Savigny) IDRC/WHO, Geneva, pp. 17-53.

Livak, K. J. (1984): Organization and mapping of a sequence on the *Drosophila melanogaster* X-chromosome and Y-chromosome that is transcribed during spermatogenesis. *Genetics*, **107:**611-634.

Lochouarn, L., Dia, I., Boccolini, D., Coluzzi, M. and Fontenille. (1998). Bionomical and cytogenetic heterogeneities of *Anopheles funestus* in Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **92:**607-612.

Lochouarn, L. and Fontenille, D. (1999). ELISA detection of malaria sporozoites: false-positive results in *Anopheles gambiae* s.l. associated with bovine blood meals.*Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93:**101-102.

Lyamiche, V., Brow, M. A. D. and Dahlberg, J. E. (1993). Structural specific endonucleolytic cleavage of nucleic acid by eubacterial DNA polymerase. *Science*, **260:**778-783.

Lyimo, E. O. and Takken, W. (1993). Effects of adult body size on fecundity and pre-gravid rate of *Anopheles gambiae* females in Tanzania. *Medical Veterinary Entomology*, **7:**328-332.

Madhora, M., Maruyama, K. and Hartl, D. L. (1991). Molecular and functional analysis of the mariner mutator element *Mosl* in *Drosophila. Genetics*, **128:**311-318.

Majambere, S., Lindsay, S. W., Green, C., Kandeh, B. and Fillinger, U. (2006). Microbial larvicides for malaria control in The Gambia. *Malaria Journal*, **6:**76.

Mallet, J. (1995). A species definition for the modern synthesis. *Trends in Ecology and Evolution*, **10**:194-299.

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur, N. and Pauron, D. (1998). Molecular characterisation of pyrethroid knockdown resistant (*kdr*) in the major malaria vector, *Anopheles gambiae s.s*. *Insect Molecular Biology*, **7:**179-184.

Mayr, E. (1942). *Systematics and the origin of Species*. Columbia University Press, New York.

Mayr, E. (1996). What is a species, and what is not? *Philosophy of Science*, **63:**262-277.

Michel, A. P., Guelbeogo, W. M., Grushko, O., Schemerhorn, B. J., Kern, M., Willard, M. B., Sagnon, N., Constantini, C. and Besansky, N. J. (2005a). Molecular differentiation between chromosomal defined incipient species of *Anopheles funestus*. *Insect Molecular Biology*, **14:**375-387.

Michel, A. P., Ingrasci, M. J., Schemerhorn, B. J., Kern, M., LeGoff, G., Coetzee, M., Elissa, N., Fontenille, D., Vulule, J. and Lehmann, T. (2005b). Rangewide population genetic structure of the African malaria vector *Anopheles funestus*. *Molecular Ecology*, **14:** 4235-4248.

Monis, P. T., Giglio, S. and Saint, C. P. (2005). Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Analytical Biochemistry*, **340:**24-34.

Morgan, J. C., Irving, H., Okedi, L. M., Steven, A. and Wondji, C. S. (2010). Pyrethroid resistance in an *Anopheles funestus* population from Uganda. PLoS ONE 5(7):e11872. doi:10.1371/journal.pone.0011872.

Mouatcho, J. C., Keith Hargreaves., K., Koekemoer, L. L., Brooke, B. D., Oliver, S. V., Hunt, R. H. and Coetzee, M. (2007). Indoor collections of the *Anopheles funestus* group (Diptera: Culicidae) in sprayed houses in northern KwaZulu-Natal, South Africa. *Malaria Journal*, **6:**30.

Mouchet, J., Manguin, S., Sircoulon, J., Laventure, S., Faye, O., Onapa, A. W., Carnevale, P., Julvez, J. and Fontenille, Didier. (1998). Evolution of malaria in Africa for the past 40 years: impact of climatic and human factors. *Journal of the American Mosquito Control Association*, **14:**121-130.

Mulla, M. S., Thavara, U., Tawatsin, A., Chomposri, J. and Su, T. (2003). Emergence of resistance and resistance management in field populations of tropical *Culex quinquefasciatus* to the microbial control agent *Bacillus sphaericus*. *Journal of the American Mosquito Control Association*, **19:**39-46.

Nauen, R. (2006). Insecticide resistance. *Bayer Environmental Science Journal*, **18:**10-15.

N'Guessan, R., Corbel, V., Akogbéto, M. and Rowland M (2007). Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. *Emerging Infectious Disease*.**13:**199-206

Olsson, K. E., Higgs, P. J., Gaines, A. M., Powers, B. S., Davis, K. I., Kamrud, J. O., Carlson, C. D., Blair. and Beaty, B. J. (1996). Genetically engineered resistance to dengue-2 virus transmission in mosquitoes. *Science,* **272:**884-886.

Paterson, H. E. H. (1985). The recognition concept of species. Species and speciation, pp. 21-29. Transvaal Museum, Pretoria.

Paterson, H. E. H. (1993). Evolution and the recognition concept of species, collected Writings of H.E.H. Paterson. Johns Hopkins University Press, Baltimore, MD.

Pates, H. and Curtis, C. **(2005).** Mosquito behaviour and vector control. *Annual Review of Entomology*, **50:**53-70.

PRESIDENT'S MALARIA INITIATIVE: Malaria Operational Plan -FY (2010) MALAWI.

PRESIDENT'S MALARIA INITIATIVE: Malaria Operational Plan -FY (2011) MALAWI.

Rehman, A. M., Coleman, M., Schwabe, C., Baltazar, G., Matias, A., Gomes, I. R., Yellott, L., Aragon, C., Nchama, G. N., Mzilahowa, T., Rowland, M. and Kleinschmidt, I. (2011). How much does malaria vector control quality matter: the epidemiological impact of holed nets and inadequate indoor residual spraying. PLoS ONE | www.plosone.org. e19205

Roberts, D. W. and Hajek, A. E. (1992). Entomopathogenic fungi as bioinsecticides *In*: Leathman, G. F. Ed. Frontiers in Industrial Mycology: Chapman and Hall Inc. Routledge, Pp. 144-159.

Rose, I. R. (2001). (Pesticides and public health): Intergrated methods of mosquito management. *Emerging Infectious Diseases*, **7:**17-23.

Rossello-Mora, R. and Amann, R. (2001). The species concept for prokaryotes. *Federation of European Microbiological Societies Microbiology Reviews*, **25:**39-67.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). DNA sequencing in: Molecular Cloning, a laboratory manual, Nolan C. (Editor). New York: New York: Cold Spring harbour Laboratory, press pp, 13.3-13.10., USA.

Scott, J. A., Brogdon, W. G., and Collins, F. H. **(1993).** Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, **49:**520-529.

Service, M. W. (1983). Biological control of mosquitoes, has it a future? *Mosquito News*, **43:**113-120.

Service, M. W. (1984). Problems of vector-borne diseases and irrigation projects. *Insect Science and Its Application*, **5:**227-231.

Simpson, G. G. (1951). The species concepts. *Evolution*, **5:**285-298.

Skovmand, O. and Sanogo, E. (1999). Experimental formulations of *Bacillus sphaericus* and *B. thuringiensis isrealensis* against *Culex quinquefasciatus* and *Anopheles gambiae* (Diptera: Culicidae) in Burkina Faso. *Journal of Medical Entomology*, **36:**62-67.

Snounou, G., Viriyakosol, S., Zhu, X. P., Jarra, W., Pinheiro, L., do Rosario, V. E., Thaithong, S. and Brown, K. N. (1993). High sensitivity of detection of human malaria parasite by the use of nested polymerase chain reaction. *Molecular and Biochemical Parasitology*, **61:**315-320.

Spillings, B., Basil, B. D., Koekemoer, L. L., Chiphwanya, J., Coetzee, M. and Hunt, R, H. **(2009).** A new species concealed by *Anopheles funestus* Gilies, a major malaria vector in Africa. *American Journal of Tropical Medicine and Hygiene*, **81:**510-515.

Sylla, E. H., Kun, J. F. and Kremsner, P. G. (2000). Mosquito distribution and entomological inoculation rates in three malaria-endemic areas in Gabon. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **94:**652-656.

Tassanakajon, A., Boonsaeng, V., Wilairat, P. and Panyim, S. (1993). Polymerase chain reaction detection of *Plasmodium falciparum* in mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **87:**273-5.

Temu, E. A., Minjas, J. N., Tuno, N., Kawada, H. and Takagi, M. (2007). Identification of four members of the *Anopheles funestus* (Diptera: Culicidae) group and their role in *Plasmodium falciparum* transmission in Bagamoyo coastal Tanzania. *Acta Tropica,* **102:**119-125.

Van Bortel, W., Trung, H. D., Roelants, P., Harbach, R. E., Backeljau, T. and Coosemans, M. (2000). Molecular identification of *Anopheles minimus s.l.* beyond distinguishing the members of the species complex. *Insect Molecular Biology*, **9:**335-340.

Van der Sande, C. A. F. M., Kwa, M., van Nues, R. W., van Heerikhuizen, H., Raue, H. A. and Planta, R. J. (1992). Functional analysis of internal transcribed spacer 2 of Saccharomyces cerevisiae ribosomal DNA. *Journal Molecular Biology*, **223:**889-910.

Weeto, M. M., Koekemoer, L. L., Kamau, L. , Hunt, R. and Coetzee, M. (2004). Evaluation of a species-specific PCR assay for the *Anopheles funestus* group from eleven African countries and Madagascar. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **98:**142-147.

Wheeler, Q. D. and Platnick, N. I. (2000). A critique from the wheeler and Platnick phylogenetic species concept perspective: Problems with alternative concepts of species. Species concepts and phylogenetic theory (Ed. Q. D. Wheeler and R. Meier), pp. 133-145, Columbia University Press, New York.

Whiteacre, D. M. and Ware, G. W. (2004). The pesticide book. 6th Edition, Meister Media Worldwide, Welloughby, Ohio.

Wilkes, T. J., Matola, Y. G. and Charlwood, J. D. (1996). *Anopheles rivulorum*, a vector of human malaria in Africa. *Medical and Veterinary Entomology*, **10:**108-110.

Wirtz R, A., Zavala, F., Charoenvit, Y., Cambell, G. H., Burkot, T. R., Schneider, I., Esser, K. M., Beaudoin, R. L. and Andre, G. R. (1987). Comparative testing of *Plasmodium falciparum* sporozoite monoclonal antibodies for ELISA development. *Bulletin of the World Health Organanization*, **65:**39-45.

Wirtz, R. A., Sattabonkot, J., Hall, T., Burkot, T. R. and Rosenberg, R. **(1992).** Development and evaluation of an ELISA for *plasmodium vivax*-VK247 sporozoites. *Journal of Medical Entomology*, **29:**854-57.

World Health Organisation. **(1998).** Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. Document *WHO/CDS/CPC/MAL/98.12*. Geneva, Switzerland.

World Health Organization (2001) Chemistry and specifications of pesticides. *World Health Organization Technical Series report*, **899:**1-68.

World Health Organization. (2004). Global strategic framework for integrated vector management. Document WHO/CDC/CPE/PVC/2004.10. Geneva, Switzerland.

World Health Organization. (2005). International Travel and Health.

World Health Organization. (2006). Malaria vector control and personal protection. WHO technical report series, No. 936.

World Health Organization. (2008). World Malaria Report.

World Health Organization. (2010). The World Malaria Report. Geneva, Switzerland.

Young, M. D., Baerg, D. C. and Rossan, R. N. (1971). Sporozoite transmission and serial blood passage of Plasmodium vivax in squirrel monkeys (Saimiri sciureus). *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **65:**835-836.

Zaim, M., Aitio, A. and Nakashima, N (2000). Safety of pyrethroid-treated mosquito nets. *Medical and Veterinary Entomolology*,**14:**1-5.