THE PREVALENCE OF *Trypanosoma congolense savannah* IN SHIMBA HILLS, KENYA AND DEVELOPMENT OF A GENOTYPING PROTOCOL

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my family, for their endless support and God for unlimited providence.

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List of abbreviations

AAT	Africa Animal Trypanosomiasis
DNA	Deoxyribonucleic acid
FFLB	Fluorescent Fragment Length Bar-coding
HRM	High Resolution Melting
ICIPE	International Centre of Insect Physiology and Ecology
ITS1/2	Internally Transcribed Spacer
RBC	Red Blood Cell
RNA	Ribonucleic acid
Mb	Mega-base
PATTEC	Pan- African Tsetse and Trypanosomiasis Eradication Committee
PCR	Polymerase chain reaction
PSG	Phosphate Saline Glucose
Q - PCR	Quantitative PCR
SHNR	Shimba Hills National Reserve
TcM	*Trypanosoma congolense* Microsatellite

ABSTRACT

Eleven species/subspecies belonging to the genus *Trypanosoma,* cause African Animal Trypanosomiasis (AAT) with *Trypanosoma vivax* and *Trypanosoma congolense* being the predominant pathogens. Annually, AAT leads to losses worth 4.5 billion dollars to livestock dependent economies in Sub-Saharan Africa. In order to manage the disease, trypanocidal drugs have been in use. However, parasites resistant to these trypanocides have emerged making the treatment of AAT difficult. Parasite resistance to trypanocides is one among the few variable phenotypes whose genetic basis is poorly understood. There is need to create a link between the genetic component, environmental component and phenotypic manifestation of the parasite. Consequently, knowledge of genotypes/diversity of species helps create this link. In this study, the ecology, identity and genetics of trypanosomes circulating between bovine hosts and *Glossina pallidipes* in sites surrounding the Shimba hills game reserve, Kenya was considered. The Internally Transcribed locus (ITS1) of ribosomal DNA was targeted for amplification to identify different species of trypanosomes. Species specific amplification of minisatellite repeats was used to confirm the presence of *T. congolense savannah* detected using the ITS1 locus. Six out of the eleven trypanosome species that are detectable by PCR were found to be present in this area. This level of species diversity was observed for both cattle hosts and *G. Pallidipes* with *T. congolense savannah* being the dominant species. These findings will greatly influence future studies in this area, whereby either the vector or host will be used to source trypanosomes. A multiplex protocol for microsatellite genotyping was developed for *T. congolense savannah*, which is the most virulent trypanosome.

CHAPTER ONE

1.0 Introduction and literature Review

1.1 General Introduction

Trypanosoma congolense is only one of a few, but perhaps the most important parasite causing African animal trypanosomiasis or Nagana, a major cause of livestock death in sub-Saharan Africa (Au-Ibar, 2010). The total loss due to this disease is estimated at about $4.5 billion every year (Au-Ibar, 2010). In order to decrease the economic burden caused by trypanosomiasis, there has been a significant research effort in the past, concentrated on the development of diagnostic tools (Desquesnes and Davila, 2002). This has resulted in the generation of several molecular diagnostic tools that have been employed in the identification of up to eleven trypanosome species/clades (Njiru *et al.*, 2005). These diagnostic tools have been used to identify three genetically distinct subtypes of *T. congolense* important to agricultural productivity. The subtypes include Savannah, Forest and Kilifi/Kenya Coast based on isoenzyme electrophoresis data (Young and Godfrey, 1983, Gashumba *et al.*, 1988) and Polycmerase chain reaction (PCR) based methods (Kukla *et al.*, 1987, Gibson *et al.*, 1988, Moser *et al.*, 1989, Masiga *et al.*, 1992).

PCR based methods are now widely used in field studies to detect and reliably identify pathogenic trypanosomes in mammals (Njiru *et al.*, 2005) and tsetse flies (Malele *et al.*, 2003). This is very useful when conducting surveys on the prevalence of trypanosome species which are a reflection of the level of disease. The relationship between the prevalence of trypanosome species and the incidence of

trypanosomiasis is explained by the cyclical transmission vectored by tsetse flies (Vickerman, 1985). This means that livestock infective trypanosomes are transmitted by the bite of infected tsetse (*Glossina* spp.) to mammalian hosts where they live and multiply extracellularly in blood and tissue fluids. The distribution of trypanosomiasis therefore corresponds to that of the tsetse fly vector (Leak, 1999). This implies that, in order to study trypanosome diversity exhaustively, both the vector and host should be considered in the same habitat.

In Kenya, *Glossina pallidipes* has been reported to be the most abundant tsetse species (Ouma *et al.*, 2006). A 2006 study carried out in Nguruman, south-western Kenya revealed that *G. pallidipes* was the more abundant species compared to *G. longipennis* which is also found in the same area (Ouma *et al.*, 2006). It was also the most abundant tsetse fly species in another study conducted along the Kenya coast (Tarimo *et al.*, 1984) and was implicated with the highest levels of trypanosome infections. This makes *G. pallidipes* an excellent target for studying trypanosome diversity. *G. pallidipes* has a wide host range which includes both bovids and suids (Glover, 1967, Allsopp *et al.*, 1972, Moloo *et al.*, 1980, Lehane *et al.*, 2000). The current study focussed on an area neighbouring the Shimba hills National Reserve which has a high density of *G. pallidipes* (Ohaga *et al.*, 2007) and high levels of trypanosome infection (*Andoke*, 2011).

1.2 Literature Review

1.2.0 *Trypanosoma congolense*

1.2.1Classification

In 1904 based on morphological observations, Broden identified a distinct protozoa species which he named *Trypanosoma congolense* (Mulligan, 1970). Broden's grouping of *T. congolense* was later revised and trypanosomes belonging to this group were placed in a new subgenus called *Nanomonas* (from the Greek word *nannos meaning* dwarf). The subgenus Nanomonas retained *Trypanosoma (Nanomonas) congolense* Broden, 1904, as a type-species (Hoare, 1970). Taxonomically, *T. congolense* belongs to the section salivaria in the subgenus *nanomonas* of the genus *Trypanosoma* (Hoare, 1970).

Although *T. congolense* appears to be monomorphic when observed morphologically, several subtypes within the species have been identified that differ genetically. These genetically distinct subtypes were initially demonstrated by way of iso - enzyme electrophoresis patterns designated as *T. congolense forest* type and *T. congolense kilifi* type (Young and Godfrey, 1983, Gashumba *et al.*, 1988). The advent of the polymerase chain reaction (PCR) facilitated the verification of these subtypes through DNA based methods. Specific DNA probes and PCR primers were used to identify the different subtypes and thereby confirm the existence of *T. congolense tsavo* type (Majiwa *et al.*, 1993), *T. congolense savannah*, *forest* and *kilifi/kenya coast* subtypes (Kukla *et al.*, 1987, Gibson *et al.*, 1988, Moser *et al.*, 1989, Masiga *et al.*, 1992).

1.2.2 Morphology

Like most trypanosomes in the subgenus *Nanomonas*, *T. congolense* is a single cell measuring between 9-22 µm in total length (Uilenberg and Boyt, 1998). Each cell has an outer limiting and protective layer known as the pellicle which encloses a fluid filled cytoplasm. Within the cytoplasm is a centrally placed nucleus **(Figure 1**) which contains DNA packaged within chromosomes. A medium-sized kinetoplast is found situated at the anterior margin of the body **(Figure 1**). The kinetoplast contains 10% of the trypanosome genome and plays a role in parasite reproduction, metabolism and cyclical transmission of the parasite (Uilenberg and Boyt, 1998). Other notable organelles of the *T. congolense* cytoplasm are small volutin granules, a parabasal body from where the flagellum emerges. The flagellum is however poorly developed and inconspicuous (Uilenberg and Boyt, 1998).

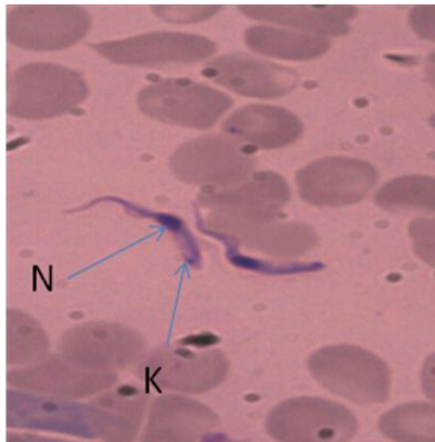

Figure 1: Morphology of *T. congolense* as revealed through a thin film of Giemsa stained mouse blood; where N is the nucleus and K is the kinetoplast (Photo courtesy of (Muthinja, 2011).

1.2.3 Lifecycle

Trypanosoma congolense has a complex developmental cycle that involves both mammalian hosts and tsetse vectors (Vickerman, 1985, Coustou *et al.*, 2010). While in the mammalian host, *T. congolense* is a bloodstream form (Roditi and Lehane, 2008); however upon ingestion by a tsetse fly vector in a blood meal the trypanosome changes its morphology as a form of adaptation to a new environment. Here it enters the midgut, becoming a motile procyclic form (Vickerman, 1985). The procyclic form becomes established in the tsetse fly and then it moves from the midgut and differentiate into a non-motile epimastigote form that adheres to the fly's mouthparts (Vickerman, 1985). It is therefore clear that, through this cyclical mode of transmission *T. congolense* interacts with both tsetse vectors and mammalian hosts thereby influencing parasite differentiation, survival, maturation and infectivity; which are important for parasite transmission and perpetuation of disease (Roditi and Lehane, 2008).

1.2.4 Genome

A partial nuclear genome shotgun sequence of *T. congolense* was carried out by the Wellcome Trust Sanger Institute's Pathogen Genomics group. The group used *T. congolense* strain IL3000 and produced a high quality draft genome sequence which has a depth of 5× (meaning the genome has been resequenced five times) (www.genedb.org/genedb/tcongolense). This genome is currently undergoing automated pre-finishing using *T. brucei* as a template. So far, the total genetic information in this genome is 34,751,073bp, arranged into 11 pseudo-chromosomes, based on the *T. brucei* karyotype. The chromosomes like in *T. brucei* are enclosed in

a nucleus and divide into three classes based on their size. The largest are the Mega-base chromosomes (1Mb to 6Mb) which are numbered 1-11 from the smallest to the largest. The smallest are the mini-chromosomes (50-150 kb) in size and in between are the intermediate chromosomes (200-900kb size range) (El-Sayed *et al.*, 2000). The use of the genome to generate molecular markers for species identification begun even before the Sanger project was conceived. In 1989, Moser described the highly abundant (5% per genome) 369bp repeats unique to *T. congolense* which were later used to generate species specific probes. These satellite repeats were also used to generate species specific primers for identification of *T. congolense* species/subtypes (Masiga *et al.*, 1992, Majiwa *et al.*, 1993). Recently, Morrison *et al* (2009) used the sequenced genome to generate microsatellite markers for use in studying mating in *T. congolense* Savannah found in the Gambia using a population genetics approach. The *T. congolense* genome will undoubtedly be valuable in further studies of this organism and its relationship to other Kinetoplastids. This is supported by the realized usefulness of the *T. brucei* genome for instance in understanding antigenic variation as a consequence of switching expression of variant surface glycoprotein genes (Barry *et al.*, 2005). The *T. brucei* genome also continues to serve as a template for other trypanosome genome projects e.g. *T. congolense* genome project.

In addition to the nuclear genome described, *T. congolense* also has the kinetoplast genome. The kinetoplast is a uniquely structured organelle containing DNA that is synonymous to the mitochondrion found in most eukaryotes. It consists of a few maxi circles encoding mitochondrial proteins and ribosomal RNA; several thousand

minicircles encoding guide RNA molecules that function in the editing of maxicircle messenger RNA transcripts (Shlomai, 2004) .

1.2.5 Distribution of *T. congolense* in Africa and Kenya

Trypanosoma congolense is vectored by tsetse flies and its distribution therefore coincides with that of *Glossina* described in the last two decades (Hendrix *et al.,* 1997, Rogers *et al.,* 1996). The distribution of tsetse flies is principally determined by the climate influenced by altitude, vegetation and presence of suitable host animals. The tsetse belt in Africa colonized by *Glossina* is estimated to occupy 11 million square kilometres between 10°N and 10°S of the equator (El-Sayed *et al.*, 2000). Although the tsetse flies can be found over some 11 million squared kilometres of the African continent, the presence of *Glossina* populations throughout the continent is not continuous. Consequently a tsetse belt is proposed to exist (Carter, 1906, Ford, 1963, Gouteux, 1987a).

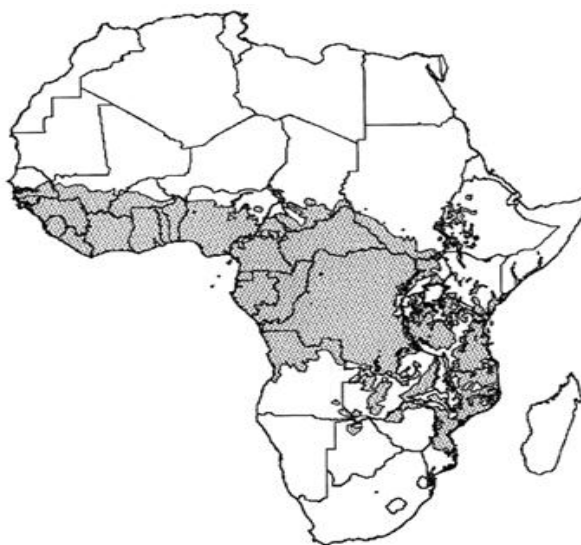

Figure 2: Map of Tsetse distribution in Africa (FAO 1998 based on the original map of (Ford and Katondo, 1975)

Tsetse flies (genus *Glossina*) are classified into three groups or subgenera, namely the fusca group (subgenus *Austenia*), the morsitans group (subgenus *Glossina*) and the palpalis group (subgenus *Nemorhina*) (Buxton, 1955). Each group of flies is subdivided into species and subspecies. In total 31 species and subspecies are found to transmit trypanosomes within the tsetse belt.

In Kenya, the tsetse belt is divided into six major parts and a few isolated patches found scattered throughout the country (**Figure 3**) (GOK, 2011). It includes: the Lake Victoria Basin; Narok - Kajiado; Lake Bogoria - Baringo - Kerio valley - Koibatek; Central Kenya; Isiolo - Samburu and Coastal Kenya fly belts (GOK, 2011). These areas are of high tsetse infestation colonized by at least eight species of *Glossina* (Glover 1967; Tarimo *et al.*, 1984; Bourn *et al.,* 2001; PATTEC 2011). Although different species of *Glossina* are known to be trypanosome vectors, *G. pallidipes* is the main vector caught along the Kenya coast (Tarimo *et al.*, 1984).

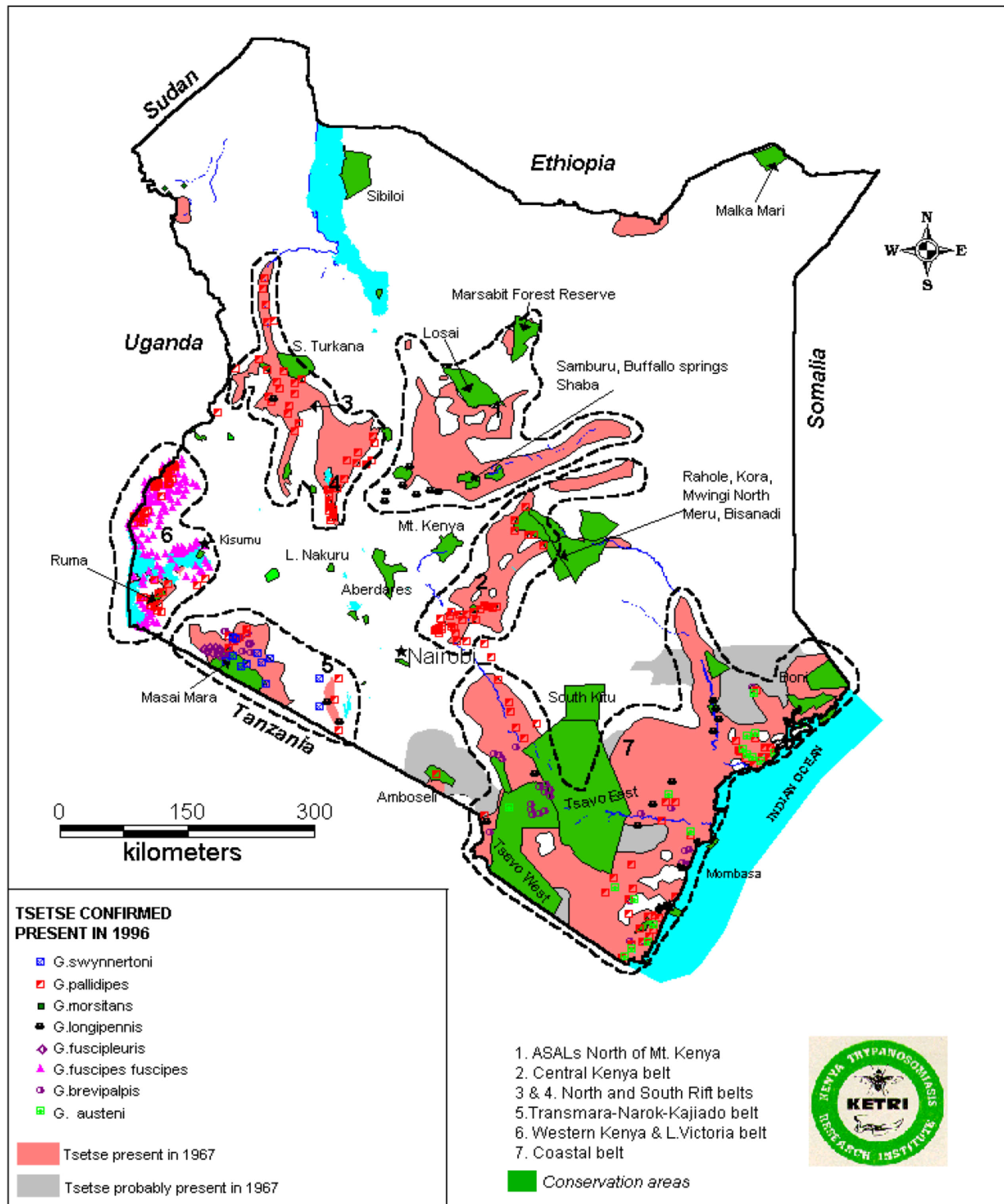

Figure 3: Tsetse distribution in Kenya (KETRI 1996, in PATTEC 2011)

1.2.6 Determining trypanosome diversity

1.2.6.0 Species specific PCR

Most livestock trypanosomes can be accurately identified using DNA sequences that are from various regions of the trypanosome genome. These DNA sequences have been used for the sensitive and specific PCR-based detection of trypanosome DNA in host blood and/or in tsetse flies (Masiga *et al.*, 1992, Majiwa *et al.*, 1993, Masake *et al.*, 1997, Morlais *et al.*, 2001). Satellite DNA found in 5% of the trypanosome genome, has been targeted for detection and identification of *Trypanosoma* belonging to the *Trypanozoon* subgenus (*Trypanosoma brucei spp., Trypanosoma evansi* and *Trypanosoma equiperdum*), the species *Trypanosoma vivax* and *Trypanosoma simiae*, and the three main *Trypanosoma congolense types* (savannah, forest, and kenya Coast) (Masiga *et al.*, 1992, Clausen *et al.*, 1998). One pair of primers is required to detect each subgenus, species or type, leading in some cases to the processing of three to five different PCRs per sample. However, internal transcribed spacer (ITS1) marker (Desquesnes *et al.*, 2001, Njiru *et al.*, 2005, Adams *et al.*, 2006) facilitates identification of up to eleven trypanosome species/subspecies in a single PCR.

1.2.6.1 Internal transcribed spacer 1 (ITS1) ribosomal DNA

The ITS1 is located in the ribosomal RNA locus of nuclear DNA and allows the detection of up to eleven trypanosome species in a single tube (Desquesnes *et al.*, 2001). ITS1 locus has several characteristics that make it suitable for application as a genetic marker for trypanosome identification. It is found in a locus that has a copy number of 100-200 per genome (Hernandez *et al.*, 1993) meaning that it is fairly

easy to target for amplification as it is not rare. Also, ITS1 is 300-800bp long (Desquesnes *et al.*, 2001) allowing for identification of different kinetoplastid species based on variable lengths. While length distinguishes one species from another, it is presumed to be constant within a given species. Trypanosome species are not always homogenous and intraspecific variations exist, these rely on sequence polymorphism an additional feature of the ITS1 marker for identification. The various properties of ITS1 make it a very versatile tool and it is also used in phylogenetic analysis, taxonomic identity and evaluation of the evolutionary process (Powers *et al.*, 1997). The ITS locus is arranged into transcriptional units separated by two non-coding regions which are ITS1 and ITS2. Each transcriptional unit has the following structure: 18S ,ITS1 ,5.8S ,ITS2, 28S (McLaughlin *et al.*, 1996). The primers used for kinetoplastid identification are based on the ITS1 sequence. The primers are designed from conserved regions (18S and 5.8S) flanking the non-coding region.

Figure 4: ITS loci of rDNA (Botelho *et al.*, 2004)

1.2.7 Other trypanosome identification tools

A more effective tool for species identification based on variable length regions within the 18S and 28S rRNA genes has been developed (Hamilton *et al.*, 2008). This method is known as fluorescent fragment length barcoding (FFLB) and it utilizes fluorescently labeled primers to identify species (Hamilton *et al.*, 2008). This method complements the ITS method in species identification (Adams and Hamilton,

2008). Other tools of detecting the genetic variability of trypanosomes are being generated. Njiru *et al* (2011) has reported the use of mobile genetic elements to genotype *T. evansi* strains (Njiru *et al.*, 2011). To generate diversity data Microsatellite markers are suitable because of their wide coverage of the genome. Currently seven highly polymorphic loci exist that might be suitable for the genotyping of trypanosomes (Morrison *et al.*, 2009). This set of highly polymorphic microsatellite loci was designed by Morrison *et al* (2009) in his study on mating in *T. congolense*. The findings of his study ascertained that sexual recombination does occur in *T. congolense*.

1.2.8 Microsatellites

Microsatellites are short segments of DNA that have a repeated sequence such as CACACACA (**Figure 5**) and they often occur in non-coding DNA (Field and Wills, 1998, Tóth *et al.*, 2000). These markers are widely distributed in the genome and they have high rates of mutation compared to the rest of the genome (Henderson and Petes, 1992, Jarne and Lagoda, 1996, Tóth *et al.*, 2000). The mutations are the main sources of genetic variation leading to multiple alleles at a single microsatellite locus. Several mechanisms explain the high mutation rates, they include unequal crossing over, errors in recombination and DNA polymerase slippage (Levinson and Gutman, 1987, Schlotterer and Tautz, 1992). Different alleles are identified by size variation between them. Microsatellites markers have been used in genetic studies (Goldstein and Schlotterer, 1999), with applications in conservation genetics (Williamson *et al.*, 2002), population genetics (Sunnucks, 2000), molecular breeding (Goldstein and Schlotterer, 1999) and paternity testing (Avise *et al.*, 1994, Goldstein and Pollock,

1997, Goldstein and Schlotterer, 1999). This range of application is due to the fact that microsatellites markers are co-dominant, multi-allelic and hyper-variable. Genotyping is highly reproducible and of high resolution based on the polymerase chain reaction (PCR) (Avise *et al.*, 1994).

Primers for microsatellite markers are designed to give coverage of the conserved flanking regions of the microsatellite, and the PCR-amplification of the repeat region. For loci that are conserved, a single pair of PCR primers will work for every individual in the species and produce different sized products for each of the different length microsatellites.

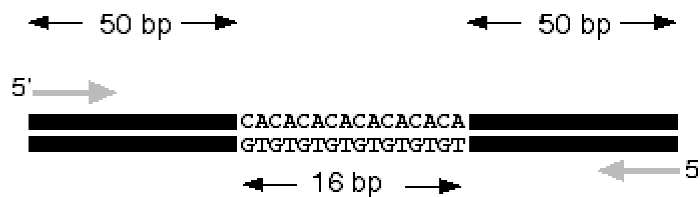

Figure 5: A sketch of a hypothetical microsatellite showing two PCR primers (forward and reverse gray arrows) designed to flank the microsatellite region. If there were zero repeats, the PCR product would be 100 bp in length. Therefore, by determining the size of each PCR product (in this case 116 bp), one can know the number of repeats present in each microsatellite (8 repeats in this example) (www.geneticmarkerservices.com).

1.2.9 High Resolution Melting (HRM) Curve Genotyping

High Resolution Melt (HRM) analysis is a technique that detects small sequence differences by melting of double stranded DNA (Wittwer *et al.*, 2003). Its basis is the generation of melting curves for different dye saturated DNA samples after subjecting them to an increasing temperature gradient (Montgomery *et al.*, 2007).

High resolution melting analysis is done immediately after PCR amplification of a sample that includes in the PCR mix non- inhibitory fluorescent dyes such as LCGreen© that bind to duplex DNAs(Montgomery *et al.*, 2007). To generate a melting curve the PCR products are heated gradually through a range of temperatures which increase at a rate of 0.1-1°C/s, while fluorescence is continuously monitored. The dyes used for HRM analysis fluoresce only when bound to double stranded DNA and cease fluoresce for single stranded DNA (Montgomery *et al.*, 2007). Dye fluorescence is the property that facilitates the generation of the melting curve (Wittwer *et al.*, 1997). The melting curve takes the form of a sigmoid curve whereby at time zero fluorescence is highest but begins to reduce gradually until at a certain characteristic temperature double stranded DNA melts and becomes single stranded. This characteristic temperature, more conventionally known as the melting temperature, is influenced by factors such as the GC content and length of the amplicon (Ririe *et al.*, 1997). The melting temperature alone can be used to distinguish between different homozygotes and heterozygotes; however the entire melting curve is more informative of variation. The curve takes on a different shape for a homozygote and a heterozygote. HRM has great potential in genotyping trypanosomes rapidly and at a minimum cost. For example HRM has been used to successfully genotype different strains of the dinoflagellate symbiodinium using primers that target the ITS2 sequence (Granados-Cifuentes and Rodriguez-Lanetty, 2011).

This technique is used in sequence matching, mutation scanning (Reed *et al.*, 2007, Smith *et al.*, 2010) and multiplex genotyping - analyses that traditionally required

processing of PCR products by electrophoresis or other non-homogeneous means. In some applications, knowing the complete genotype of a DNA is less important than knowing whether DNA sequences match. This occurs in tissue transplantation, genotype/phenotype correlations, and forensics. The ability to scan mutations eliminates expensive sequencing and re-sequencing steps because wild/normal genotypes are readily identified. This technique has therefore found wide application in disease diagnosis for personalized medicine (Dobrowolski *et al.*, 2009).

1.3 Statement of the problem

African Animal Trypanosomiasis remains a problem in most livestock dependent economies in Sub - Saharan Africa causing economic loss in terms of agricultural productivity worth 4.5 billion dollars annually (AU – IBAR, 2010). Among the economies affected is that of farmers that live adjacent to the Shimba Hills National reserve, on the coastal belt that lies in the south east of Kenya. Knowledge on the trypanosome species diversity circulating between, hosts, vectors and reservoirs is lacking making AAT management challenging (Ohaga *et al.*, 2007).The problem of this study was to determine the trypanosome richness and diversity in both tsetse fly vectors and cattle hosts in the Shimba Hills area. The incidence of *T. congolense savannah*, the most virulent trypanosome (Bengaly *et al.*, 2002) is also unknown. In *T. congolense* virulence, infectivity and resistance to trypanocides are quantitative traits that demonstrate phenotypic variability; however the genetic bases responsible for these traits remain largely unknown. A cost effective and efficient within-species genotyping tool is also lacking. This study aims to develop a genotyping protocol for

T. congolense savannah, which will be used to study diversity at a higher resolution. Knowledge of these *T. congolense* genotypes will potentially help in the local control of AAT which depends on understanding the local epidemiology and impact of disease on livestock (Snow & Rawlings, 1999).

1.4 Justification

For many years control of AAT has been achieved through the use of trypanocidal drugs among other methods. However, in the recent past, resistance to trypanocidal drugs has emerged among trypanosomes making the treatment of AAT difficult (Bourn *et al.,* 2001; Verlinde *et al.,* 2001). This resistance has been caused by the continued use/overuse of the few available trypanocides and has led to multiple drug resistance (Verlinde *et al,* 2001) which can be attributed to changes in the trypanosome genome (Masumu *et al.,* 2006). These changes in the genome and their underlying causes such as recombination or mutation are best understood when the detailed genotype of an organism is known. The interaction between the genotype and environment gives rise to the phenotype for most quantitative traits (Nei, 1975). In *T. congolense* virulence, infectivity and resistance to trypanocides are quantitative traits that demonstrate phenotypic variability; however the genetic bases responsible for these traits remain largely unknown. A cost effective and efficient within-species genotyping tool is also lacking. This study aims to develop a genotyping protocol for *T. congolense savannah*, which will be used to study diversity at a higher resolution. Knowledge of these *T. congolense* genotypes will potentially help in the local control of AAT which depends on understanding the local epidemiology and impact of disease on livestock (Snow & Rawlings, 1999).

1.5 Research Questions

1. What trypanosome species/clades are found in the Shimba Hills, Kubo division, Kwale County?
2. Are *G. pallidipes* flies and *T. congolense savannah* trypanosomes present?
3. Are there tools that can be used to study *T. congolense* subtype *savannah* genetic diversity?

1.6 Null Hypothesis

There is no diversity in trypanosome species/cledes and *T. congolense savannah* is not the most prevalent species in Shimba Hills, Kubo division, Kwale County.

1.7 Objectives

1.7.1 General objective

To determine the trypanosome interspecific diversity found at Shimba hills, Kubo division, Kwale County Kenya

1.7.2 Specific objectives

1. To describe tsetse fly distribution in Shimba Hills, Kubo division, Kwale County.
2. To identify the trypanosome species/clades found in Shimba Hills, Kubo division, Kwale County.
3. To optimize an efficient tool for studying the diversity of *T. congolense* subtype s*avannah.*

CHAPTER TWO

2.0 General Materials and methods

2.1 Sampling area

The study was conducted in Shimba Hills, Kubo Division, and Kwale County of Kenya. Shimba Hills lies between latitudes 4°20′S and longitudes 39°31′E in the Coastal lowlands agro-ecological zones 2-4 (Jaetzold and Schimdt, 1983). The vegetation in the Shimba Hills National Reserve is diverse and is characterized by Savannah grasslands, shrubs and a coastal rainforest. Much of the vegetation in the sites surrounding the park was cleared for farming and it comprises of palm, pine, cashew and mango trees (Ohaga *et al.*, 2007). The area receives rainfall averaging 500–900 mm per year, mean annual minimum and maximum temperatures are 24 and 36 °C, respectively (Ohaga *et al.*, 2007). Kubo Division is a hilly area with an elevation that lies between 180m and 250m above sea level. Sampling in this area was done in the month of May which falls in the middle of the long rains and September which marks the beginning of the short rains.

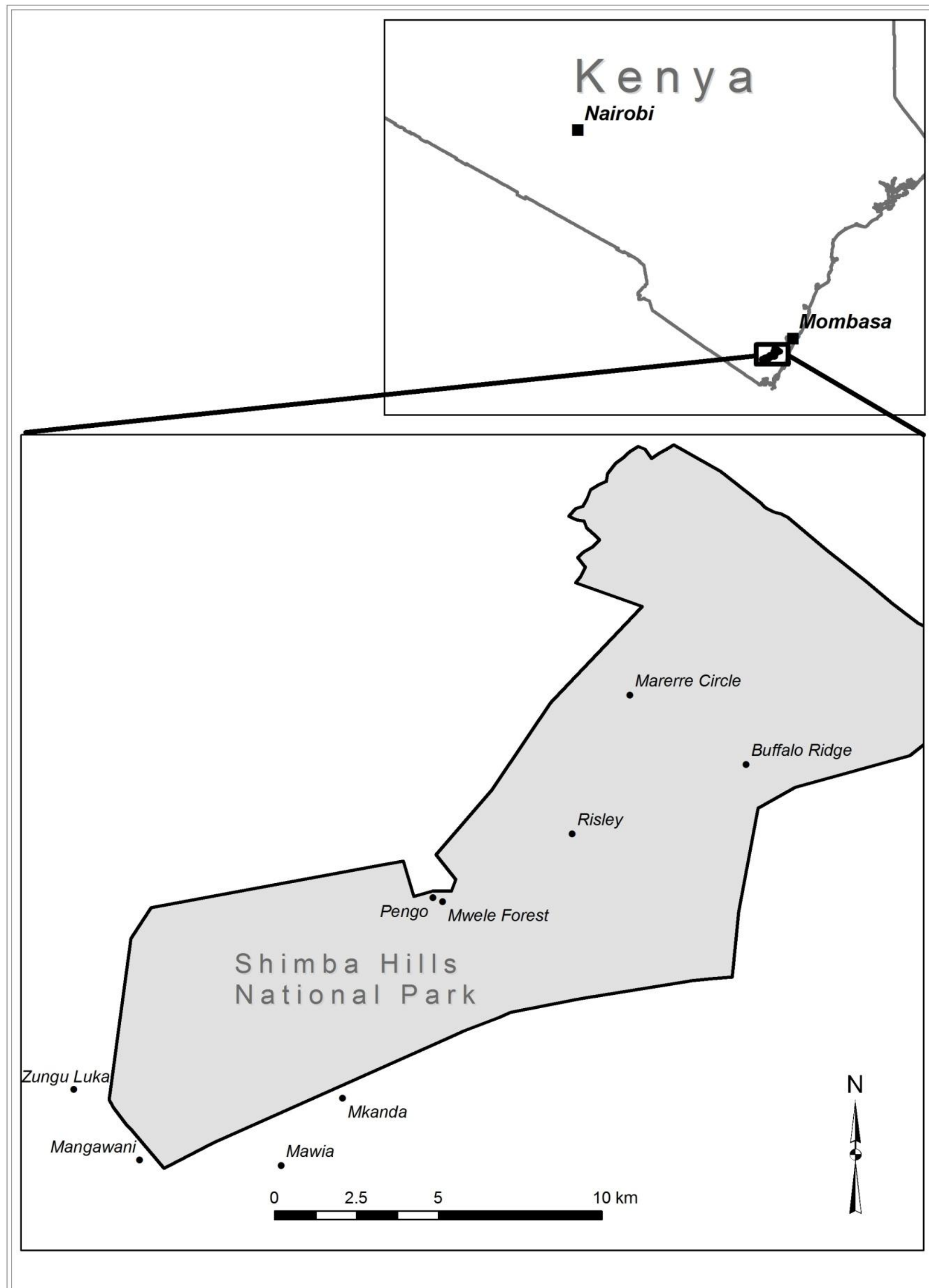

Figure 6: Map of sampling sites (marked with dots) within and around the Shimba Hills National Reserve

2.1.0 Sampling of tsetse flies

Tsetse flies were collected using geo-referenced NG2G traps (Brightwell *et al.*, 1991) baited with acetone and cow urine. One to four traps were deployed per site at an inter-trap distance of approximately 100m. Four trapping sites, 10 kilometres apart, were used within the Shimba Hills National Reserve; they include Risley Ridge, Marere Circle, Buffalo Ridge and Mwele Forest (**Figure 6**). The first three sites were characterised by Savannah grasslands while the last site was at the edge of a coastal rainforest. Mkanda, Zunguluka and Pengo were trapping sites in villages approximately less than five Kilometres from the Shimba Hills National Reserve fence (**Figure 6**). Flies were collected twice a day from each trap in the morning between 0900-1000hrs and in the evening between 1600-1800hrs for five consecutive days. This was done to coincide with peak activity periods for *G. pallidipes* which is most active early in the morning and late in the evenings (Brady, 1972). Upon collection, flies were aspirated into labelled cages then transported in a cool box humidified using a moist blanket to a laboratory at the *icipe* field station, Muhaka. Those flies that were found alive in traps were maintained on rabbits at the Muhaka station. In order to be certain of their identity, the flies were identified using morphological keys (Reinhardt, 2002) and sorted. In this study the non-pallidipes flies were discarded in the field. The non-pallidipes flies included stomoxys, tabanids and *G. brevipalpis*. The flies were then transported to the insectary at the *icipe* Duduville campus. Dead flies from the field were preserved in bottles containing 95% ethanol.

2.1.1 Sampling cattle blood

Cattle blood was sampled from five sites close to the park fence; Mangawani, Mkanda, Mawia, Zunguluka and Pengo. Sampling was done in the morning at 0700-1100hrs by collecting blood from the ear vein into capillaries. Blood from all the animals collected in capillaries was tested for trypanosomes using the buffy coat technique (Murray *et al.*, 1977). The capillaries were cut using a diamond tipped pencil then the buffy coat extruded onto a clean glass slide. A cover slip was placed on the buffy coat creating a wet mount that was examined under a light microscope at × 40 magnifications for the presence of trypanosomes. Positive animals were noted and a larger volume of blood was collected from their jugular veins into heparinised vacutainers™. The blood that was positive for *Trypanosoma* was cryopreserved by first mixing with 10% glycerol in Phosphate Saline Glucose (PSG) in a ratio of 1:1 in 1.5ml cryovials. The cryoprotected samples were cooled slowly in the vapour phase of nitrogen inside a plasticine-insulated protective jacket overnight followed by preservation in nitrogen indefinitely. In order to ease transportation, surplus positive and all negative blood samples that were collected in vacutainers™ were subjected to a Red Blood Cell (RBC) lysis buffer (Matovu *et al.*, 2010) then the pellet of white blood cells and possibly concentrated trypanosomes was suspended in 95% ethanol. The lysis buffer contains two stock solutions that were prepared as follows: stock 1 was 8.3g ammonium chloride in 1,000 ml water, while stock 2 was 20.59g Tris base in a total volume of 1,000 ml water after adjusting pH to 8.0 with 1M hydrochloric acid. To make the lysis solution, 9 parts of stock 1 were added to 1 part of stock 2 and the pH adjusted to 7.65 with 1M hydrochloric acid. To lyse RBCs with this solution, 1 part of blood was added to 9 parts of the lysis solution, mixed gently and

allowed to stand for 5-10 minutes. The mixture became more translucent over time, as the RBCs lysed and released haemoglobin into the solution. The role of this buffer was to selectively lyse the red blood cells leaving behind intact trypanosomes if present. The lysis technique was therefore part of a preservation step to allow for easy transportation to the laboratory where PCR screening was done. For some sites, blood sampled from the ear vein in capillaries was blotted onto FTA cards to ensure the preservation of sample, while in others; capillary tubes were used to collect and store blood by cryopreservation.

2.2 Sample Size Determination

A simple sampling design approach was applied where subsamples needed to be used for example in determination of the sex ratio between flies. The Yamane (1967) formula was used (Yamane, 1967). For the formula, a 95% confidence level and P =.5 were assumed. Formulae: $n = N/1+ (0.05)^{2.}$

Cattle samples were collected using the census approach whereby all the individuals in the population were sampled. Fly samples were also sampled using the same approach. As many flies as the NG2 trap could accommodate were sampled.

2.3 Cattle blood sampling site inclusion criteria

Cattle blood was randomly sampled from select villages around the reserve. The criterion for selection of a village to sample cattle blood was the corresponding tsetse fly density i.e. those with high fly densities were chosen.

2.4 Data analysis

The significance of the data that were in counts was determined using the Chi square test.Genetic variation was assessed by determining the mean number of alleles per locus (A). A was calculated with Geneclass 2 ver. 2.0.g (Piry *et al.*, 2004). For comparisons of A values between population samples, estimation of allelic richness (AR) was on the basis of minimum sample size, using the rarefaction method (Petit *et al.*, 1998) implemented in Fstat 2.9.3 (Goudet, 2001).

CHAPTER THREE

3.0 Tsetse Fly distribution in Shimba Hills, Kubo division, Kwale County.

3.1 Introduction

Kubo division in Kwale County is an area that has a high prevalence of bovine trypanosomiasis whose causative agents are the tsetse borne trypanosomes. A cross sectional survey of the area in 2004 to determine the epidemiology of bovine trypanosomiasis showed that there was an infection prevalence of 18% predominantly due to *T. congolense* as detected by microscopy (Ohaga *et al.*, 2007). The survey also examined the distribution and density of tsetse flies whereby *G. austeni, G. brevipalpis* and *G. pallidipes* were caught in the area; with *G. pallidipes* found to be the most abundant (2 -738 flies/trap) (Ohaga *et al.*, 2007).

An important feature of this location is that the Shimba Hills National reserve (SHNR) covers >90% of the total land surface in Kubo Division of Kwale County refer to map (**Figure 6**). Shimba Hills National Reserve contains high densities of game animals such as buffalo, antelopes, elephants, snakes, lizards and birds etc: that can potentially act as reservoirs of trypanosomes and vegetation cover that is suitable for tsetse fly survival and propagation. The remainder of the land area is owned by small holder cattle farmers affected by trypanosomiasis. It was therefore possible to sample tsetse flies from within the Shimba Hills National Reserve and also in villages approximately less than five Kilometres from the park's fence. Cattle blood was sampled from sites surrounding the national park. Although there have been larger scale ecological and epidemiological studies in the past (Tarimo *et al.*, 1984),

a local focus on tsetse fly and trypanosome genetics has been minimal. In this study, trypanosomes from cattle blood and tsetse flies were sampled to facilitate population genetics analysis.

3.2 Materials and Methods

2.2.0 Sampling of tsetse flies

Tsetse flies were collected using geo-referenced NG2G traps (Brightwell *et al.*, 1991) baited with acetone and cow urine. One to four traps were deployed per site at an inter-trap distance of approximately 100m. Four trapping sites, 10 kilometres apart, were used within the Shimba Hills National Reserve; they include Risley Ridge, Marere Circle, Buffalo Ridge and Mwele Forest (**Figure 6**). The first three sites were characterised by Savannah grasslands while the last site was at the edge of a coastal rainforest. Mkanda, Zunguluka and Pengo were trapping sites in villages approximately less than five Kilometres from the Shimba Hills National Reserve fence (**Figure 6**). Flies were collected twice a day from each trap in the morning between 0900-1000hrs and in the evening between 1600-1800hrs for five consecutive days. This was done to coincide with peak activity periods for *G. pallidipes* which is most active early in the morning and late in the evenings (Brady, 1972). Upon collection, flies were aspirated into labelled cages then transported in a cool box humidified using a moist blanket to a laboratory at the *icipe* field station, Muhaka. Those flies that were found alive in traps were maintained on rabbits at the Muhaka station. In order to be certain of their identity, the flies were identified using morphological keys (Reinhardt, 2002) and sorted. The non-pallidipes flies included stomoxys, tabanids and *G. brevipalpis*. The flies were then transported to the

insectary at the *icipe* Duduville campus. Dead flies from the field were preserved in bottles containing 95% ethanol.

3.3 Results

3.3.1 *Glossina pallidipes* density in seven sampling sites

The density of flies indicated by the number of trapped flies per site and per trap was higher in the reserve than in surrounding villages. The Shimba Hills National Reserve had a total of 3012 flies collected from 12 traps (**Table 2**); surrounding villages had 1995 (**Table 1**) flies collected from 13 traps. A significantly larger proportion (52.7%) of the flies caught were from within the reserve than outside the reserve (47.3%), (Chi sq. = 21.7691, df = 1, $P<0.0001$). The highest and lowest fly catches per day was 218.6 and 0.2 respectively for traps within the SHNR (**Table 2**). Fly mortality at Zunguluka was at 92% per day. It is worth noting that Zunguluka did not have vector control measures put in place whereas the other sites had either traps or insecticide treated targets. The fly collections in this site were also probably the best estimation of fly density in villages surrounding the park.

Table 1: *G. pallidipes* trapping sites and numbers of tsetse caught outside the Shimba Hills National Reserve.

Site	Trap no.	No. of flies caught	Average daily catches	% of flies caught per site
Mkanda	1	138	27.6	15.9%
	2	48	9.6	
	3	17	3.4	
	4	115	23.0	
Pengo	1	89	17.8	4.5%
	2	0	0.0	
	3	0	0.0	
	4	0	0.0	
	5	0	0.0	
Zunguluka	1	184	36.8	79.6%
	2	400	80.0	
	3	685	137.0	
	4	319	63.8	

NB: These proportions were significantly different from one another (Chi sq.=2941.61, df=2, $p < 0.0000001$)

Table 2: *G. pallidipes* trapping sites and numbers of tsetse caught in five days within the Shimba Hills National Reserve

Site	Trap	# flies caught	Av. daily catches	% caught per site
Buffalo Ridge	1	53	10.6	93.4
	2	206	41.2	
	3	1093	218.6	
	4	132	26.4	
	5	189	37.8	
Marere circle	1	94	18.8	5.2
Mwele Forest	2	1	0.2	0.3
Mwele Forest	4	5	1.0	
Risley	1	6	1.2	1.0
	2	1	0.2	
	3	11	2.2	

NB: These proportions were significantly different from one another (Chi sq.== 5974.181, df = 3, p-value < 0.000000001)

3.3.2 *Glossina pallidipes* sex ratios

One hundred and forty two flies from several traps were randomly sampled to estimate the male to female sex ratios of the flies caught in Shimba Hills area. In all instances, there were more female flies than males caught. An average of 12 males was caught in all sites for every 59 females (Table 3); i.e. in an approximate sex ratio of 1:5.

Table 3: Ratio of male to female flies caught within and around the Shimba Hills Game Reserve

Site from which trap was chosen	No. of females	No. of males	Ratio of males to females
Buffaloes ridge	27	4	1:6.75
Pengo	26	4	1:6.5
Zunguluka	36	9	1:4
Total	118	24	1:4.92

3.4 Discussion

The Shimba Hills area exhibits different patterns of fly distribution and density both within and around the game reserve. These differences in fly density can be explained by the long recognized fact that a suitable fly habitat is dependent on the vegetation cover along with climate and availability of hosts (Cecchi *et al.*, 2008). This spatial variability in tsetse fly density is also directly related to variability in fly catches which is influenced by factors such as site visibility and shading (Morris and Morris, 1949). Shimba Hills is composed of lowland rainforest woodland, bushed grassland and *Hyphaene* Savannah (Tarimo *et al.*, 1984). Savannah habitats characterised by deciduous woodland and deciduous shrubland account for over 50% of fly distribution in Africa (Cecchi *et al.*, 2008) therefore qualifying the suitability of the Shimba Hills vegetation for tsetse habitation. Many wild animals are also present especially but not exclusively in the game reserve. They include elephant (*Loxodonta africana*), bushpig (*Potamochoerus porcus*), warthog (*Phacochoerus aethiopicus*), buffalo (*Syncerus cafer*), bushbuck (*Tragelaphus scriptus*), sable antelope (*Hippotragus niger*) (Tarimo *et al.*, 1984, Snow *et al.*, 1988). These hosts and others have been associated with tsetse in blood meal analysis studies aimed at determining fly feeding habits around East Africa (Tarimo *et al.*, 1984, Snow *et al.*, 1988, Lehane *et al.*, 2000, Muturi *et al.*, 2011). They are therefore important for the maintenance of fly populations.

The highest density of tsetse flies within the reserve is found at the Buffaloes Ridge, an area best described as open Savannah grassland that is heavily colonized by buffaloes and small grazers. Fly movement was observed to be dependent on the

movement of buffaloes at this site and was evidenced by considerable variation in densities in catches between traps. Similar observations were made in an earlier study where the movement of flies in relation to host movement was cited as a reason for daily fluctuations in fly catches (Morris, 1960, Glasgow, 1961b). Therefore the buffalo movement determined where the fly catches would be greatest. The abundance of tsetse flies has also been linked to the numbers and diversity of wild herbivores (Snow *et al.*, 1988). Snow *et al* (1988) in their study on "the feeding habits of tsetse, *G. pallidipes* Austen on the south Kenya coast, in the context of its host range and trypanosome infection rates in other parts of East Africa" suggested that any factor including host rarity or anti-feeding responses that reduced the feeding frequency or feeding success, could result in increased mortality or reduced fecundity. This implied that each host population had a carrying capacity which could act in such a way as to regulate fly numbers. This was an explanation offered for their observations at the Kenya coast where the greatest numbers of flies were caught in the game reserve which was home to an equally large number of diverse hosts. This also explains similar observations by this study in the same area more than twenty years after Snow *et al* (1988) reported them.

The lowest density of flies within the reserve was observed at the Risley Ridge where the vegetation was composed of riverine-scrub with a few large grazers such as elephants present. Host availability may have been the limiting factor in this area and not so much the vegetation type. *G. pallidipes* flies are very adaptable and in areas such as Nguruman they inhabit the riverine shrubs where it is cooler and moist

(Jordan, 1995). Therefore, although vegetation can influence fly abundance, it may not be the dominant factor here.

In the villages surrounding the Shimba Hills National Reserve, fly catches also differed from site to site. The differences may have occurred due to changes in land use patterns such as clearance of bushes for farming purposes. A general observation was that sites with the least disturbance to their flora and fauna presented the best breeding grounds for the flies as was reflected by high numbers of flies caught. An example of such an area is Zunguluka which had the highest fly density. The villages around the reserve have substantial numbers of cattle and a much lower density of flies compared to the Shimba Hills National Reserve. This low fly density in areas where cattle are somewhat numerous may mean that feeding is poor on local livestock and that they are not completely satisfactory alternative hosts for *G. pallidipes* (Snow *et al.*, 1988).

Approximately 10% of flies were caught alive in traps and attempts were made to maintain these alive with a view to potentially establishing laboratory tsetse colonies. However, high fly density was accompanied by high fly mortality in the traps, making the maintenance of flies alive challenging. The high mortality was catalysed by high levels of humidity that may have exceeded the recommended 70% suitable for tsetse. In drier areas such as Nguruman it is routine practice to place a wet blanket over tsetse cages so as to provide humidity. However, Shimba hills is humid and when flies were covered using a moist blanket, it is probable that the humidity was too high leading to high levels of mortality. The flies, also through an action similar to vomiting, were excreting a large volume of fluid through the proboscis and

depositing it onto the cage surface. This would affect their mobility as their wings would get wet leading to their deaths.

The sex ratios observed in this study varied from trap to trap, but there were generally more females tsetse flies compared to males (**Table 3**). This has been observed consistently in past studies, whereby although the sex ratio is near parity when pupae emerge, it varies for field catches with females being more readily caught than males (Dean *et al.*, 1969, Tarimo *et al.*, 1984, Dransfield *et al.*, 1990). Seasonal variation in fly density, trap related selection and migration of flies has also been used to explain the differences in sex ratios.

Several trypanosomiasis/fly management practices were on-going around Shimba Hills area that may have affected our sampling of both flies and cattle. Thus the numbers of flies caught and prevalence of infected cattle will reflect this. Some of the tsetse fly/trypanosomiasis management practices used included: treatment of trypanosome-positive cattle with Diamezene diaceturate/phenazone; deployment of Deltamelthrin treated targets; use of conventional NG2G traps and a trial being run by *icipe* using of a collar laced with a tsetse repellant. The fly and trypanosome diversity in Shimba Hills has been skewed by these practices. A good example of an area where fly management was in place is Pengo, which had a total of only 89 flies caught using five traps in five days. This was the lowest fly catch probably influenced by the presence of insecticide treated targets deployed every 100m along the reserve fence. There are, however, two sites that do not have any fly management practice in place: Mkanda and Zunguluka. Mkanda area was therefore chosen as the preferred study site for future work due to high trypanosome infection prevalence

(approximately 30%) observed in a survey conducted in July 2011 (*Andoke*, 2011) and substantive numbers of flies caught.

CHAPTER FOUR

4.0 Identification of trypanosome species/clades found in Shimba Hills, Kubo division, Kwale County

4.1 Introduction

Trypanosomiasis continues to be a burden to the livestock farmers living adjacent to Shimba Hills National Reserve (SHNR) in Kwale County (Ohaga *et al.*, 2007). The disease is rampant due to close proximity of the small holder cattle farms in Mawia, Mkanda and Mangawani locations to the tsetse infested SHNR. Domestic animals are usually in close contact with trypanosome-laden tsetse flies and their inability to tolerate infection makes them succumb to infections that culminate in death if they are not treated (Tarimo *et al.*, 1984). *Glossina pallidipes* is the most abundant and widespread tsetse fly implicated in vectoring trypanosomes in this area where cattle constitute the dominant domestic hosts (Tarimo *et al.*, 1984), personal field observation).

This chapter compares the level of trypanosome diversity and species abundance in cattle hosts and *G. pallidipes* vectors. Trypanosomes sampled from both cattle blood and *G. pallidipes* were screened rapidly at minimum cost using PCR that targeted the internal transcribed spacer locus (ITS1) of Ribosomal DNA. The ITS1 locus is used as the target for identification because it exhibits size and sequence polymorphism between trypanosome species (Desquesnes *et al.*, 2001). An additional species specific test that targeted the amplification of satellite repeats in *T. congolense savannah* was performed. This is because the ITS1 locus alone would yield the same amplicon size of 700bp for *T. congolense* subtypes *forest* and *savannah*. The TCS1

primer pair was therefore used to target the amplification of 316bp satellite repeats in *T. congolense* subtype *savannah* (Majiwa *et al.*, 1993). This chapter describes the different species and of trypanosomes causing infections and the percentage prevalence encountered among hosts and vectors. Comparisons have been drawn on: infection levels/types between the vectors and hosts; infections in the proboscis and tsetse abdomen.

Parasitological surveys, using microscopy have in the past demonstrated the presence of up to three trypanosome species causing disease in this area namely *T. brucei* spp., *T. vivax* and *T. congolense* (Tarimo *et al.*, 1984, Ohaga *et al.*, 2007). In this study, a more precise breakdown of the number of: circulating trypanosomes (abundance), distinct species/subtypes (diversity) and ecology in relation to their immediate niche i.e. bloodstream or fly mouthparts and gut is provided. This gives a better picture of the disease problem in the Shimba Hills area.

4. 2 DNA Extraction

4.2.1 Extraction of trypanosome DNA from Cattle blood

Blood samples were preserved in two ways, on FTA cards and as lysed pellets preserved in 95% ethanol. For the second method, whole blood was treated with a buffer that differentially lyses red blood cells thereby improving microscopic visualization of trypanosomes (Matovu *et al.*, 2010). In this case red blood cell lysis was used to concentrate trypanosomes for screening by microscopy and for PCR amplification. Lysed blood was centrifuged forming a pellet that contained trypanosomes. The lysis procedure was described in greater detail in *Chapter two,*

Section 2.2.1. Trypanosome DNA was isolated from Lysed blood samples using the ammonium acetate precipitation procedure (Bruford *et al.*, 1998).

Isolation of trypanosome DNA from blood stored on Whatman© FTA cards was performed by the cutting of 0.1 by 0.1cm pieces of the FTA on a self-healing mat using a scalpel blade. The small pieces of FTA cards carrying sample DNA were then placed in 1.5ml eppendorf tubes. In order to avoid cross contamination between samples when cutting, the scalpel blade, forceps and self healing mat were cleaned using 10% commercial bleach after processing each individual FTA blood sample. Lysed blood preserved in absolute ethanol was blot dried before extraction. Digsol lysis buffer at a volume of 250µl, 0.5M EDTA (pH 8.0), 0.2M NaCl, 1M, Tris. HCl (pH 8.0), 20% Sodium Dodecyl Sulphate) (Bruford *et al.*, 1998) was added to each sterile 1.5ml eppendorf tube followed by 10µl of 10mg/ml proteinase K and the samples were incubated overnight at 37°C or for three hours at 55°C. After incubation, 300 µl of 4M ammonium acetate was added to each tube to precipitate the proteins. This was followed by a centrifugation step at 15,000×g for 15 minutes at 4°C. The supernatant was transferred into a new sterile labelled 1.5ml eppendorf tube, whereas the tubes containing the protein precipitate were discarded. To each supernatant 1ml of 100% ethanol was added then vortexed for two seconds and inverted 10-20 times gently to precipitate the DNA. This was followed by centrifugation at 15,000×g at 4°C for 15 minutes. Ethanol was carefully discarded leaving a white DNA pellet at the bottom of each eppendorf tube. The pellet was subjected to two rounds of washing using 70% ethanol and 5 minute centrifugation steps at 15,000×g at 4°C. After the second wash, the pellets were dried and then

resuspended in 50µl of T.E buffer pH 8.0 (10 mM Tris. HCl (pH 8.0), 0.1mM EDTA (pH 8.0), and distilled autoclaved water). The tube containing DNA sample was flicked briefly, vortexed for two seconds then incubated at 37°C for 10 minutes to ensure dissolution of pellet. 4ul of each DNA extract was run on a 1% agarose gel at 80 V for one hour to evaluate the success of the extraction then stored at -20°C. The presence of high molecular weight DNA run against a DNA size standard indicated successful extraction. A spectrophotometer (Shimadzu mini-biospec (Shimadzu Corporation, Japan) was also used to quantify DNA.

4.2.2 Extraction of trypanosome DNA from tsetse flies

The sex of flies collected and preserved in 95% ethanol in the Mkanda area of Shimba Hills was determined by examining their genitalia under a dissecting microscope at ×100 objective. The males were identified by looking for the presence of a folded hypophygium at the posterior tip of the abdomen, while the females lack equivalent obvious structure, but simply have a small opening surrounded by a variable number of small flat chitinous plates (Pollock, 1974). The male and female flies were separated then each individual fly was placed on a sterile Petri dish, and sterile forceps were used to pluck the abdomen and proboscis. The forceps were cleaned using bleach, then rinsed in distilled water to prevent cross contamination between the organs and also between different flies. The proboscis, abdomen and carcass were put in separate autoclaved 1.5ml eppendorf tubes. The carcass was stored in an eppendorf tube containing 95% ethanol to serve as future reference material. The proboscis and abdomen were snap frozen in liquid nitrogen and then

homogenized using sterile pestles. The homogenates were then extracted using the ammonium acetate precipitation protocol (Bruford *et al.*, 1998).

4.2.3 PCR screening of trypanosome samples

PCR was performed in a 10µl reaction volume containing 0.2mM Magnesium chloride, 5× Phusion® HF (Thermo scientific), 0.02U/µl Phusion® DNA polymerase (Thermo scientific), 200µM dNTPs (GenScript), 0.5µM of each primer, ~10ng template DNA and nuclease free water to a final volume of 10µl. Negative (Template free) and positive (*icipe* 001 reference strain) controls were included in each PCR experiment. The role of the negative control was to check for carryover DNA contamination in PCR reagents, while the positive control was used as an indicator of the success of the PCR reaction. Cycling conditions were as follows: 98°C for 2 minutes and then 98°C for 2 seconds, 64°C for 30 seconds, 72°C for 30 seconds and a final hold at 72°C for 7 minutes performed in a 9800 Fast™ (ABI) thermal cycler. All samples were screened using generic ITS1 primers (ITS1 CF 5'- CCGGAAGTTCACCGATATTG -3'; ITS1 BR 5' TTGCTGCGTTCTTCAACGAA -3') (Njiru *et al.*, 2005) and TCS1 primers(TCS1 5'- CGAGCGAGAACGGGCAC - 3'; TCS2 5'- GGGACAAACAAATCCCGC -3') (Majiwa *et al.*, 1993) that are specific for *T. congolense* subgroup *savannah*. Specific primers were used to confirm the presence/absence of *T. congolense savannah* which is the main focus for genotyping studies. Five microlitres of each PCR product was separated by electrophoresis for one and half hours at 80V in a 2% (w/v) Ethidium-bromide stained agarose gel. Separated products were then visualized under ultraviolet light in a transilluminator (KODAK Gel Logic 200 Imaging System (Raytest GmbH,

Straubenhardt) to determine the presence of the bands associated with different trypanosome species **(Table 4)**. The reference strains were donated from Tanga in Tanzania except for IL 1180 from ILRI in Nairobi (**Table 4**).

Table 4: PCR amplification of trypanosome DNA from reference strains

Reference strain	Specificity	Product size	Source
IL 1180	*T. congolense savannah*	700bp	ILRI, Kenya
1	*T. congolense forest*	700bp	Tanzania
2	*T. congolesense kilifi*	620bp	Tanzania
3	*T. vivax*	250bp	Tanzania
4	*T. godfreyi*	300bp	Tanzania
5	*T. simiae tsavo*	370bp	Tanzania
6	*T. simiae*	400bp	Tanzania

4.3 Results

4.3.1 Trypanosome species and clades in cattle in Shimba Hills

All trypanosome screening experiments used an ethidium bromide-stained agarose gel to resolve different species as is shown in the sample gel images in **Figures 7**, **8** and **9**. Several gel images of the kind displayed here were analysed in order to generate the trypanosome frequency data in **Figures 10**, **11**, **12** and **13**. **Figure 7** shows the expected fragment sizes when ITS1 primers are used to amplify known trypanosome reference strains of different species and clades. When field caught *G. pallidipes* were screened, electrophoresis revealed several trypanosome species (**Figure 8**). In the case of the species-specific TCS1 test a typical positive result shows bands at 315bp (**Figure 9**).

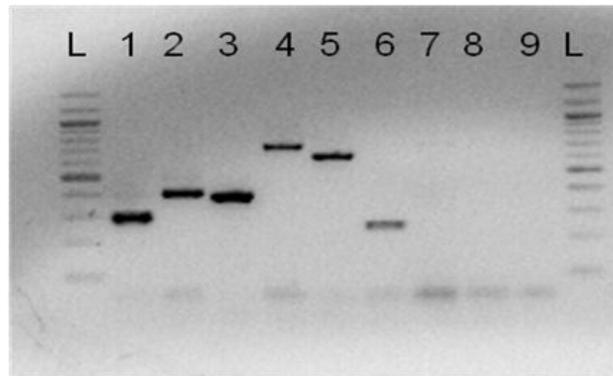

Figure 7: PCR amplification of trypanosome DNA from reference strains of some species and clades. Molecular size Marker L (New England Biolabs). Lane 1: *T. godfreyi* (280bp), 2: *T. simiae* (400bp), 3: *T. simiae tsavo* (380bp), 4: *T. congolense forest* (700bp) 5: *T. congolense* Kilifi (620bp), 6: *T. vivax* (250bp), 7: *T. congolense savannah* (700bp), 8& 9: negative control

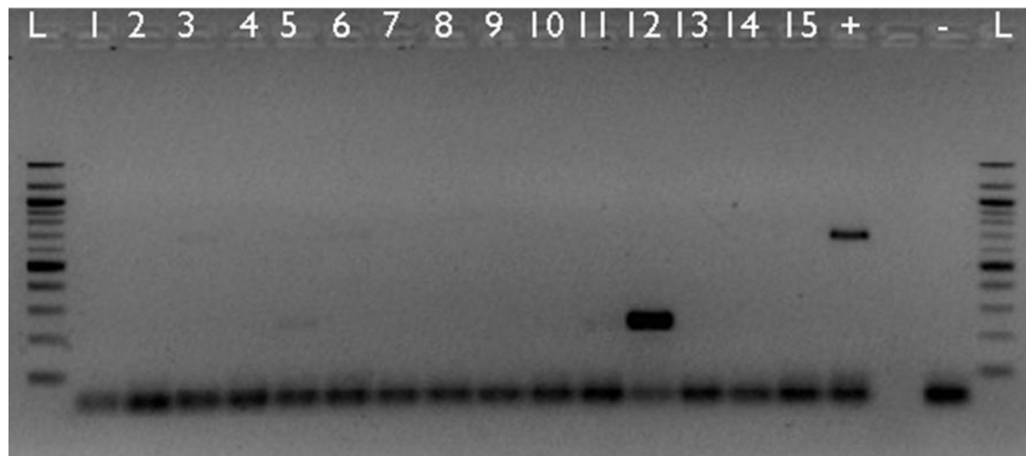

Figure 8: Ethidium stained agarose gel showing PCR products of *G. pallidipes* proboscis samples (1–15) amplified using the ITS1 primer pair. Lane L contains a molecular weight marker of 100bp (New England Biolabs). Lanes marked + (positive: icipe 001) and – (negative: water) show PCR controls. The fragment sizes observed here and their corresponding species are as follows: 250bp (lanes 5, 11 and 12) *T. vivax*; 700bp *T. congolense savannah/forest* (lanes 3 and 6) .

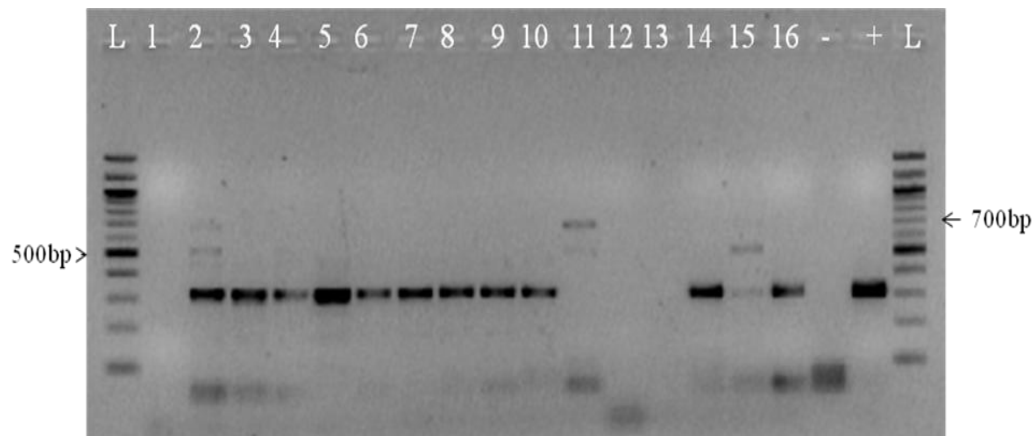

Figure 9: PCR amplification of trypanosome template DNAs using *T. congolense savannah* specific primer pair (TCS1). 100bp size marker, L (New England Biolab). Lanes marked + (positive: Icipe 001) and – (negative: water) show PCR controls. Lanes 2,3,4,5,6,7,8 9, 10, 14, and 16: *T. congolense savannah* (315bp); Lanes 2, 11: Dimer (`~700bp) and lane 15 unknown fragments 500bp.

4.3.2 ITS1 Screening to estimate trypanosome species diversity in parasitologically positive cattle in Shimba Hills

In the field study conducted in Mkanda, Mangawani and Mawia several species of trypanosomes were identified using ITS1 screening of parasitologically positive cattle blood samples. They included *T. vivax, T. congolense kilifi*, *T. congolense savannah/forest* and *T. brucei* spp. The infections were single, double or triple. The species in order of prevalence levels from highest to lowest were as follows: *T. congolense savannah*, *T. vivax*, *T. brucei* spp. and *T. congolense kilifi* (**Figure 10**). However, it is important to note that only parasitologically positive samples have been screened and so these are not true prevalence values. Three double infections were observed and they consisted of the following pairs *T. congolense savannah/T. vivax* and *T. vivax/T. brucei* Spp. (**Figure 11**). Interestingly, two triple infections were also observed; the first had *T. congolense savannah*/*T. congolense kilifi/T. vivax*, while the second had *T. congolense savannah/ T. vivax/T. brucei* Spp. (**Figure**

11). Eighty seven cattle blood samples were screened and the area with the highest numbers of *T. congolense savannah* infections was Mkanda. It is also worth reporting that samples that had been confirmed positive through PCR amplification were at 71.3% for the 87 samples (**Figure 11**) that were extracted from lysed blood pellets and 47.5% for 65 FTA card samples (**Figure 12**). This therefore indicates that lysed pellets have a higher probability of detecting signal than FTA cards.

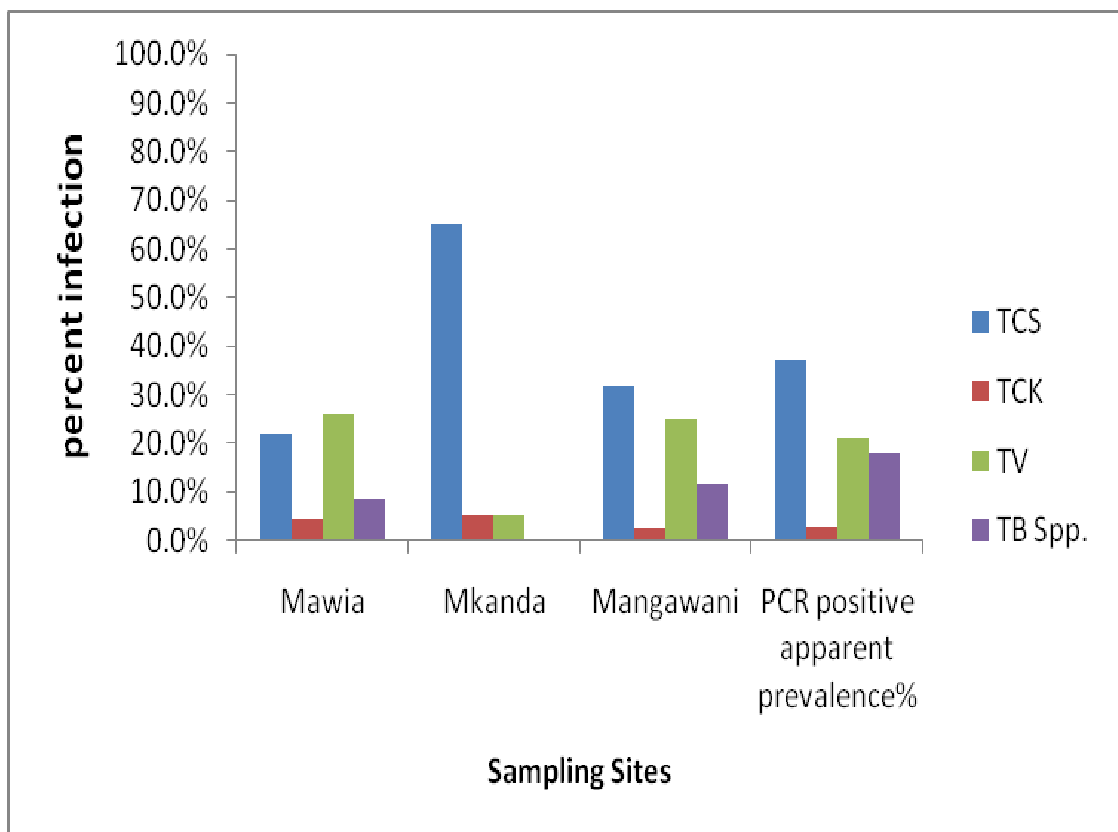

Figure 10: PCR identification of Trypanosome single infections found in Shimba Hills in cattle blood. Trypanosome species/subtype/subgenus: TCS, *T. congolense savannah* (confirmed using TCS1); TCK, *T. congolense kenya coast/kilifi*; TV, *T. vivax;* TB spp., *T. brucei* spp.

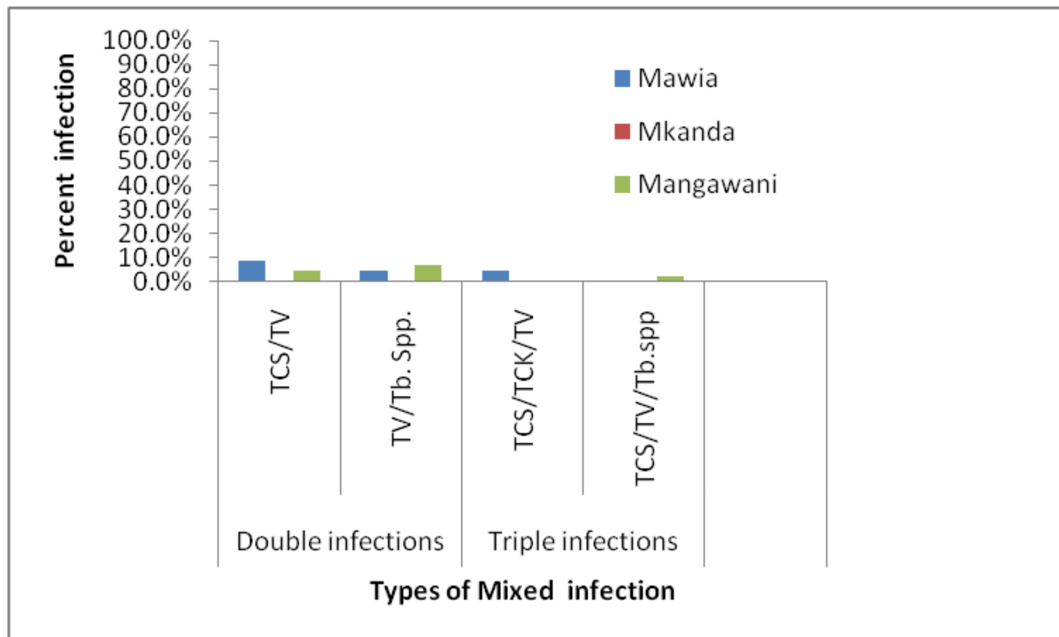

Figure 11: PCR identification of Trypanosome mixed infections found in Shimba Hills from cattle blood. Trypanosome species/subtype/subgenus: TCS, *T. congolense savannah* (confirmed using TCS1); TCK, *T. congolense kenya* coast/*kilifi*; TV, *T. vivax;* TB spp., *T. brucei* spp.

This study also investigated whether the *T. congolense savannah* samples were distributed independently of the *T. vivax* samples. If they were distributed independently the expected number of mixed infections of the two species would be 6.8. This compares with the observed number of 6 and there was no statistical difference between expected and observed values (Chi squared = 0.06, df = 1, 0.8>p>0.7). Mixed infections with the other species all have insufficient sample sizes to permit equivalent calculations.

4.3.3 Prevalence of livestock trypanosomes in Mkanda area, Shimba Hills

In order to investigate more accurately the prevalence of infection, a larger number of samples were screened from one of the sites, Mkanda, irrespective of the infection status as diagnosed by microscopy. A total of 65 cattle blood samples were screened using ITS1 primers. Trypanosomes in mixed infections were considered singly making the total number of infections 31/65 samples. Mkanda therefore had a 48% trypanosome prevalence comprising of four different trypanosome species (**Figure 12**). Two trypanosome species causing single infections were observed with *T. congolense savannah* having a high prevalence compared to *T. congolense kilifi* which had the lowest prevalence. For all the double infections there was a pattern of an equal and very low level of prevalence at 1.5% (**Figure 12**).

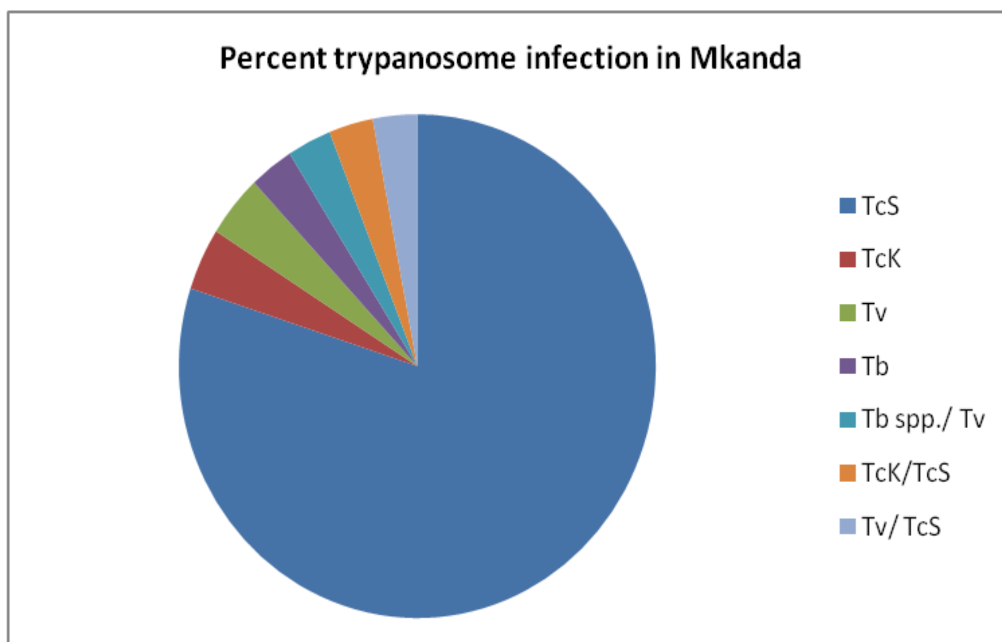

Figure 12: PCR identification of trypanosomes found in 65 cattle at Mkanda, Shimba Hills. Trypanosomes species/subtype/subgenus: TCS, *T. congolense savannah* (confirmed using TCS1);

TCK, *T. congolense kenya coast/kilifi*; TV, *T. vivax*; TB spp., *T. brucei* spp. There are three infection types which include single, double and triple infections.

4.3.4 Prevalence of trypanosomes in *G. pallidipes* in Mkanda area, Shimba Hills

Figure 13 presents a breakdown of the trypanosome species found in 128 *G. pallidipes* in the Mkanda area of Shimba Hills. Five different trypanosome species are present here either as single infections, or part of a combination in a double infection (**Figure 13**). The species with the highest prevalence is *T. congolense savannah*, followed by *T. godfreyi*, *T. vivax* and the species with the lowest prevalence were *T. congolense kilifi* and *T. simiae tsavo*. There are also three different combinations of double infections as shown in **Figure 13**. The trypanosome combinations found in the double infections are similar with those found in cattle blood (**Figures 10** and **11**) with one exception the *T. vivax/T. godfreyi* pair. The *T. godfreyi* single infection is unique to *G. pallidipes* because it is not observed in cattle blood (**Figure 10** and **11**). Additionally, *T. simiae tsavo* was found only in tsetse but not in cattle blood. The presence/absence of trypanosome infections varied between the two different body parts that were examined, with the proboscis having the greater number of trypanosome infection compared to the abdomen (**Figure 14**). Three species of trypanosomes were detected in the proboscis namely *T. vivax*, *T. congolense savannah* and *T. godfreyi* in addition to three different combinations of double infections (**Figure 14**). Only 1.6% mature *T. congolense savannah* infections constituted the total number of trypanosomes detected in the abdomen. Whether double infections occurred with the frequency expected for independent distribution of each species was not investigated because expected values were less than five in all cases.

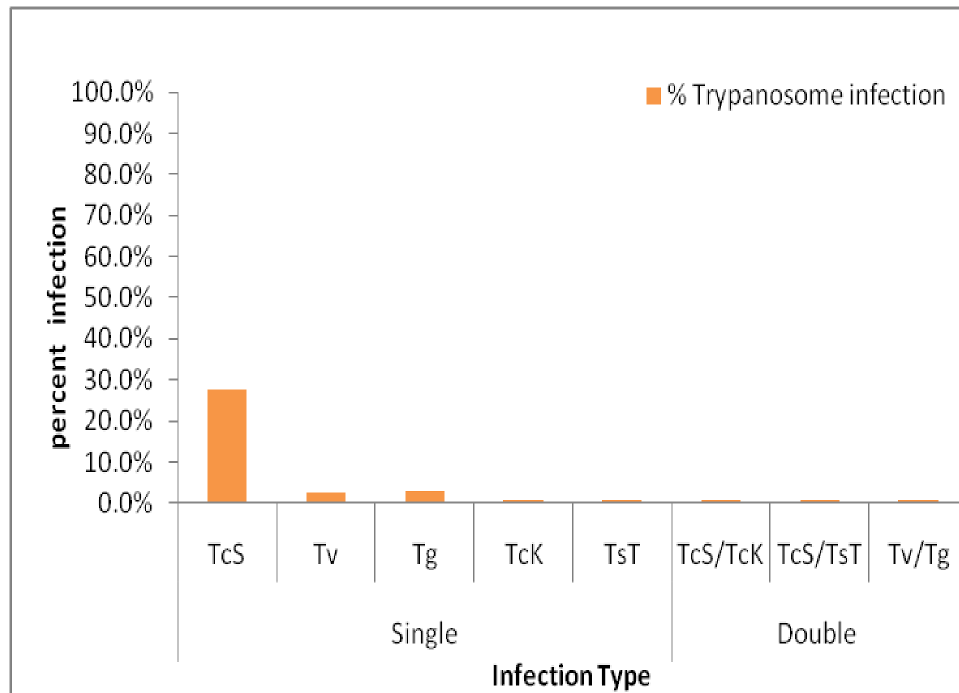

Figure 13: PCR identification using ITS1 and TCS1 primers of trypanosomes found in 120 *G. pallidipes* fly samples at Mkanda, Shimba Hills. Trypanosomes species/subtype: TCS, *T. congolense savannah*; TCK, *T. congolense kenya coast/kilifi*; TV, *T. vivax*; TST, *T. simiae tsavo*; TG, *T. godfreyi*.

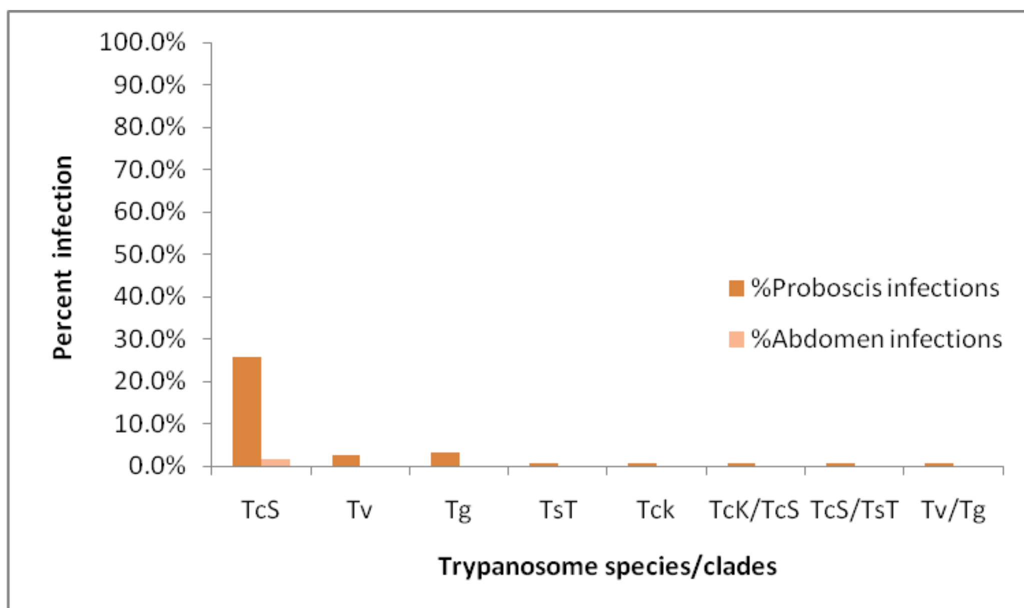

Figure 14: Proportion of proboscis and abdomen infections among 120 *G. pallidipes* fly samples that were screened in Mkanda area, Shimba Hills. Trypanosome species: TST, *T. simiae tsavo*; TG, *T. godfreyi*; TCK, *T. congolense kilifi*.

4.4 Discussion

This study was successful in identifying several species/subspecies/genera of trypanosomes by targeting the amplification of the ribosomal DNA ITS1 locus developed by Njiru *et al.* (2005) which shows length and sequence polymorphism for different trypanosome species. Six of the 11 livestock pathogenic trypanosomes studied by Njiru *et al*. (2005) were detected. This diversity appears to be similarly distributed between the flies and the cattle with the cattle lacking *T. godfreyi* and *T. simiae tsavo* which were only detected in a small proportion of flies (**Figure 13**). This is presumably because *T. godfreyi* and *T. simiae tsavo* are associated mainly with suid hosts (McNamara *et al.*, 1994) rather than cattle. The flies also lacked infections from the *T. brucei* Spp. (Figure 12), perhaps due to the low infection rate of 0.1% or less that is usually encountered for *T. brucei* Spp. (Lehane *et al.*, 2000). This means that a greater number of flies should be screened in order to improve the probability of detecting *T. brucei*. Another possible explanation for this low prevalence of *T. brucei* spp. is that alternative insect species might be the favoured vectors of these trypanosomes. Two subtypes of *T. congolense*, *kilifi* and *savannah* were detected. *T. congolense* Savannah was by far the most prevalent trypanosome species at a prevalence of 27.5% in *G. pallidipes* at Mkanda and 40% in cattle at the same location. Since *T. congolense savannah* is considered to be the most virulent in the *T. congolense* group (Bengaly *et al.*, 2002) then such high prevalence levels imply equally high levels of livestock trypanosomiasis in this area. The high level of trypanosome diversity and infection found among *G. pallidipes* in Shimba Hills correlates well with the findings observed by Tarimo *et al.* (1984) at the Kenya coast. Tarimo *et al.* (1984) observed there were higher infection rates in *G. pallidipes* found

in sites where domestic animals were kept due to their high parasitaemia and the inability to tolerate infection.

Trypanosoma congolense savannah samples detected by amplification of the ITS1 locus of ribosomal DNA were confirmed by a species-specific test to rule out the possibility of the samples being *T. congolense forest* which has a similar band size of 700bp. The clade specific test which targeted satellite DNA, using TCS1 primer pair (Majiwa *et al.*, 1993) confirmed the identity of *T. congolense savannah* and additionally exhibited a consistent banding pattern at 500bp (Figure 4) for most of the samples. Sequencing and alignment using nucleotide BLAST (www.ncbi.org) revealed that the 500bp amplicon was a hypothetical variable surface glycoprotein likely a product of non-specific amplification. An additional *T. congolense forest* species specific test may be necessary to rule out a savannah/forest double infection.

Lehane *et al.* (2000) found that trypanosomes in mixed infection divided into groups, possibly associated with the feeding preferences of groups or individual *G. pallidipes* on suids and bovids. In the present study trypanosomes found in *G. pallidipes* were in several combinations which include *T. congolense savannah*/*T. congolense kilifi* both are associated with many hosts (Lehane *et al.*, 2000); while *T. congolense savannah* is linked to a wide host range and *T. simiae tsavo* with suids; *T. vivax*, on the other hand, is associated with many hosts and *T. godfreyi* with suids. From these data we can therefore conclude that flies feed on diverse hosts found in the Shimba Hills National Reserve. A study in Nguruman located in south-western Kenya revealed that *G. pallidipes* took almost similar proportions of blood meals from diverse hosts such as the bushbuck, ostrich, elephant, buffalo, warthog and giraffe

(Sasaki *et al.*, 1995). All these hosts except for the ostrich were present in the Shimba Hills National Reserve and may be the favoured hosts/reservoirs of the trypanosomes observed here.

Trypanosome mixed infections were also found in cattle (**Figure 11** and **12**) in similar combinations as those found in flies. The exceptions were that *T. godfreyi* and *T. simiae tsavo* which favour suid hosts were not found in the cattle that were screened. Triple infections were only found in cattle in two combinations of trypanosomes that favour bovine hosts. The first combination was *T. congolense savannah/ T. congolense kilifi/ T. vivax* from a cattle sample collected in Mawia. The second combination consisted of *T. congolense savannah/T. vivax/* genus *T. brucei* Spp. from a cow sampled in Mangawani. Several researchers have demonstrated that in the field a substantial proportion of the infected tsetse flies carry mixed trypanosome infections (Majiwa and Otieno, 1990, McNamara *et al.*, 1994, McNamara *et al.*, 1995, Masiga *et al.*, 1996, Woolhouse *et al.*, 1996, Morlais *et al.*, 1998, Lehane *et al.*, 2000, Jamonneau *et al.*, 2004). Such mixed infections can be contracted simultaneously from a single animal supporting a mixed trypanosome infection (Van den Bossche *et al.*, 2004) or could be the result of sequential infections over a number of feeds on different infected animals. Moloo *et al.* (1982),as well as Gibson and Ferris (1992) have shown that teneral tsetse flies can become infected with different trypanosome species during consecutive blood meals.

Polymerase chain reaction analysis revealed the presence of more trypanosomes in proboscides compared to fly abdomens (**Figure 14**). The proboscid infections had no accompanying midgut infections and the midgut infections als had no accompanying

proboscid infections. Only two *T. congolense savannah* isolates were observed in tsetse abdomens with no accompanying proboscid infections. The lack of accompanying proboscid for infected midguts can be explained by the fact that, the midgut is a developmental site for some trypanosomes such as *T. congolense savannah*. Consequently, this observation may be an indicator of immature infections, whereby trypanosomes had not yet migrated to the mouthparts from the midgut to complete their fly developmental cycle. It is also known that not all blood meals eventually give rise to mature trypanosomes in tsetse (Aschcroft, 1959) as some infections are eliminated. Therefore, the discordant midgut/abdomen infections observed here could potentially be the result of cellular debris of non-established trypanosomes after ingestion in a blood meal. Several species of salivarian trypanosomes were detected as single or double infections in the proboscis such as *T. vivax* (two isolates), *T. godfreyi* (three isolates) and *T. congolense savannah* (29 isolates). *T. vivax* infections are known to be restricted to the cibarium/oesophageal region of the flies (Moloo and Gray, 1989) therefore this sufficiently explains their absence from the abdomen. For *T. godfreyi* (McNamara *et al.*, 1994) and *T. congolense savannah*, rapid multiplication of procyclic forms occurs in the midgut before their migration through the proboscis lumen to the salivary glands for maturation as infective epimastigotes. This essentially means that, the presence of these trypanosomes in the proboscis indicates a mature infection (Vickerman *et al.*, 1988).

However, the lack of an accompanying infection in the midgut has been shown before and can be attributed to two things. Firstly, tsetse flies infected with *T.*

congolense savannah have been shown to lose midgut infections while retaining mouthpart infections (Jordan, 1964). Secondly, a study to investigate whether there was PCR failure to identify parasitologically positive tsetse showed that identification, irrespective of trypanosome species present, failed in 40% of the samples examined (Ravel *et al.*, 2004). This however changed when DNA samples were diluted; this showed that there was a factor in the DNA preparation from abdomens/midguts that inhibited the PCR reaction (Ravel *et al.*, 2004). This was consistent with other surveys(Masiga *et al.*, 1992, Solano *et al.*, 1995, Masiga *et al.*, 1996, Woolhouse *et al.*, 1996) The factor was speculated to be related to the cellular environment of the midgut (Ravel *et al.*, 2004). In this current study the 37 proboscis infections and only two abdomen infections in random *G. pallidipes* field samples can therefore be attributed to either PCR inhibition or loss of infection in flies.

CHAPTER FIVE

5.0 Optimization of a Trypanosome genotyping protocol

5.1 Introduction

In order to identify *Trypanosoma congolense savannah* genotypes two methods were tested. Firstly, microsatellite markers were tested for genotyping trypanosomes grown in laboratory mice. Over the last decade, several polymorphic microsatellite markers specific for *T. brucei* complex, *T. vivax* and *T. congolense* species have been developed for the characterization of trypanosome isolates and for the assessment of the population structure and reproductive mode of the parasites (Biteau *et al.*, 2000, Koffi *et al.*, 2007, Morrison *et al.*, 2009). Here, a panel of ten microsatellite primers used in a study on "the mating in *Trypanosoma congolense* in the Gambia" was assessed for transferability and polymorphism (Morrison *et al.*, 2009). A multiplex PCR protocol was optimized for genotyping using the panel of ten microsatellite markers. Multiplex manager software version 1.0 (Holleley and Geerts, 2009) was used to design and optimize reactions where the multiple genetic loci were amplified in a single reaction.

Secondly, High resolution melting (HRM) was evaluated as a potential trypanosome genotyping tool. The basis of this technique is the melting of double stranded DNA which is dependent on the sequence length and composition (Ririe *et al.*, 1997, Reed *et al.*, 2007, Montgomery *et al.*, 2007). HRM analysis of the ITS1 locus was tested as a possible tool for rapid genotyping of trypanosomes. The ITS1 locus was chosen because it had the potential to genotype different trypanosomes species

simultaneously (Desquesnes and Davila, 2002). In this chapter these two methods of genotyping will be presented.

5.2 Materials and methods

5.2.1 Microsatellite Genotyping: Multiplex PCR Protocol

A set of 10 primer pairs designed by Morrison *et al* 2009, based on nucleotide sequences flanking microsatellites found in the *T. congolense* partial genome were used in multiplex reactions. Morrison *et al* 2009, designed primers to facilitate Nested PCR reactions for samples with low trypanosome DNA concentration. In this experiment, the second round primers similar to those used by Morrison *et al* 2009 with the exception of the reverse primers targeting amplification of the 3545 locus were used. The first and second round PCR reverse primers for TcM3545 were redesigned by manual inspection of the sequence enabling the production of 250 - 400bp amplicons whilst retaining primer quality. This range of products allowed for better primer combinations during multiplexing. Multiplex manager software version 1.0 (Holleley and Geerts, 2009) was used to generate the best primer combinations resulting in two PCR reactions for the ten Loci (**Figure 15**). The PCR primers were first tested in mono-plex PCR, on a *T. congolense savannah* laboratory isolate (*icipe* 001) originally isolated in Nguruman, Kenya and field DNA samples of trypanosomes originating from different sites in Shimba Hills, extracted using Ammonium acetate precipitation (Bruford *et al.*, 1998). All the primers produced good quality PCR products for all samples and were retained. These primers were then labelled with fluorescent dyes (Applied Biosystems) and integrated in a

multiplex panel consisting of two multilocus PCR sets each with five loci, (**Table 5**). Multiplex PCR was performed using the QIAGEN Multiplex kit and a thermocycler 9800Fast (Applied Biosystems). One microlitre of genomic DNA (~10-50 ng) was added to nine microlitres of mix consisting of 1X QIAGEN buffer, water and primers at final concentration of 0.2 µM. PCR conditions for both multilocus set were as follows: initial denaturation at 94 °C for 15 min; 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 60 s) and elongation (72 °C, 2 min); final extension at 60 °C for 30 min. The annealing temperature was determined using a PCR gradient ranging between 51-57°C. Amplification was done in triplicate to minimise genotyping error. 1.2 µL of PCR product was mixed with 0.25 µL of 500 LIZ Size Standard (Applied Biosystems) and 8.75 µL of formamide (Applied Biosystems). Products were then electrophoresed using an ABI PRISM 3130 sequencer (Applied Biosystems) at the Segolilab facility (the Biosciences East and Central Africa hub).

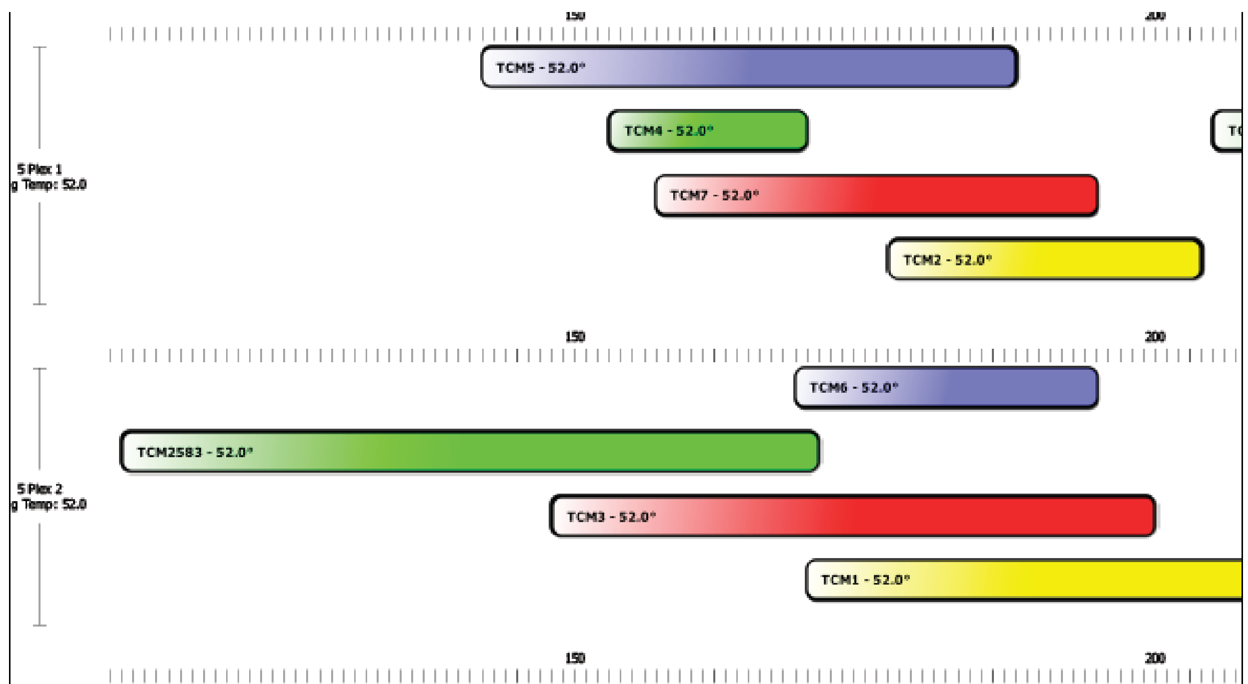

Figure 15: Multiplex setting eight out of ten loci that were evaluated divided into two reactions containing five primers each obtained with Multiplex Manager 1.0 software (Holleley and Geerts,

2009). This software was used to identify combinations of markers suitable for two multiplex reactions. For each of the ten microsatellite primers, one of the four dyes 6-FAM(Blue), VIC(Green), NED(Yellow) and PET(Red) is assigned and the allele size range is provided along the main axis (in base pairs).

Table 5: Primer pairs used for the two PCR sets of the multiplex panel. For each of the 10 loci, the table includes the repeat motifs in the sequence used to design primers, sequence and fluorescent dye label used for each primer and allelic size range. Loci 3545- R was modified to produce amplicons of 250-400bp.

PCR set	Locus	Repeat Motif	Primer Sequence	Fluorescent dye	Size Range(bp)
PCR set 1	TcM5	(AAT]n	F- CAATGGTTCAATAAGCGCACC R- AAGGCAAGTAAGTTACGC	6-FAM	159-189
	TcM4	[ATT]n	F-GTCTCTTTCCGCACAGTGAC R- GGGGGAAGATATTAAAGACAC	VIC	150-177
	TcM7	[TTA]n	F-GTGTAGTTTGTTATACTTCG R-GTTAAATACTTGTGAGAGCCAGC	PET	161-179
	TcM2	[TTA]n	F-CAGTCATGTATATGTTTGTG R- CCTGAAATGGGTCTACTGAG	NED	204-206
	1654	[TTA]n	F- CTGTAAGCACACATTCATTTGG R- CCCTCAATTGGGTATATTGCC	VIC	204-237
PCR set 2	TcM6	[TTA]n	F- GAATGCGAGACCTGCTTCTTGG R- CATTTAGACTCTCACTTTCCG	6-FAM	165-198
	2583	[GAA]n	F- AAGGACGAGGGCCATGTCTCG R- GTCCTCCAGCTCAGACAAGTC	VIC	134-143
	TcM3	[TAT]n[C] [TAT]n	F-CATGCTCTTAGGTTCCATCGG R- AGCATCCGACATTGAAACGAC	PET	146-191
	3545	[TAA]n[G] [TAA]n	F- AGGTGAGATAGATAAGAGCACG R- GCTTCTTTACTGGTTGAACGC	6-FAM	270-306
	TcM1	[TA]n[TG]	F- CTAGAAGCGAGTAACAGCC R- AAGGGTTCGTACCACAGCCC	NED	171-207

5.2.2 High resolution DNA melting analysis

Conventional PCR was performed using a Solis Biodyne HRM kit in 10µl reactions as follows: 5µl of HRM mix (containing LCG green dye), 0.5 µl of each primer (ITS1 in Table.1), 1µl template DNA and water was used to top up to 10ul. To check for precision, two technical replicates were performed per sample. The PCR cycling conditions were as follows initial PCR activation at 95 ºC for 5minutes, denaturation at 95 ºC for 10 seconds, annealing at 61 ºC for 30seconds and a final extension at 72 ºC for 10seconds. The PCR products were then subjected to HRM in a Rotor gene Q (Qiagen). The HRM step consisted of a temperature ramp between 65 and 95 ºC, rising by 0.1 to 1 ºC/s. Results were displayed in form of curves showing the different melting profiles of different trypanosome species. Template DNA (10-50ng concentration) used consisted of that from *T. vivax* (Tanzanian reference isolate), *T. congolense savannah* (icipe 001, IL 1180) and *T. brucei* (KETRI 807, 2710*)* strains. As part of optimization protocol, amplification was carried out in a Biorad thermocycler using the conditions described above. This was done to compare the amplification efficiency with that of the Rotor gene QPCR machine. 10 µl of each PCR product was then dispensed into tubes which were placed on a rotor for HRM using the earlier described temperature ramp and increment. The rest of the product was analysed using gel electrophoresis to check for successful amplification.

5.3 Results

5.3.1 Microsatellite genotyping

The multiplex PCR setting allowed the successful amplification of up to five loci in a single PCR reaction (**Figure 16**). All but TcM 2 and 5 microsatellite loci were successfully amplified. Among the eight successfully amplified loci, all were polymorphic including TcM 1654, 2583, 4, 3545, and 1 showed greater polymorphism compared to Morrison's findings in 2009. TcM 7, 3 and 6 had a lower number of alleles compared to Morrison's findings (**Table 6**). For the 35 field *T. congolense savannah* samples that were genotyped there were missing data at different loci (**Table 7** and **8**).

Table 6: The number of observed alleles for the multiplex reactions in comparison with the number of alleles obtained by Morrison *et al*., 2009. NB: (-) represents no alleles.

PCR set	Locus	No. of alleles observed	No. of alleles (Morrison *et al*., 2009)
PCR set 1			
	TcM 5	-	13
	TcM 4	7	6
	TcM 7	7	8
	TcM 2	-	9
	TcM 1654	7	1
PCR set 2			
	TcM 6	4	9
	TcM 2583	7	1
	TcM 3	5	9
	TcM 3545	8	1
	TcM 1	14	8

Table 7: Alleles scored at loci: TcM 1, TcM 3, TcM 4, TcM 6 and TcM 7, for 35 *T. congolense savannah* isolates sampled from flies and cattle in the Shimba Hills area**.**

Sample name	TcM1	TcM1	TcM1	TcM1	TcM3	TcM3	TcM4	TcM4	TcM4	TcM4	TcM6	TcM6	TcM6	TcM7	TcM7	TcM7
SH.KZ.5.11#2	181	181	0	0	149	155	161	170	0	0	173	173	0	166	169	0
SH.M.7.11#378	185	187	0	0	149	149	161	161	0	0	173	173	0	000	000	0
SH.MA.5.11#284	185	193	0	0	149	149	161	161	0	0	000	000	0	166	178	0
SH.MA.5.11#289	187	187	0	0	155	155	000	000	0	0	167	176	0	000	000	0
SH.MA.7.11#282	195	195	0	0	161	161	161	161	0	0	170	176	0	166	166	0
SH.MA.7.11#284	185	193	0	0	149	149	161	161	0	0	173	188	0	166	178	0
SH.MA.7.11#289	187	187	0	0	155	155	167	173	0	0	167	173	176	166	166	0
SH.MA.7.11#297	000	000	0	0	000	000	000	000	0	0	176	176	0	000	000	0
SH.MA.7.11#298	000	000	0	0	000	000	000	000	0	0	000	000	0	000	000	0
SH.MA.7.11#322	171	187	0	0	149	149	155	161	0	0	180	180	0	166	175	0
SH.MA.7.11#359	000	000	0	0	149	149	161	167	0	0	167	173	0	166	166	0
SH.MA.7.11#360	187	193	0	0	000	000	161	161	0	0	167	176	0	163	163	0
SH.MA.7.11#378	000	000	0	0	000	000	161	161	0	0	176	176	0	166	175	0
SH.MA.7.11#382	191	191	0	0	149	149	161	161	0	0	000	000	0	166	166	0
SH.MA.7.11#383	189	193	0	0	149	149	161	161	0	0	167	176	0	000	000	0
SH.MA.7.11#393	185	187	0	0	000	000	152	152	0	0	176	176	0	163	166	0
SH.MA.7.11-FlyAB#2	179	181	183	0	149	161	161	173	152	0	176	183	0	166	166	0
SH.MA.7.11-FlyAB#55	000	000	0	0	000	000	161	161	0	0	000	000	0	000	000	0
SH.MA.7.11-FlyP#45	181	185	187	193	149	161	158	161	170	0	167	176	0	166	169	0
SH.MA.7.11-FlyP#107	171	179	187	0	000	000	158	161	167	173	173	180	0	166	169	178
SH.MA.7.11-FlyP#112	171	181	187	0	000	000	000	000	0	0	167	173	180	000	000	0
SH.MN.5.11#47	000	000	0	0	000	000	152	161	0	0	000	000	0	166	166	0
SH.MN.7.11#7	185	185	0	0	185	185	000	000	0	0	000	000	0	000	000	0
SH.MN.7.11#32	189	193	0	0	149	149	161	161	0	0	167	176	0	163	166	0
SH.MN.7.11#44	171	187	0	0	000	000	155	161	0	0	173	180	0	166	175	0
SH.Mts.5.11#2	000	000	0	0	161	161	161	167	173		000	000	0	166	166	0
SH.MW.5.11#7	000	000	0	0	000	000	000	000	0	0	173	173	0	000	000	0
SH.MW.5.11#26	181	193	0	0	149	149	161	167	170		176	176	0	166	166	0
SH.MW.5.11#142	189	201	0	0	161	191	000	000	0	0	167	176	0	000	000	0
SH.MW.5.11#153	183	193	0	0	149	149	000	000	0	0	173	176	0	000	000	0
SH.MW.7.11#10	185	201	0	0	149	149	161	161	0	0	188	188	0	175	175	0
SH.MW.7.11#23	183	193	0	0	000	000	158	161	0	0	173	176	0	166	169	0
SH.PN.5.11#1	187	187	0	0	149	149	000	000	0	0	000	000	0	000	000	0
SH-MK-P#106	000	000	0	0	000	000	000	000	0	0	000	000	0	000	000	0

Table 8: Alleles scored at loci: TcM 1654, TcM 2583, TcM 3545, for 35 *T. congolense savannah* isolates sampled from flies and cattle in Shimba Hills area.

Sample name	TCM1654	TCM1654	TCM2583	TCM2583	TCM3545	TCM3545	TCM3545
SH.KZ.5.11#2	212	214	137	140	297	297	0
SH.M.7.11#378	214	214	137	140	276	300	0
SH.MA.5.11#284	214	214	140	140	300	300	0
SH.MA.5.11#289	000	000	140	140	276	297	0
SH.MA.7.11#282	214	221	137	140	294	294	0
SH.MA.7.11#284	214	214	140	140	300	300	0
SH.MA.7.11#289	214	214	140	140	276	297	0
SH.MA.7.11#297	000	000	000	000	000	000	0
SH.MA.7.11#298	000	000	137	140	000	000	0
SH.MA.7.11#322	212	214	137	140	294	297	0
SH.MA.7.11#359	212	212	137	140	000	000	0
SH.MA.7.11#360	214	214	140	140	000	000	0
SH.MA.7.11#378	214	214	000	000	300	300	0
SH.MA.7.11#382	214	214	140	140	000	000	0
SH.MA.7.11#383	214	214	140	140	294	294	0
SH.MA.7.11#393	212	214	140	140	276	276	0
SH.MA.7.11-FlyAB#2	214	237	140	140	273	297	0
SH.MA.7.11-FlyAB#55	000	000	137	140	000	000	0
SH.MA.7.11-FlyP#45	212	212	137	140	276	294	297
SH.MA.7.11-FlyP#107	214	237	140	140	276	294	297
SH.MA.7.11-FlyP#112	000	000	140	140	276	294	297
SH.MN.5.11#47	000	000	000	000	000	000	0
SH.MN.7.11#7	000	000	137	140	000	000	0
SH.MN.7.11#32	214	214	140	140	294	306	0
SH.MN.7.11#44	214	214	137	140	294	294	0
SH.Mts.5.11#2	214	224	140	140	294	297	0
SH.MW.5.11#7	000	000	000	000	000	000	0
SH.MW.5.11#26	214	214	137	140	294	294	0
SH.MW.5.11#142	000	000	140	140	276	276	0
SH.MW.5.11#153	000	000	137	140	297	297	0
SH.MW.7.11#10	221	221	140	140	297	297	0
SH.MW.7.11#23	214	214	137	140	000	000	0
SH.PN.5.11#1	000	000	137	140	297	297	0
SH-MK-P#106	000	000	140	140	000	000	0

5.3.1.1 Multilocus Genotypes of reference strains

Four complete multilocus genotypes were obtained at eight loci for the four *T. congolense savannah* samples isolated from Mice. Locus 2583 appears monomorphic while the rest of the loci appear polymorphic.

Table 9: Alleles scored at eight loci for *T. congolense* Savannah reference strains grown in mice. Four multilocus genotypes were observed, one for each strain

Sample name	TCM1	TCM1	TCM3	TCM3	TCM4	TCM4	TCM6	TCM6	TCM7	TCM7	TCM1654	TCM1654	TCM2583	TCM2583	TCM3545	TCM3545
ICIPE001	179	183	149	161	161	173	176	183	166	166	214	237	140	140	273	297
IL1180 G	171	187	149	185	161	161	167	176	166	169	212	214	140	140	276	282
MC4.8	185	185	149	149	161	179	167	180	166	172	212	212	140	140	294	300
RTCS	179	183	149	161	161	173	176	183	166	166	214	237	140	140	273	297

5.3.1.2 Mean number of alleles

Genetic variation was assessed by determining the mean number of alleles per locus (A). A was calculated with Geneclass 2 ver. 2.0.g (Piry *et al.*, 2004). For comparisons of A values between population samples, estimation of allelic richness (AR) was on the basis of minimum sample size, using the rarefaction method (Petit *et al.*, 1998) implemented in Fstat 2.9.3 (Goudet, 2001). Differences in allelic richness and the mean number of alleles were not statistically significant among the following: all samples, all field samples, all cattle samples, all Mkanda flies and all Mkanda cattle samples (**Table 10**).

Table 10: Average number of alleles per locus (A). Given by direct counts (DC) and as allelic richness (AR). AR is based on minimum sample size (N=16 in Friuli for locus DVV-ET1)

No. of alleles per locus	All samples	All field samples (cattle + flies)	All cattle samples	All fly samples (from Mkanda)	Cattle (Mkanda)
Loci					
TCM1	11	11	10	7	7
TCM3	5	5	5	2	3
TCM4	8	7	7	6	5
TCM6	7	7	6	5	6
TCM7	6	5	5	3	4
TCM1654	5	5	4	3	3
TCM2583	2	2	2	2	2
TCM3545	7	6	5	4	4
Average No. of alleles	6.375	6	5.5	4	4.25
Allelic richness based on N=2 for TCM3 in the flies	5.714286	5.285714	4.857143	3.571429	3.857143

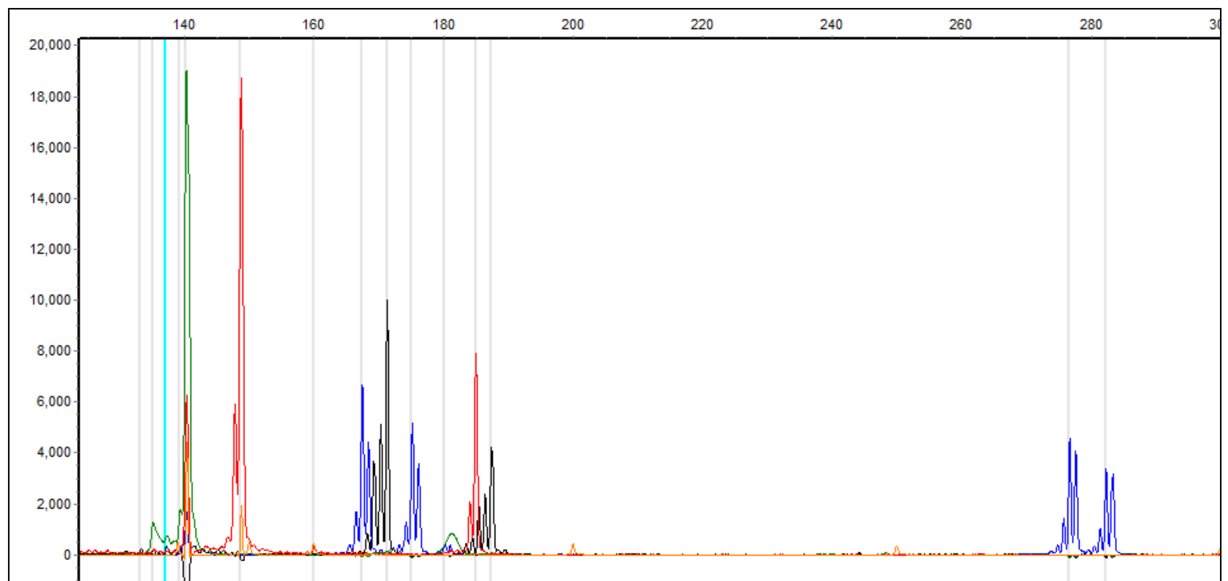

Figure 16: Electropherogram of a PCR set showing successful amplification of five microsatellite loci in a single run of IL1180 *T. congolense savannah* isolate. Different fluorescently tagged loci with each colour representing a single locus (From left to right):Green (134-143-bp, VIC, TcM2583), Red (146-191bp, PET, TcM3), Blue (165-306bp, 6-FAM, TcM6/TcM3545), Yellow (171-207bp, NED, TCM 1) and Orange(size marker).

5.3.2 High resolution melting

Successful high resolution melting was achieved for the four *T. congolense savannah* characterised strains that were amplified by targeting the ITS1 locus of ribosomal DNA. The curves of all the four strains numbered 1-4 appeared staggered and did not have the conventional sigmoid shape obtained during melting. The curves clustered together for all the four strains (**Figure 17**). **Figure 18** shows the distinct melting curves of three different species *T. vivax* (250bp amplicon), *T. brucei* (500bp amplicon) and *T. congolense savannah* (700bp amplicon) in duplicate. Four different replicates of *T. vivax* divided into two melting profiles each containing a pair of curves.

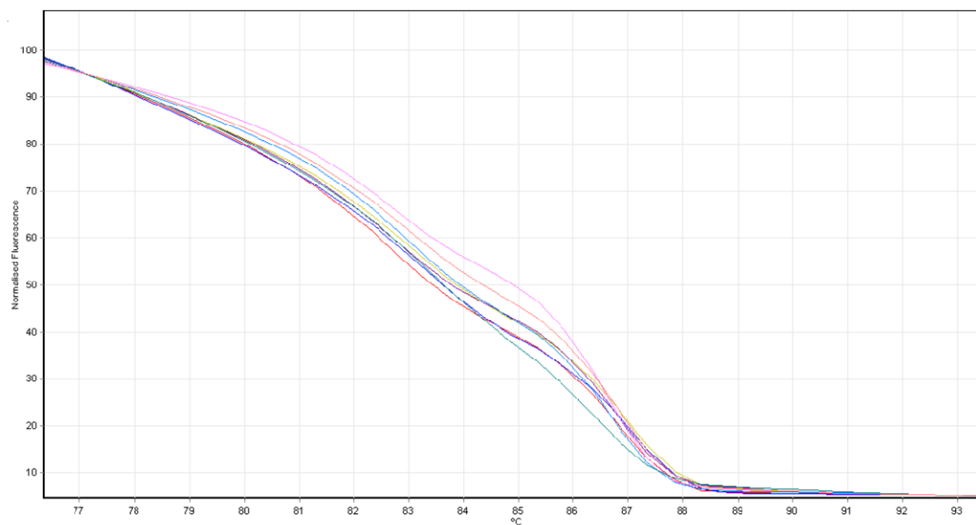

Figure 17: Normalized HRM curves of 4 different strains (*icipe* 001, ILRI 1180, Tanzanian isolate, 1 field isolate) of *T. congolense savannah*. The y- axis has normalized fluorescence units (with intervals of 10 units between 0-100) while the x-axis shows the HRM temperature range (77-93°C)

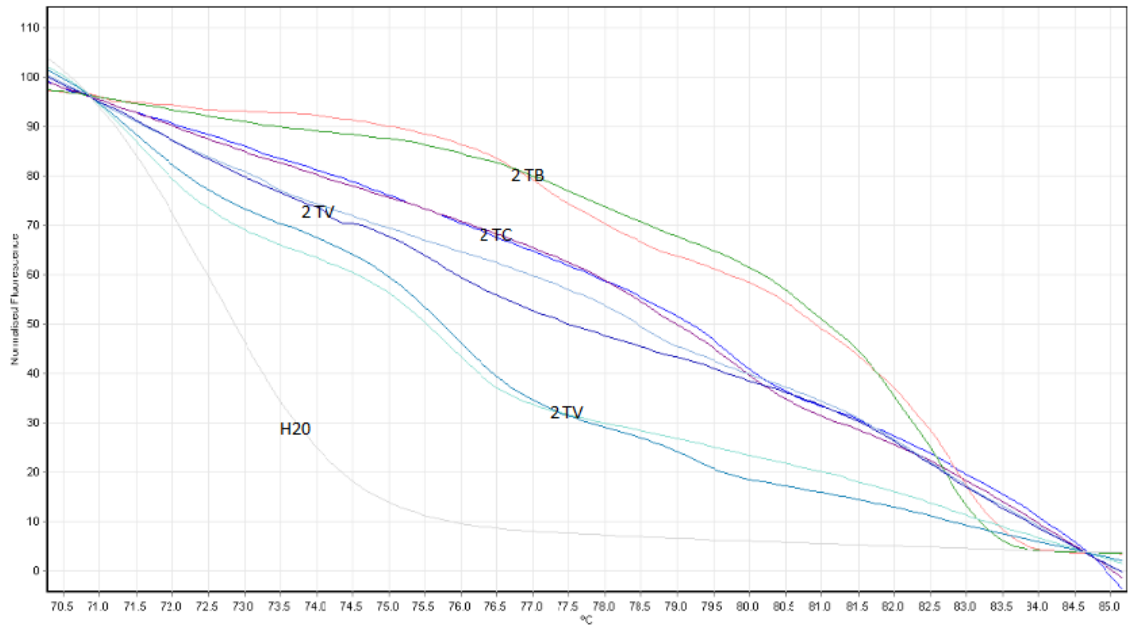

Figure 18: Normalized HRM curves of three different species/strains: TB, *T. brucei* (KETRI 807), TC, *T. congolense savannah* (*icipe* 001), 4TV, *T. vivax* (Tanzanian isolate) and H_20 (Negative control). The y- axis has normalized fluorescence units (with intervals of 10 units between 0-110) while the x-axis shows the HRM temperature range (70.5 - 85°C)

5.4 Discussion

A multiplex genotyping protocol for ten microsatellite loci in two sets was successfully optimized for reference DNA of trypanosomes grown in laboratory animals (**Table 9**). This was possibly due to the high trypanosome concentration that is attainable in laboratory rodents. A high trypanosome DNA concentration increases the template available for primers to work on thereby elevating the concentration of target DNA. Although multiplexing of the ten loci was possible, there was a problem when working with field isolates because of low starting DNA concentrations. This was demonstrated by the incomplete data observed at the different loci for the 14 field isolates that were tested (**Table 7** and **8**). Previously, better amplification of poor templates was achieved through whole genome amplification followed by nested PCR reactions in single - locus PCR for all loci (Morrison *et al*., 2009). This approach, however, is not practical for high throughput screening of *T. congolense savannah* due to the cost and time involved.

Eight out of the ten loci that were evaluated were polymorphic and amplified successfully except for TcM 2 and TcM 5 which failed. Failure of these loci may be due to mutations in the primer binding site flanking the microsatellite or lack of this locus in the isolates examined. Mutations occurring in some individuals in the conserved primer region have been reported to have one allele amplify or a complete failure in amplification (Paetkau and Strobeck, 1995) further explaining the TcM 2/TcM 5 observations. Morrison *et al* (2009) found seven of the ten microsatellite markers tested in this study to be polymorphic and used them to study mating in *T. congolense savannah* isolates collected in the Gambia. In the current study, three

additional markers from the batch of 25 initially evaluated by Morrison *et al* (2009) were included and they all appeared to be polymorphic. The eight markers instead of seven are an advantage, because each marker locus can be considered a sample of the genome (Selkoe and Toonen, 2006). Thereby increasing the number of samples of the genome by combining the results from numerous loci provides a more accurate and statistically powerful way of comparing individuals (Selkoe and Toonen, 2006). The varying patterns of polymorphism among the loci evaluated in the Gambia in comparison to this study may be due to genetic differentiation in the *T. congolense savannah* population genotyped (in the Gambia and Kenya) and/or the small number of isolates genotyped in the present study.

More than two alleles were genotyped at several loci (**Table 10**) indicating multiple infections of *T. congolense savannah* in a single cow and flies. For the tsetse flies, it is the first time that multiple genotypes of a single trypanosome species are being identified in a single proboscis. This protocol therefore offers an excellent cost and time saving way of high throughput genotyping of *T. congolense savannah* from the East African region.

The multiplex genotyping protocol in this study can be used in verifying the identity of a trypanosome reference strain. This is because; it is sometimes difficult to rely on the recorded history of trypanosome stabillates. Moreover when they are transferred from one research institution to another, trypanosome strains are often given new names creating confusion with regards to their identity. Genotyping of trypanosome strains is therefore essential for the characterization of laboratory trypanosome stabillates to avoid confusion between strains. Using a relatively costly genotyping

protocol (involving nested PCR and single locus PCR, Morrison *et al*.,(2009) was able to genotype *T. congolense savannah* laboratory strains at seven microsatellites loci. This genotyping led to the conclusion that IL1180 and KETRI2885 share the same genotype at the 7 microsatellites loci used (Morrison personal communication). It is interesting to see that both strains also have a similar history. IL1180 and KETRI2885 were described by independent sources, to have been isolated from a lion in Tanzania in 1971. The present work thus provides a cost effective and high-throughput protocol enabling the genotyping of *T. congolense savannah* laboratory isolates at 8 microsatellites loci in two PCR. This is a useful tool to more accurately characterize strains and to avoid duplicating work. The duplication occurs when one works on the same samples all the while considering them different erroneously.

High resolution melting analysis, genotyping of organisms using ITS2 has been demonstrated in *Symbiodinium* (Granados-Cifuentes and Rodriguez-Lanetty, 2011) however it was quite challenging to do the same for trypanosomes using ITS1 in this study. There were variations within replicates of a single sample, which were seen in the melting curve profiles (**Figure 17** and **18**). The within sample variations can be explained by the nature of the ITS1 region. The region is described as hyper-variable; with a high copy number (100-200copies) (Desquesnes and Davila, 2002, Hernandez *et al.*, 1993). This implies that for a single amplification several different copies serve as templates. These different templates have internal sequence variability and may be the reason why single samples yielded different melting curve profiles. Each curve was also observed to have two staggered melting temperatures (**Figure 17**), which may indicate two different amplicons in a single reaction. These

differences in melting curve profiles may also be due to varying concentrations of the amplicon which have been found to result in varying entropy changes and therefore curve shapes (Schutz *et al*., 2009). This is supported by the above explanation on copy numbers and their amplification behaviour. A more preferred target for future HRM genotyping of trypanosomes would be a single copy locus.

The HRM melting curves did not have the acceptable and recommended S-shape that is typical of these reactions. This may be due to factors such as purity and size of amplicons. The size range of PCR products obtained using ITS1 primers is between 250-700bp. For HRM analysis optimal results are obtained when amplicons are <200bp (Montgomery *et al.*, 2007), however fragments of 400-1000 bp can used for analysis at lower resolution. The large fragments yielded by ITS1 may therefore be to blame for the poor resolution of products and therefore resulting in atypical curves. The peculiar melting curves observed in this study may be accurate under the prevailing conditions however, more optimization steps are necessary to determine typical trypanosome curves. There is no known typical shape for trypanosome melting though Njiru *et al* (2011) demonstrated that HRM can be used to verify isothermal Loop DNA mediated amplification results for *T. vivax* identification. Njiru's work however, may not be considered to be of high resolution because of the large temperature increment at 1 ºC/s it was a confirmatory test. This therefore means that considerable further technical development is required before HRM analysis can be applied to genotype trypanosomes.

CHAPTER SIX

6.0 GENERAL DISCUSSION

Shimba Hills, Kubo division in Kwale County, presents an excellent area to study vector- parasite interactions both in the domestic and sylvatic cycles. This is as a result of the high fly density, incidence of at least six trypanosome species of veterinary importance and availability of a variety of hosts.

Trypanosoma congolense savannah was found to be the most prevalent trypanosome species and it being the most virulent strain means that the disease burden is high for the livestock found adjacent to the game reserve. This finding informs the management of the disease, by giving a more precise and accurate picture of the trypanosome prevalence which is directly proportional to the incidence of the disease.

In addition to having more knowledge on the occurrence of trypanosomes of veterinary importance, this study provides a tool for studying diversity at a refined scale. A multiplex protocol for the genotyping of *T. congolense savannah* isolates grown in laboratory rodents is in place. This protocol will be useful in characterizing trypanosomes in cryobanks. The current protocol is rapid and relatively cost effective. Attempts were made to use HRM, an even more effective tool to genotype trypanosomes. In order to use this tool a new single copy gene needs to be identified and optimization done.

CHAPTER SEVEN

7.0 Conclusions and recommendations

7.1 Conclusions

1. There is a significant population of *G. pallidipes* around and in the Shimba Hills National Reserve to facilitate studies on trypanosome genetics.

2. *Glossina pallidipes* presents great trypanosome diversity with *T. congolense savannah* being the lead agent causing Nagana in this focus.

3. This study developed a multiplex PCR protocol which is now available for the rapid genotyping of *T. congolense savannah* isolates at concentrations greater than 10^7 cells. This protocol successfully amplified nine polymorphic microsatellite loci. This protocol therefore facilitates the study of *T. congolense savannah* genotypes circulating in this and other foci in the East African region.

7.2 Recommendations

The following recommendations can be made from this study:

1. The multiplex PCR protocol should be adopted for rapid characterization and identification of *T. congolense savannah*

2. Evaluate a single copy gene for the possible genotyping of trypanosomes using HRM analysis.

3. Map the *T. congolense savannah* genotypes in the Shimba Hills area and correlate them with existing disease phenotypes.

4. Further optimization of the genotyping protocol on field isolates which have a low concentration of trypanosome DNA (Less than 10^7).

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