Analysis of tsetse fly (diptera: glossinidae) blood meals using mitochondrial cytochrome genes for vertebrate host identification

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Thesis submitted to the Graduate School in partial fulfillment of the requirements for the award of the degree of Master of Science in Biochemistry of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

I declare that this Thesis is my original work and has not been presented for a degree award in any other university.

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DEDICATION

I dedicate this thesis to my husband, Stephen Muturi and family members for being there for me and giving me moral support that enabled me to carry out the study.

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May the Almighty God bless you!

ABSTRACT

Information on the vertebrate hosts of blood feeding vectors forms a useful decision-making tool in the planning of tsetse fly control and eradication operations. The objective of this study was to investigate the utility of mitochondrial Cytochrome c oxidase I and Cytochrome b gene sequences in the identification of tsetse fly blood meals in order to provide a basis for more rational control of trypanosomosis in East Africa. Blood fed *Glossina swynnertoni* were sampled from the Serengeti (Tanzania) and *G. pallidipes* were from Nguruman and Busia in Kenya. Tsetse blood meal analysis was carried out by amplification and sequencing, to score polymorphisms in the partial COI and Cytb genes with diagnostic value. Sequences obtained were used to query the BOLD and GenBank databases, and the percent identities used to identify hosts. Hosts identified from blood fed *G. swynnertoni* collected in Serengeti ecosystem, included twenty five buffaloes, nine had fed on giraffes, three on warthogs and other three had fed on elephants and one on spotted hyena. In Nguruman, the analyzed *G. pallidipes* were found to have obtained their blood meals from different hosts which included six elephants, five warthogs, a buffalo and a baboon. Only cattle blood was detected in flies caught in Busia in western Kenya. Regarding the hosts identified in the different sampling sites, insecticides on cattle will form an integral part of a control strategy for trypanosomosis in Busia, while different approaches, for example traps and targets are needed for Serengeti and Nguruman ecosystems, where wildlife abound and are the major component of the tsetse fly food source. These results demonstrate the feasibility of the COI and Cytb gene sequences for the identification of the vertebrate hosts of tsetse fly species.

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LIST OF ABBREVIATIONS

AAT	African animal trypanosomosis
BecANet	Biosciences east and central Africa
Bp	Base pairs
BLAST	Basic local alignment search tool
BOLD	Barcode of life data systems
BOLD-ID	Barcode of life data systems-identification engine
COI	Cytochrome c oxidase I gene
Cytb	Cytochrome b gene
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetra-acetic acid
FAO	Food and Agriculture Organization
FTA	Flindes technology associates
HAT	Human african trypanosomosis
HDA-PCR	Hetero-duplex analysis-polymerase chain reaction
mtDNA	Mitochondrial DNA
NCBI	National Centre for Biotechnology Information

PCR	Polymerase chain reaction
TAE	Tris-acetate ethylenediaminetetra-acetic acid
Taq	*Thermus aquaticus*
TE	Tris- ethylene diamine tetra-acetic acid
VSG	Variant surface glycoprotein
PBS	Phosphate buffered saline
UV	Ultra violet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Tsetse flies (Diptera: Glossinidae) are medically and economically important insects confined to sub-Saharan Africa. They are exclusively hematophagous insects and are the vectors of African trypanosomes that infect both man and animals. Sleeping sickness is a zoonosis caused by the flagellated protozoa *Trypanosoma brucei rhodesiense* in East Africa and *T. brucei gambiense* in West and Central Africa. Gambian sleeping sickness has a long asymptomatic stage, which is eventually succeeded by a sub-acute febrile illness followed by late-stage chronic meningoencephalitis; death might occur several years after onset of the disease. Rhodesian sleeping sickness progresses much more rapidly, with >80% of deaths occurring within six months of the onset of illness. Initially, after the bite from an infected tsetse fly, patients may experience only a few symptoms. From the site of inoculation, the trypanosomes disseminate into the bloodstream via the lymphatic system. The parasites proliferate in waves, evading the host's immune responses by continuously changing their antigenic coat of variant surface glycoproteins (VSG). During this period, there are systemic symptoms of fever and gradually worsening debilitation and wasting. Encephalitic stage is the second stage that follows when parasites transverse the blood brain barrier. Patients waste and succumb, often by developing secondary bacterial infections (Stich *et al.,* 2003).

Human African trypanosomosis is a major threat to human health in 36 countries in sub-Saharan Africa (Kennedy, 2004). According to Pepin and Meda (2001), HAT epidemics in central Africa destroyed whole village communities and forced people to leave endemic areas. World Health Organisation (WHO, 2001) reported that within tsetse fly-infested areas, sixty million people live at risk of infection. In 2005, major outbreaks were observed in Angola, the Democratic Republic of Congo and Sudan. In Central African Republic, Chad, Congo, Côte d'Ivoire, Guinea, Malawi, Uganda and United Republic of Tanzania, sleeping sickness remains an important public health problem. Following the epidemics, surveillance have been reinforced

and the number of new cases reported throughout the continent have substantially reduced and currently the estimated number of cases is between 50,000 and 70,000 (WHO, 2006).

African trypanosomes cause African animal trypanosomosis also referred to as nagana in livestock, a wasting and fatal disease in cattle. *T. vivax* and *T. congolense* are regarded as major pathogens of cattle and other ruminants, while *T. simiae* causes high mortality in domestic pigs. Nagana has restricted agricultural development and nutritional resources in sub-Saharan Africa such that approximately 70% of the humid and semi-humid zones are devoid of cattle (Aksoy, 2003). Rural households in Africa benefit from livestock through their provision of draught power, manure, milk, meat and skin (Masiga *et al.,* 2002). Livestock also provide income through their sale to meet exceptional expenses or to mitigate the effects of crop failure (Ellis and Mdoe, 2003). There are significant financial losses arising from the effects of trypanosomosis with losses in cattle being estimated to be about 4.5 billion US dollars per year, with about 3 million cattle deaths (Reinhardt, 2002).

Trypanosome species are transmitted cyclically by tsetse flies or mechanically by biting flies depending on the species, leading to death of cattle, camels, pigs, sheep and goats. *T. evansi* and *T. vivax* are transmitted mechanically by hematophagous flies like Tabanids (Hoare, 1972). During cyclical transmission, tsetse flies acquire infection of bloodstream trypomastigotes from the mammalian host blood. The trypanosomes enter the vector midgut where they transform into procyclics. Later they penetrate into the peritrophic membrane and the midgut epithelium into the haemoceal then to the salivary glands or proboscis of the fly where they develop into infective metacyclics. Pathogenesis of trypanosomosis is initiated when infected tsetse flies inoculate metacyclic trypanosomes into the skin of the host animal as they take a blood meal (Aksoy *et al.,* 2001). Parasites multiply for a few days and may result in a localized swelling (chancre) which is an inflammatory response after inoculation of the infective metacyclics. They subsequently invade lymph nodes and then the bloodstream, where they divide rapidly by binary fission.

The drugs available for treating trypanosomosis in domestic ruminants include isometamidium and homidium, which have both prophylactic and therapeutic effects and also diminazene which has therapeutic properties (Geerts *et al.,* 2001). Approximately, 35 million

doses of these drugs are used in Africa each year, with about 50-70 million animals at risk from trypanosomosis (Geerts and Holmes, 1998). Prolonged usage of these drugs has led to development of drug resistance since they have been in the market for over forty years (Geerts *et al.,* 2001).

Tsetse fly control has been undertaken to reduce the incidence of trypanosomosis that tsetse flies transmit. This has been by oduor-baited traps and treatment of animals with insecticides (Esterhuizen *et al.,* 2006). Control of the trypanosomes has been mainly through the use of trypanocidal drugs. However, long term sustainable control has not been achieved (Bauer *et al.,* 1995). Due to the increased development of trypanocide resistance in the parasites, it is proposed that the future control of trypanosomosis lies in the effective control of the tsetse fly vectors (Geerts *et al.,* 2001). For significant integration into tsetse fly control planning, it is therefore imperative that vector hosts are identified in the target area. Results of blood meal analysis can provide precise information on hosts fed on by tsetse flies and other hematophagous arthropods (Mukabana *et al.,* 2002). In the past, serological techniques have been applied for blood meal identification. These techniques include precipitin and haemagglutination test (Weitz, 1963), complement fixation test based upon the detection of host antigens (Staak *et al.,* 1986) and enzyme-linked immunosorbent assays (ELISA) using polyclonal antibodies raised against blood components from potential hosts (Clausen *et al.,* 1998; Wamwiri *et al.,* 2007). These methods require preparation of immune sera against the blood of each potential host species which is a difficult and laborious process. Pre-absorption procedures are also needed to avoid cross reactions when using the ELISA technique (Hunter and Bayly, 1991). However, difficulties have been experienced in the identification of phylogenetically closely related species, resulting in a high percentage of samples being identified only to the family level (Clausen *et al.,* 1998). By using the ELISA technique, Clausen *et al.,* (1998) found that *G. longipennis, G. austeni* and *G. fuscipes* feed on bushpigs; G. *morsitans* feeds on warthogs; *G. longipalpis* and *G. fusca* feed mainly on bushbucks; *G. pallidipes* feeds mainly on ruminants such as buffalo, bushbuck and cattle, while hippopotamus was the main host for *G. brevipalpis*. Using the same technique, Wamwiri *et al.,* (2007) reported that *G. f. fuscipes* collected in Mageta Island fed on monitor lizards while *G. pallidipes* in Busia had fed on bovids.

Developments in molecular biology techniques have enhanced accuracy and the reliability of analytical methods for the identification of vertebrate species. These techniques include modified heteroduplex polymerase chain reaction (PCR) method (Njiokou *et al*., 2004) and polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) whereby a short conserved region of Cytb gene was applied for the identification of origins of blood meals in tsetse flies (Steuber *et al*., 2005). In these techniques the mitochondrial DNA is the preferred target for identifying the species origin since it contains high proportion of nucleotide substitutions that have evolutionary significance, making it a particularly valuable molecule in studying the relationships between closely related vertebrates. In the mitochondrial genome, cytochrome c oxidase I (COI) have been proposed as the 'global standard' barcode for species identification and this has been termed as DNA barcoding (Hajibabaei *et al.,* 2007). Species identification through barcoding is usually achieved by the retrieval of a short DNA sequence of approximately 650 bp fragment of the 5' end of the mitochondrial COI (Hebert *et al.,* 2003a). The barcode sequence from each unknown specimen is compared with a library of reference barcode sequences derived from individuals of known identity. A species is identified if its sequence closely matches one in the barcode library. When a test specimen does not return a close match to existing records probably due to inadequate representation in the library, such specimens are flagged for thorough taxonomic analysis but not designated as new species (Hajibabaei *et al.,* 2007).

Cytochrome b (Cytb) gene is thought to be divergent enough to distinguish between different host species, but conserved enough for identification of each potential host species and thus it has been widely used in systematic studies to resolve divergences at many taxonomic levels (Bartlett and Davidson, 1992). Moreover, identification of a standard set of universal primers directed towards conserved regions of the mitochondrial Cytb gene from vertebrates (Kocher *et al.,* 1989) enables an adequate PCR amplification of relevant nucleotide sequences especially from highly processed foodstuff or largely digested DNA samples found in haematophagous arthropods (Lenstra *et al.,* 2001).

Information on the source of blood meals of the tsetse fly vectors is essential in understanding the relationship between hosts and vectors, and their respective roles in the transmission cycle (Tempelis, 1975). The source of tsetse fly blood meal provides important information relating to

the epidemiology of trypanosomosis and natural feeding habits of different species of *Glossina*. Identification of the vertebrate hosts for tsetse flies would be valuable in the design and implementation of disease and vector control strategies (Bauer *et al.,* 1995).

1.2 Statement of the Problem

Tsetse fly blood meal analysis has been founded on serological techniques that are costly, time consuming and are not always applicable for species level identification of vertebrate hosts for tsetse flies. Commercial reagents are also not available to facilitate a standardized globally reliable outcome. The need therefore exists for a method that is reliable and relatively inexpensive to allow its application in different endemic settings.

1.3 Main objective

To accurately determine the sources of tsetse fly blood meals in order to provide a basis for more rational control of trypanosomosis in East Africa.

1.4 Specific objectives

1. To investigate the utility of Cytochrome c oxidase I and Cytochrome b gene sequences in the identification of tsetse fly blood meals
2. To apply these loci in identifying origins of tsetse fly blood meals from different regions in East Africa.

1.5 Hypothesis

1. Analysis of tsetse fly blood meals using Cytochrome oxidase I and Cytochrome b gene sequences can reveal the vertebrate hosts for tsetse fly species.

2. Loci in cytochrome genes can be used in identification of tsetse fly blood meals from different regions in East Africa

1.6 Justification

Information on the host species of the tsetse fly vectors of diseases is essential in understanding the relationship between hosts and vectors, and their respective roles in disease transmission cycle. Identification of the host range of tsetse fly species is considered a prerequisite for a successful tsetse fly and trypanosomosis control by aiding in the designing and implementation of integrated pest management (IPM) system. Identification of blood meals taken by hematophagous insects have been carried out extensively using serological techniques like complement fixation tests or by enzyme- linked immunosorbent assay. ELISA technique is generally based upon the detection of host antigens using polyclonal antibodies raised against blood components from potential vertebrate hosts. It is also necessary to carry out pre-absorption steps against the blood antigens of other species before the specificity of the antibody reagent can be ensured. In some cases, this is impossible, given that the supply of antigen obtained from a given insect specimen may be limited and also identification of a particular blood meal may not be possible if a suitable antibody is not available. Thus, serological procedures are difficult and laborious. In addition, they are not applicable to species level identification of vertebrate hosts. Analysis of DNA sequences of the host species can provide a more specific and accurate approach as compared to serological techniques. Therefore analysis of COI and Cytb gene sequences was applied to determine the host species for some *Glossina* species.

1.7 Scope and limitations

The study was undertaken to identify tsetse fly blood meals in different locations of East Africa. Application of COI and Cytb genes allowed host identification to the species level. However, only a few sampling sites were considered and this might not be an accurate representation of the tsetse flies population in East Africa. In addition, it was not possible to trap many engorged tsetse flies since the traps used as sampling tools are biased towards hungry flies.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 General overview of trypanosomosis

Trypanosomosis is a disease that affects people and animals and is caused by parasitic protozoan trypanosomes of the genus *Trypanasoma.* There are two types of African trypanosomosis affecting humans, East African and West African, named according to the region of Africa in which they were historically found. The sub- species responsible for East African trypanosomosis is *Trypanosoma brucei rhodesiense* that causes an acute illness leading to death within weeks or months. *T. b. gambiense*, responsible for West African trypanosomosis, causes a more chronic form of the illness which may last several years. Both forms of sleeping sickness are fatal if left untreated (Stich *et al.,* 2003). Trypanosomes are transmitted by the tsetse fly, whereby trypanosomes are taken up in a blood meal and grow and multiply within the tsetse fly gut. After 2-3 weeks, depending upon environmental conditions, they migrate into the salivary glands, where they become mature infective metacyclic forms, and are then transmitted by the injection of infected saliva into a host during a blood meal (Radostits *et al.,* 2000).

2.2 Classification and distribution of tsetse flies

Tsetse flies belong to the insect order Diptera, family Glossinidae and genus *Glossina*. The genus *Glossina* is divided into three different groups based on a combination of distributional and morphological characteristics. These are: morsitans (savannah species), fusca (forest species) and palpalis (riverine species). There are about 33 species and subspecies of *Glossina* mostly restricted to sub-Saharan Africa (Gooding and Krafsur, 2005). They are distributed discretely throughout their range and each taxon is restricted to a relatively specific habitat. The morsitans group species are present throughout the savannah (grassy woodland) of Africa. Where climatic conditions are less severe, their distribution may be limited by scarcity of game animals on which to feed and by lack of vegetation. *Glossina morsitans* is the most widespread species (Leak, 1999). The palpalis group is limited to the very humid areas of Africa, the mangrove swamps, the rain forest, the lakeshores and the gallery forests along rivers. They are found in the

more humid areas of West Africa. The fusca group species are widely scattered in forest areas throughout eastern parts of Africa (FAO, 1982).

2.3 Life cycle of tsetse flies

Tsetse flies have an unusual life cycle in that a fertilized egg is retained within the uterus such that the offspring develop internally during the first three larval stages, a strategy referred to as adenotrophic viviparity. During this time, the female feeds the developing offspring with a milky substance secreted by a modified milk gland in the female's reproductive accessory glands until it completes its development. In the third larval stage, the larva leaves the uterus and begins its independent life and is laid onto the soil. The new larva crawls into the ground, forms a puparial case in which it completes its morphological transformation into an adult fly. The larva relies on stored resources during this time. The importance of the richness of blood to this development can be seen since all development prior to the emergence from the puparial case as a full adult occurs without feeding. Female tsetse flies must therefore obtain enough energy for her needs, for the needs of her developing offspring, and to store the resources which her offspring will require until it emerges as an adult (FAO, 1982; WHO, 1988). Figure 1 represents a tsetse fly taking a blood meal from a host. The first mature offspring is produced when the female is about 16-17 days old from the time of emergence of the fly from the puparium (FAO, 1982) and subsequent progeny are produced maximally at approximately 9-day intervals at a temperature of about 25°C and 80% relative humidity. Tsetse flies are thus considered to have a low reproductive rate (Gooding and Krafsur, 2005).

2.4 Vector control methods

Tsetse fly control was carried out in the early days using methods that involved clearing of vegetation in large tracts of land, killing of wild game and installation of game fences to prevent the game hosts from carrying flies into the tsetse fly free areas. Insecticide-based control techniques, which involve ground and aerial spraying, were introduced to complement the methods employed to destroy the vector habitat (Budd, 1999). In view of environmental concerns regarding chemical use and the destruction of forests, these methods gave way to other techniques. Currently trypanosomosis is managed by the use of stationary tsetse fly traps and

targets, especially when odour-baited and impregnated with insecticides. The flies are attracted to visual cues provided by large expanses of blue or black cloth and chemical cues such as acetone, a tsetse fly-attracting component of cow's breath and also treatment of animals with insecticides (Wamwiri *et al.*, 2007). The major shortcomings of these methods lie in the limited size of area for which they can be economically deployed relative to the total size of the tsetse fly affected area and the continued costs associated with preventing re-invasion and also the fact that they do not lead to complete eradication (Budd, 1999).

Sterile insect technique (SIT) which is a genetic population suppression approach involves sustained and a systematic release of sterile male insects among the wild population has also been used to reduce tsetse fly populations. The sterile males fertilize wild females, which then are unable to produce progeny. The insects are mass reared in large-scale insectaries where the males are sterilized by irradiation and then taken to the targeted area and released by air. Sufficient sterile males need to be released in order to achieve an over-flooding ratio sufficient to cause a decline in population size. By continually releasing sterile males in these numbers over a period of three or four generations, the target population can be eradicated. While the low-reproductive rate of tsetse flies makes this a highly desirable approach, it has been criticized due to the relatively large costs that would be associated with its implementation (Aksoy, 2003). However, this strategy led to successful eradication of *G. austeni* from the island of Zanzibar using an integrated approach of population suppression through trapping and insecticide spraying followed by SIT. This has demonstrated the feasibility and applicability of this technology in area wide vector-control programs (Vreysen *et al*., 2000).

The use of trypanocidal drugs to control trypanosomosis is well established and represents the most widely used approach (d'Ieteren *et al.*, 1998). As a control measure, drug therapy strategies are currently protecting more cattle against the disease than any other method (Budd, 1999). However, the most serious setback in the use of drugs to control trypanosomosis is the increasing trend in drug resistance (Geerts *et al.,* 2001). A study by the International Livestock Research Institute (ILRI) and partner institutions discovered serious drug resistance in Kenedougou, a southern region of Burkina Faso (McDermott *et al.*, 2003). The use of trypanocides is increasingly left to farmers and extension workers rather than veterinary authorities, which raises

concerns regarding under dosing and the development of drug resistance to animal trypanocides (Matovu *et al.,* 1997).

The anticipated breakthrough in the development of vaccines to control trypanosomosis appears unattainable owing to the antigenic variation of trypanosomes and the complexity of their antigenic repertoire. New strategies that integrate several options in controlling trypanosomosis are being developed (FAO, 1998). There has been exploitation of trypanotolerant livestock as an option for trypanosomosis control but this strategy has been hampered by the reduced productivity of the animals (FAO, 2003). It is thus proposed that only a combination of several methods in an integrated pest management approach can lead to sustainable reductions of tsetse flies and eventually lead to a viable agriculture in vast regions of Africa (Holmes, 1997).

2.5 Blood meal analysis

Blood meal analysis has been conducted before, mainly using various serological techniques to generate information regarding the epidemiology of trypanosomosis and natural feeding habits of different species of *Glossina*. However, difficulties have been experienced in the identification of phylogenetically closely related species. With developments in molecular biology techniques, accuracy and the reliability of analytical methods have been enhanced for the identification of origins of blood meals in hematophagous insects. These techniques include heteroduplex analysis (HDA-PCR) based method which relies upon the mobility of the heteroduplex molecule formed by hybridizing sample and driver PCR products to give the identification. Thus, with an optimized heteroduplex driver and a collection of reference DNA samples, it is possible to identify the blood meal source using a single assay (Boakye *et al.,* 1999). However, in cases where reference DNA samples are not available identification could be difficult unless identity of products is further analyzed by DNA sequence determination. Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) has also been previously applied whereby a short conserved region of Cytb gene was applied for the identification of origins of blood meals in tsetse flies down to the species level (Steuber *et al*., 2005).

Information on the vertebrate hosts of tsetse flies is essential in understanding the relationship between hosts and vectors and consequently their roles in the disease transmission cycle (Tempelis, 1975). Identification of the host range of tsetse fly species is considered a prerequisite for successful tsetse fly and trypanosomosis control by aiding in the designing and implementation of effective integrated pest management (IPM) system.

Fig.1: Image of a tsetse fly taking a blood meal (Courtesy of University of California in Los Angeles)

2.6 Mitochondrial DNA

The mitochondrion is a cytoplasmic organelle of the eukaryotes that is involved in oxidative phosphorylation and the formation of ATP. Mitochondria contain their own genome, the mitochondrial DNA (mtDNA). Animal mtDNA is a small circular double stranded molecule with a size of about 15-20 kilo bases. It is composed of 37 genes coding for 13 proteins mainly involved in electron transport and oxidative phosphorylation, 22 transfer RNA genes responsible for translation of proteins and two ribosomal RNA genes (Boore, 1999). MtDNA genes have long dominated the field of molecular systematics because of their maternal inheritance, limited

recombination and rapid evolution, making them ideal markers for many species level questions (Avise *et al.,* 1987). In addition, mtDNA sequences occur in high copy numbers per cell and therefore, it suits situations where extracted DNA is low in quantity or significantly degraded, which is likely the case for extracts from tsetse fly blood meals. Different regions of the mtDNA evolve at different rates allowing suitable regions to be chosen for various studies (Saccone *et al.*, 1999). Robust primers also enable the routine recovery of specific segments of the mitochondrial genome (Folmer *et al.,* 1994; Simmons and Weller, 2001). Past phylogenetic work has often focused on mitochondrial genes encoding 12S or 16S rDNA, but their use in broad taxonomic analyses is constrained by the prevalence of insertions or deletions (indels) that greatly complicate sequence alignments (Doyle and Gaut, 2000).

DNA barcoding is a technique for characterizing species of organisms using a short DNA sequence from the COI. It provides an efficient method for species level identifications and thus is useful for taxonomic and biodiversity research (Hajibabaei *et al.,* 2007). The proof that DNA barcoding can distinguish at least some species has been provided by analysis of mitochondrial COI sequences among closely related species across diverse phyla in the animal kingdom (Hebert *et al.,* 2003b). The COI gene has advantages in that, the universal primers for this gene enable the recovery of its 5' end from representatives of most, if not all, animal phyla (Folmer *et al.,* 1994). In addition, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene. In common with other protein coding genes, its third-position nucleotides shows a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA (Knowlton and Weigt, 1998). In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species (Cox and Hebert, 2001; Wares and Cunningham, 2001). Although other mitochondrial genes in resolving cases of recent divergence may match COI, this gene is more likely to provide deeper phylogenetic insights because changes in its amino acid sequence occur more slowly than those in any other mitochondrial genes (Simmons and Weller, 2001).

Species identification through barcodes is achieved by the retrieval of a short DNA sequence from a standard part of the genome, from the specimen under investigation. Approximately 650 base fragment of the 5' end of the COI gene is the barcode region for animals (Hebert *et al.,*

2003b). Additionally, smaller fragments of about 100 bp (mini-barcodes) of the standard COI barcode have been shown to be effective for species identification in specimens whose DNA is degraded or in other situations where obtaining a full-length barcode is not feasible especially for museum specimens. Mini-barcodes may produce data comparable to full-length barcodes but somewhat less effective in discriminating among species in large assemblages (Hajibabaei *et al.,* 2006). Pilot projects have now established the effectiveness of this approach in several large groups of animals, such as fish (Ivanova *et al.,* 2007), spiders (Barrett and Hebert, 2005), Lepidoptera (Hebert *et al.,* 2004a; Janzen *et al.,* 2005) and in birds (Hebert *et al.,* 2004b).

The Cytb gene has been used for phylogenetic work and although it evolves slowly in terms of non-synonymous substitutions, the rate of evolution in silent positions is relatively fast (Irwin *et al.,* 1991). Cytb is thought to be variable enough for population level questions, and conserved enough for clarifying deeper phylogenetic relationships. However, it is under strong evolutionary constraints because some parts of the gene are more conserved than others due to functional restrictions (Meyer, 1994). Most of the variable positions seem to be located within the coding regions for transmembrane domains or in the amino- and carboxy-terminal ends. The mitochondrial Cytb gene is widely used in systematic studies to resolve divergences at many taxonomic levels. Blood meal analysis in the tick *Ixodes ricinus* (L.) (Acari:Ixodidae) was carried out based upon PCR of a 638 bp fragment of the Cytb gene encoded in the host vertebrate mitochondrion in combination with restriction fragment length polymorphism (RFLP) and hybridization analysis. Classification of tick blood meals was attained to the genus level (Kirstein and Gray, 1996). This study will therefore involve the application of COI and Cytb gene sequences in the analysis of tsetse fly blood meals from different regions.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

Tsetse fly collection was undertaken in Kenya, Uganda and Tanzania (Fig. 2) using biconical traps (Challier and Laveissie`re, 1973) baited with acetone (Torr *et al.,* 1997) and cow urine. In Kenya, samples were obtained from Busia, Suba and Kajiado districts. Tsetse flies (*G. fuscipes fuscipes*) from Mbita in Suba district were fed on Monitor lizards and these were used as standard controls for the applicability of the molecular approach. Blood fed *G. pallidipes* were trapped in Nguruman and Busia. Nguruman is located in Kajiado district, south-West Kenya and tsetse flies inhabit approximately 600 km^2. The vegetation consists of patches of woodland and thicket infested with two tsetse fly species, *G. pallidipes* and *G. longipennis*, with the former being the predominant species. In Nguruman, lowland woodland patches are surrounded by open savannah (Brightwell *et al.,* 1997) and there is abundant game in Nguruman (Tarimo-Nesbitt *et al.,* 1999). Busia on the other hand is moderately settled with small-scale mixed agricultural activities and is infested by *G. f. fuscipes* and *G. pallidipes*. There are no game reserves and wildlife is scarce (Wamwiri *et al.,* 2007). Samples from Serengeti National Park (Tanzania) and Uganda were obtained through collaborators based in these countries.

3.2 Sample collection

Trapped tsetse flies were sorted out and those with blood meals in their midguts were preserved in absolute ethanol and stored at –20 ^{o}C until used. In other cases, a pair of forceps was used to pull the gut out of the abdomen of blood fed flies, so that the blood meal could be spread onto a section of a disc of Whatman® filter paper or Flinders Technology Associates (FTA) classic cards. After air-drying, they were stored in air tight desiccators with silicon beads and stored at 4°C.

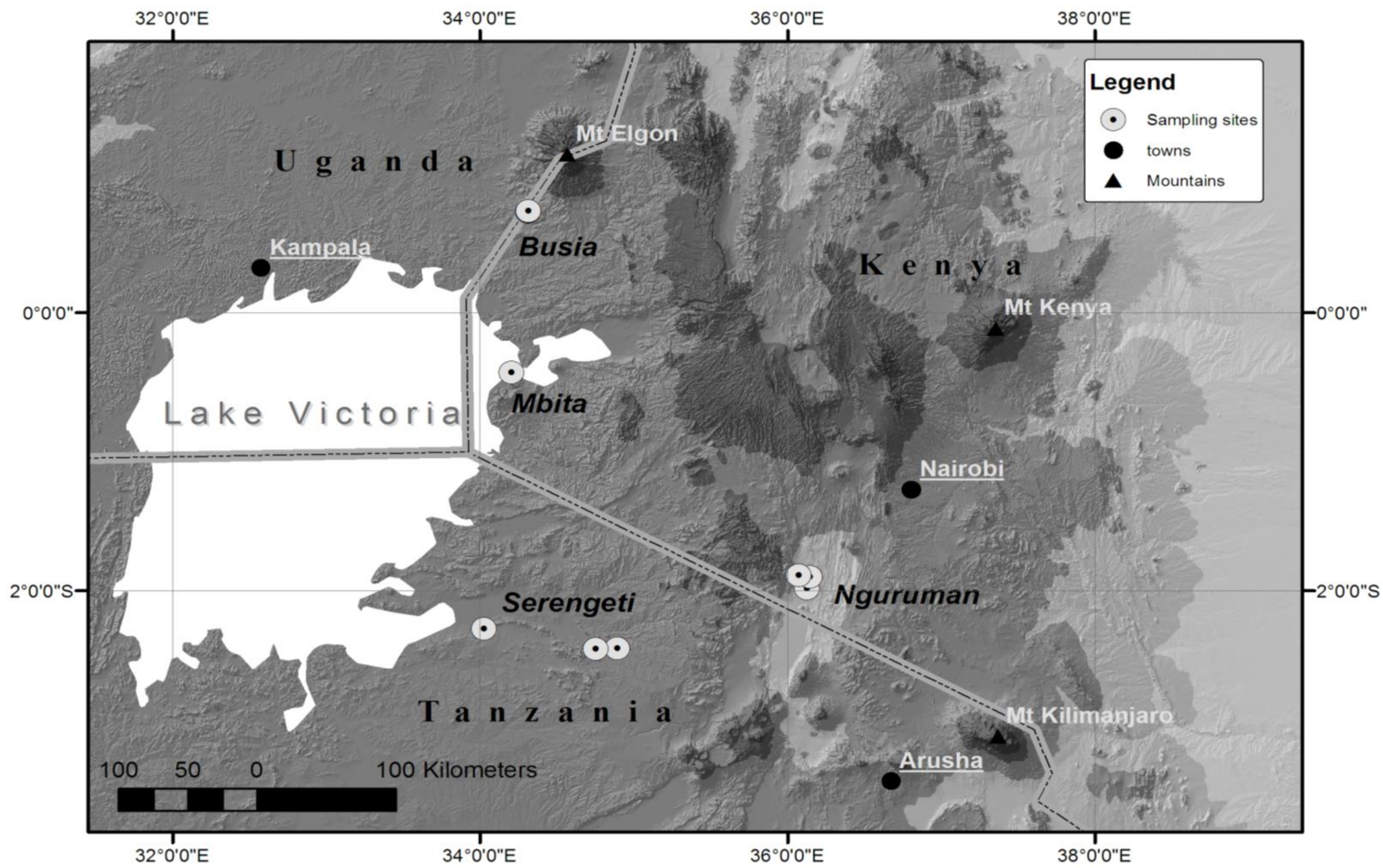

Fig. 2: Map showing sampling sites in East Africa

3.3 DNA extraction

Host genomic DNA was extracted from the midgut of blood fed ethanol preserved tsetse flies using QIAGEN DNeasy extraction kits (Qiagen inc., Valencia, CA, USA), following the manufacturer's instructions. The quality of DNA extracted using the DNeasy kit was better compared to that extracted from either FTA classic cards or filter papers.

DNA was also extracted from spotted Flinders Technology Associates (FTA®) classic cards (Whatman no. 1®). Briefly, five 1.2 mm discs were punched out of the spotted area and placed in a 1.5 ml tube. Four hundred micro litres FTA® purification reagent was added and the mixture incubated at room temperature for 30 min, vortexing was done twice between this period that is after 10 min and after 20 min. The FTA® reagent was then pipetted out and discarded. This was repeated twice for a total of three washes. Then a 300 µl aliquot of Tris-ethylene diamine tetra-acetic acid (TE) buffer (10Mm Tris-HCl, 0.1 mM EDTA, pH 8.0) was added to the discs and incubated at room temperature for 15 min, vortexing was done twice between this period that is after 5 min and after 10 min. TE buffer was then pipetted out and discarded. This was repeated once for a total of two washes. The discs containing the DNA were then incubated overnight at 37°C for them to dry completely, before using a quarter of the disc directly for PCR.

DNA was also extracted from spotted filter papers (Whatman no. 1®) using the chelex® 100 method (Walsh *et al.,* 1991) whereby approximately 10-20 punches were made on the spotted area and placed in a 1.5 ml tube and 500 µl of distilled water was added and kept at room temperature for one hour, vortexing was done after every 20 min. The supernatant was pipetted out and 200 µl of 5% Chelex®-100 (Bio- Rad laboratories, Hercules, California, USA) was added and the tubes were incubated in a water bath at 56°C for 2 hours. This was followed by 20 min incubation at 99°C in the heating block. After cooling, the samples were vortexed briefly and centrifuged at 12,000 rpm for 3 min. The supernatant was used as template in PCR reactions.

3.4 DNA amplification by polymerase chain reaction

Amplifications were carried out for COI using primers with a broad target group including mammals, reptiles and fishes (Ivanova *et al.,* 2007). Typical conditions for COI amplification

were as follows: Initial denaturation was done for 2 min, at 94°C, followed by two steps PCR of 20 cycles each of denaturation at 94°C for 30s, annealing at 45°C for 45 sec, and primer extension at 72°C for 1 min with a final extension at 72°C for 10 min. PCR was carried out for a total volume of 25 µl and the final concentration of each component was as follows; 10 pmoles of each primer, 1X PCR buffer comprising of (10mM Tris-HCl, pH 8.3 and 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 2 µl of the DNA template and 1unit *Taq* DNA polymerase (Genescript corporation). The primers used for COI amplification were the last two in the table below, which represent the forward and reverse primers and their sequences are given.

The primers used for amplification of the 359 bp fragment of the Cytb gene were the first two, which represent the forward and reverse primers (Table 1). PCR was carried out for a total volume of 25 µl and the final concentration of each component was as stated above. Typical conditions for Cytb amplification were as follows: Initial denaturation was done for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 45 sec, and primer extension at 72°C for 1 min with a final extension at 72°C for 10 min.

Table 1: PCR primers for amplification of partial COI and Cytb genes of different tsetse fly hosts.

Primer identity	Primer sequence 5'-3'	Target group	Reference
Cb1	CCATCCAACATCTCAGCATGATGAAA	Mammals	Kocher *et al.,* (1989)
Cb2	GCCCCTCAGAATGATATTTGTCCTCA	Mammals	Kocher *et al.,* (1989)
VF1d_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG	Mammals, reptiles and fishes	Ivanova *et al.,* (2007)
VR1d_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA	Mammals, reptiles and fishes	Ivanova *et al.,* (2007)

3.5 Agarose gel electrophoresis

A 1.5% Agarose gel (w/v) was prepared by dissolving 1.5g of agarose powder into 100 ml of 1X Tris-acetate ethylene diamine tetra-acetic acid (TAE) buffer. The gel solution was stirred, brought to boil in a microwave for 2 min to completely dissolve the powder then the cooled gel solution was poured into a gel casting tray. Ethidium bromide (50mg/µl) was incorporated in the gel to facilitate visualization of DNA under ultra violet (UV) light. PCR product was mixed 5:1 with the Blue/Orange 6X loading buffer which comprises of 15% Ficoll®400, 0.03% Bromophenol blue, 0.03% xylene cyanol FF, 0.4% Orange G, 10mM Tris-HCl (pH 7.5) and 50mM EDTA. A 100bp plus ladder (Fermentas) molecular size marker was loaded in the first well to co-migrate with the samples in order to determine the size of the fragments. A voltage of 80 volts was applied and the samples ran for 1 hour.

3.6 DNA precipitation

For the samples that had low genomic DNA concentration as observed after agarose gel electrophoresis, a further precipitation using ethanol to concentrate the DNA was undertaken. Briefly, one tenth of 3M sodium acetate, pH 5.2 was added to the DNA solution in 1.5 ml tubes and mixed by vortexing. Then 2.5 volumes of ice-cold 100% ethanol was added, vortexed and kept at -20°C for 1 hour. The samples were then centrifuged at 12,000 rpm for 20 min and the ethanol was pipetted out. The pellet was washed with 70% ethanol, centrifuged at 12,000 rpm for 5 min and ethanol was discarded. DNA was dried for 1 hour in the incubator at 37°C with the tubes inverted. DNA was then dissolved in 30 µl of TE buffer (10Mm Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20°C until used.

3.7 DNA purification

DNA purification before sequencing was carried out either through gel extraction by excising the DNA of the expected band size from the gel or by direct purification of the PCR products. In both cases the appropriate kits were employed to clean up DNA. For gel extraction, QuickClean 5M gel extraction kit was used (GenScript Corporation) and Quickclean 5M PCR purification kit (Genscript Corporation) was used for purification of the PCR products.

3.7.1 Gel extraction

Briefly, DNA band was excised from the gel using a clean scalpel blade and the gel slice placed in a preweighed tube to determine the weight of the gel slice. Three volumes of binding solution II was added to the gel slice and placed in a water bath at 50°C with occasional vortexing for 10 min to dissolve it. One volume of isopropanol was added and the mixture was transferred to QuickClean column and centrifuged at 12,000 rpm for 30 sec. The flow through was discarded. Then 500 µl of wash solution was added to the column and centrifuged at 12,000 rpm for 1 min. This was repeated once for a total of 2 washes. The column was transferred to a clean 1.5 ml micro centrifuge tube. Thirty micro litres of elution buffer was added to the centre of the column and incubated at room temperature for 3 min. Finally, the mixture was centrifuged at 12,000 rpm for 1 min to elute and collect DNA. A 1% agarose gel electrophoresis of 5µl of each of the purified DNA sample was carried out to confirm the recovery of the purified DNA. This was then ready for sequencing. Gel purified DNA samples that were sequenced gave quality chromatographs as compared to the PCR products that were purified directly using the PCR purification kit and subsequently sequenced.

3.7.2 PCR product purification

The PCR products were purified of primers, unused nucleotides and salts using the Quickclean 5M PCR purification kit (Genscript Corporation). The protocol was as follows.
Fifty micro litres of binding solution I was mixed with 20 µl of each of the PCR products in a 1.5 ml tube and then transferred into a quick spin column with a collection tube, spun for 60 sec at 12,000 rpm in a centrifuge to bind the DNA onto the matrix. The flow through was discarded. Then 500 µl of ethanol-based wash solution was added to the column and spun for 30 sec at 12,000 rpm to wash the bound DNA. The flow through was discarded and washing step was repeated for a total of two washes. Samples were centrifuged for an additional 60 sec at 14,000 rpm to remove any residual of wash solution. The spin column was then placed in a clean 1.5 ml tube and 30 µl EB buffer was added at the centre of the column membrane. This was allowed to stand for 3 min and then centrifuged for 60 sec at 12,000 rpm to elute and collect purified DNA ready for sequencing.

3.8 DNA sequencing

DNA sequencing involves the determination of the nucleotide sequence in a sample of DNA. The advantage of sequencing methods coupled with PCR is that little quantities of DNA template are required like hairs (Morin *et al.,* 1992). There are two methods of determining the DNA sequence. One approach by Maxam and Gilbert (1977) relies on chemical cleavage reactions specific to individual bases. This method is not frequently used but it has an advantage in that it permits direct sequencing of small fragments. The other method by Sanger *et al.,* (1997) is based on controlled interruption of *in vitro* DNA replication. The Sanger method which is also referred to as the dideoxynucleotide chain termination method is the basis of the ABI 3730 DNA analyzer. In this method, there are synthetic nucleotides that lack the hydroxyl group at the 3' carbon atom therefore during DNA synthesis a dideoxynucleotide can be added to the growing DNA strand causing the chain elongation to stop due to lack of a free 3'-hydroxyl group for the next nucleotide to attach. The ratio of deoxynucleotides to dideoxynucleotide is high thus enhancing several nucleotides to be added to the growing DNA strand before insertion of a dideoxynucleotide which stops the replication process. The fragments are then separated by length on a gel giving individual bands. Each of the four dideoxynucleotides fluoresces in a different colour when illuminated by a laser beam and an automatic scanner provides a print out of the sequence.

The purified products that had a DNA concentration of at least 50 ng/µl were sequenced to determine the nucleotide sequence. Sequencing was done using the Big Dye® Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, USA) which is based on the dideoxynucleotide chain termination method (Sanger *et al.,* 1997). The reactions were analyzed using ABI 3730 DNA analyzer. The sequencing primers were the respective forward and reverse primers used for COI and Cytb amplifications.

3.9 Data analysis

Nucleotide sequences were edited using BioEdit, Version 7.0.9 (Hall, 1999). By using the software, chromatograms were viewed and manually edited. Matching of each forward sequence with the reverse compliment of the reverse sequence enabled better viewing of gaps and

mismatches and these were corrected on the chromatograms. The start of each forward sequence had a lot of background noise, those sequences were deleted and the sequences on the reverse compliment that were more reliable were adopted. The same case applied for reverse compliment sequence whereby the last few sequences were unreliable due to background noise and they were deleted and those of the forward sequence were used to fill up the gaps. Each of the chromatogram sequences was copied in fasta format and pasted as a word document with the details of sample name and orientation of sequencing as either forward or reverse direction. The edit - find tool in Microsoft word was used to check for sequence similarity. The forward sequences and reverse compliment sequences were assembled into a consensus sequence for each analyzed sequence and both primer sequences were trimmed. This was done for all the sequences in which both partial COI and Cytb genes had been sequenced.

The obtained COI consensus sequences of approximately 658 bp without both forward and reverse primer sequences were queried in barcode of life data systems-identification engine (BOLD-ID) site and a BLAST (Basic Local Alignment Search Tool) search was carried out for comparison of the sequences obtained with all the sequences available in the database. A species was identified if its sequence closely matched one in the databases giving the highest percentage sequence similarity and identity up to the species level.

Cytb consensus sequences of about 307bp without both forward and reverse primer sequences were submitted in fasta format to National Centre for Biotechnology Information (NCBI) and a nucleotide BLAST search with parameters selected as megablast, for highly similar sequences. This was carried out for comparison of the sequences obtained with those sequences present in GenBank. A species was identified if its sequence closely matched one in GenBank by yielding the highest percentage sequence similarity and the lowest e-value. Identification was made to the species level in all the queried sequences.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 PCR and agarose gel electrophoresis

Amplifications of COI and Cytb genes were carried out on the extracted genomic DNA and this was followed by an agarose gel electrophoresis for confirmation of the amplifications. The COI amplifications gave the expected band size of approximately 700bp in about sixty four samples from a total of ninety samples that were analyzed from all the sampling sites. The figures of gel photos shown were obtained after several PCR optimization reactions which involved modifying the annealing temp, $MgCl_2$ concentration and the number of cycles. The gel photos are representatives of samples obtained from different localities whose COI and Cytb genes were amplified. Tsetse fly blood meals from Uganda had been spotted on filter papers and genomic DNA extractions from the samples was not successful and subsequently their amplifications. This was because the amount of blood meal present in the midguts of the trapped tsetse flies was little and DNA extraction of samples spotted on filter papers require sufficient amount of tsetse fly blood meal.

4.1.1 Blood meal identification using COI

As shown in Figure 3, eight blood fed tsetse fly samples (*G. fuscipes fuscipes*) from Mbita had DNA extracted from their blood meals and were amplified using the COI primers. Six samples were successfully recovered and sequenced. These tsetse flies had been fed on monitor lizards and were used to standardize the process of using COI to identify blood meals sources present in the tsetse fly midguts. Positive COI amplicons were sequenced and gene sequences analyzed. The sequences were queried in BOLD-ID and as expected, gave an output as *Varanus niloticus* (Monitor lizard) as shown in Table 2.

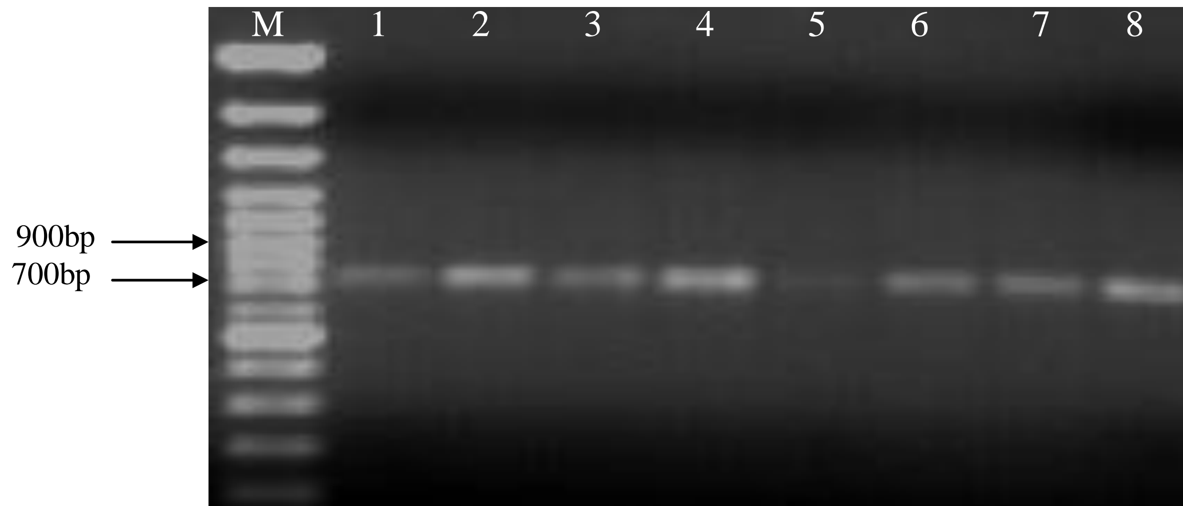

Fig 3: 1.5% Agarose gel electrophoresis for COI amplified *G. fuscipes fuscipes* blood meals from Mbita. M- 100bp plus DNA ladder (Fermentas). Lanes 1-8: sample DNA with sample identity as Mb1-Mb8 (~700 bp).

As shown in Figure 4, eighteen partially blood fed tsetse fly samples (*G. swynertonni*) from Tanzania had DNA extracted from their blood meals and were amplified using the COI primers. Gel extraction was carried out using the Gel extraction kit (Genscript Corporation) and subsequently DNA samples were sequenced. Sequence analysis and blast search results yielded identity as *G. swynertonni* (as shown in table 3) indicating that only insect DNA had been amplified instead of tsetse fly host DNA.

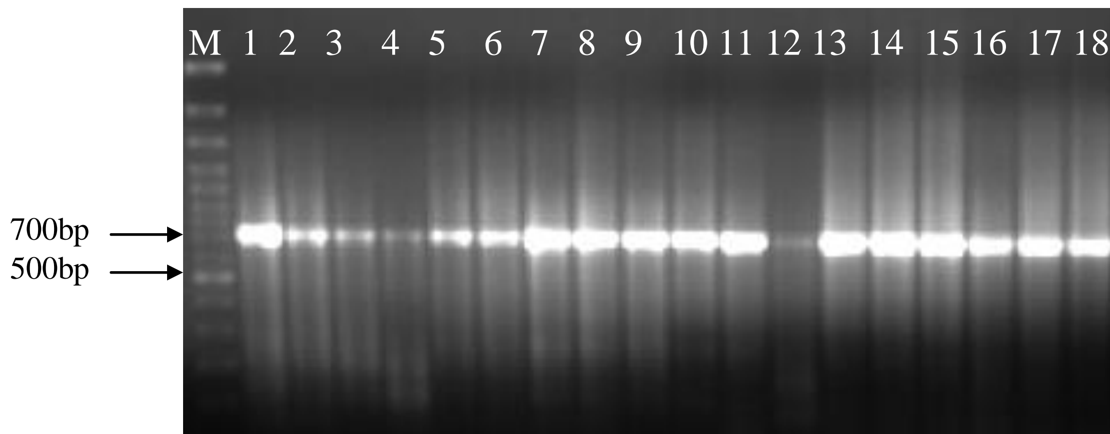

Fig 4: 1.5% Agarose gel electrophoresis for COI amplified *G. swynertonni* DNA from Tanzania. M- 100bp plus DNA ladder (Fermentas). Lanes 1-18 sample DNA with sample identity as TP11-TP80 (~700 bp).

Twenty partially blood fed tsetse fly samples (*G. pallidipes*) from Busia had DNA extracted from their blood meals and only twelve gave positive COI amplifications (Figure 5) possibly because the recovery of genomic DNA was poor where flies had very little of their last blood meal still present. The origins of their blood meals were determined after sequence analysis which indicated that they had fed on cattle blood meals as shown in Table 2.

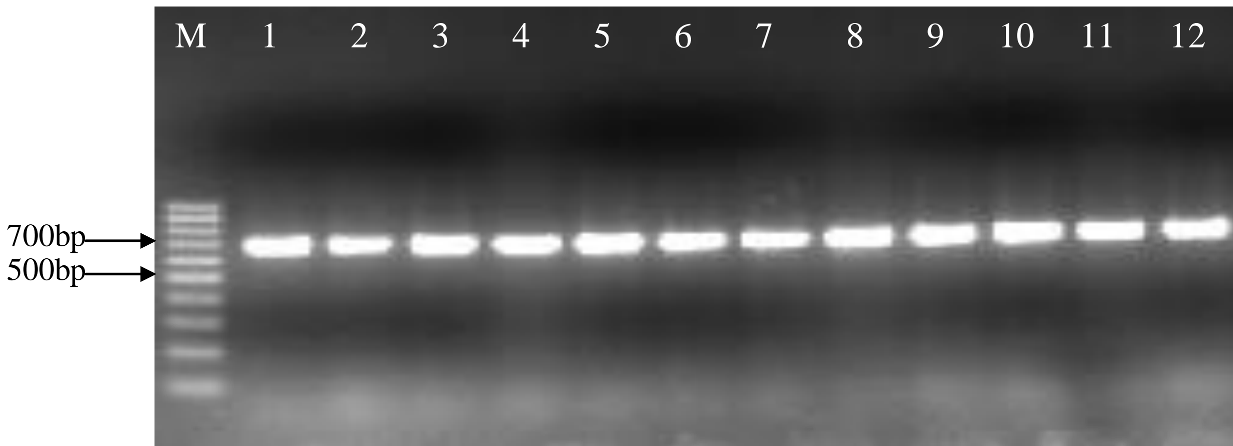

Fig 5: 1.5% Agarose gel electrophoresis for COI amplified *G. pallidipes* blood meals from Busia. M- 100bp plus DNA ladder (Fermentas). Lanes 1-12 sample DNA whose sample identity was BS4, BG4, KA1 and AKP-KKP (~700 bp).

4.1.2 Blood meal identification using Cytb

Samples from Nguruman had very little of their last blood meals still present. The blood meals were squashed on FTA classic cards and extractions were later carried out as described on section 3.2. Amplifications were carried out using the Cytb primers and later agarose gel electrophoresis for confirmation of the amplifications (Figure 6). Positive amplicons were purified and subsequently sequenced. The results of sequence analysis and blast search in GenBank gave identity as buffalo, warthog, elephant and baboon as shown in Table 4.

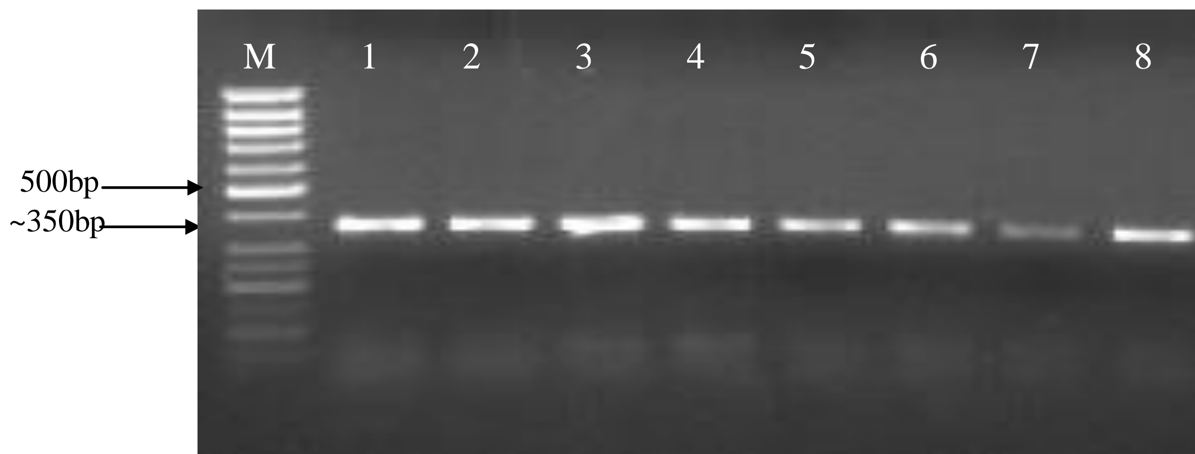

Fig 6: 1.5% Agarose gel electrophoresis for Cytb amplified *G. pallidipes* blood meals from Nguruman. M- 100bp plus DNA ladder (Fermentas). Lanes 1-8 sample DNA whose sample identity was NG21-OT15 (~350 bp).

Most of the Cytb amplified DNA from *G. swynertonni* blood meals sampled from Tanzania gave good amplifications with the vertebrate specific Cytb PCR primers (Figure 7). Gel purification and sequencing was done. Sequence analysis was later carried out giving various vertebrate hosts as shown in Table 4.

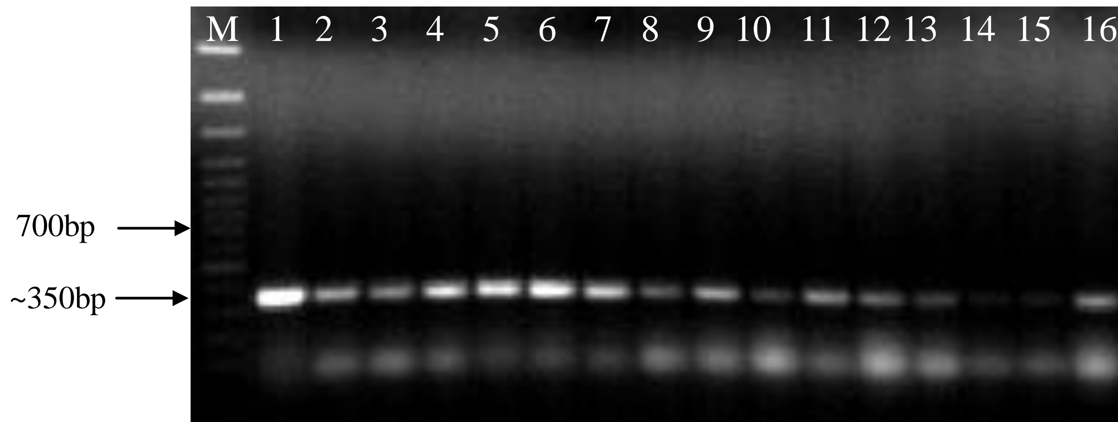

Fig 7: 1.5% Agarose gel electrophoresis for Cytb amplified *G. swynertonni* blood meals from Tanzania. M- 100bp plus DNA ladder (Fermentas). Lanes 1-16: sample DNA whose sample identity was TP15-TP93 (350 bp).

4.2 Sequence analysis

The sequences obtained for each sample were edited using BioEdit version 7.0.9 (Hall, 1999) and a consensus sequence was obtained by assembling sequences from both the forward and reverse sequences and eliminating the primer sequences. Each of the test COI consensus sequences was used to query the barcode of life data systems identification engine (http://www.barcodinglife.org) whereby each sequence was matched to the closest sequence in the database and identity was given to the species level as shown in Table 2 and 3.

In the case of Cytb sequences, each of the consensus sequences was subjected to a nucleotide blast search in GenBank at NCBI. Nucleotide sequence matches gave identity of the vertebrate host species to the species level as illustrated in Table 4 below. All the sequence searches were carried out between 31st March and 1st April 2009.

Table 2: Search results obtained from the barcode of life data systems-identification engine (BOLD-ID) for samples from different sampling sites in Kenya whose partial COI gene was amplified. The sequences of the vertebrate host species listed matched closely with those present in the database and their respective percentage similarities were as stated in the table below.

Sampling Sites	Sample name	Genus and species	Common name	% Similarity
Nguruman	NG25	*Loxodonta africana*	Elephant	99.39
Nguruman	NG33	*Loxodonta africana*	Elephant	98.47
Nguruman	OT15	*Phacochoerus africanus*	Warthog	100
Nguruman	SN1	*Loxodonta africana*	Elephant	99.39
Nguruman	SN8	*Phacochoerus africanus*	Warthog	99.85
Mbita point	Mb1	*Varanus niloticus*	Monitor lizard	97.29
Mbita point	Mb2	*Varanus niloticus*	Monitor lizard	97.29
Mbita point	Mb4	*Varanus niloticus*	Monitor lizard	97.29
Mbita point	Mb5	*Varanus niloticus*	Monitor lizard	97.29
Mbita point	Mb6	*Varanus niloticus*	Monitor lizard	97.29
Mbita point	Mb8	*Varanus niloticus*	Monitor lizard	97.29
Busia	BS4	*Bos taurus*	Cattle	99

Busia	BG4	*Bos taurus*	Cattle	99
Busia	KA1*	*Bos taurus*	Cattle	95
Busia	AKP	*Bos taurus*	Cattle	96.12
Busia	BKP	*Bos taurus*	Cattle	97.16
Busia	CKP	*Bos taurus*	Cattle	100
Busia	EKP	*Bos taurus*	Cattle	97.74
Busia	FKP	*Bos taurus*	Cattle	99.44
Busia	GKP	*Bos taurus*	Cattle	99.21
Busia	HKP	*Bos taurus*	Cattle	100
Busia	IKP	*Bos taurus*	Cattle	100
Busia	KKP	*Bos taurus*	Cattle	100

The reverse sequencing of sample KA1* from Busia failed and only the forward sequence was used to query the BOLD data base thus accounting for the low percentage of sequence similarity as shown in the table above. In all the other samples, a consensus sequence was obtained from assembling edited sequences from both the forward and reverse direction and then queried in the data base to give output as stated above.

Table 3: Search results obtained from the barcode of life data systems identification engine (BOLD-ID) for Serengeti samples whose partial COI gene was amplified. The generated sequences were matched closely with those present in the database and their respective percentage similarities were as stated in the table below.

Sampling Sites	Sample name	Genus and species name	Common name	% Similarity
Serengeti ecosystem	TP 11	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP14	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP 15	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP 21	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP24	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP34	*Glossina swynnertoni*	Tsetse fly	99.49
Serengeti ecosystem	TP41	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP44	*Glossina swynnertoni*	Tsetse fly	99.83
Serengeti ecosystem	TP50	*Glossina swynnertoni*	Tsetse fly	99.83
Serengeti ecosystem	TP56	*Glossina swynnertoni*	Tsetse fly	99.83
Serengeti ecosystem	TP58	*Glossina swynnertoni*	Tsetse fly	99.78
Serengeti ecosystem	TP59	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP64	*Glossina*	Tsetse fly	99.83

		swynnertoni		
Serengeti ecosystem	TP65	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP66	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP39*	*Glossina swynnertoni*	Tsetse fly	95.75
Serengeti ecosystem	TP76	*Glossina swynnertoni*	Tsetse fly	99.32
Serengeti ecosystem	TP80	*Glossina swynnertoni*	Tsetse fly	99.49
Serengeti ecosystem	TP86	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP88	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	TP93	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA342	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA343	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA354	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA356	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA358	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA369	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA372	*Glossina swynnertoni*	Tsetse fly	100

Serengeti ecosystem	HA376	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	HA378	*Glossina swynnertoni*	Tsetse fly	99.66
Serengeti ecosystem	HA388	*Glossina swynnertoni*	Tsetse fly	99.83
Serengeti ecosystem	DV429	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV449	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV452	*Glossina swynnertoni*	Tsetse fly	99.32
Serengeti ecosystem	DV457	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV489	*Glossina swynnertoni*	Tsetse fly	99.49
Serengeti ecosystem	DV496	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV501	*Glossina swynnertoni*	Tsetse fly	99.83
Serengeti ecosystem	DV618	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV620	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV622	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV624	*Glossina swynnertoni*	Tsetse fly	99.66
Serengeti ecosystem	DV635	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV637	*Glossina*	Tsetse fly	99.83

		swynnertoni		
Serengeti ecosystem	DV639	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV645	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV650	*Glossina swynnertoni*	Tsetse fly	100
Serengeti ecosystem	DV454	*Glossina swynnertoni*	Tsetse fly	99.49
Serengeti ecosystem	DV474	*Glossina swynnertoni*	Tsetse fly	100
Nguruman	OT14	*G. pallidipes*	Tsetse fly	99.66

The forward sequencing of sample TP39* from Serengeti failed and only the reverse sequence was used to query the BOLD data base thus accounting for the low percentage of sequence similarity as shown in the table above. In all the other samples, a consensus sequence was obtained from assembling edited sequences from both the forward and reverse direction and then queried in the data base to give output as stated above.

Table 4: A nucleotide blast search result at NCBI for the Cytb amplified samples from different sampling locations. The sequences of the vertebrate host species listed matched closely with those in the databases and their respective percentage similarities were as stated below.

Sampling Sites	Sample name	Genus and species name	Common name	% Similarity	e-value
Serengeti ecosystem	TP 15	*Syncherus caffer*	Buffalo	98	1e-152
Serengeti ecosystem	TP23	*Syncherus caffer*	Buffalo	99	4e-156
Serengeti ecosystem	TP24	*Syncherus caffer*	Buffalo	99	2e-154
Serengeti ecosystem	TP29	*Syncherus caffer*	Buffalo	99	2e-154
Serengeti ecosystem	TP34	*Syncherus caffer*	Buffalo	99	4e-156
Serengeti ecosystem	TP46	*Syncherus caffer*	Buffalo	99	4e-156
Serengeti ecosystem	TP50	*Syncherus caffer*	Buffalo	98	1e-152
Serengeti ecosystem	TP58	*Syncherus caffer*	Buffalo	99	2e-154
Serengeti ecosystem	TP59	*Syncherus caffer*	Buffalo	98	4e-151
Serengeti ecosystem	TP64	*Syncherus caffer*	Buffalo	99	4e-156
Serengeti ecosystem	TP65	*Syncherus caffer*	Buffalo	98	2e-159
Serengeti ecosystem	TP66	*Syncherus caffer*	Buffalo	98	2e-159
Serengeti ecosystem	TP78	*Syncherus caffer*	Buffalo	99	6e-150

Serengeti ecosystem	TP86	*Syncherus caffer*	Buffalo	99	4e-156
Serengeti ecosystem	TP88	*Syncherus caffer*	Buffalo	97	1e-147
Serengeti ecosystem	TP93	*Syncherus caffer*	Buffalo	98	1e-152
Serengeti ecosystem	TP39	*Syncherus caffer*	Buffalo	98	3e-148
Serengeti ecosystem	TP41	*Syncherus caffer*	Buffalo	99	2e-154
Serengeti ecosystem	TP44	*Syncherus caffer*	Buffalo	98	1e-152
Serengeti ecosystem	HA343	*Syncherus caffer*	Buffalo	99	4e-156
Serengeti ecosystem	HA358	*Syncherus caffer*	Buffalo	99	2e-154
Serengeti ecosystem	DV449	*Syncherus caffer*	Buffalo	99	1e-157
Serengeti ecosystem	DV474	*Syncherus caffer*	Buffalo	98	2e-154
Serengeti ecosystem	DV637	*Syncherus caffer*	Buffalo	99	1e-157
Serengeti ecosystem	DV650	*Syncherus caffer*	Buffalo	99	2e-154
Serengeti ecosystem	HA366*	*Giraffa camelopardalis tippelskirchi*	Giraffe	93	4e-111
Serengeti ecosystem	HA376	*Giraffa camelopardalis tippelskirchi*	Giraffe	97	1e-152
Serengeti ecosystem	DV489	*Giraffa*	Giraffe	100	7e-159

		camelopardalis tippelskirchi			
Serengeti ecosystem	DV618	*Giraffa camelopardalis tippelskirchi*	Giraffe	99	6e-150
Serengeti ecosystem	DV622	*Giraffa camelopardalis tippelskirchi*	Giraffe	96	3e-128
Serengeti ecosystem	DV635	*Giraffa camelopardalis tippelskirchi*	Giraffe	99	7e-154
Serengeti ecosystem	DV639	*Giraffa camelopardalis*	Giraffe	91	4e-117
Serengeti ecosystem	DV645*	*Giraffa camelopardalis tippelskirchi*	Giraffe	99	7e-154
Serengeti ecosystem	DV454	*Giraffa camelopardalis tippelskirchi*	Giraffe	100	7e-159
Serengeti ecosystem	Tp11	*Phacocherus africanas*	Warthog	96	8e-139
Serengeti ecosystem	HA381	*Phacocherus africanas*	Warthog	99	3e-157
Serengeti ecosystem	DV620*	*Phacocherus africanas*	Warthog	91	3e-108
Serengeti ecosystem	HA356	*Loxodonta africana*	Elephant	100	7e-149
Serengeti ecosystem	DV496	*Loxodonta africana*	Elephant	100	1e-166
Serengeti ecosystem	DV457	*Loxodonta cyclotis*	Elephant	100	7e-159

Serengeti ecosystem	DV501	*Crocuta crocuta*	Spotted hyena	99	2e-155
Nguruman	NG21	*Syncherus caffer*	Buffalo	98	7e-180
Nguruman	NG22	*Phacochoerus africanus*	Warthog	97	1e-151
Nguruman	NG25	*Loxodonta africana*	Elephant	99	0.0
Nguruman	NG26	*Loxodonta africana*	Elephant	99	0.0
Nguruman	NG28	*Papio hamadryas*	Baboon	97	1e-172
Nguruman	SN1	*Loxodonta africana*	Elephant	99	0.0
Nguruman	SN8	*Phacochoerus africanus*	Warthog	99	1e-177
Nguruman	OT15	*Phacochoerus africanus*	Warthog	99	1e-177

The quality of sequences of samples HA366*, DV620* and DV645* was poor because the quality of the DNA that was recovered after purification was low thus accounting for their low percentage of sequence similarity as shown in the table above. In all the other samples, a consensus sequence was obtained from assembling edited sequences from both the forward and reverse direction and then queried in the data base to give output as stated above.

For the tsetse fly (*G. pallidipes*) samples from Nguruman it was evident that the blood meal sources were mainly from the wild animals with *Loxodonta africana* (elephants), *Phacochoerus africanus* (warthogs), *Syncherus caffer* (buffaloes) and *Papio hamadryas* (baboon) as the important hosts for tsetse fly population in that area. In this study, warthogs and elephants accounted for the highest number of tsetse fly host species.

Blood-fed tsetse fly (*G. pallidipes*) samples were obtained from Busia and analysis of their blood meals gave an output as *Bos taurus* (cattle) which is an indication that livestock kept by the Busia inhabitants play an important role in the survival of the tsetse fly populations in the area.

Fifty partially blood fed tsetse flies (*G. sywnertonni*) from Tanzania had DNA extracted from their midguts and amplifications were carried out for both the COI and Cytb genes. DNA sequencing was carried out after gel purification and the sequences obtained for both genes yielded varying results. All the fifty sequences for COI amplification gave species identification in BOLD-ID as *G. swynertonni* as shown in table 3. For Cytb sequences, nucleotide blast search gave identification of tsetse flies hosts as wild animals in the Serengeti ecosystem which included *Syncherus caffer* (African buffalo), *Phacochoerus africanus* (warthog), *Loxodonta africana* (elephants), *Giraffa camelopardalis tippelskirchi* (giraffe) and C*rocuta crocuta* (spotted hyena) as shown above (Table. 4).

4.3 Discussion

The present study applied molecular markers as a method for accurate identification of vertebrate host species of tsetse flies through analysis of their blood meals. For identification purposes we chose to use both COI and Cytb genes. Amplification of COI gene was by the use of primers described by Ivanova *et al.,* (2007) designed to amplify the locus from mammals, fishes and reptiles. The generic primers were chosen since the target group would cover the species that previous studies had shown to be hosts for tsetse flies. Amplifications with these primers worked well for most of the samples. However, COI amplification of the blood meals from *G. swynnertoni* collected in Serengeti failed.

A short variable region of the mitochondrial Cytb gene was amplified using universal primers complementary to a conserved region of the gene (Kocher *et al.,* 1989). Previous studies had shown that the amplified sequence of the Cytb gene was divergent enough to distinguish between different hosts (Kocher *et al.,* 1989; Bartlett and Davidson, 1992). The advantage of using Cytb gene is the availability of its nucleotide sequence entries from a constantly growing number of vertebrate's sequences in the public databases (Parson *et al.,* 2000) allowing rapid sequence matches for identification purposes.

For COI gene, DNA barcodes have proved to be excellent tools for identification of species whose sequences are already in the COI library. Thus, the prospects of identifying a sequence to the correct genus or species group are not good unless a near perfect match is already represented in the DNA sequence library (Torbjørn *et al.,* 2007). This was incidentally confirmed by the results of the identification requests made via the BOLD-ID site (http://www.barcodinglife.org) in April 2009 for a sample obtained from Nguruman where identification was made to the species level as *Phacochoerus africanus* (warthog) with a 100% sequence similarity. The fact that a perfect match was found between the COI sequence of a pre-identified specimen sequence already present in BOLD is clearly a success for the current state of biotechnology and a demonstration of the potentials of identification via barcoding. A similar perfect sequence match was observed using the generated Cytb sequences for five samples from Tanzania with three samples being identified as elephant and the rest as giraffe in GenBank. Therefore, if a species is

represented in a DNA sequence library, there is a high probability for correct identification (Torbjørn *et al.,* 2007).

PCR optimization conditions were necessary to ensure that the amplified DNA was from the blood meal and not from the insect itself. However, *G. sywnertonni* was given as identity for fifty samples from Tanzania and one from *G. pallidipes* caught in Nguruman. This case was only observed for the Ivanova primers for COI amplification whose target group did not include insects yet the primers amplified DNA from the tsetse flies. It was likely that the generic primers used matched better to the tsetse fly DNA than that from the blood meal. This could have been a more significant problem since the amount of blood meal was very small. Therefore, more specific primers should be used for the identification of blood meal sources in tsetse flies and other hematophagous insects since the host DNA in the blood meal is likely to be contaminated with insect DNA. Genomic DNA extraction method should also be carried out in a way that limits contamination parts of the insect itself. The fact that the COI amplicons that were sequenced for the samples from Nguruman and Tanzania gave an output with the correct identification of the tsetse fly species further adds to the pool of knowledge that DNA barcodes can be used for species identification. The barcode data obtained for *G. swynertonni* and *G. pallidipes* will be uploaded to the BOLD database.

The major problem of trapping wild tsetse flies for blood meal analysis is that the traps used as sampling tools are biased towards hungry flies (Dransfield and Brightwell, 1992) and therefore field samples of tsetse flies usually contain little of their last blood meals in their midgut (Rurangirwa *et al.,* 1986). However, valuable data have been obtained from this study and will add to the pool of knowledge on the feeding patterns of different species of *Glossina* relevant to the understanding of the epidemiology of African trypanosomosis. Blood meal analysis provides information on the hosts involved in tsetse flies survival and therefore leads to the selection of the most effective method for tsetse fly control to be applied in a given target area. However, for better understanding of the roles of the various blood meal sources in the transmission of trypanosomes that cause HAT and AAT, detailed studies of each local population is necessary (Clausen *et al.,* 1998).

In the present study, samples obtained from Busia were shown to have obtained their blood meals from cattle (*Bos taurus*). This is consistent with previous studies that had indicated domestic livestock as important hosts for tsetse flies in Busia (N'gayo *et al.,* 2005). It is therefore apparent that tsetse fly control in western Kenya can be achieved by the large scale use of insecticide-treated cattle that will consequently lead to a reduction of HAT and AAT incidences. A cost-effective way of controlling sleeping sickness in people and nagana in cattle is being applied in Uganda. The techniques involve treating infected cattle with trypanocidal drugs that kill blood-borne trypanosomes and restricted application of insecticide to the legs, ears and belly of the cattle that tsetse fly often bite (Thomieres *et al.,* 2006).

In Nguruman, the analyzed *G. pallidipes* were found to have obtained their blood meals from different hosts which included six elephants, five warthogs, a buffalo and a baboon. In a previous study using ELISA technique, it was reported that bushbuck, ostrich, warthog, elephant and buffalo are the blood meal sources for *G. pallidipes* and *G. longipennis* in Nguruman (Sasaki *et al.,* 1995). This is an indication that wildlife in Nguruman is vital for the survival of the tsetse fly population in this area and therefore control targeting a reduction of tsetse fly population in the savannah and forested region of Nguruman would be achieved by deploying traps and targets especially when odour-baited and impregnated with insecticides. The same control measure could also be undertaken in the Serengeti ecosystem (Tanzania) since the tsetse fly host species are also wild animals which included twenty five buffaloes, nine giraffes, three warthogs, three elephants and one on spotted hyena. This will ultimately lead to the reduction of the tsetse fly populations in these regions. The findings are consistent with a study that was carried out at the Serengeti National park which showed that sleeping sickness parasites may persist in both domestic livestock and wildlife reservoirs in and around the Serengeti national park (Kaare *et al.,* 2007). In the Serengeti and other wildlife-protected areas, wild species have the potential to serve as a parasite reservoir which can also get to domestic livestock when they share habitat with wildlife and tsetse flies. Disease control can then be achieved by limiting interactions between livestock and wildlife, use of chemotherapeutic drugs in cattle and controlling tsetse flies through 'pour on' insecticides on cattle. In addition, stationary tsetse fly traps and targets at the boundaries of wildlife protected areas can lead to a reduction of tsetse fly populations. These results show the importance of wildlife in the epidemiology of trypanosomosis.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

The molecular approach reported in this study, has illustrated the feasibility of the COI and Cytb gene sequences for the identification of the vertebrate hosts of the tsetse fly species. In order to prevent amplification of tsetse fly host DNA, it is recommended that more specific primers are designed. It may also be necessary to develop a panel of 'group specific' primers for groups of closely related hosts. At the same time a larger sample size of fully fed tsetse flies should be considered to representing the varied tsetse fly species.

A larger scale study is required to include more hosts to enable development of methods such as PCR-RFLP, which can circumvent the need for sequencing.

CHAPTER SIX

6.0 REFERENCES

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APPENDICES

Appendix 1: DNeasy extraction kit contains the following reagents:

Kit Contents	**250 preps**
DNeasy mini spin columns (colorless) in 2 ml collection tubes	250
Collection tubes (2 ml)	500
Buffer ATL	50 ml
Buffer AL	54 ml
Buffer AW1 (concentrate)	95 ml
Buffer AW2 (concentrate)	66 ml
Buffer AE	2 @ 60 ml
Proteinase K	6 ml

Appendix 2: QuickClean 5M Gel extraction kit contains the following:

LOO199 Components	**250 Preps**
Binding solution II	2 @ 110 ml
Wash solution	55 ml
Elution buffer	25 ml
QuickClean collumns	250
2 ml Collection tubes	250

Appendix 3: Quickclean 5M PCR purification kit (Genscript Corporation) contains the following reagents

LOO198 Components	250 Preps
Binding solution I	2 @ 75 ml
Wash solution	55 ml
Elution buffer	25 ml
QuickClean collumns	250
2 ml Collection tubes	250

Appendix 4: Solutions

6X Gel- loading buffer

The stock solution of the loading dye comprised:

0.42% Bromophenol blue

0.42% Xylene cyanol FF

50% Glycerol in water

50X TAE Buffer

242g Tris base

57.1ml Glacial acetic acid

18.6 g EDTA

Adjust volume to 1L with additional distilled H_2O.

Dilute to 1X TAE working solution with distilled water

Appendix 5: Bench Protocol: Animal Blood using DNeasy extraction kit (Qiagen inc., Valencia, CA, USA).

Procedure

1. Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 50–100 µl anticoagulated blood. Adjust the volume to 220 µl with PBS.

2. Add 200 µl Buffer AL. Mix by vortexing. Incubate at 56°C for 10 min.

3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.

4. Pipet the mixture into a DNeasy Mini spin column in a 2 ml collection tube.
Centrifuge at _6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1.
Centrifuge for 1 min at _6000 x g. Discard flow-through and collection tube.

6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard flow-through and collection tube.
Remove the spin column carefully so that it does not come into contact with the flow-through.

7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube, and add 200 µl Buffer AE for elution. Incubate for 1 min at room temperature. Centrifuge for 1 min at _6000 x g.
Recommended: Repeat this step for maximum yield.