MOLECULAR DETECTION, GENETIC AND PHYLOGENETIC ANALYSIS OF TRYPANOSOME SPECIES IN UMKHANYAKUDE DISTRICT OF KWAZULU-NATAL PROVINCE, SOUTH AFRICA

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that it has not, previously in its entirety or in part, been submitted at any university for a degree. I therefore cede copyright of this dissertation in favour of the University of the Free State.

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DEDICATION

‘To my nephews and nieces to inspire them in reaching their dreams and goals’

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TABLE OF CONTENTS

CHAPTER TWO

2. THE PREVALENCE OF TRYPANOSOME SPECIES IN UMKHANYAKUDE DISTRICT OF KWAZULU-NATAL PROVINCE, SOUTH AFRICA

CHAPTER THREE

3. GENETIC DIVERSITY WITHIN AND AMONG *TRYPANOSOMA* SPECIES IN KWAZULU-NATAL PROVINCE, SOUTH AFRICA

CHAPTER FOUR

4. PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN TRYPANOSOMES DETECTED IN KWAZULU-NATAL PROVINCE

List of figures

List of tables

List of plates

ABBREVIATIONS

A: Adenine

ARC: Agricultural Research Council

AFLP: Amplified fragment length polymorphisms

AMOVA: Analysis of molecular variance

AAT: African animal trypanosomiasis

BARP*: brucei* alanine-rich proteins

Bst: *Bacillus stearothermophilus*

Bp: Base pair(s)

BLAST (bl2 seq): Alignment of two sequences using BLAST

BLAST: Basic local alignment search tool

BLAST (n): Nucleotide BLAST

CAT: Canine African trypanosomiasis

CATT: Card agglutination test for trypanosomiasis

CNS: Central nervous system

CSF: Cerebrospinal fluid

cyt b: Cytochrome b

C: Cytosine

DDT: Dichlorodiphenyltrichloroethane

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide triphosphate

ddH_2O: Double distilled water

ELISA: Enzyme-linked immunosorbent assay

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

E-value: Expect value

FTA card: Fast technology for analysis of nucleic acids

G. austeni: Glossina austeni

G. brevipalpis: *Glossina brevipalpis*

G. m. morsitans: *Glossina morsitans morsitans*

G. pallidipes: Glossina pallidipes

GARP: Glutamic acid/alanine-rich proteins

gGAPDH: glycosomal Glyceraldehyde 3-phosphate dehydrogenase

G: Guanine

HAT: Human African trypanosomiasis

H-trap: Harris/ horizontal trap

IFAT: Indirect fluorescence antibody test

ITS: Internal transcribed spacer

KZN: KwaZulu-Natal Province

LAMP: Loop-mediated isothermal amplification

$MgCl_2$: Magnesium chloride

mtDNA: Mitochondrial DNA

MEGA: Molecular evolutionary genetic analysis

NCBI: National center for biotechnology information

NTS: Non-transcribed spacers

NaCl: Sodium chloride

PCV: Packed cell volume

PCl: Phenol-chloroform-isoamyl alcohol

PCR: Polymerase chain reaction

Pro-K: Proteinase K

RAPD: Randomly amplified polymorphic DNAs

RFLP: Restriction fragment length polymorphism

RPM: Revolutions per minute

RNA: Ribonucleic acid

SSU rRNA: Small subunit ribosomal RNA

SDS: Sodium dodecyl sulphate

SPR: Subtree-pruning-regrafting

Taq: Thermus aquaticus

TU: Transcriptional units

T. b. brucei: *Trypanosoma brucei brucei*

T. b. gambiense: *Trypanosoma brucei gambiense*

T. b. rhodesiense: Trypanosoma brucei rhodesiense

T. congolense: *Trypanosoma congolense*

T. cruzi: *Trypanosoma cruzi*

T. equiperdum: Trypanosoma equiperdum

T. evansi: *Trypanosoma evansi*

T. theileri: *Trypanosoma theileri*

T: Thymine

Tris-HCl: Tris-Hydrochloric acid

UV light: Ultra violet light

VSG: Variant surface glycoprotein

WBC: White blood cell

ABSTRACT

African animal trypanosomiasis (AAT) is a disease caused by haemoparasites of the genus *Trypanosoma* and its vectors are tsetse flies of the genus *Glossina* which are endemic to the African continent. In South Africa the disease is restricted to the north eastern parts of KwaZulu-Natal Province and it is transmitted to susceptible vertebrate hosts by *Glossina brevipalpis* and *G. austeni*. The current study aimed at determining the prevalence, genetic diversity and the phylogenetic position of the South African trypanosome species in the north eastern KwaZulu-Natal as well as determining preferred feeding host by tsetse flies from their blood meal. A total of 296 blood samples were collected from the north eastern parts of KwaZulu-Natal Province whereby 137 were from cattle; 101; 9; 49 were from goats, sheep and dogs respectively and 376 tsetse flies (375 *G. brevipalpis* and 1 *G. austeni*) were also collected. PCR with universal KIN primers was used to detect the trypanosome parasites in both blood and tsetse flies. From 137 cattle samples 23.4% (32/137) were positive for the presence of trypanosome infections whilst none were positive for sheep, goat and dog samples. A total of 15.4% (54/375) *G. brevipalpis* tested positive for trypanosomes. Detected trypanosome species with KIN primers were *Trypanosoma congolense* (Savannah) and *T. theileri* for blood samples and for tsetse flies *T. congolense* (Savannah and Kilifi) types were detected. Nested PCR targeting 18S rRNA gene detected *T. congolense* (Savannah) and *T. theileri* species. The sequences from this gene revealed great genetic diversity within these *Trypanosoma* species. Amplification of gGAPDH gene detected *T. congolense* (Savannah) and *T. brucei brucei* species when subjected to BLAST. Sequences obtained from this gene also revealed great genetic diversity and showed that the detected trypanosomes are different genotypes from the known species in other countries outside South Africa. Phylogenetic analysis revealed that South African *Trypanosoma* species were more genetically related to east African trypanosomes however, they formed isolated clusters with each other indicating that indeed they are different genotypes from the trypanosome species on the NCBI database. Blood meal analysis showed that *G. brevipalpis* preferred to feed on small mammals, birds and humans in the absence of livestock or other large wild reservoir hosts. This study showed that there are active trypanosomes circulating amongst livestock and tsetse flies in KwaZulu-Natal Province as well as the prevalence of *T. theileri* and *T. b. brucei* which were never documented in previous studies. Further research is needed to investigate the pathogenicity of these detected *Trypanosoma* parasites in domestic animals.

Key words: African animal trypanosomiasis, PCR, prevalence, genetic diversity, phylogenetic position, host preference, KwaZulu-Natal Province

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Classification of trypanosomes

Trypanosomes belong to the phylum Sarcomastigophora and order Kinetoplastida (Stevens and Brisse, 2004). Members of kineplastids are flagellated protozoans that are distinguished by the presence of a DNA-containing region, known as a kinetoplast in their single large mitochondrion (Stuart *et al*., 2008). They are monophyletic and parasitize almost all animal groups ranging from fish to humans as well as plants and insects. African trypanosomes are protozoan blood parasites of the genus *Trypanosoma* that infect most vertebrates. They are widely dispersed throughout the sub-Saharan Africa primarily in tropical areas covering an area of 10 million km² (OIE, 2013). Tsetse flies (genus *Glossina*) act as vectors for the transmission of these haemoparasites whereby, transmission occurs when an infected fly feeds on a susceptible mammalian host (Esterhuizen *et al*., 2005; Mekata *et al.,* 2008). Due to the parasite site of development in the carrier and mode of transmission by the vector arthropods, pathogenic and economically important trypanosome species infecting mammals are divided into two different groups namely; the Stercoraria (subgenera *Schizotrypanum, Megatrypanum* and *Herpetosoma*), in which the infective forms of trypanosomes are formed in the hindgut and are then passed on to the host by contaminative transmission from the posterior of the vector. Secondly, the Salivaria (subgenera *Duttonella, Nannomonas, Pycnomonas* and *Trypanozoon*), in this group transmission occurs at the anterior position of the vector and it is inoculative (Stevens and Brisse, 2004). However, the Salivaria species are more abundant in Africa due to that characteristically they possess a variant surface glycoprotein (VSG) gene and are the only trypanosomes to show antigenic variation (Stevens and Brisse, 2004). Meaning they can alter their surface proteins to evade their host's immune response. Subgenera and species of medical and veterinary importance are: (i) *Duttonella*: *Trypanosoma vivax* and *Trypanosoma uniforme*; (ii) *Nannomonas*: *Trypanosoma congolense* and *Trypanosoma simiae*; (iii) *Pycnomonas: Trypanosoma suis* and (iv) *Trypanozoon*: *Trypanosoma brucei brucei, Trypanosoma brucei gambiense, Trypanosoma brucei rhodesiense*, *Trypanosoma evansi* and *Trypanosoma equiperdum* (Gibson, 2007).

During an infection by a Salivaria parasite, an individual trypanosome only expresses a single VSG gene at a time and this enables the immune system to respond against this particular VSG resulting in the release of antibodies that will neutralize the parasites (Wiser, 2011). However, some trypanosomes will switch to the expression of a different VSG gene leading to the replacement of the surface coat with a protein not recognised by the antibodies that are present in the serum at that particular moment in time. Therefore, resulting in a new wave of parasitemia and these new parasites will increase in numbers since the expressed VSG genes are not familiar to the host's immune response (Wiser, 2011). Due to the ability of the Salivaria trypanosomes to switch their expressed VSG genes, in sub-Saharan Africa these parasite pose serious threats to the wellbeing of both domestic animals and humans. Whereby, more than 50 million cattle and more than 60 million people in 37 countries are likely to be infected by animal or human African trypanosomiasis (Esterhuizen *et al*., 2005; Mekata *et al.,* 2008; OIE, 2013).

Classification of trypanosomes of economic, medicinal and veterinary importance (Stevens and Brisse, 2004)

Subkingdom : Protozoa
Phylum : Sarcomastigophora
Class : Zoomastigophorea
Order : Kinetoplastida
Family : Trypanosomatidae
Genus : *Trypanosoma*
Subgenus : *Megatrypanum* (*T. theileri*) } Stercoraria
: *Herpetosoma* (*T. lewisi*)
: Schizotrypanum (*T. cruzi* and *T. rangeli*)
: *Duttonella* (*T. vivax*) } Salivaria
: *Nannomonas* (*T. congolense; T. simiae* and *T. godfreyi*)
: *Pycnomonas* (*T. suis*)
: *Trypanozoon* (*T. brucei brucei; T. b. gambiense; T. b. rhodesiense; T. evansi* and *T equiperdum*)

1.2 Life cycle of African trypanosomes

1.2.1Tsetse transmitted trypanosomes

Different trypanosome species develop in different organs within the tsetse fly. Inside the flies, parasites undergo a number of developmental stages starting from the midgut migrating to the mouthparts. Transmission of both African animal trypanosomiasis (AAT) and human African trypanosomiasis (HAT) starts when a susceptible mammalian host is bitten by an infected fly vector therefore injecting metacyclic trypomastigote form of the parasite together with its saliva during a blood meal (Chappuis *et al.,* 2005). Subsequently parasites multiply locally by binary fusion at the site of the bite before migrating to the blood stream and lymphatic system of the mammalian host. The parasites will then migrate to other organs including the CNS and at this stage they occur in two forms of trypomastigotes, firstly as a long slender form that can reproduce by asexual division and secondly a non-replicating, short stumpy form (Chappuis *et al.,* 2005). The ratio of the long slender form to the short stumpy form of trypomastigotes varies with each wave of parasitemia and regularly more stumpy form of trypomastigotes are observed later in the infection (Vassella *et al.,* 1997; Wiser, 2011). This is because the stumpy forms play a dual functional role by limiting the parasitemia wave in the infected mammalian host and preadaptation for effective transmission to vector tsetse fly (Vassella *et al.,* 1997).

According to Roditi and Lehane (2008), when trypomastigotes are sucked up during a blood meal, they migrate to the midgut of the tsetse fly vector. In the fly's midgut the trypanosome parasites are most likely to encounter different consequences whereby, the slender forms are rapidly killed by proteases. Stumpy forms survive and differentiate to procyclic trypomastigotes (Roditi and Lehane, 2008). This differentiation is characterized by changes in the expression of the surface proteins as well as changes in metabolism (Wiser, 2011). This is then accompanied by loss of the surface coat and replacement of the variant surface protein (VSG) with another membrane surface protein called procyclin (Wiser, 2011).

Proteases are abundant in the fly posterior midgut, and provide at least one of the natural triggers; however, additional signals such as cold shock may also contribute to differentiation *in vivo*. These additional signals reduce the trypanosome parasites to low concentrations of citrate or cis-aconitate (Roditi and Lehane, 2008). In addition, starvation may also contribute to

decreased immune gene expression as a result leading to an increase in susceptibility of the nutritionally stressed tsetse flies in developing a trypanosome infection (Akoda *et al.*, 2009). As described by Roditi and Lehane (2008), for a trypanosome parasite to complete its life stages it must colonize the salivary glands and generate metacyclic trypomastigotes that are infectious to mammals. The migratory forms found in the proventriculus include long trypomastigotes that replicate their nuclear DNA and shift the position of the kinetoplast to give rise to long epimastigotes. Subsequently, the long epimastigotes then undergo an asymmetric division and in doing so, generating short epimastigotes that are alleged to be the parasitic form colonizing the salivary glands. Under optimum conditions many as half of the flies with a midgut infection will give rise to infected salivary glands (Akoda *et al*., 2009). These epimastigotes will then multiply in the salivary glands to produce infective metacyclic trypomastigotes that will be transmitted to a mammalian host during the next blood meal (Roditi and Lehane, 2008). Inside the mammalian host these metacyclic trypomastigotes transform into bloodstream trypomastigotes (Figure 1). The bloodstream trypomastigotes will also migrate to spinal fluid and lymph whereby they will again multiply by binary fusion. Re-infection to other cells will result due to a high number of trypomastigotes in the blood and spinal fluid. These trypomastigotes will then transform into slender and stumpy forms inside the host. In the host the slender form trypomastigotes will cause acute symptoms and the stumpy form parasite will be ingested by the vector flies during another blood meal and the cycle will be repeated all over again (Roditi and Lehane, 2008).

The life cycle of *T. vivax* is an exception in its mode of transmission in the tsetse fly vector. All the life cycle stages (trypomastigotes, epimastigotes and the infective metatrypomastigotes) are formed in the proboscis (Stevens and Brisse, 2004). *Trypanosoma vivax* differs from other Salivaria trypanosomes by its elongated and granular bloodstream form with a large kinetoplast and a centrally placed nucleus (Uilenberg, 2011). In addition, *T. vivax* has been reported to be congenitally transmitted from mother to foetus during pregnancy via the placenta or when bleeding occurs during birth (Uilenberg, 2011). Stevens and Brisse (2004) suggested that all these features enable this species to better adapt to development in its host than any other Salivaria species.

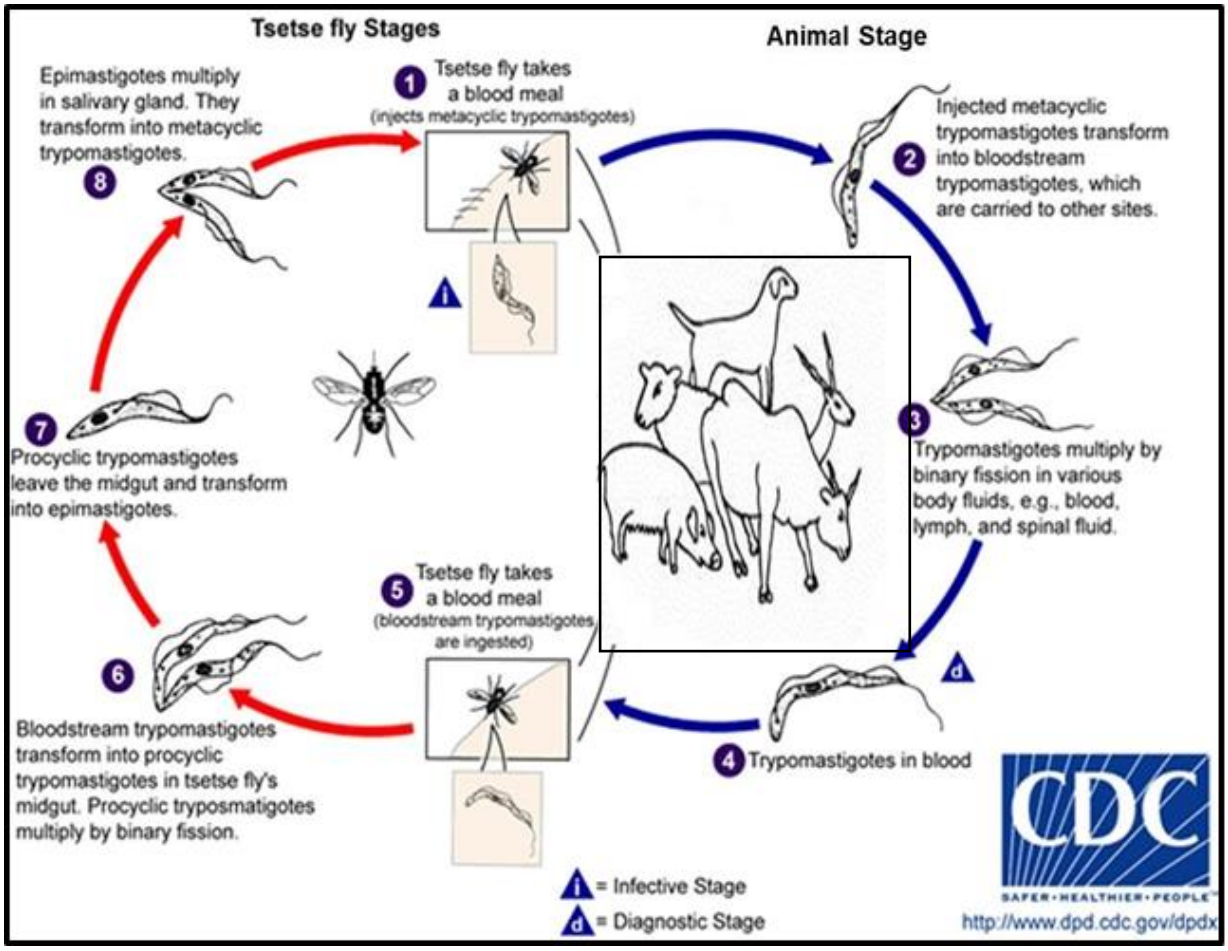


Figure 1: Life cycle of African trypanosomes. Figure extracted from, http://www.cdc.gov/parasites/trypanosomiasis/biology.html with modifications.

1.2.2 Non-tsetse-transmitted trypanosomes

However, not all trypanosome parasites are cyclically transmitted by tsetse flies, and some are mechanically transmitted by contaminative transmission through faeces of other haematophagous arthropods during their blood meal. The geographic distribution of these non-tsetse trypanosome parasites is more widespread ranging from Africa, Asia, and Central as well as South America respectively and they include all members of Stercoraria and a few Salivaria parasites (Lia *et al.,* 2007). From the Stercoraria group, in Central and South America *T. cruzi* and *T. rangeli* (*Schizotrypanum*) are transmitted to humans and animals by triatomine insects and in human *T. cruzi* is responsible for a disease known as Chagas disease. Secondly is *T. lewisi* (*Herpetosoma*) which parasitic to rats is solely transmitted by rat fleas worldwide (Stevens and Brisse, 2004).

The cosmopolitan *T. theileri* (*Megatrypanum*) is transmitted by various haematophagous arthropods ranging from insects to arachnids. In Africa, *T. theileri* is mechanically transmitted by tsetse flies (*Glossina*), black (*Chrysops*), horse (*Tabanus*) and stable (*Stomoxys*) flies from the families Tabanidae and Muscidae respectively (Leak, 1999). Furthermore, *Hyalomma anatolicum* is responsible for transmitting *T. theileri* in North Africa, southern Europe, Middle East, Russia, and China as well as in India (Latif *et al*., 2004). However, *T. theileri* is not pathogenic to both animals and humans mainly because, it doesn't possess a VSG gene which makes it exposed to antibodies released by the immune system (Stevens and Brisse, 2004).

Lastly, *T. evansi* and *T. equiperdum* which belong to the subgenus *Trypanozoon* are responsible for surra in dogs, livestock as well as horses and dourine in camels and equines (Taylor and Authié, 2004; OIE, 2013). Both parasites are morphologically similar and they are both widely distributed in Africa, Asia and South America (Lia *et al*., 2007). *T. evansi* however, is mechanically transmitted by blood sucking insects such as *Tabanus, Stomoxys, Lypersoia* and *Haematopota* (Taylor and Authié, 2004). Surra, which is caused by *T. equiperdum* on the other hand, is transmitted during coitus and it is only lethal in equines as they are the only known hosts (Stevens and Brisse, 2004; Taylor and Authié, 2004).

1.3 Vectors of African trypanosomes

1.3.1 The genus *Glossina*

The only species that are capable of cyclically transmitting African trypanosomes are grouped within the family Glossinidae with 31 species and subspecies. In addition, members of this family are characterised by the presence of a hatchet cell on both wings (Roditi and Lehane, 2008). Tsetse fly species are then arranged into three subgenera, namely *Austenina, Nemorhina* and *Glossina* that correspond to the structural complexity of genitalia, body hairs as well as locality and ecological settings required by the flies (Dyer, *et al.,* 2008). According to Krinsky (2009) these subgenera are regularly cited by their group names, each designated by one of the better known species in each subgenus; namely, the *fusca* group (*Austenina*), the *palpalis* group (*Nemorhina*) and the *morsitans* group (*Glossina*) (Leak, 1999). Species in the *fusca* group occur in forest habitats such as, rain, swamp and mangrove forests respectively. Those in *palpalis* are found mainly in vegetation around lakes and along rivers and streams. Lastly the *Glossina* group, with the exception of forest dwelling *G. austeni* are found in dry thickets, scrub vegetation and Savannah woodland areas (Krinsky, 2009).

In South Africa only 4 tsetse fly species are present namely *Glossina morsitans morsitans, G. pallidipes, G. austeni* and *G. brevipalpis* (Kappmeier *et al.*, 1998). However, previous studies on tsetse flies in South Africa reported that *G. m. morsitans* and *G. pallidipes* have been eradicated in South Africa, leaving only *G. brevipalpis* and *G. austeni* restricted to the north eastern part of KwaZulu-Natal Province (Kappmeier *et al.*, 1998). These two tsetse species are said to be the vectors of *Trypanosoma congolense*, *T. suis, T. simiae* and *T. vivax,* which are the disease causing agents of nagana (Leak, 1999). *T. theileri* and *T. vivax* on the other hand are transmitted mechanically among cattle by tabanid flies (Tabanidae), their distribution is cosmopolitan and they may not cause any clinical symptoms on their own (Krinsky, 2009; OIE, 2013).

1.3.2 Tsetse-trypanosome interaction

The life cycle of trypanosomes is fairly simple and as such it is expected of them to have a high infection rate in both the mammalian host and the tsetse fly, however, this is not the case with these parasites. During the development in the tsetse carrier, the trypanosome parasites change their respiratory pathway from a non-Krebs cycle to a Krebs cycle. This transformation

is due to a change from an oxygen-rich environment in the mammalian host to an oxygen-deficient environment in the tsetse fly (Leak, 1999). Most research has been conducted to understand the interaction of *T. brucei* and *T. congolense* in the vector tsetse fly and it has been shown that for the parasites to fully mature into infectious metacylic forms they must migrate from the midgut to colonize the salivary glands of the tsetse fly which is often problematic due to proteases which are abundant in the fly's midgut. However, both *T. brucei* and *T. congolense* parasites have a series of glycoproteins namely *brucei* alanine-rich proteins (BARP) for *T. brucei* and glutamic acid/alanine-rich proteins (GARP) for *T. congolense* which are both resistant to the proteases in the midgut (Roditi and Lehane, 2008).

Additionally, Roditi and Lehane (2008) stated that some studies also reported to have found genes which are closely related to GARP in *T. simiae* and *T. godfreyi* respectively. Other factors that might influence the infection rate of trypanosomes in tsetse flies include: the tsetse fly species involved, the gender of the fly, the genetic variation within and among tsetse species, host preference of the fly as well as concurrent infections such viruses, bacteria and fungi in the tsetse vector. In addition to that, the tsetse fly age might also have a profound impact on the susceptibility to *T. brucei* and *T. congolense*, with young flies being more susceptible however, the age factor has no effect on the susceptibility of the fly infected with *T. vivax* (Leak, 1999; Roditi and Lehane, 2008). Leak (1999) also included ecological factors that influenced the rate of infection in the vectors which are climatic factors, availability of infected hosts and the number of host available for subsequent feeds. These factors lead to a variation in the feeding behaviour between infected and non-infected tsetse flies whereby, infected tsetse flies depending on the strain of the parasite tend to feed more ravenously when infected with either *T. congolense* or *T. b. brucei*. Moreover, infected tsetse flies tend to live longer as compared to non-infected flies and this is because there is a competition between the tsetse vector and *Trypanosome* parasite for the partial oxidation of proline, which is the main source of energy for the tsetse flight (Leak, 1999).

1.4 Epidemiology of African animal trypanosomiasis

1.4.1 Virulence of animal trypanosomes

There are three types of African animal trypanosomiasis (AAT), namely nagana which affects ruminants (cattle, goats, sheep as well as dogs and pigs) and horses (Taylor and Authié, 2004). Nagana is said to be derived from a Zulu word meaning to be depressed or unfit (Bigalke, 2002). Nagana or AAT in Africa is caused by *T. congolense*, *T. vivax, T. uniforme, T. simiae* as well as *T. b. brucei* and tsetse flies are responsible vectors for the cyclic transmission of the disease in these domesticated animals (Steverding, 2008). Additionally, African mammals may also harbour non-pathogenic trypanosomes namely *T. theileri* and *T. ingens* commonly found in both domestic and wild animals (Biryomumaisho *et al*., 2013). Surra which is caused by *T. evansi* is widely distributed in Africa, Asia and South America. *T. evansi* is transmitted mechanically by bloodsucking insects, from the genera *Tabanus* and *Stomoxys* as well as vampire bats such as *Desmodus rotondus* (Claes *et* al., 2004; Taylor and Authié, 2004). Lastly dourine is a venereally transmitted disease caused by *T. equiperdum* that commonly affects equines and has a wider geographical range as compared to the other two diseases (Taylor and Authié, 2004).

Nagana only creates severe symptoms in domesticated animals since in wild animals it only causes mild infections and infected animals show no clinical symptoms at all therefore, making them reservoir hosts (Steverding, 2008). The pathogenesis of AAT evolves in two forms, chronic and acute, depending on the susceptibility status of the animal and the virulence of the *Trypanosoma* strain involved. In cattle, dogs and sheep the pathogenesis of the disease establishment depends on the damage caused to the visceral organs and the degree of anaemia. Acute or chronical stage of the disease may be fatal following a short period of illness, however chronic illness can endure for months to years. In goats acute disease causes high fever, mucous membrane turn pale and there is a rapid weight loss in the affected goat host. The pathogenesis in horses may vary as compared to donkeys. This is due to that horses do not survive for long in the presence of trypanosome infected flies, but donkeys are more resistant (Taylor and Authié, 2004). The common major clinical symptoms in nagana consist of fever, listlessness, emaciation, hair loss, and discharge from the eyes (Leak, 1999; OIE, 2013). Additionally they may include hyperthermia, anaemia, poor body condition, mucous pallor,

miscarriage, 'petering out', pica which involves the consumption of non-nutritive substances by pregnant female livestock, splenomegaly, oedema, cachexia, paralysis and eventually death (Taylor and Authié, 2004; Uilenberg, 2011).

Nagana causes an economic loss of more than US$ 4.5 billion per year in agriculture and also leads to a reduction in food production, low milk yield as well as decreased livestock reproduction rate either through mortality, abortion and low growth rates as well as effecting fertility on domesticated animals in affected countries in Africa (Leak, 1999; Farikou *et al.,* 2011; Biryomumaisho *et al*., 2013). Due to an increase in game farming, where wild animals are being held in captivity for either meat, hunting or for tourism attraction has also led to the establishment of trans-frontier game parks with unrestricted movement of wildlife across national boundaries (Mamabolo *et al.*, 2009). Given the role of wildlife as reservoir hosts, this may result to an increase in infections to the livestock population in such areas (Mamabolo *et al.*, 2009).

1.4.2 Diagnosis of animal trypanosomiasis

Microscopic diagnosis of animal trypanosomiasis: Traditionally the identification of trypanosomes has been based on microscopic observations, host range, geographic area, and the presence of the parasite in specific organs of the tsetse fly and lastly the ability of these parasites to grow *in vivo* or *in vitro* (Desquesnes and Dávila, 2002). Microscopic observations have been based on morphology, morphometry and mobility of the parasite in host tissues. Thick or thin blood films observed under a light microscope were used for the identification of the trypanosome parasites. Successively, to increase the sensitivity of microscopic diagnosis, a heparinised microhaematocrit tube are used whereby the trypanosome parasites are concentrated in the buffy coat layer and examined directly at low power under a light microscope (OIE, 2013). In some cases the buffy coat may be smeared on a slide and stained for observations under a light microscope where the low pack cell volume (PCV) could be determined and the level of anaemia on infected cattle be estimated (Uilenberg, 2011; OIE, 2013).

Serological diagnosis of animal trypanosomiasis: Vast amount of research has been conducted on different species of trypanosomes using serological based assays such as Card Agglutination Test for Trypanosomiasis (CATT), antibody–enzyme linked immunosorbent assay (ELISA) and indirect fluorescence antibody test (IFAT) (Stevens and Brisse, 2004; Uilenberg, 2011). These techniques rely on the detection of antibodies released by the immune system in response to foreign pathogens (Chappius *et al*., 2005). They have high sensitivity and mostly are genus specificity, but their species specificity is generally low and at present they can only be used for presumptive diagnosis of trypanosomiasis (Uilenberg, 2011). However, these techniques can also produce false-negative results in cases of low parasitemia. These tests cannot distinguish between current infections and residual antibody from previous vaccination or infection (Chappius *et al*., 2005; Uilenberg, 2011).

Molecular diagnosis of animal trypanosomiasis: The introduction of molecular techniques such as restriction enzymes, sequencing and synthesis of DNA, DNA probing and polymerase chain reaction (PCR), have increased the specificity and sensitivity in trypanosome diagnosis, compared to the above mentioned diagnostic tools (Desquesnes and Dávila, 2002). Various molecular techniques have been used to identify and manipulate DNA. The first methods to be developed were DNA sequencing techniques and synthesis of DNA-probes, followed by PCR then finally combination of both techniques (Desquesnes and Dávila, 2002). DNA probe is basically a known DNA sequence which can be obtained by cloning or by PCR with labelled nucleotides either using enzymes or isotopes (Desquesnes and Dávila, 2002). These probes however, have been developed for the most pathogenic trypanosomes and the sensitivity of this technique is limited to a few numbers of parasites (about 100 parasites). This is not enough to detect the trypanosome infection in the mouthparts of the flies or host blood when there is low parasitemia (Desquesnes and Dávila, 2002). Other approaches to investigate the molecular variation between various *Trypanosoma* spp include methods such as; restriction enzyme fragment length polymorphism (RFLPs), randomly amplified polymorphic DNAs (RAPD), and amplified fragment length polymorphisms (AFLP). According to Eisler *et al.* (2004), these methods may be effective for characterization of trypanosomes though they have not been applied largely in the detection of parasites because they generally require large amounts of purified parasite DNA.

These DNA-based methods were later modified to species specific DNA probes then eventually improved to species-specific PCR tests (Adams *et al.,* 2008). Species-specific PCR tests greatly improved the accuracy of identification and increased our understanding and knowledge of trypanosome diversity. In particular, the high prevalence of mixed infections with multiple trypanosome species was documented for the first time using PCR techniques (Adams *et al.,* 2008).

The use of quantitative PCR techniques has been shown to be of potential value for other types of parasitic infections in domesticated animals. Conventional PCR techniques simply indicate the presence or absence of parasite DNA when compared to the quantitative PCR methods which are able to give an indication of the level of parasite load (Desquesnes and Dávila, 2002; Eisler *et al.,* 2004). This may be important with trypanosome infections in terms of their effect on the productivity of livestock.

For diagnostic purposes, PCR must be performed with various biological materials in both vectors and host. In vectors, it is generally recommended to dissect out the organs of the insect where the parasite is thought to occur (in tsetse fly: mouth parts, salivary glands and midgut) and these organs have to be homogenized prior to DNA extraction (Desquesnes and Dávila, 2002). In mammalian hosts, the parasites are most often present in the blood, but other tissues such as lymph, cerebrospinal fluid (CSF), genital secretion (in terms of *T. equiperdum*), or any material derived from other organs can be investigated as well for the presence of trypanosome parasites (Desquesnes and Dávila, 2002). It is also recommended that the samples be obtained from fresh material and if possible the fresh samples may be fixed on either filter paper or on slides. This allows the delaying of preparation for PCR which might not be possible in the field.

Loop-mediated isothermal DNA amplification (LAMP) method is simple, rapid, highly specific and sensitive, requires simple equipment for amplification reaction and is cost effective (Notomi *et al*., 2000). LAMP depends on auto-cycling strand displacement DNA synthesis that is performed by a *Bacillus stearothermophilus* (*Bst*) DNA polymerase and unlike *Taq* DNA polymerase it is barely inhibited by impurities, such as haemoglobin and/or myoglobin

contaminated blood and tissue derived DNA samples which are known to be inhibitors in PCR (Thekisoe and Inoue, 2011). All these advantages noted above indicate that LAMP has the potential to be used as an alternative molecular diagnostic method particularly at the under resourced laboratories in trypanosome endemic areas. LAMP assays have been developed for the detection of *T. congolense*, *T. evansi*, *T. cruzi* as well as *T. b. gambiense* (Njiru *et al.,* 2005; Thekisoe *et al*., 2007b).

1.5 Control measures of the vectors

Several methods have been used to prevent human and animal trypanosomiasis and the best approach is to control the tsetse fly vectors (Leak, 1999; Krinsky, 2009). Control measures to eradicate these vectors in Africa often included aerial and ground spraying with insecticides such as Dichlorodiphenyltrichloroethane (DDT) and dieldrin. Secondly is the removal of wild reservoir hosts by selective hunting (Leak, 1999). Other methods include the application of insecticides or insect repellents to livestock either by dipping or pour-on technique. Another non biological method was to destroy the habitats of the tsetse flies, a process known as bush clearing. Targets, baits and traps have also proved to be effective. According to Krinsky (2009), one of the most effective targets is a black and blue cloth baited with attracted components of ox breath or urine. Attractants include acetone, 1-octen-3-ol, and phenols (4-methyl-and 3-n-propyl). The target is designed in such a manner that it can be used either with an electrocution device or an insecticide (Leak, 1999; Esterhuizen *et al*., 2005; Krinsky, 2009). Therefore, an unattended trap charged with a residual insecticide can be employed to remove flies from the environment for 12 to 18 months, which is long enough to eradicate local populations of tsetse flies (Krinsky, 2009).

Natural enemies of tsetse include puparial parasites, such as ants, beetles, wasps and over 10 species of bombyliids (*Thyridanthrax* spp), predators such as spiders, dragon and may flies, asilids, sphecid and vespid wasps also aid in controlling tsetse populations by killing more than 20% of the puparia (Krinsky, 2009). Lastly sterile insect technique is also applicable for the integrated pest management control, whereby reproductively sterile insects are released among indigenous target population, sustained over several generations of the pest population. Males are sterilised by radiation at the appropriate developmental stage and when

these males mate and inseminate female insects the female will become effectively infertile for the remainder of their lifespan (Fledmann, 2004).

In the north eastern regions of KwaZulu-Natal Province in South Africa, which is dominated by *G. austeni* and *G. brevipalpis*, the utilization of targets and H-traps baited with odour proved to be effective in different ways (Esterhuizen *et al*., 2005). This is because firstly, *G. austeni* has a low dispersal rate, whereas *G. brevipalpis* disperses more readily and moves between habitats. Secondly, there is no effective odour-bait known for *G. austeni* (Esterhuizen *et al*., 2005). With regard to the possible future control of tsetse flies in the Zululand district, it is evident that targets deployed in well wooded habitats at a relatively low density can be effective in the control of *G. austeni.* This may be important in the numerous game reserves and natural areas that form part of the KwaZulu-Natal Province tsetse belt (Esterhuizen *et al*., 2005; Krinsky, 2009). In contrast, the control of *G. brevipalpis* require odour baited targets to be deployed in all habitats, and lastly special attention needs to be focused to seemingly unsuitable habitats such as open grasslands to effectively eradicate tsetse populations.

1.6 Genotyping of trypanosome parasites

African trypanosomes have two genomes, one within the nucleus and the other enclosed within the kinetoplast (Melville *et al.,* 2004). Nuclear DNA bears genes coding for ribosomal RNA and ribosomal DNA cistron genes which occur in multiple copies in cycle arrays (Desquesnes and Dávila, 2002). Desquesnes and Dávila (2002), indicated that these genes are made of transcriptional units (TU), separated by non-transcribed spacers (NTS). The TU is composed of an 18S ribosomal subunit, internal transcribed spacer 1 (ITS-1), 5.8S ribosomal subunit and ITS-2, 28S ribosomal subunit. The length of ITS-1 is about 300-800 base pairs (bp) and has a variable length size depending on the *Kinetoplastida* species (Desquesnes and Dávila, 2002). The length of ITS-1 is assumed to be constant within a species. Previous studies indicated that KIN-1 and KIN-2 primers, used to amplify the ITS-1 of *Kinetoplastida* gave rise to variable size products in *Leishmania* and *Trypanosoma* in a single PCR reaction (McLaughlin *et al*., 1996; Desquesnes and Dávila, 2002; Njiru *et al*., 2005). Further assessments have indicated that the following *Trypanosoma* species can be identified through a single PCR process (even in the case of mixed species-specific DNA) namely: *T. vivax, T. theileri, T. simiae, Trypanozoon* spp*,*

T. congolense Savannah, *T. congolense* Forest and *T. congolense* Kilifi. It should be noted that these primers allow for the detection and identification of *T. theileri*, a non-pathogenic trypanosome of cattle for which specific primers had never been described before. A similar PCR assay based on ITS rDNA which detects trypanosomes of economic importance has also been developed by Njiru *et al*. (2005).

Molecular analysis of the genomic or mitochondrial DNA by RFLP and PCR-RAPD, or the utilization of microsatellite and minisatellite DNA probes have been used successfully for the detection and identification of trypanosomes (Agbo *et al.,* 2001). It was noted by Agbo *et al.* (2001) that, PCR based RFLP and sequence analysis of the internal transcribed spacer (ITS-1, ITS-2) and the intervening 5.8S ribosomal subunit can be used to successfully determine and identify genome relatedness in trypanosome species (either human or non-human trypanosomiasis).

Nonetheless, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an ubiquitous and essential glycolytic enzyme and these GAPDH genes has a slow rate of molecular evolution making them appropriate for the studying of evolution over a large time scale (Hamilton *et al.,* 2004). According to Hamilton *et al.* (2004), the SSU rRNA gene neither strongly support nor reject trypanosome monophyly, as different alignments give different tree topologies when tested. Hamilton *et al.* (2004) concluded that, all trees based on GAPDH gene support monophyly of trypanosomes and show them as a relatively late-evolving lineage within the family Trypanosomatidae, which is also monophyletic.

The ITS-1 and ITS-2 genes can be successfully used to differentiate different species in the genus *Trypanosoma* either using ITS or KIN primers, additionally 18S rRNA and gGAPDH genes can be used to confirm monophyly in the trypanosome evolution however much detailed application of these genes has not been well documented for South African trypanosomes which are restricted to the tsetse belt in north eastern KwaZulu-Natal.

1.7 Host species identification of blood meals from vectors

Detailed knowledge of the source of an insect's blood meal provides important information relating to the epidemiology of vector borne diseases on their various vertebrate hosts and this is considered to be a prerequisite for a successful tsetse and trypanosomiasis control programme (Steuber *et al.,* 2005; Torr *et al.,* 2001). According to Steuber *et al.* (2005), serological techniques like the precipitin and haemagglutination test the complement fixation test and the enzyme-linked immunosorbent assay (ELISA) have been developed to identify the source of vertebrate blood in the intestinal tracts of wild tsetse flies. Up to now, however, some problems remain with the identification of phylogenetically closely related species, which may result in a high percentage of samples being identified only to the family level (e.g. Suidae, Bovidae) but not to the exact species taxon. Mitochondrial DNA (mtDNA) is the ideal gene target in indicating the origin of species. This is due to that; mtDNA contains a high proportion of evolutionary-caused nucleotide replacement making it particularly valuable as a discriminatory molecule in studying the relationships between closely related vertebrates. Additionally, the early identification of a standard set of universal primers aimed at conserved regions of the mitochondrial cytochrome b (cyt b) gene from vertebrates enables an adequate PCR amplification of relevant nucleotide sequences especially from highly processed foodstuff or largely digested DNA samples found in haematophagous arthropods. In particular, the combination of the polymerase chain reaction with the restriction fragment length polymorphism analysis (PCR-RFLP) is a widely used method for the accurate determination of species origin of samples taken from meat and foodstuff (Steuber *et al.,* 2005). Torr *et al.* (2001), used microsatellite DNA analysis to detect blood meals in tsetse flies, however this technique was less reliable to detect blood meals that were consumed by flies 2 or more days prior capture.

In South Africa, recent molecular studies on tsetse flies revealed that both *G. austeni* and *G. brevipalpis* were mostly infected with two genotypes of *T. congolense* Savannah and Kilifi types respectively which were found in midgut as well as in the proboscis (Mamabolo *et al.,* 2009). Further studies on tsetse flies to investigate their blood meals are needed to determine the preferred mammalian feeding hosts as well as which trypanosome parasites are harboured by the flies.

1.8. Statement of the problem

The prevalence of animal trypanosomiasis and the distribution of tsetse vectors in South Africa have been documented and the findings published by most scientists in the previous years (Esterhuizen *et al*., 2005; Van den Bossche *et al*, 2006; Mamabolo *et al*., 2009). Most of trypanosome detection conducted in South Africa was based on microscopy. Studies by Van den Bossche *et al*. (2006) and Mamabolo *et al.* (2009) were the first to report on molecular techniques for the detection of trypanosomes in South Africa.

The current study is aimed at improving the current knowledge on the prevalence status of trypanosomes in the north eastern KwaZulu-Natal Province of South Africa. Additionally this study is aimed at determining the nucleotide diversity, phylogenetic position of South African trypanosomes and identifying the host preference by the tsetse flies. This will further assist in understanding the phylogeny of the parasites found in both blood and tsetse fly vectors in KwaZulu-Natal Province, consequently increasing the information on the relatedness of the trypanosome parasites found in the study areas as well as other affected countries in Africa and the world in general. It also assists in determining the feeding range of the flies and host preference of the vector flies in the study area. Lastly, the study will be identifying the phylogeny of South African trypanosome strains when compared to other strains in other affected nations in Africa in terms of how different or similar are they genetically by comparing their nucleotide sequences. As such the following hypotheses were drawn: (i) the prevalence of AAT will not differ among sampled local municipalities in KwaZulu-Natal Province. (ii) there will be great genetic diversity among the sequences of South African trypanosomes (iii) South African trypanosomes will be more genetically to related east African trypanosomes when compared to other trypanosome parasites in Africa.

1.9 Objectives of the study

1.9.1 General objective

To use PCR techniques to detect and genotype the trypanosome parasite species found in the blood of domestic animals (cattle, sheep, goats and dogs) and tsetse flies (*G. austeni* and *G. brevipalpis*) which inhabit uMkhanyakude district of KwaZulu-Natal Province of South Africa and to determine possible feeding preferences of tsetse flies in that region.

1.9.2 Specific objectives of the study

1. To determine the prevalence of trypanosome species in uMkhanyakude district of KwaZulu-Natal Province, South Africa using PCR.

2. To determine genetic diversity of trypanosome species detected in uMkhanyakude district of KwaZulu-Natal Province, South Africa using semi-nested PCR.

3. To conduct phylogenetic analysis of South African trypanosomes detected in uMkhanyakude district of KwaZulu-Natal Province, South Africa by constructing phylogenetic trees using trypanosome parasite sequences generated in this study together with other trypanosome sequences found on the NCIB gene bank.

4. To determine the possible mammalian host of the tsetse flies from their blood meals using PCR.

CHAPTER 2

PREVALENCE OF TRYPANOSOME SPECIES IN UMKHANYAKUDE DISTRICT OF KWAZULU-NATAL PROVINCE, SOUTH AFRICA

2.1 Introduction

The problems that resulted from African animal trypanosomiasis which are caused by haemoprotozoan parasites *Trypanosoma* spp have been known to livestock herders for many years before the exact description of its causes and its mode of transmission by tsetse flies (*Glossina* spp) was understood (Boyt, 1988). The development of effective diagnostic methods has been of vital importance in affected nations. Diagnostic methods for trypanosomes in the field differ from the ones used in the laboratory (Uilenberg, 2011). In the field, diagnostics are based on observations of poor body condition score and microscopy using thin or thick blood smears as well as low PCV from the susceptible hosts and these methods are said to be reliable in providing direct results mainly used in poorly resourced areas that are endemic to the disease (Picozzi *et al*., 2002). It has been noted that the only problems with microscopy is the lack of sensitivity and inability to differentiate between morphologically similar species of the same genus as in the case of *T. brucei brucei, T. b. gambiense* as well as *T. b rhodesiense* (Nakayima *et al.,* 2012). In cases of low parasitemia and lack of anaemic symptoms from infected hosts resulting to normal PCV and regular body condition, these field methods have limitations as they cannot pick up the presence of parasites as well as to differentiate between single and mixed infections therefore this may lead to uncertainties in terms of accurate diagnosis of infected livestock (Boyt, 1988; Uilenberg, 2011; OIE, 2013).

Serologically-based assays such as CATT, ELISA IFAT can also be employed for the detection of trypanosomes in the laboratory and these methods rely on either antigen-detection assays or antibody-detection assays (Ndao, 2009; Uilenberg, 2011; OIE, 2013). These assays are more sensitive and specific as compared to microscopy and are also effective in monitoring the parasite clearance succeeding therapy. However, they also have limitations in that they do not have standardized test procedures and they cannot distinguish between mixed infections as well as past and current infections (Ndao, 2009).

Molecular diagnostic techniques such as PCR-based methods have been proven to be more specific and sensitive for the detection of trypanosome parasites in both livestock blood samples, and wild tsetse flies as they could single out the causal agent alone and excluded other organisms that are of no pathological significance (Desquesnes *et al*., 2001).The KIN 1 and KIN 2 trypanosome universal PCR primers were developed by McLaughlin *et al*. (1996) and these primers reacted specifically with kinetoplastid species and amplified the internal transcribed spacer one (ITS 1) gene which is situated between 18S and 5.8S genes in the mitochondrion. These primers are able to amplify significant livestock trypanosomes and distinguish mixed infections all in one PCR reaction (Desquesnes *et al*., 2001; Nakayima *et al*., 2012). Furthermore, because they amplify the ITS 1 gene, these primers are species-specific in size and produce different base pairs for all trypanosomes with the exception of *T. vivax* because they are less sensitive in detecting the latter parasite For members of the subgenus *Trypanozoon* (*T. b. brucei, T. b. gambiense, T. b. rhodesiense, T. evansi* and *T. equiperdum*) the KIN primes produced amplicons of approximately 480 bp (base pairs); *T. congolense* Savannah subgroup 700 bp, *T. congolense* Kilifi subgroup 620 bp, *T. congolense* Forest subgroup 710; *T. simiae* 400 bp, *T. simae* Tsavo 370 bp and for the less sensitive *T. vivax* 250 bp respectively (Desquesnes *et al.,* 2001; Nakayima *et al.,* 2013). By reducing the number of reactions per sample, these KIN primers are ideal for PCR-based tests in effectively reducing the costs of PCR and the time required for accurate diagnosis (Nakayima *et al.,* 2013).

Previous research in southern most boundary of the tsetse belt, north eastern KwaZulu-Natal Province was conducted using PCR-based molecular techniques and their findings strongly supported the absence of *T. brucei* and its subspecies in the area, this statement was supported by the fact that *G. austeni* and *G. brevipalpis* are poor vectors of *T. b. brucei* and its subspecies *T. b. gambiense* and *T. b. rhodesiense* respectively (Mamabolo *et al.,* 2009). Secondly they showed that there were mixed infections of *T. congolense* (Savannah) type with *T. vivax* in diptanks of Ekophindisweni, Mahlambanyathi as well as Mvutshini (Van den Bossche, 2006; Mamabolo *et al.,* 2009). However, these previous studies were focused only on cattle and tsetse samples. They did not include goats, sheep as well as dog samples. Hence the current study was conducted to cover the areas that were previously not sampled also increasing the variety of sampled domesticated animals. A hypothesis which stated that the prevalence of

AAT will not differ among sampled local municipalities in KwaZulu-Natal Province was formulated.

2.2 Objectives

1. To determine prevalence of animal trypanosomes infecting cattle, sheep, goats and dogs in the uMkhanyakude district of KwaZulu-Natal Province using PCR.

2. To determine the prevalent trypanosome parasites infecting tsetse flies in the uMkhanyakude district of KwaZulu-Natal Province using PCR.

3. To identify the trypanosomes infecting livestock and tsetse flies in KwaZulu-Natal to species level by sequencing analysis.

2.3 Materials and methods

2.3.1 Study area

The distribution of African animal trypanosomiasis is limited to the north eastern parts of KwaZulu-Natal Province, South Africa (Mamabolo *et al.*, 2009). The uMkhanyakude district (28°01'25"89 S, 32°17'30"30 E) is situated in the north eastern parts of KwaZulu-Natal Province (Figure 2) with altitude ranging between 450 - 900 m. It is dominated by three structural vegetation types which include dense bushveld thickets and the dominant tree genera are *Afzelia*, *Balanites*, *Combretum*, *Ficus* and *Pseudobersama*, which form canopies ranging between 5 to 20 meters giving heavy shade, and there is little undergrowth (Pooley, 1993; Mucina and Rutherford, 2006). The second is sour grassland and the dominant grass genera are *Heteropogon*, *Perotis*, *Setaria* and *Tragus* (Esterhuizen *et al.*, 2005; Mucina and Rutherford, 2006). There is little shade present, except under large isolated trees (*Syzygium* spp.) and inside the forest patches. The last vegetation type is the wooded grassland which consists of plantations of exotic *Eucalyptus* and *Pinus* trees which are mainly used for wood and paper (Esterhuizen *et al.*, 2005). It has frequent rains in the summer than in winter with mean annual precipitation ranging between 600 - 1 050 mm (Mucina and Rutherford, 2006). Its weather conditions are characterized by hot summers and some frost during the winter months, and lastly it has well-drained as well as shallow soil types (Pooley, 1993). However, due to erosion urbanization, drainage of wetlands for housing and agriculture, competition from invasive alien plants, deforestation as well as commercial farming, conservation in these areas is crucial (Pooley, 1993). In the uMkhanyakude district, three local municipalities were sampled for blood from domesticated animals namely: the Big 5 False Bay, Hlabisa and Mtubatuba respectively and tsetse fly samples were collected from Charter's Creek game reserve and Boomerang commercial farm.

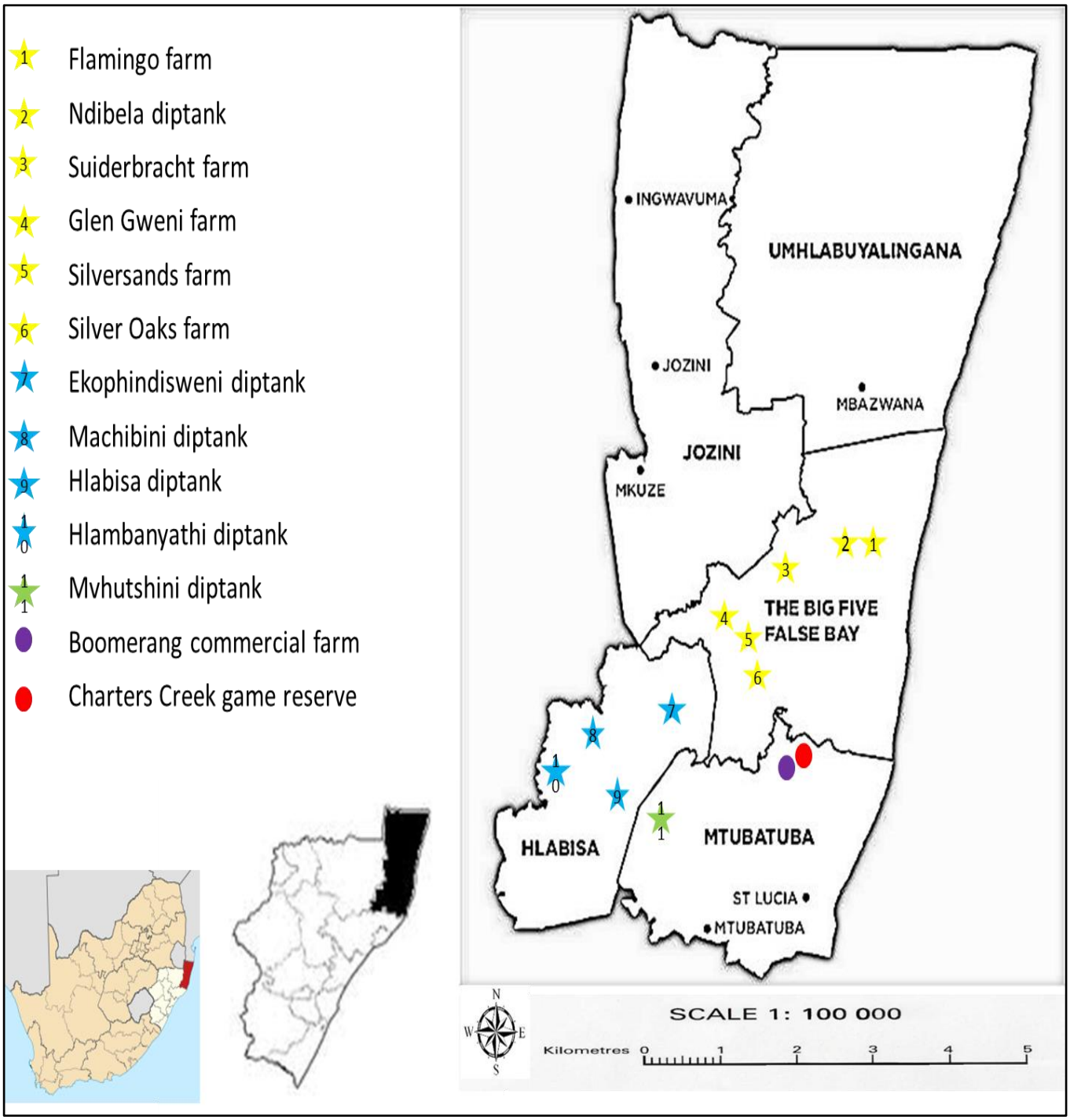


Figure 2: A map of uMkhanyakude district in KwaZulu Natal Province, South Africa. The stars represent the local municipalities where blood samples were collected and the spherical shapes represent the areas where tsetse flies (*Glossina brevipalpis*) were collected. Source: www.localgovernment.co.za

2.3.2 Collection of samples

2.3.2.1 Collection of blood samples

Blood samples were collected from domestic animals in the uMkhanyakude district by jugular vein puncture using EDTA-coated vacutainer tubes with 18 gauge needles as of February 2013 to June 2013. A total of 296 samples were collected from cattle (n=137), sheep (n=9), goats (101) and dogs (n=49) respectively. For dog samples only 5 samples were collected in vacutainers and the remaining 44 were preserved on FTA cards. All blood samples were then labeled accordingly, packed in a cooler bag with ice packs at the field before they were couriered to the University of the Free State, Qwaqwa Campus for DNA extraction and PCR analysis. In the uMkhanyakude district, 3 local municipalities (Big 5 False Bay, Hlabisa and Mtubatuba) with 11 sites (5 farms and 6 diptanks) were sampled. The names of the sampled farms and diptanks are given in figure 2. The sampled diptanks and farms are situated near Hluhluwe-uMfolozi game reserve, Phinda private game reserve and St. Lucia wetlands respectively. Consequently, these conserved areas harbor most if not all reservoir hosts of animal trypanosomiasis in north eastern KwaZulu-Natal Province and they are more or less the preferred habitat for the vector tsetse flies therefore, posing huge threats to the wellbeing of the domesticated animals in the surrounding regions.

2.3.2.2 Collection of tsetse fly samples

Tsetse flies (*Glossina brevipalpis* and *G. austeni*) were collected in two areas namely Boomerang commercial farm and Charter's Creek game reserve which are located between Hlabisa and Mtubatuba local municipalities. The flies were captured using H-traps baited with acetone, methyl phenol and octanol (Plate 1) that were placed in the two study areas for 5 days. A total of 18 traps were set in the two areas, 6 H-traps at Boomerang commercial farm and 12 traps at Charter's Creek game reserve respectively. The H-traps were monitored and changed daily to collect the captured flies. A total of 376 tsetse samples were collected from the traps whereby 375 were *G. brevipalpis* and only one was identified as *G. austeni* (Table 1). Car samples were only collected at Charter's Creek game reserve as the tsetse fly samples were collected from the traps. Figure 3 represents the exact localities of the traps in the two study sites. Firstly the flies were sorted according to the number of the H-trap they were captured in and taken to the ARC-Kuleni tsetse station. On arrival at the station, the flies were immobilized

by placing them in a freezer set at -4°C then they had their wings and legs removed with forceps. This was done to remove the amount of exoskeletons that might affect the enzymatic activity of the reactions to follow on a later stage (Meketa *et al*., 2008). The tsetse flies were then sorted according to gender and recorded. From the 376 tsetse samples collected 26 were dissected under a dissecting microscope where the proboscis as well as the midgut were removed and placed on a glass slide for further analyses under a light microscope. Twenty six tsetse samples that were dissected 25 were from *G. brevipalpis* and only one from *G. austeni.* However, no active trypanosomes were observed from the 26 tsetse samples that were dissected. The remaining flies were placed in labelled 1.5 ml Eppendorf tubes filled with small blue silicon stones separated by a cotton wool dipped with ethanol, this was done to retain moisture in the tubes in order for the files no to desiccate. All samples were also sent to the University of the Free State, Qwaqwa Campus for further analysis.

Table 1: The number of tsetse fly species captured by H-traps and total number of positive samples by PCR

Species	Collection Site	Trap no	Latitude	Longitude	Male	Female	Total
G. brevipalpis	Charter's Creek	1	28°11'02.6"S	032°24'43.2"E	10	5	15
G. brevipalpis	Charter's Creek	2	28°11'04.3"S	032°24'45.6"E	27	24	51
G. brevipalpis	Charter's Creek	3	28°11'11.4"S	032°24'47.8"E	7	9	16
G. brevipalpis	Charter's Creek	4	28°11'19.4"S	032°24'46.0"E	6	5	11
G. brevipalpis	Charter's Creek	5	28°11'45.8"S	032°24'39.0"E	18	25	43
G. brevipalpis	Charter's Creek	6	28°12'14.4"S	032°23'57.4"E	9	10	19
G. brevipalpis	Charter's Creek	7	28°12'51.6"S	032°24'18.3"E	21	28	49
G. brevipalpis	Charter's Creek	8	28°12'56.6"S	032°24'16.5"E	14	16	30
G. brevipalpis	Charter's Creek	9	28°13'04.7"S	032°24'14.7"E	26	28	54
G. brevipalpis	Charter's Creek	10	28°13'15.7"S	032°24'07.2"E	8	8	16
G. brevipalpis	Charter's Creek	11	28°13'19.8"S	032°24'04.0"E	21	13	34
G. brevipalpis	Charter's Creek	12	28°13'26.8"S	032°23'56.8"E	10	5	15
G. brevipalpis	Boomerang	1	28°14.682'S	032°18.909'E	1	0	1
G. brevipalpis	Boomerang	2	28°15.049'S	032°18.848'E	2	3	5
G. brevipalpis	Boomerang	3	28°14.530'S	032°18.840'E	3	0	3
G. brevipalpis	Boomerang	4	28°13.780'S	032°20.856'E	1	1	2
G. brevipalpis	Boomerang	5	28°14.143'S	032°19.093'E	1	1	2
G. brevipalpis	Boomerang	6	28°13.690'S	032°19.381'E	2	2	4
G. brevipalpis	Charter's Creek	Car	NR	NR	3	2	5
G. austeni	Charter's Creek	2	28°11'04.3"S	032°24'45.6"E	0	1	1
Total PCR Positives					**19.2% (32/167)**	**12.0% (22/183)**	**15.4% (54/350)**
Total					**190**	**186**	**376**

NR= No co-ordinates recorded

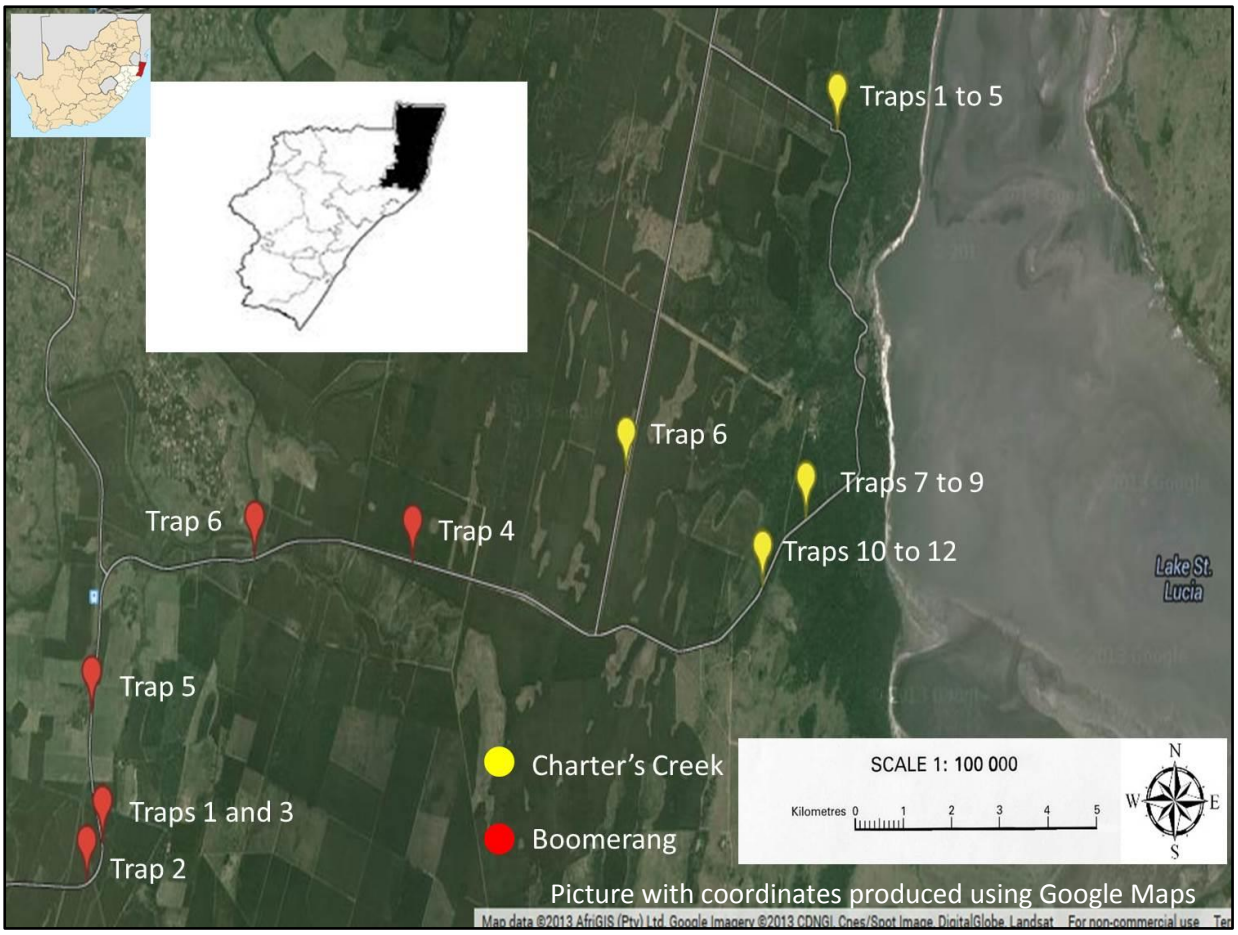


Figure 3: A map showing the 18 H-traps sites set in Boomerang and Charter's Creek. Traps; traps 1-5, traps 7-9 and traps 10-12 in Charter's Creek are represented by 1 yellow point. In Boomerang only traps 1 and 3 are represented by one red point.
Source:www.google.co.za/maps/place/Umkhanyakude+District+Municipality/

Plate 1: H-trap used to capture tsetse flies. The traps were baited with acetone for odour and sacks filled with 4-methyl phenol and octanol for visual attractant. The traps were set in a shaded area and vegetation around the traps was removed to make it visible to the flies. Source: Picture taken during sampling by Taioe M. O. (2013).

2.3.3. DNA extraction from blood and tsetse flies by salting out method (Nasiri *et al*., 2005) with modifications

On arrival at the University of the Free State, Qwaqwa Campus, the samples were unpacked and sorted according to their local municipalities and divided into batches before they were stored at -35°C until DNA extraction was conducted. DNA was extracted from 252 blood samples (137 cattle, 9 sheep, 101 goats and 5 dogs) collected in EDTA vacutainers as well as 350 *Glossina brevipalpis* flies.

Prior to extraction DNA was lysed by adding 50 µl of blood samples into 1.5 ml Eppendorf tubes. The tubes containing blood were filled with 410 µl extraction buffer which contained 10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulphate (SDS). In a separate experiment tsetse flies were homogenized (Diallo *et al*., 1997) and 500 µl of DNA extraction buffer added to the 1.5 ml Eppendorf tubes containing each fly. Then, 80 µl of 10% SDS was added followed by 10 µl of Proteinase K (Pro-K) and the samples were incubated at 55°C for 1 hour. After an hour additional 10 µl of Pro-K was added and the samples were incubated again at 55°C and left overnight to complete the digestion of DNA.

DNA was extracted the following day as follows: Samples were centrifuged for 5 minutes at 12 000 rpm (Biocen 22R centrifuge, Ortoalresa, Spain). Using a micro pipette 600 µl of the supernatant was transferred to the second set of 1.5 ml Eppendorf reaction tubes and 180 µl of 5 M NaCl was added to the supernatant. The tubes were vortexed for 30 seconds, centrifuged at 13 500 rpm for 5 minutes. The supernatant was transferred to the final third 1.5 ml Eppendorf tube where 420 µl of ice cold isopropanol (Propan-2-ol) was added to the supernatant. The mixture in the tubes was mixed by inverting the tubes 50 times followed by centrifugation at full speed (14 000 rpm) for 5 minutes at 4°C to precipitate the DNA. Subsequent to centrifugation, the supernatant was discarded and the pellet containing the DNA was washed twice by adding 250 µl of 75% ethanol. Tubes were vortexed for 30 seconds followed by centrifuging the samples at full speed for 5 minutes and the supernatant was discarded. The wash was done twice to remove excess cellular and chemical content that might inhibit PCR. The samples were left opened to air dry for an hour at room temperature to evaporate the 75% ethanol. Finally, the DNA pellet was dissolved in 200 µl of double distilled

H_2O then incubated at 37°C for 30 minutes. The presence of DNA was confirmed by gel-electrophoresis using 1% agarose stained with 1 µl ethidium bromide then visualized under UV light before being stored at -35°C for PCR analysis.

A total of 11 FTA cards with 44 blood spot samples representing 44 canines were extracted using the methanol fixation technique according to Johanson *et al*. (2009). Approximately 3 - 6 mm circles in diameter were punched on each blood spot from each FTA card. The puncher was cleaned with 70% ethanol in between the punches to avoid cross contamination. Using a pasture pipette 4 drops of methanol were pipetted on the sample spot and the samples were allowed to dry. This step was done 3 times during which the samples were air dried for 20 minutes in the initial step dried in an incubator at 37°C for 40 minutes each in the subsequent steps. Using forceps, the dried sample spots were then transferred to 0.2 ml PCR tubes. A 5 µl 10X PCR buffer containing 15 mM $MgCl_2$, Tris-Cl, KCl and $(NH_4)_2SO_4$ (QIAGEN, USA) and 45 µl of dd H_2O were added to each tube. The tubes were then placed in a multigene optimax thermo cycler (Labnet, USA) with the following temperature conditions: one cycle at 60°C for 30 minutes, followed by 99.9°C for 10 minutes and lastly cooled to 4°C. The punched FTA paper was then left in the PCR tube with the elute DNA and stored 4°C until PCR was conducted.

2.3.4. PCR using KIN universal primers

For the detection of trypanosomes in the blood of domestic animals, FTA filter cards and tsetse flies, PCR was conducted using *Trypanosoma* genus specific primers which are known to anneal to the conserved regions of 18S and 5.8S rRNA genes to amplify internal transcribed spacer regions (Desquesnes *et al.,* 2001). The KIN 1 (GCG TTC AAA GAT TGG GCA AT) and KIN 2 (CGC CCG AAA GTT CAC C) primers were used to detect the presence of trypanosome DNA in the blood and tsetse fly samples. For the amplification trypanosome DNA using KIN universal primers the final reaction mixture was 25 µl and consisted of 3 µl of template DNA, 4.5 µl double distilled water, 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase (Thermo Scientific, USA). The primer mix contained 10 µM of each oligonucleotide primer. PCR conditions for KIN universal primers were set as follows: denaturation at 94°C for 3 minutes subjected to 35 cycles at 94°C for 1 minute, annealing at 58°C, 56°C and 54°C each annealing

temperature was set for 1 minute, the first extension at 72°C for 1 minute and final elongation at 72°C for 5 minutes with the holding temperature at 4°C (Desquesnes *et al.,* 2001). Following the amplification, 5 µl amplicon was resolved by gel electrophoresis using 1% agarose gel stained with 10 µl GR – Green nucleic acid and visualized under UV (ultra violet) light.

2.3.5 Sequencing and genetic analysis

Twenty micro litres of all positive PCR products were sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa. Dideoxy-mediated chain termination sequencing also known as shortgun sequencing was conducted with an ABI Prism 3130*x* genetic analyser (Sambrook and Russell, 2001b). This sequencing procedure enables users to run large numbers of reactions in parallel, rather than in series whereby, artificial DNA primers are used to start the reaction. The unknown recombinant DNA sequence is cloned and the clones are inserted into *Escherichia coli* (*E. coli*) vector sequence. Finally the sequencing results are then arranged and assembled on the computer into a contiguous sequence of overlapping fragments (Sambrook and Russell, 2001b).

2.3.6 Statistical analysis

Standard deviation and Chi-square (χ^2) test were used for statistical analysis. The standard deviation was used to calculate the average prevalence of AAT observed and *Trypanosoma* species present in domestic animals found in the three sampled local municipalities of the uMkhanyakude district of KwaZulu-Natal Province. Then χ^2 was used to determine the significant difference in *Trypanosoma* species infection rate between male and female tsetse flies found in both Charter's Creek game reserve and Boomerang commercial farm in north eastern KwaZulu-Natal Province.

2.4 Results

2.4.1 Overall prevalence of animal trypanosomiasis infection in uMkhanyakude district

PCR was used for the detection of prevalent trypanosome species found in the blood samples from the domestic animals in the study area. The overall prevalence of trypanosomes by PCR in tested domestic animals was 23.4% (32/137), 0% (0/101), 0% (0/9) and 0% (0/49) in cattle, goats, sheep and canines respectively (Table 2). Hlabisa local municipality showed to have high prevalence of the disease with 12.7% (20/158) followed by 10.2% (5/49) and 7.9% (7/89) in Mtubatuba and Big 5 False Bay local municipalities respectively. The prevalence of AAT in the three sampled local municipalities of uMkhanyakude district of KwaZulu-Natal Province is represented in figure 4 and uncertainties that occurred during sampling are represented by error bars. The narrow error bar from the Big 5 False Bay local municipality indicates that there was no sampling bias as compared to the other two sampled local municipalities with fairly large error bars.

Table 2: The overall prevalence of African animal trypanosomiasis infection in blood samples collected from cattle, sheep, goats and dogs in the three sampled localities in KwaZulu-Natal Province

Local municipality	Cattle	Sheep	Goats	Dogs	Total
Big 5 False Bay	19.4% (7/36)	0% (0/9)	0% (0/24)	0% (0/20)	7.9% (7/89)
Hlabisa	24.7% (20/81)	0% (0/0	0% (0/60)	0% (0/17)	12.7% (20/158)
Mtubatuba	25% (5/20)	0% (0/0)	0% (0/17)	0% (0/12)	10.2% (5/49)
Total	23.4% (32/137)	0% (0/0)	0% (0/101)	0% (0/49)	10.8% (32/296)

Average standard deviation was conducted to determine the variation or similarity in the average prevalence of AAT infection in bovine samples only. Therefore the variation in the overall prevalence of AAT infection among all tested bovine samples was high in Hlabisa (10±2.828), followed by Mtubatuba (2.5±2.121) and lastly the Big 5 False Bay (3.5±0.707) local municipalities respectively.

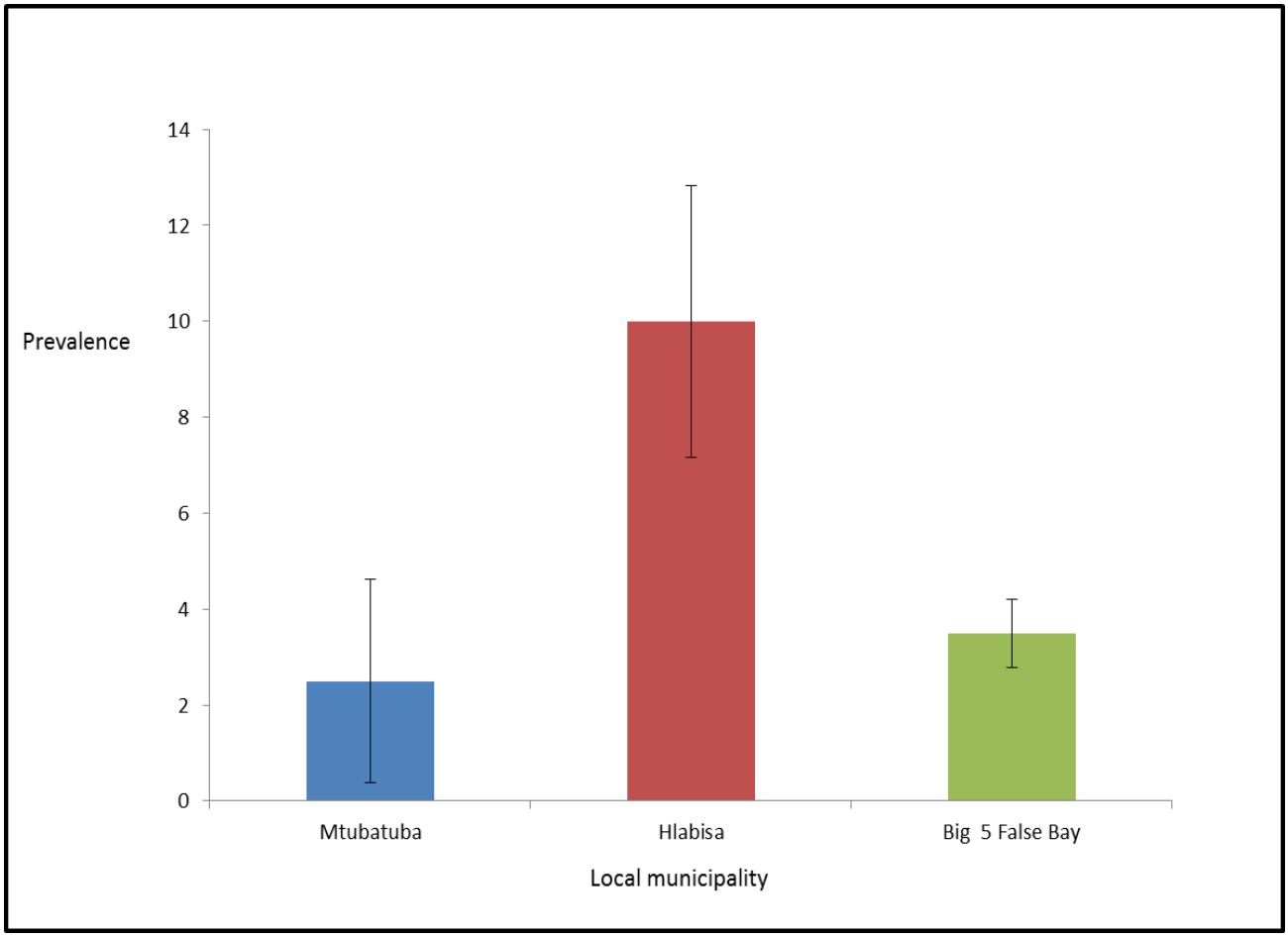


Figure 4: Average prevalence of AAT in the three sampled local municipalities in uMkhanyakude district of KwaZulu-Natal

2.4.2 Prevalent trypanosome parasites among the three local municipalities in uMkhanyakude district

Trypanosoma parasites were only documented in bovine samples as they are the only domestic animals that tested positive by PCR. From the 137 tested bovine samples only 32 were PCR positive. Only *T. congolense* and *T. theileri* species were detected by PCR and both occurred as single infections among the tested cattle samples. No mixed infections and *T. vivax* parasites were detected by PCR from the tested cattle samples in this study. The two prevalent trypanosome parasites in the three sampled localities are summarized in table 3. In plate 2 and 3 are the agarose gel images which show the detected *Trypanosoma* species by PCR using KIN 1 and KIN 2 primers. Sequences obtained from positive PCR products of 450-455 bp were aligned and subjected to BLAST (bl2 seq) and they revealed identity matches of 90%-98% (E-value: 0.0) with *T. theileri* isolates from the NCBI database (Figure 5a). However, from the alignment of the two sequences for *T. theileri* there are 7 polymorphic sites with 5 gaps, 1 transition and 1 transversion. Additional PCR results had sequences ranging between 704 bp to 716 bp and were also subjected to BLAST (bl2 seq) which showed identity matches of 95%-98% (E-value: 1^{-77} and 4^{-78}) with *T. congolense* isolates (Figure 5b). For *T. congolense* there are 48 polymorphic sites with 14 gaps, 25 transitions and 23 transversions. *Trypanosoma theileri* was most prevalent in Hlabisa local municipality by 14.8% (12/81) followed by 8.3% (3/36) and 5% (1/20) from Big 5 False Bay and Mtubatuba local municipalities respectively. *Trypanosoma congolense* on the other hand was more prevalent in Mtubatuba local municipality by 20% (4/20) then followed by Big 5 False Bay local municipality with 11.1% (4/36) and lastly Hlabisa local municipality with 9.9% (8/81). Figure 6 represents the prevalence of the two *Trypanosoma* species in the three sampled localities. Mtubatuba had higher prevalence of 25% (5/20) when compared to the other two local municipalities, due to small sample size and there was no significant difference at $p > 0.05$ ($\chi^2=2.88$; df=2) in the overall distribution of the two species in the three sampled municipalities. The accession numbers JN673389, JN673388 JX853185, JX178166, AJ009164 and AB007814 were used to compare sequences obtained in this study to those that have been submitted on the database (http://blast.ncbi.nlm.nih.gov).

Table 3: Summary of prevalent *Trypanosoma* parasites in bovine samples that tested positive by PCR using KIN primers in the three local municipalities.

Local municipality	*Trypanosoma theileri*	*Trypanosoma congolense*	Total
Big 5 False Bay	8.3% (3/36)	11.1% (4/36)	**19.4%(7/36)**
Hlabisa	14.8% (12/81)	9.9% (8/81)	**24.7% (20/81)**
Mtubatuba	5% (1/20)	20%(4/20)	**25% (5/20)**
Total	**11.7% (16/137)**	**11.7% (16/137)**	**23.4% (32/137)**

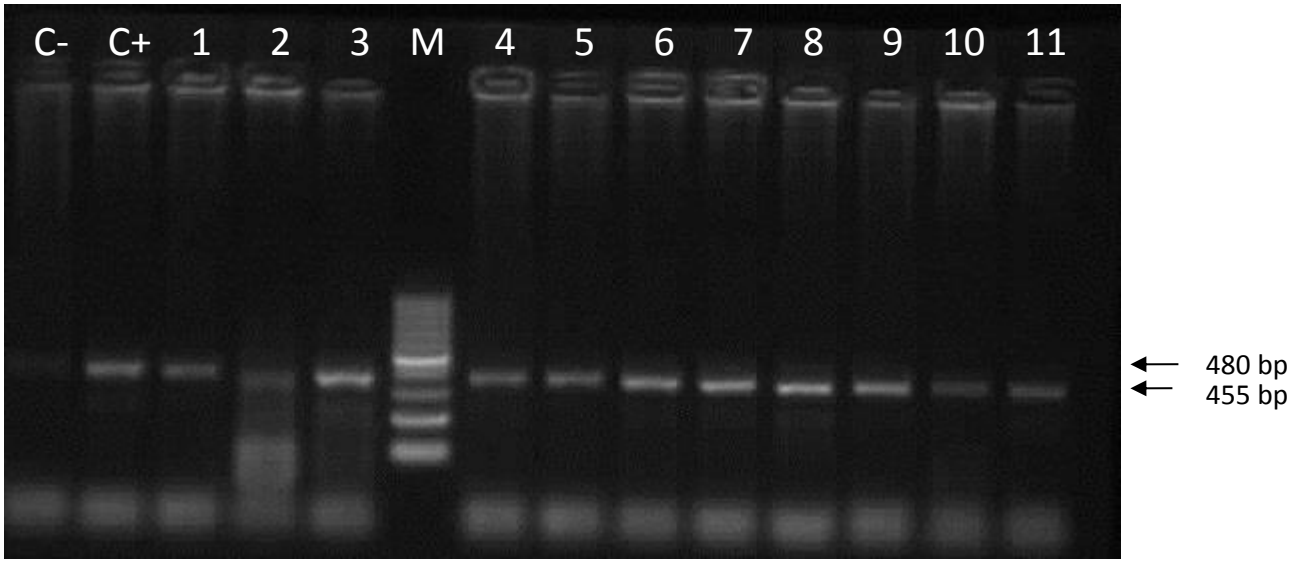


Plate 2: Agarose gel showing amplification of *T. theileri* from cattle samples using KIN primers. Molecular marker of 100 bp, positive control of *T. brucei* 3.1 with 480 bp and positively amplified DNA of *T. theileri* at 455 bp.

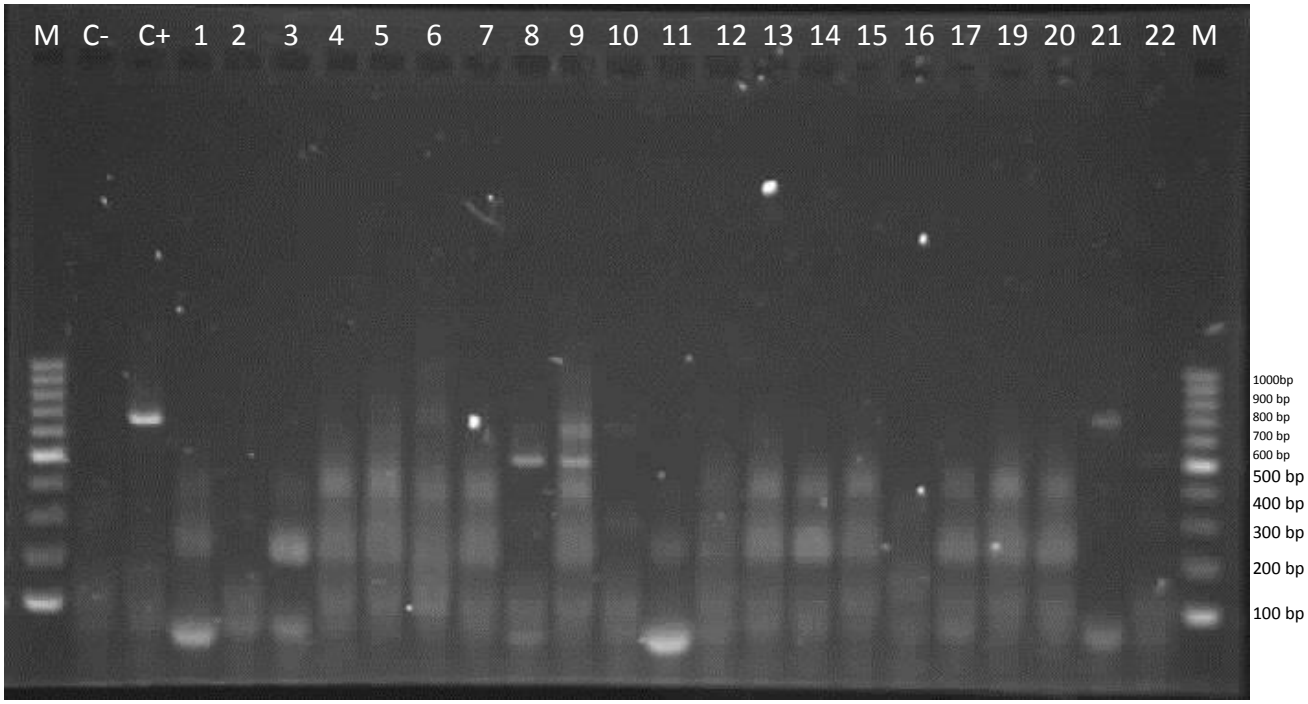


Plate 3: Agarose gel showing amplification of *T. congolense* from cattle samples using KIN primers. Molecular marker of 100 bp, positive control of *T. congolense* IL 3000 with positive samples of *T. congolense* ranging from 700 bp to 720 bp in lanes 8, 9 and 20. The remaining lanes show below detection limit for trypanosomes.

Figure 5a: BLAST (bl2 seq) results showing the alignment of *T. theileri* and one of the sequences from this study which was from a cattle sample from Ndibela diptank, Big 5 False Bay local municipality. The subject sequence (*T. theileri,* isolate cow 138 clone) covered 99% of the query sequence (KZN_B5FB-B11 Bovine) and it had 99% match score with 5 gaps and 1131 maximum score. The black stars indicate transversions as well as transitions that occurred between sequences and red stars show gaps between sequences.

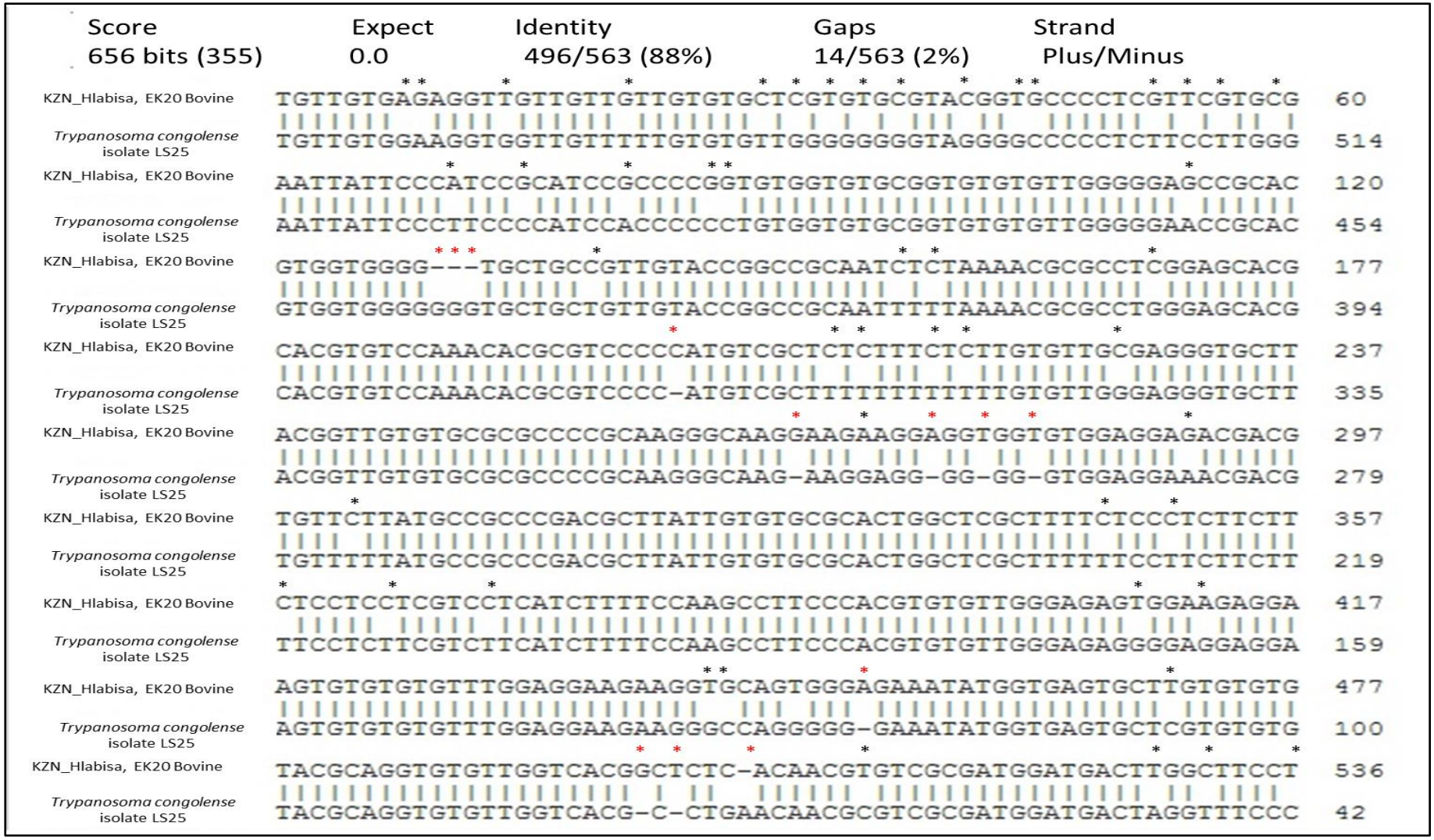


Figure 5b: BLAST (bl2 seq) results showing the alignment of *T. congolense* isolate and one of the sequences from this study which was from a cattle sample in Ekophinsweni diptank, Hlabisa local municipality. The subject sequence (*Trypanosoma congolense* isolate LS 25) covered 100% of the query sequence (KZN_Hlabisa, EK 20 Bovine) and it had 88% match score with 14 gaps and 656 maximum score The black stars indicate transversions as well as transitions that occurred between sequences and red stars show gaps between sequences.

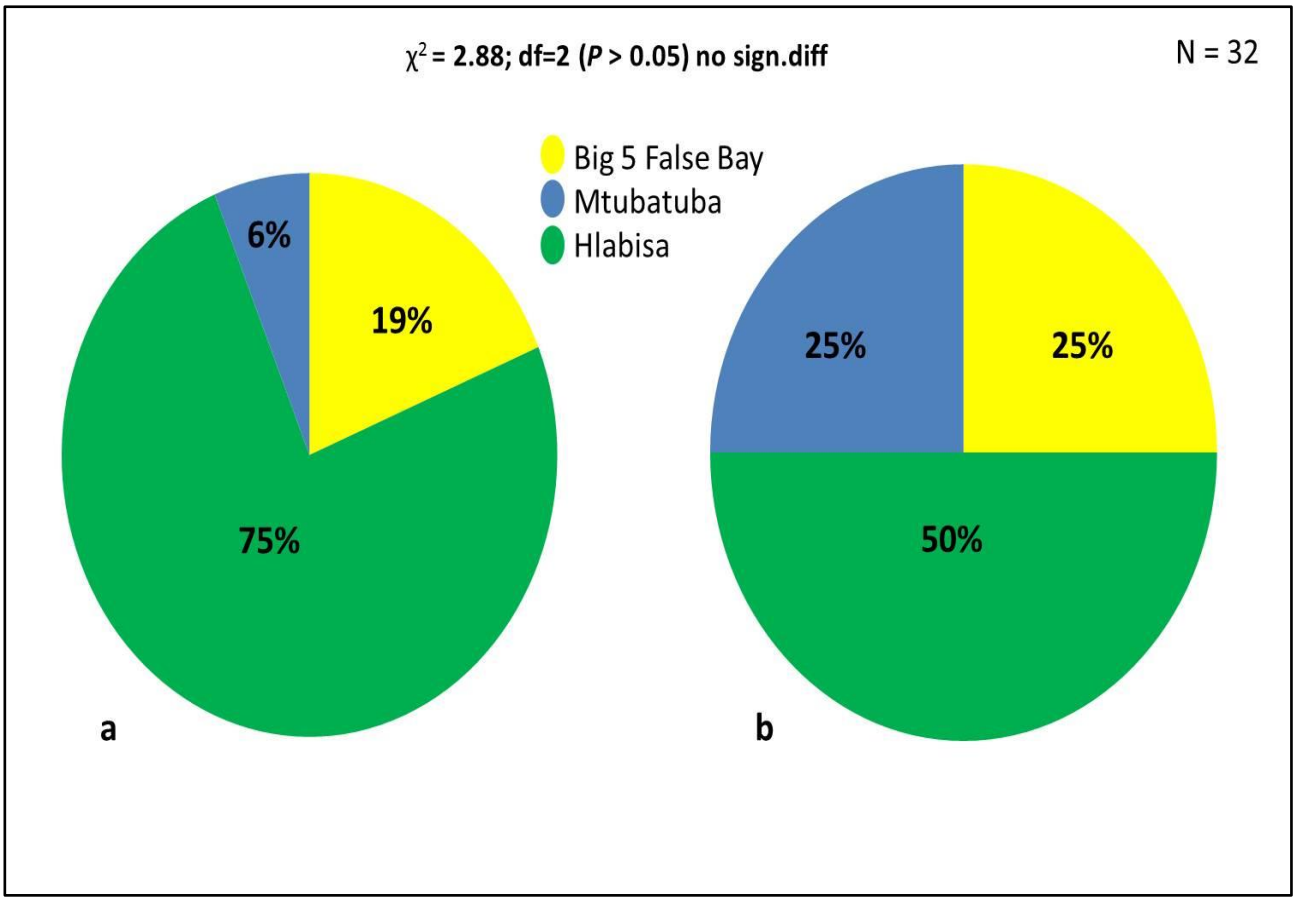


Figure 6: Prevalence of the two *Trypanosoma* species in the three sampled local municipalities in uMkhanyakude district of KwaZulu-Natal Province. Chart a) represents the prevalence of *T. theileri* and Chart b) represents the prevalence of *T. congolense.*

2.4.3 Prevalent *Trypanosoma* parasites in *Glossina brevipalpis* collected from Boomerang commercial farm and Charter's Creek game reserve

A total of 350 *Glossina brevipalpis* were screened by KIN 1 and KIN 2 primers to detect trypanosome parasites. A total of 15.4% (54/350) were positive for trypanosomes and all positive *Glossina brevipalpis* samples were from Charter's Creek game reserve and non from Boomerang commercial farm. Female *G. brevipalpis* had higher infection prevalence of 19.7% (32/162) and the males had 12.6% (22/174) infection prevalence. A summary of this data is given in figure 7 which represents the prevalence of trypanosome parasites between different genders of *G. brevipalpis*. Chi square test (χ^2) was conducted to determine the significant difference of trypanosome infections between male and female tsetse flies. There was significant difference observed between the two genders with the χ^2=232.283; df=1 (p<0.05) and the prevalence of infection among the tested *G. brevipalpis* samples does vary among traps in Charter's Creek. From the 54 *G. brevipalpis* samples that tested positive for PCR dominant *Trypanosoma* species were 77.7% (42/54), 16.6% (9/54) and 5.55% (3/54) for *T. congolense* (Savannah), *T. b. brucei* as well as *T. congolense* (Kilifi) respectively. However, these finding are only based on DNA bands observed on agarose gel after gel electrophoresis. *Trypanosoma congolense* (Savanna) positives had 750 bp bands, *T. congolense* (Kilifi) had 680 bp bands and *T. b. brucei* had 540 bp bands. Plate 4 shows the positive bands of *T. b. brucei* obtained by gel electrophoresis. Sequences were retrieved from the 54 samples and only 28 sequences had significant matches when subjected to BLAST (n) however, none of the 28 sequences had produced any significant matches to *Trypanosoma* species submitted in the NCBI database. They either matched with the vector clone used during sequencing or other blood feeding arthropods which might be due contamination of samples during sequencing. Likewise, as in cattle samples no *T. vivax* and mixed infections were observed.

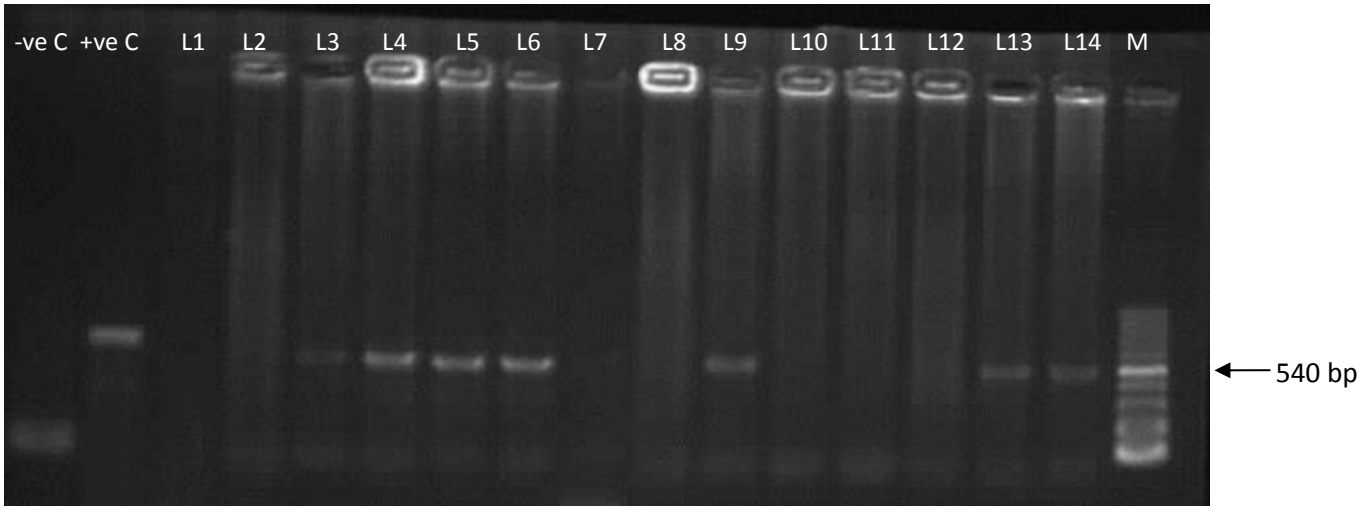


Plate 4: Agarose gel showing amplification of trypanosome parasites from *Glossina brevipalpis* DNA collected from Charters Creek using KIN primers. Lanes 3, 4, 5, 6, 9, 13 and 14 show positive amplification of *T. brucei* and lanes 1, 2, 7, 8, 10, 11 and 12 show samples that were below the detection limit

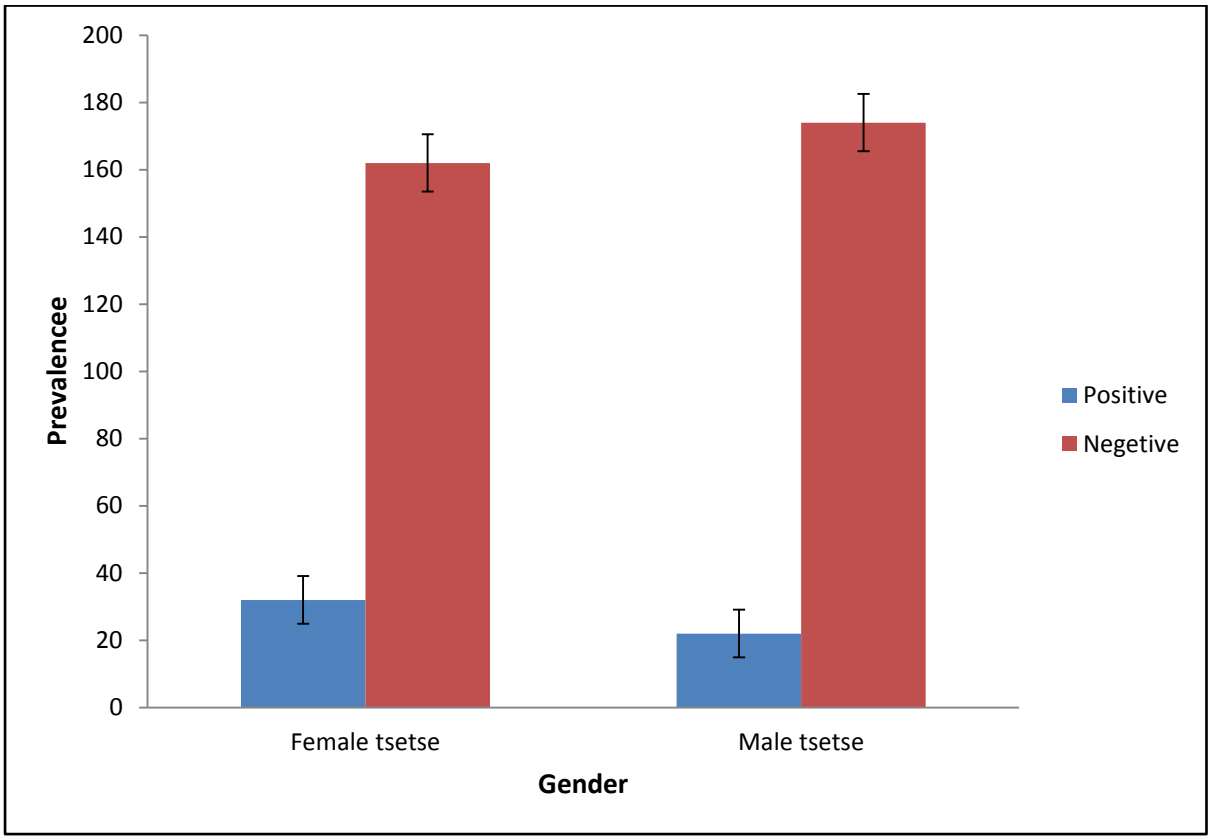


Figure 7: Data representing the prevalence of trypanosome parasites in *Glossina brevipalpis* between different genders that tested positive for *Trypanosoma* parasites by PCR in the uMkhanyakude district of KwaZulu-Natal Province.

2.5 Discussion

Although no mixed infections and *T. vivax* parasites were detected in this study, the findings do correspond to recent findings made by Mamabolo *et al.* (2009) of (*Nannomonas*) *T. congolense* (Savannah) parasites circulating in the north eastern parts of KwaZulu-Natal and they further show that a Stercoraria parasite (*Megatrypanum*) *T. theileri* is likewise prevalent in the district. However, most studies on animal trypanosomiasis conducted either on domestic animals or tsetse vectors in South Africa (Van den Bossche *et al*., 2006; Mamabolo *et al*., 2009; Motloang *et al*., 2012) have never documented the prevalence of *T. theileri* before, reasons being because it of less concern as it is non-pathogenic to both animals and humans or the primers they were using could not detect the parasites. Secondly *T. theileri* is mechanically transmitted by tabanid flies (*Tabanus*) as well as *Hyalomma anatolicum* ticks (Latif *et* al., 2004) and not tsetse flies therefore, making the current study one of the few to document the presence of *T. theileri* parasites in the country. These findings also prove what has been noted by Mamabolo *et al*. (2009) that *T. congolense* is more widespread mainly in diptanks (Ekophindisweni and Hlambanyathi) of Hlabisa local municipality. However, there was a limited sample size particularly in the Mtubatuba local municipality which explains why there were few *Trypanosoma* parasites detected in the sampled diptank in the current study. One of the reasons why in this study no *T. vivax* parasites were detected is because as noted by Desquesnes *et al*. (2001), KIN 1 and KIN 2 primers used for this study were less sensitive in detecting *T. vivax* parasites and therefore requires more optimization in future research.

Cattle are the preferred host by the tsetse fly vectors as compared to other domesticated animals in the country and this has been proven in previous studies on AAT in South Africa (Boyt, 1988; Van den Bossche, 2006; Mamabolo *et al*., 2009). As noted by Van den Bossche (2001), the grazing sites near tsetse infested zones such as national parks and game reserves increase the likelihood of livestock being infected by the parasite however, merely 23.4% (32/137) of tested cattle blood samples were infected by *Trypanosoma* parasites therefore, indicating that cattle in the north eastern KwaZulu-Natal Province partially encounter the vector tsetse flies that feed on reservoir host or it may be due to a small sample size. These findings are quite higher as compared to the most recent study on the prevalence of AAT in KwaZulu-Natal Province by Mamabolo *et al*., (2009) which had lower prevalence of the disease

with 18.6% (125/673). This means that although the AAT infections are currently of least concern in South Africa an outbreak of the disease may occur if no effective control measures are taken.

Sheep and goats are only susceptible to AAT in the absence of cattle and this supported by the fact that no trypanosomes were detected in both animals. Both animals are covered by thick fur which might make them least preferred hosts by the tsetse flies. However, due to small sample sizes of sheep and goats species in the three sampled local municipalities this might not be true if sufficient blood samples were collected. Canine African trypanosomiasis (CAT) is uncommon and rarely reported, in South Africa a documented case of CAT was back in 2006 where a six year old male Jack Russell terrier was infected with *T. congolense* (Savannah) (Gow *et al.*, 2007). However, the dog got the disease when it was visiting a tsetse infested area with its owner in Mozambique and was only diagnosed with the disease 3 years later when it had relocated with its owner to the United Kingdom (Gow *et al*., 2007). The dog eventually died as a result to the acute symptoms infection meaning *T. congolense* is very lethal in canines. In this study no canine samples were positive for the presence of trypanosomes, even though the sample size for canines was fairly reasonable. This might be due to that canine samples used in this study were never in contact with tsetse flies, which explains why none tested positive by PCR for the presence of trypanosomes. Nonetheless, the overall rate of infection among all tested domesticated animal samples in the uMkhanyakude district municipality showed significant difference and there was variation in the overall prevalence of infection.

The findings showed that *T. congolense* and *T. theileri* were the two prevalent species in all positively tested domestic animals. *T. congolense* (57%) was more dominant as compared to *T. theileri* (43%). The BLAST results were able to validate that the sequenced positive amplicons were from *T. congolense* and *T. theileri* and both had strong identity scores ranging from 90% to 98% additionally, the E-values (expect value) were quite low. In BLAST the E-value is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size and they decrease as the identity match scores increase. The lower the E-value, or the closer it is to zero, the more "significant" the match between the

query and the subject sequence are to one another (http://blast.ncbi.nlm.nih.gov). This confirmed that indeed the sequences obtained were from *T. congolense* and *T. theileri*.

From tsetse samples that were collected 99% were *G. brevipalpis* and only 1% belonged to *G. austeni.* These findings are supported by previous studies on tsetse flies in South Africa that *G. brevipalpis* catches were higher than *G. austeni* (Esterhuizen *et al*., 2005; 2006; Mamabolo *et al*., 2009; Motloang *et al*., 2012). This is because currently there are no effective baited traps designed to catch *G. austeni* (Esterhuizen *et al*., 2006). No active trypanosome parasites were found by microscopy from the dissected *G. brevipalpis* and one *G. austeni* samples. There were more male tsetse flies captured by H-traps than female tsetse flies however, both sexes are likely to be equally infected by the trypanosome parasites (Leak, 1999).

The distribution of the trypanosome species in the three local municipalities also showed some variation with no significant difference observed whereby, *T. congolense* was more prevent in Mtubatuba local municipality and *T. theileri* was prevailing in Hlabisa local municipality. However, due to limited sample size especially in Mtubatuba local municipality these findings may not represent the current situation there since only one diptank was sampled in that particular local municipality. In terms of individual counts of positive samples in both Hlabisa and Mtabatuba local municipalities respectively most positives of both *T. congolense* and *T. theileri* were documented in Hlabisa local municipality. Observations made on agarose gels showed that dominant *Trypanosoma* species circulating between *G. brevipalpis* are *T. congolense* (Savannah and Kilifi) types with 77.7% and 5.55%, lastly *T. brucei* by 16.6%. Although a study by Mamabolo *et al*. (2009) concluded that South Africa, in particular KwaZulu-Natal Province is free from *T. brucei* parasites but, this was not the case in current study meaning, the *T. brucei* species might have been misdiagnosed or it is a re-emerging infection.

The chi square test showed that there was no statistical significant difference observed in the overall distribution of the disease in the sampled local municipalities and this is supported by the fact that there were insufficient number of blood samples of ovine in all three municipalities and few samples for bovine and caprine in Mtubatuba local municipality. The uMkhanyakude district municipality has five local municipalities and in this study only three

were sampled. Previous studies by Mamabolo *et al*. (2009) included Jozini which is the fourth local municipality in uMkhanyakude district. These authors showed that indeed there is *T. congolense* and *T. vivax* parasites circulating among livestock, wild animals and wild tsetse flies in this area but these parasites did not occur as mixed infections in the area. As indicated in the current study all the sampled areas are located either near or in between nature reserves such as Hluhluwe-IMfolozi Game Reserve, St Lucia Wetland and St Lucia Marine Reserve, Phinda Private Game Reserve as well as Ndumo Game Reserve which is not included in the map but it is found in Jozini local municipality. All these reserves are preferred habitat for both tsetse species of AAT and wild animals which act as reservoir hosts for the parasites. This explains why there is prevalence of AAT documented in this study.

Trypanosoma vivax parasites were not detected by PCR but this does not prove the parasites are not prevalent in north eastern KwaZulu-Natal because they were previously reported by Mamabolo *et al*. (2009). *T. congolense* parasites are most prevalent in the area as compared to non-pathogenic *T. theileri* therefore effective control measures must be formulated to prevent the spread of *T. congolense* since *T. theileri* is of least concern and all animals that tested positive for AAT should be treated with the necessary drugs to prevent loss of agricultural productivity and income to the affected owners. More research is needed in the north eastern parts of KwaZulu-Natal Province which will have sufficient samples that will cover all five local municipalities of uMkhanyakude district and to include all domesticated animals such as equids (horses and donkey) as well as felids (cats). The utilization of PCR techniques was quite successful even though further optimization of KIN 1 and KIN 2 primes is a necessity to detecting all *Trypanosoma* parasites that are believed to be circulating among domestic and wild animals as well as in the tsetse vectors. The current study showed that AAT is still prevailing in KwaZulu-Natal Province. Global warming might increase the distribution of the vector flies and the trypanosome parasites and thus making domestic animals susceptible if effective eradication or control measures of the vector flies are taken to consideration.

CHAPTER 3

GENETIC DIVERSITY WITHIN AND AMONG *TRYPANOSOMA* SPECIES IN KWAZULU-NATAL, SOUTH AFRICA

3.1 Introduction

Taxonomy, genetic exchange and molecular epidemiology are the major subjects of interest in understanding the population biology of trypanosomes. For the effective control of trypanosomes it is crucially important to define as well as identify pathogenic species, subspecies, strains and trypanosome populations circulating in both hosts and vectors to enable epidemiological investigations in affected nations (Hide and Tait, 2004). This approach is achieved by developing molecular markers that can describe and classify distinct taxonomic units such as species, subspecies, strains, populations as well as variants, with the objective of tracking parasites through different hosts and vectors. Secondly determining the link between the parasites and geographic distribution, and lastly by investigating the association between diseases and particular groups of parasites (Hide and Tait, 2004).

Previous studies during the 1970's on trypanosomes which mainly relied on the development of clinical symptoms, the type of host preference as well as microscopic techniques to analyse morphological and morphometric characters made it somehow difficult and large scale analysis impossible for the distinction of populations and subspecies (Hide and Tait, 2004). For this reason, development of new molecular techniques was essential. The study of isoenzyme variation by multilocus enzyme electrophoresis (MLEE) resulted in a breakthrough for molecular biology and opened up new possibilities on the study of trypanosomes. MLEE was mainly used to develop markers to distinguish members of the subgenus *Trypanozoon* (Gibson, 2007). The technique was used on the basis of allele frequencies, in terms of similarities of shared traits and lastly on the distinction based on the presence or absence of a specific isoenzyme band in a given taxonomic group (Gibson, 2007). This method gave a clear distinction between parasite strains of *T. b. brucei* and *T. b. gambiense* but they were not able to differentiate between *T. b. brucei* and *T. b. rhodesiense* (Hide and Tait, 2004). Restriction fragment length polymorphism (RFLP) also revealed great heterogeneity in both *T. b. brucei*

and *T. b. rhodesiense* however, like most analytical methods it did not differentiate between the two strains (Radwanska *et al*., 2002).

Molecular techniques such as restriction enzymes, sequencing and synthesis of DNA, DNA probing and polymerase chain reaction (PCR) have been developed (Desquesnes and Dàvila 2002). These methods have made a significant contribution into trypanosome identification, characterisation as well as accurate and reliable diagnosis at various taxonomic levels mainly at species and subspecies level as well as to distinguish between closely related species. In most cases, PCR diagnosis aims to identify the parasites at the species level, which is achieved by using preferred targets which are present in high copy numbers in the genome of trypanosomes. As noted by Desquesnes and Dàvila, (2002), intra-specific genetic diversity and variations found in trypanosomes can be defined at the level of both genomic level by aligning whole genome sequence, and at individual genes by detecting single mutations that arise in a sequence of a species. Consequently, for both diagnostic work and population genetics DNA sequences make it possible to identify clonal-specific sequences or even point out mutations. These mutations are responsible for the resistance detected in some *Trypanosoma* parasites. A novel study by Radwanska *et al*. (2002) used serum resistance-associated (SRA) gene in a SRA gene-based PCR to clearly distinguish between *T. b. brucei* and *T. b. rhodesiense* and *T. b. gambiense*-specific glycoprotein (TgSGP) gene to distinguish *T. b. gambiense* from other *Trypanozoon* species. These discoveries lead to advances in the diagnosis and treatment of African sleeping sickness in both East and West Africa (Hide and Tait, 2004; Mwandiringana *et al*., 2012).

The 18S ribosomal RNA (rRNA) and glycosomal Glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) were used to detect genetic diversity, homogeneity as well as heterogeneity among detected trypanosome species (Stevens and Gibson, 1999; McInnes *et al*., 2009). In the current study the two proposed genes were amplified from DNA samples obtained from cattle blood samples in the uMkhanyakude district of KwaZulu-Natal Province, South Africa. Therefore it was hypothesised that there will be great genetic diversity among the sequences of South African *Trypanosoma* species.

3.2 Objectives

1. To determine the genetic diversity within *Trypanosoma* species in uMkhanyakude district of KwaZulu-Natal Province

2. To determine nucleotide polymorphism and conserved regions of *Trypanosoma* species in sampled localities using 18S rRNA and gGAPDH genes

3.3 Materials and methods

3.3.1. Experimental procedure

Trypanosoma positive bovine samples (n=32) were subjected to two nested PCRs which amplified the 18S ribosomal RNA (rRNA) and glycosomal Glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) genes respectively (Stevens and Gibson, 1999; Hamilton *et al.*, 2004; Mamabolo *et al.*, 2009; McInnes *et al*., 2009).

3.3.2 Nested PCR using 18S rRNA primers

To amplify 800-900 base pairs (bp) fragments of trypanosome 18S rRNA gene, two rounds of PCRs were done. In the first round, the primer set 18ST nF2 (CAA CGA TGA CAC CCA TGA ATT GGG GA) and 18ST nR3 (TGC GCG ACC AAT AAT TGC AAT AC) were used and in the second reaction round, 18ST nF2 was used with 18ST nR2 (GTG TCT TGT TCT CAC TGA CAT TGT AGT G) as a reverse primer (Mamabolo *et al*., 2009). This amplification method is referred to as a semi-nested PCR amplification because in the first reaction normal template DNA is used for the amplification and in the second reaction test the amplicon from the first reaction is used as template DNA (Sambrook and Russell, 2001a). The reaction volume was 25 µl and consisted of 2 µl of template DNA, 10 µl of double distilled water, 10 µl of 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase (Thermo Scientific, USA), the primer mix contained 10 µM of each oligonucleotide primer. PCR conditions for the first round of 18S primers were set as follows: denaturation at 94°C for 4 minutes subjected to 30 cycles at 94°C for 1 minute, annealing at 60°C for 1 minute, the first extension at 72°C for 1 minute and final elongation at 72°C for 5 minutes with the holding temperature at 4°C (Mamabolo *et al.,* 2009). After the reaction was complete the second round of amplification was prepared with the final volume of 25 µl which contained 1 µl of the amplicon as template DNA, 11.5 µl of double distilled water, 9.5 µl of 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase (Thermo Scientific, USA), the primer mix containing 10 µM of each oligonucleotide primer. The PCR conditions for the second amplification round were the same as the ones used in the first round (Mamabolo *et al*., 2009). After completion 5 µl amplicon was resolved by gel electrophoresis using 1 % agarose gel stained with 10 µl GR – Green nucleic acid and visualized under UV light.

3.3.3 Nested PCR using gGAPDH primers

Amplification of approximately 880- 900 bp fragment of gGAPDH gene was conducted using a semi-nested PCR (Hamilton *et al*., 2004; McInnes *et al*., 2009). The primary PCR was done using a primer pair of GAPDHF (CTY MTC GGN AMK GAG ATY GAY G) and GAPDHR (GRT KSG ART ADC CCC ACT CG). The secondary PCR reaction was conducted using the forward primer GAPDHF and the reverse primer G4a (GTT YTG CAG SGT CGC CTT GG) respectively (Hamilton *et al*., 2004; McInnes *et al*., 2009). The final reaction volume of gGAPDH amplification was 25 µl and consisted of 2 µl of template DNA, 10 µl of double distilled water, 10 µl of 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase (Thermo Scientific, USA), the primer mix containing 10 µM of each oligonucleotide primer. The PCR conditions were as follows: pre-PCR step with 95°C for 5 minutes, 50°C for 2 minutes and an extension of 72°C for 4 minutes followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 2 minutes and 20 seconds with the holding temperature at 4°C, these conditions were for the primary PCR. The secondary PCR amplification also had the final volume of 25 µl which contained 1 µl of the amplicon from the primary PCR, 11.5 µl of double distilled water, 9.5 µl of 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase (Thermo Scientific, USA), the primer mix contained 10 µM of each oligonucleotide primer. The PCR conditions for the secondary PCR were the same as the ones used for the primary PCR except the annealing temperature was 52°C (McInnes *et al*., 2009). Thereafter 5 µl amplicon was resolved by gel electrophoresis using 1% agarose gel stained with 10 µl GR – Green nucleic acid and visualized under UV light.

3.3.4 Purification of PCR products

Polymerase chain reaction products of both 18S rRNA and gGAPDH were subjected to gel extraction and purification before they were sent to Inqaba Biotechnical Industries for sequencing. The protocol was adopted from QIAquick Spin Handbook (QIAGEN, USA). The remaining 20 µl of the amplicon was again resolved by gel electrophoresis on agarose gel for 30 minutes and placed on a UV light, then using a clean sharp scalpel the DNA fragments on the gel were sliced and placed into a 1.5 ml Eppendorf tube. A 600 µl volume of buffer QG was poured onto >400 mg of gel with DNA fragment. To dissolve the gel slice, the 1.5 ml tubes were heated at 50°C for 10 minutes in a heat block. The tubes were vortexed every 2 minutes during incubation and this was done to completely dissolve the gel. Then 200 µl of isopropanol were added into the reaction tubes. Then the mixture in the 1.5 ml tube was transferred into a QIAquick spin column with a 2 ml collection tube. To bind the DNA the spin columns were centrifuged for 1 minute at 13 000 rpm. The flow through in the collection tubes were discarded and 500 µl of buffer QG was added to the QIAquick column and centrifuged for 1 minute at 13 000 rpm. The DNA in the QIAquick column was washed with 750 µl of buffer PE and centrifuged for 1 minute. The flow through was discarded again and the QIAquick column was centrifuged to remove excess ethanol in the buffer PE. The QIAspin columns were placed into new clean 1.5 ml tubes and 50 µl of buffer EB was used to elute the DNA. The new purified DNA was stored at -20°C until they were sent for sequencing at Inqaba Biotechnical Industries.

3.3.5 Genotyping and nucleotide diversity

Sequences obtained from Inqaba Biotechnical Industries were retrieved and edited using molecular evolutionary genetics analysis 5 (MEGA 5). Sequences were first converted from AB1 format to FASTA format and the mixed bases (R, Y, M, S, W, H, B, V, D, and N) were also converted to their appropriate base pairs (A, C, G, and T) (Hall, 2008; Tamura *et al*., 2011). Then they were subjected to BLAST (n) to determine which *Trypanosoma* strains they represented and also to determine the number of nucleotide polymorphisms. Additional homologous sequences of other related species were downloaded from NCBI (National centre for biotechnology information) and added to alignment explorer. Thereafter, using MEGA 5 the names of the nucleotide sequences were changed to represent the sample batch number of the positive sample as well as the local municipality and the trypanosome species involved, the

names of the downloaded sequences were also reduced to few characters that were significant for distinguishing the downloaded sequences from the sequences obtained from positive samples.

The newly edited sequences were aligned by ClustalW (Chaichoompu *et al*., 2006; Tamura *et al*., 2007), which introduces gaps according to its algorithm and makes pairwise alignments along all sequences. For each pair of sequences it introduces gaps into each sequence in attempt to maximize the number of characters that match (Chaichoompu *et al*., 2006; Hall, 2008). Therefore as it does so it assigns positive scores for each match and the score for the alignment is the sum of those individual character match scores (Hall, 2008). MEGA was used to determine the nucleotide difference between sequences by computing pair-wise distances by evaluating nucleotide frequencies (Hall, 2008). The overall nucleotide composition between aligned sequences and the nucleotide diversity between *T. congolense* (Savannah) strains and *T. theileri* strains as well as within the two strains were also estimated using the program (Hall, 2008; Tamura *et al*., 2011). From the aligned sequences within *Trypanosoma* strains conserved regions between sequences were determined using BioEdit sequence alignment editor (Hall, 1999). All these nucleotide analysis were used in both 18S rRNA gene and gGAPDH gene for trypanosome strains detected in South African cattle.

3.4 Results

Trypanosoma positive bovine samples (n=32) that were screened by nested-PCR for both 18S rRNA and gGAPDH genes, only 14 (10 for 18S rRNA gene and 4 for gGAPDH gene) samples produced sequences that had significant matches when subjected to BLAST and the remaining were either too short (<600 bp) to be considered significant or they matched with the *E. coli* vector that was used when cloning during sequencing. The 10 18S rRNA sequences used represented the three sampled localities. The gGAPDH (n=4) sequences represented Mtubatuba and Hlabisa local municipalities.

3.4.1 Nested PCR amplifying 18S rRNA gene

The desired product sizes ranged between 800 to 850 bp were as observed from gel electrophoresis (Plate 5), and the multiple bands were removed by gel purification. The sequences obtained from the purified PCR products were subjected to BLAST (bl2 seq) (http://blast.ncbi.nlm.nih.gov) and 70% (7/10) of the sequences matched with *T. congolense* (Savannah) accession number AJ009146 with identity match scores of 99% (E-value: 0.0) (Figure 8a). The subject sequence was from *Trypanosoma congolense* (Savannah) 18S rRNA gene, isolate WG 81 and it covered 87% when compared to the sequence of *T. congolense* (Savannah) from KwaZulu-Natal Province with 99% identical match support and only one nucleotide polymorphism. The remaining 30% (3/10) sequences were from *T. theileri* and BLAST (bl2 seq) results showed that these 3 *T. theileri* species matched with the submitted sequences on the database accession number JX853185 by a match score of 95% (E-value: 0.0) (Figure 8b). The subject sequence was from *Trypanosoma theileri* isolate Cow 2095 18S ribosomal RNA gene which covered 91% of the sequence of *T. theileri* from KwaZulu-Natal Province with 95% identical match support and 23 nucleotide polymorphisms whereby 8 were transitional nucleotide changes and 15 were transversional nucleotide changes.

Plate 5: Gel image showing amplified DNA from 18S rRNA genes from bovine samples collected in uMkhanyakude district of KwaZulu-Natal. M is the molecular marker, +ve and –ve are positive and negative controls; L1- L6 and L12-L13 are positively amplified DNA. Lanes L7, 8, 9, 10 11 and 13 are samples that amplified below detection limit.

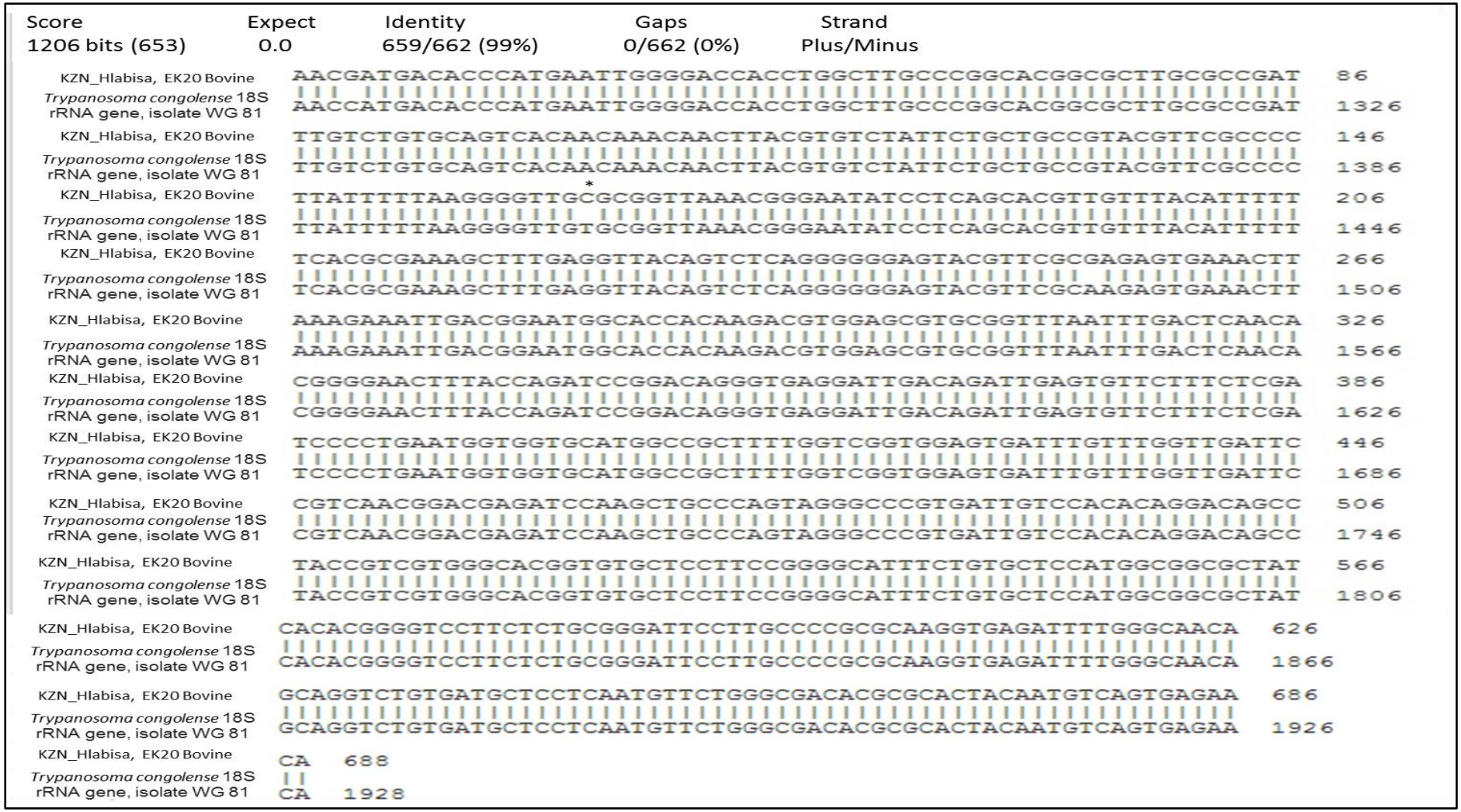


Figure 8a: BLAST (bl2 seq) results showing alignment of 18S rRNA *T. congolense* (Savannah) type with *T. congolense* strain obtained from this study. The black star indicates the nucleotide polymorphism that occurred between the two sequences no gap was observed.

Figure 8b: BLAST (bl2 seq) results showing alignment of 18S rRNA *T. theileri* with *T. theileri* strain obtained from this study. The black star indicates the nucleotide polymorphism that occurred between the two sequences and the red stars indicate where gaps were observed.

3.4.2 Genetic diversity of trypanosomes using the 18S rRNA gene

Seven *T. congolense* (Savannah) 18S rRNA sequences obtained from cattle blood samples from the three localities were aligned by ClustalW on MEGA 5. Alignments revealed significant difference in their overall nucleotide composition (Table 5a). Pair-wise distance to determine the number of differences per site between sequences as well as evolutionary divergence is shown in table 4a and the standard error estimates are shown in every other column. Analysis involved 7 sequences with 571 bp and 1^{st}, 2^{nd}, 3^{rd} and noncoding codon positions were also included. Positions containing gaps and missing data were eliminated from this analysis following the example of Tamaru *et al*. (2011). Nucleotide polymorphisms were observed between sequences and nucleotide diversity was estimated. Multiple nucleotide polymorphisms were observed between sequences with one conserved region that was 44 bp long at position 554 to 597 bp. Conserved regions in the alignment are represented by dots which represent homologous nucleotides in the alignment of the 7 *T. congolense* (Savannah) sequences (Figure 9a). Nucleotide diversity within *T. congolense* (Savannah) species in the three sampled municipalities which represented the mean evolutionary diversity for the entire population was d=0.310 and SE=0.012, the parameters on the MEGA 5 software were set as follows: bootstrap procedure with 1000 replicates, 1^{st}, 2^{nd} and 3^{rd} codon positions as well as non-coding positions were also included in the analysis however, gaps and missing data in the alignments were eliminated in this evolutionary analysis (Tamaru *et al*., 2011). Therefore, the second hypothesis which stated that there will be great genetic diversity among the sequences of South African trypanosomes was accepted as there was great genetic diversity within the *T. congolense* sequences from South Africa.

Three *T. theileri* 18S rRNA strains were detected and their alignment also showed high significant difference for overall nucleotide composition between the sequences (Table 5b). Pair wise-distance to determine the base difference per site from between sequences as well as evolutionary divergence is shown in table 4b with the standard error estimates shown above the diagonal and were obtained by a bootstrap procedure of 1000 replicates. The parameters for the analysis involved 3 nucleotide sequences; codon positions included were 1^{st}, 2^{nd}, 3^{rd} and noncoding. Additionally, all positions containing gaps and missing data were eliminated as before and there were a total of 679 positions in the final dataset (Tamaru *et al*., 2011). Nucleotide polymorphisms were also observed between sequences from *T. theileri* and they

represented only two municipalities namely Big 5 False Bay and Hlabisa local municipalities respectively. There was highly significant nucleotide polymorphisms observed between sequences however, there were 5 conserved regions observed all with a minimum length of more than 15 bp at sequence positions number 72 to 91 with 20 conserved bp, position 140 to 396 with 257 conserved regions, position 398 to 500 with 103 conserved sites, position 502 to 551 with 50 conserved bp and lastly position number 591 to 610 with 20 conserved regions (Figure 9b). From the three sequences that were analysed the mean evolutionary diversity for the entire population was d=0.072 and SE=0.008 the parameters were set as follows: bootstrap procedure with 1000 replicates, 1^{st}, 2^{nd} and 3^{rd} codon positions as well as non-coding positions were also included in the analysis and like before gaps as well as missing data in the alignments were eliminated (Tamaru *et al*., 2011). Again in this case we accept the null hypothesis which stated that there will be great genetic diversity among the sequences of South African trypanosomes.

On average there were more conserved regions observed among the *T. theileri* alignment sequences as compared to the *T. congolense* (Savannah) sequences. The genetic diversity of *T. congolense* (Savannah) was much higher as compared to that of *T. theileri.* Therefore these findings showed that the genotypes of all seven *T.* congolense (Savannah) strains are more or less genetically distinct from each other and *T. congolense* sequences submitted on the database, even though they share some similarities with one conserved site for *T. congolense* (Savannah). The remaining *T. theileri* species from KwaZulu-Natal Province had 20 conserved regions and had less genetic diversity.

Table 5a: Estimates of evolutionary divergence by 18S rRNA between *T. congolense* (Savannah) type South African sequences

Sequence Name	1	2	3	4	5	6	7
1. KZN_HLB_EK20_ Bovine_T.con.		0.00608	0.00386	0.00444	0.02018	0.02034	0.02018
2. KZN_HLB_EK01_ Bovine_T.con.	0.02102		0.00644	0.00686	0.02018	0.02034	0.02016
3. KZN_HLB_MH02 _Bovine_T.con.	0.00876	0.02277		0.00283	0.02037	0.02017	0.02037
4. KZN_MTB_M16 _Bovine_T.con.	0.01226	0.02627	0.00525		0.02027	0.02007	0.02027
5. KZN_HLB_H15_ Bovine_T.con.	0.53240	0.52890	0.53590	0.54116		0.00332	0.00236
6. KZN_BFB_SS12_ Bovine_T.con.	0.53065	0.52715	0.53065	0.53590	0.00701		0.00236
7. KZN_BFB_SS02_ Bovine_T.con.	0.53065	0.52715	0.53415	0.53940	0.00350	0.00350	

KZN=KwaZulu-Natal Province; HLB=Hlabisa; MTB=Mtubatuba; BFB=Big 5 False Bay local municipalies; EK=Ekophindisweni; MH and M=Mahlabanyathi; H= Hlabanyathi; SS=Silversands farm; T.con=*T. congolense* (Savannah)

Table 5b: Estimates of evolutionary divergence by 18S rRNA between *T. theileri* South African sequences

Sequence Name	1	2	3
1. KZN_BFB_B03_18S T.theileri		0.010	0.010
2. KZN_BFB_B03_18S T.theileri	0.082		0.007
3. KZN_BFB_B03_18S T.theileri	0.088	0.044	

KZN=KwaZulu-Natal Province; BFB=Big 5 False Bay local municipality; T.theileri=*T. theileri*

Table 4a: Nucleotide composition from 18S rRNA between *T. congolense* strains from uMkhanyakude district of KwaZulu-Natal.

Sample No	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos: 1	T-2	C-2	A-2	G-2	Pos: 2	T-3	A-3	C-3	G-3	Pos: 3
KZN_HLB_EK20_Bovine-18S_T.con_(S)	25.8	23.7	22.0	28.5	590.0	22	23.1	28.1	26.6	199.0	27	24.4	19.7	29.0	193.0	28	18.2	23.7	29.8	198.0
KZN_HLB_EK01_Bovine-18S_T.con_(S)	26.4	23.8	22.1	27.7	588.0	22	23.1	28.6	26.1	199.0	28	24.0	19.3	28.6	192.0	29	18.3	24.4	28.4	197.0
KZN_HLB_MH02_Bovine-18S_T.con (S)	25.9	23.9	22.2	28.1	591.0	22	23.6	28.1	26.6	199.0	27	23.8	19.7	29.0	193.0	29	18.6	24.1	28.6	199.0
KZN_MTB_M16_Bovine-18S_T.con_(S)	25.9	23.2	22.0	28.9	591.0	22	23.6	27.6	27.1	199.0	27	23.2	19.6	29.9	194.0	29	18.7	22.7	29.8	198.0
KZN_HLB_H15_Bovine-18S_T.con_(S)	21.9	28.8	26.6	22.7	594.0	20	26.0	29.5	25.0	200.0	22	31.8	24.1	22.1	195.0	24	26.1	28.6	21.1	199.0
KZN_B5FB_SS12_Bovine-18S_T.con_(S)	22.3	28.3	26.1	23.3	593.0	20	26.0	29.5	25.0	200.0	23	31.3	23.6	22.6	195.0	25	25.3	27.8	22.2	198.0
KZN_B5FB_SS02_Bovine-18S_T.con_(S)	22.1	28.7	26.1	23.1	593.0	20	26.1	29.1	25.1	199.0	23	31.3	23.6	22.6	195.0	24	25.6	28.6	21.6	199.0
Average composition.	**24.3**	**25.8**	**23.9**	**26.0**	**591.4**	**21**	**24.5**	**28.7**	**25.9**	**199.3**	**25**	**27.1**	**21.4**	**26.2**	**193.9**	**27**	**21.5**	**25.7**	**25.9**	**198.3**

Table 4b: Nucleotide composition from 18S rRNA between *T. theileri* strains detected from uMkhanyakude district of KwaZulu-Natal.

Sample No	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos: 1	T-2	C-2	A-2	G-2	Pos: 2	T-3	C-3	A-3	G-3	Pos: 3
KZN_B5FB_B03_Bovine_18S_T.th	26.1	21.7	24.8	27.3	681.0	23	23.8	24.2	29.1	227.0	30	19.7	24.1	25.9	228.0	25	21.7	26.1	27.0	226.0
KZN_B5FB_B11_Bovine_18S_T.th	26.1	22.5	24.4	27.0	685.0	24	25.4	23.2	27.6	228.0	30	21.0	24.9	24.5	229.0	25	21.1	25.0	28.9	228.0
KZN_HLB_EK19_Bovine_18S_T.th	26.5	21.8	24.0	27.7	683.0	24	23.6	23.6	28.8	229.0	30	20.6	23.2	26.3	228.0	26	21.2	25.2	27.9	226.0
Avg.	**26.3**	**22.0**	**24.4**	**27.3**	**683.0**	**24**	**24.3**	**23.7**	**28.5**	**228.0**	**30**	**20.4**	**24.1**	**25.5**	**228.3**	**25**	**21.3**	**25.4**	**27.9**	**226.7**

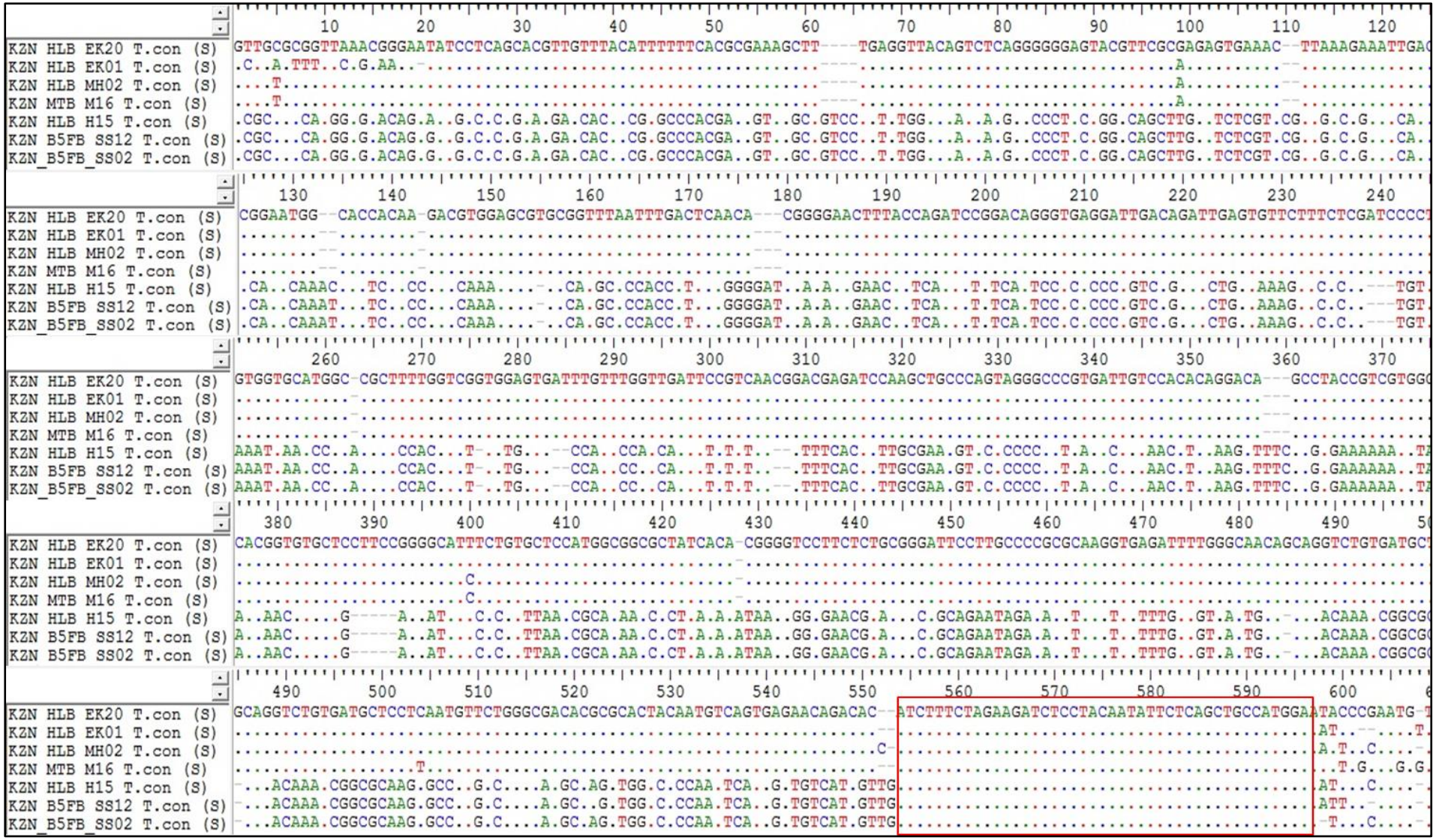


Figure 9a: Alignment of South African 18S rRNA *T. congolense* (Savannah) strains from the three sampled local municipalities (HLB: Hlabisa, B5FB: Big 5 False Bay, MTB: Mtubatuba). The highlighted area indicates the conserved sequences among all strains.

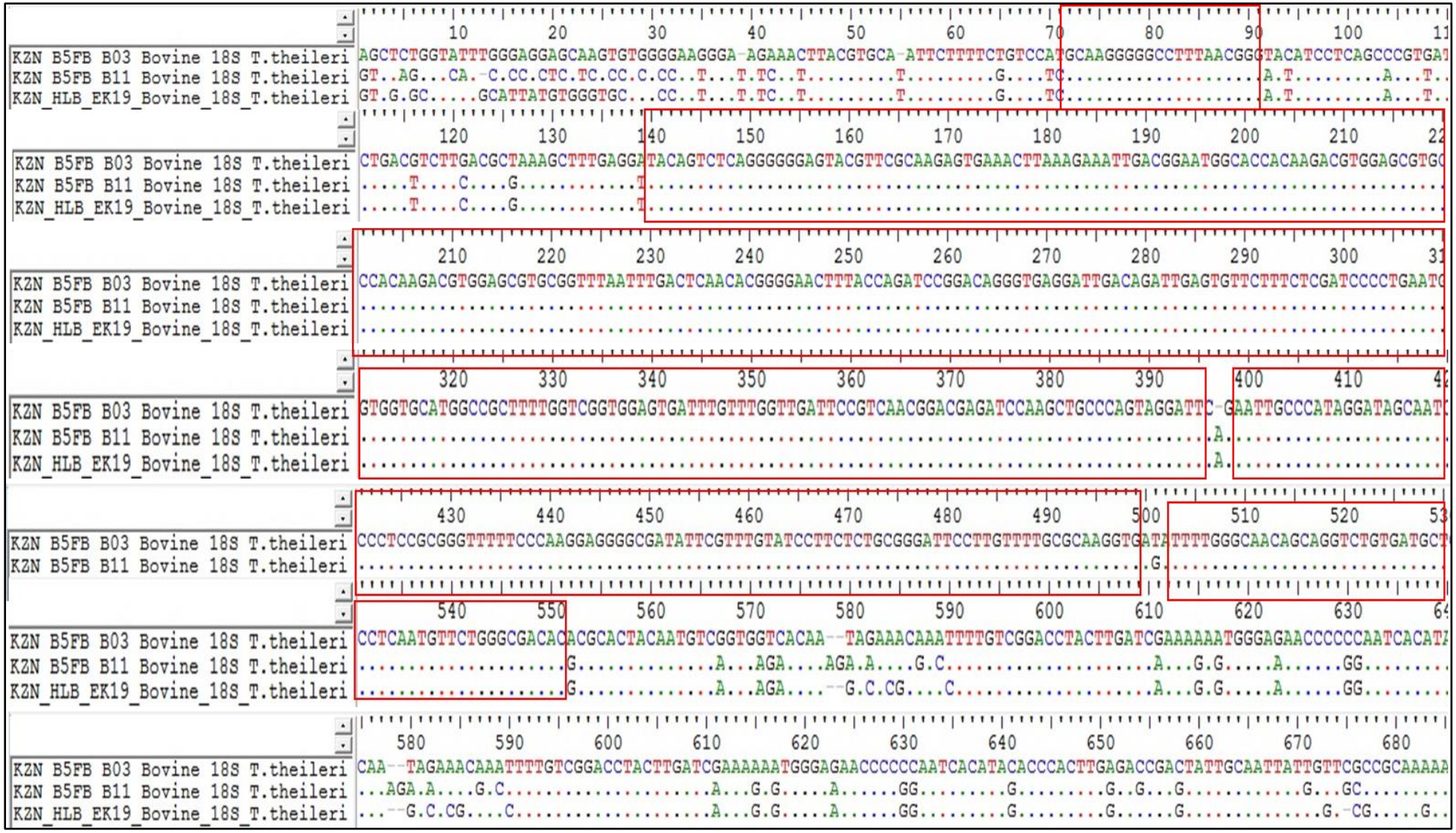


Figure 9b: Alignment of South African 18S rRNA *T. theileri* strains from the two sampled local municipalities (HLB: Hlabisa and B5FB: Big 5 False Bay). The highlighted area indicates the conserved sequences among all strains.

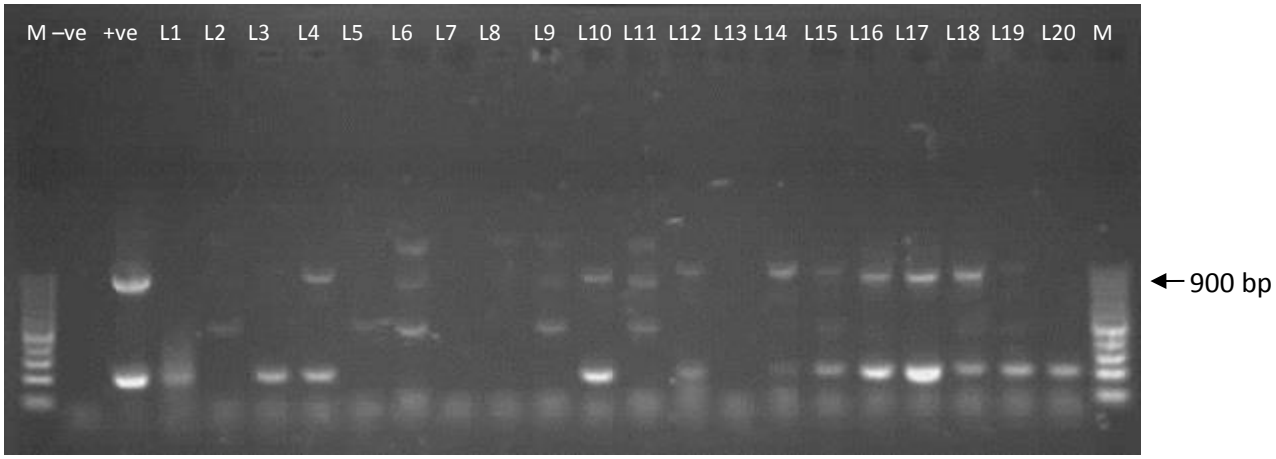
3.4.3 Genetic diversity of trypanosomes using the gGAPDH gene

For this analysis only 4 samples were used due to the reasons mentioned above in section 3.4, first paragraph. Only 25% (1/4) was from *T. congolense* (Savannah) and 75% (3/4) were from *T. b. brucei* and no *T. theileri* gGAPDH sequences were obtained. The samples represented only 2 local municipalities namely Hlabisa and Mtubatuba as sequences from positively amplified PCR products from Big 5 False Bay local municipality had no significant matches with the sequences on the NCBI database. Plate 6 is an agarose gel image showing both positively amplified DNA with multiple bands and the desired product size ranging between 880 to 900 bp respectively. The *T. congolense* (Savannah) species had 99% identity match score (E-value: 0.0) when aligned with *T. congolense* partial gGAPDH gene of *T. congolense* (Savannah) GAM 2 isolate sequence accession with number AJ620290. The subject sequence (*T. congolense* partial Savannah isolate GAM 2) from the NCBI data base covered 90% of the query sequence produced in this study (KZN_EK20_HLB_Bovine_GAPDH) with 0% (1/876 bp) gaps between alignments. Nucleotide polymorphisms were determined between the subject sequence and query sequence with only 2 transversional sites (Figure 10a). However, because only one *T. congolense* (Savannah) sequence was detected most of the nucleotide analysis such as pair-wise distance, genetic diversity as well as the number and position conserved sites among sequences were not determined. Only the nucleotide composition within this *T. congolense* sequence was estimated and its data is represented in table 7a.

The *T. b. brucei* sequences were also subjected to BLAST and they had 99% identity match score with E-value: 0.0 and the subject sequence covered 90% of the query sequence which was produced in this study. Because these findings were not expected in table 6 is showing sequences producing significant alignments when compared to the query sequence from KwaZulu-Natal. To validate these findings one *T. b. brucei* strain (KZN_MTB_M01_Bovine_*T. brucei*) was aligned with a published sequence from the database (*T. b. brucei* strain 927/4_GUTat_10.1 gGAPDH) accession number XM840454 (Figure 10b). Alignment of these two sequences had 7 transitional sites, only one transversional site and only one gap, therefore demonstrating that indeed the query sequence is from *T. b. brucei*. The nucleotide composition among the 3 *T. b brucei* sequences from South Africa revealed significant differences and the results are represented in table 7b. Pair-wise showing number of base differences per site from

between sequences were also determined by bootstrap procedure of 1000 replicates (Table 7). Analysis involved 3 nucleotide sequences and the codon positions included were 1st, 2nd, 3rd and noncoding. All positions containing gaps and missing data were eliminated. There was a total of 937 bp in the final dataset (Tamaru *et al*., 2011). All three *T. b. brucei* strains generated in this study were aligned and they had only one conserved site which was 16 bp long at positions 909 to 924 (Figure 11). Sequences also showed some degree of polymorphism when compared with one another indicating that even though they are the same species they are different genotypes. Therefore the null hypothesis is accepted which states that there will be great genetic diversity among the sequences of South African trypanosomes as indicated by genetic diversity observed within the *T. b. brucei* sequences obtained in the current study.

Plate 6a: Gel image showing amplified DNA from gGAPDH genes from bovine samples collected in uMkhanyakude district of KwaZulu-Natal. M is the molecular marker, +ve and –ve are positive and negative controls; L4, 10, 14, 15, 16, 17,18 and 19 are positively amplified DNA and L1, 2, 3, 5, 6, 7, 8, 9, 11, 12, 13 and 20 are samples below detection limit



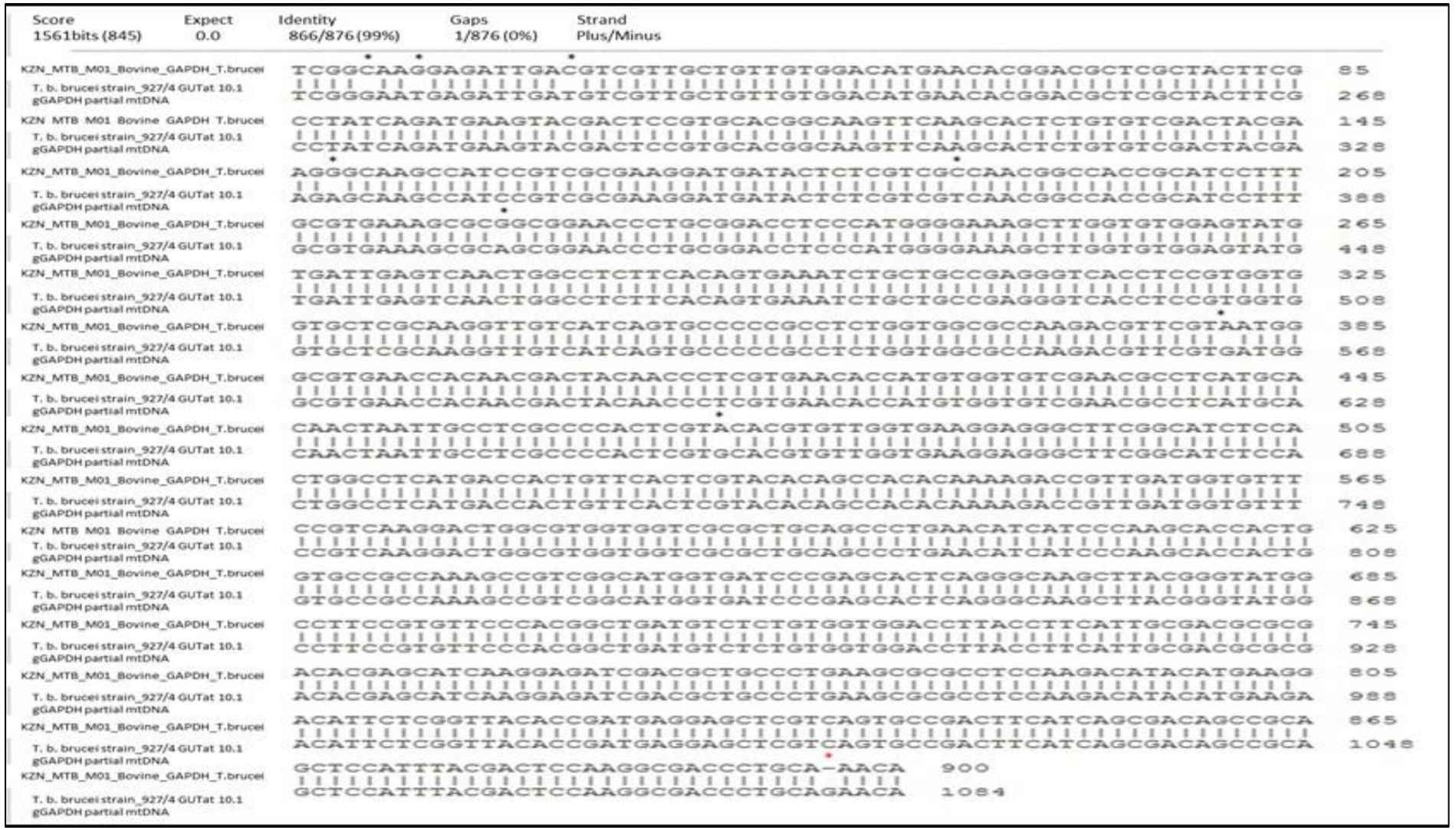


Figure 10a: BLAST (bl2 seq) results showing alignment of gGAPDH from *T. brucei* with *T. brucei* strain obtained from this study. The black star indicates the nucleotide polymorphism that occurred between the two sequences and the red stars indicate where gaps were observed.

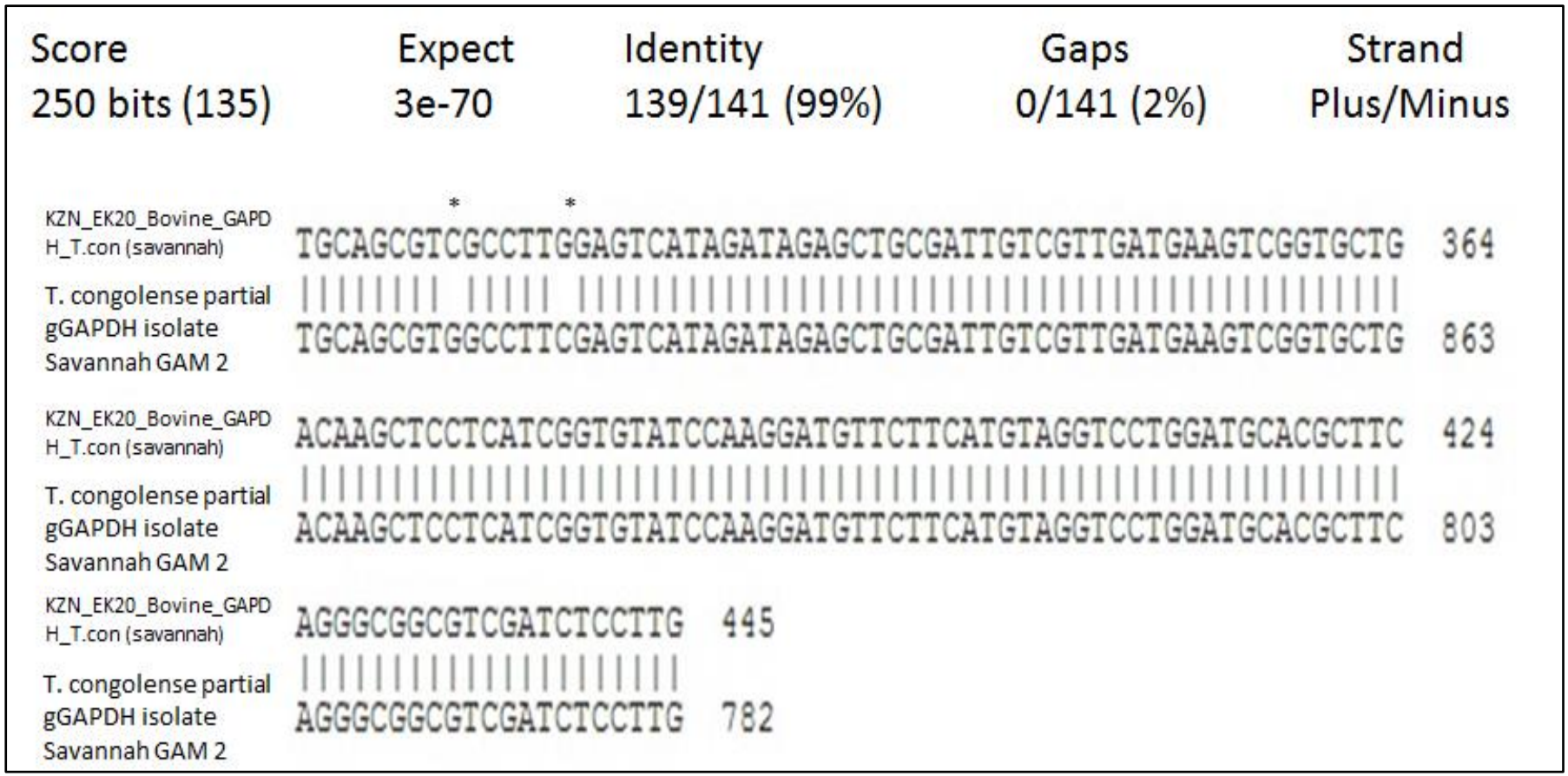


Figure 10b: BLAST (bl2 seq) results showing alignment of gGAPDH from *T. congolense* (Savannah) with *T. congolense* strain obtained from this study. The black star indicates the nucleotide polymorphism that occurred between the two sequences and no gaps were observed

Table 6: BLAST (n) results showing significant matches of *T. b. brucei* from the query sequence from Mtubatuba local municipality obtained from gGAPDH positive PCR products. The black blocks highlight *T. b. brucei* as well as *T. theileri* to indicate that the sequence obtained from PCR using gGAPDH when subjected to BLAST it matched with *T. b. brucei* and not *T. theileri.*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Trypanosoma brucei brucei strain 927/4 GUTat10.1 glyceraldehyde 3-phosphate dehydrogenase, glycosomal partial mRNA	1557	1557	96%	0.0	99%	XM_840454.1
Trypanosoma brucei brucei strain 927/4 GUTat10.1 glyceraldehyde 3-phosphate dehydrogenase, glycosomal partial mRNA	1557	1557	96%	0.0	99%	XM_840453.1
Trypanosoma brucei chromosome 6 clone RPCI93-26G9, complete sequence	1557	3115	96%	0.0	99%	AC007863.15
Trypanosoma brucei gambiense DAL972 chromosome 6, complete sequence	1552	3104	96%	0.0	99%	FN554969.1
Trypanosoma brucei rhodesiense partial gGAPDH gene for glycosomal glyceraldehyde phosphate dehydrogenase, isolate 058	1552	1552	96%	0.0	99%	AJ620284.1
T.brucei gap genes (GAPDH1 & GAPDH2) for glyceraldehyde-3-phosphate dehydrogenase	1546	3093	96%	0.0	99%	X59955.1
T.brucei glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA, complete cds	1546	1546	96%	0.0	99%	M26816.1
Trypanosoma evansi glycosomal glyceraldehyde-3-phosphate dehydrogenase (gadhg) gene, partial cds	1541	1541	96%	0.0	99%	AF053743.1
Trypanosoma theileri partial gGAPDH gene for glycosomal glyceraldehyde phosphate, isolate uganda166_717	1498	1498	94%	0.0	99%	HF545652.1
Trypanosoma brucei gambiense glycosomal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, partial cds	1042	1042	64%	0.0	99%	AF047499.1

Table 7a: Nucleotide composition from gGAPDH from one *T. congolense* strain from uMkhanyakude district of KwaZulu-Natal

Sequence Name	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos: 1	T-2	C-2	A-2	G-2	Pos: 2	T-3	C-3	A-3	G-3	Pos: 3
KZN_EK20_HLB_Bovine_gGAPDH	26.1	23.2	25.0	25.8	652.0	26	26.6	20.6	27.1	218.0	20	20.6	27.5	31.7	218.0	32	22.2	26.9	18.5	216.0

Table 7b: Nucleotide composition from gGAPDH between *T. b. brucei* strains from uMkhanyakude district of KwaZulu-Natal

Sequence Name	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos: 1	T-2	C-2	A-2	G-2	Pos: 2	T-3	C-3	A-3	G-3	Pos: 3
KZN_MTB_M01 Bovine_GAPDH_T.brucei	21.2	29.6	22.8	26.4	963.0	23	27.1	23.1	26.5	321.0	23	32.5	21.9	22.2	320.0	17	29.2	23.6	30.4	322.0
KZN_MTB_M13 Bovine_GAPDH_T.brucei	21.4	29.4	23.1	26.2	963.0	23	26.7	23.3	26.7	322.0	24	32.5	21.9	21.9	320.0	17	29.0	24.0	29.9	321.0
KZN_MTB_M19 Bovine_GAPDH_T.brucei	21.8	26.8	22.1	29.3	952.0	20	23.2	24.8	32.1	315.0	22	27.3	25.4	25.4	319.0	24	29.9	16.0	30.5	318.0
Avg.	**21.5**	**28.6**	**22.7**	**27.3**	**959.3**	**22**	**25.7**	**23.7**	**28.4**	**319.3**	**23**	**30.8**	**23.0**	**23.1**	**319.7**	**19**	**29.3**	**21.2**	**30.3**	**320.3**

Table 8: Estimates of evolutionary divergence by gGAPDH between *T. b. brucei* South African sequences

Name of sequence	1	2	3
1. KZN_MTB_M01_Bovine_gGAPDH_*T.brucei*		0.006	0.015
2. KZN_MTB_M13_Bovine_gGAPDH_*T.brucei*	0.035		0.016
3. KZN_MTB_M19_Bovine_gGAPDH_*T.brucei*	0.567	0.565	

KZN=KwaZulu-Natal Province; MTB=Mtubatuba local municipality; M=Mvutshini diptank

Figure 11: Alignment of South African gGAPDH *T. b. brucei* strains from only one local municipality (MTB: Mtubatuba). The highlighted area indicates the conserved sequences among all strains

3.5 Discussion

Genotype variability was conducted using 18S rRNA and gGAPDH genes of different *Trypanosoma* species detected from cattle blood sample collected in uMkhanyakude district of KwaZulu-Natal Province, South Africa. The two genes were selected because 18S rRNA gene sequences make it feasible for better observation and differentiation of different trypanosome species (Eisler *et al*., 2004). Glyceraldehyde 3-phosphate dehydrogenase gene on the other hand was a preferred maker due to the fact that it has a slow rate of molecular evolution making them suitable for studying evolution over large time-scales (Stevens and Gibson, 1999). Dominant species obtained from both genes included the lethal *T. congolense* (Savannah), *T. b. brucei* and non-lethal *T. theileri*. The presence of *T. congolense* (Savannah) circulating in livestock in KwaZulu-Natal validates findings made by Van den Bossche *et al.* (2006) and Mamabolo *et al.* (2009) whereby they found that there are two strains of *T. congolense* (Savannah and Kilifi) types circulating in both livestock and tsetse flies in north eastern parts of KwaZulu-Natal Province. However, it is for the first time that non-lethal *T. theileri* and *T. b. brucei* species are found among South African livestock and this might be due to the lack of sensitive tools to analyse genotype polymorphism in previous studies conducted in South Africa (Masumu *et al.*, 2009) or these two species were misdiagnosed in previous studies.

Results obtained from 18S rRNA PCR revealed that *T. congolense* (Savannah) and *T. theileri* species are the dominant *Trypanosoma* species in all the three sampled local municipalities. They also showed that there was great genetic diversity within both *T. congolense* (Savannah) and *T. theileri* strains. Seventy percent (7/10) of the *T. congolense* (Savannah) species matched by 80% to 90% with *T. congolense* sequences submitted on the NCBI database however, showed significant genotype difference when compared to one another. Nucleotide polymorphisms with a few number of transversions and transitions between sequences from different municipalities indicated that there was minor genetic diversity between the different sequences meaning that these species shared a great number of genetic similarities and they were not too divergent from each other. There was no significant differences observed in the overall nucleotide composition at positions 1, 2 and 3 and this further attested the slight diversity observed between sequences. The evolutionary divergence among South African *T. congolense* (Savannah) revealed minimal

difference in the 2nd and 3rd codon positions as shown in table 4a. Additionally, there was one conserved region with 44 bp long in the alignment of all South African *T. congolense* (Savannah) sequences which indicated some degree of genetic similarity amongst these *T. congolense* (Savannah) sequences from KwaZulu-Natal Province. The remaining sequences 30% (3/10) belonged to *T. theileri* and also showed significant match support to the *T. theileri* species when subjected to BLAST (bl2 seq). There was great genetic and nucleotide diversity observed amongst the *T. theileri* sequences as compared to the *T. congolense* (Savannah) sequences. There overall nucleotide composition was higher in *T. theileri* sequences and this might be due to the limited samples that were used in this study. From the alignment of all 3 *T. theileri* sequences there were 5 conserved regions observed all with a minimum length of more than 15 bp. This indicated that there are multiple genotypes of the non-lethal *T. theileri* species circulating among livestock in KwaZulu-Natal. Similar studies have been conducted on *T. brucei* and *T. vivax* isolates from different localities in Africa by PCR-RFLP for *T. congolense* and nested PCR using cathepsin L-like species-specific primers for *T. vivax* where they revealed similar results whereby it was observed that these two species also showed great genetic diversity between isolates from different geographical areas (Van den Bossche *et al*., 2006; Nakayima *et al*., 2013). Observations made by these authors further indicated that the genetic diversity within the same *Trypanosoma* species affects the virulence, epidemiology and drug resistance of these species, which might also be case with the *T. theileri* species, if more research is conducted with the samples from this current study. Masumu *et al.* (2009), made observations on the low genotype variability of *T. congolense* (Savannah) species using AFLP whereby the authors came to a conclusion that livestock can still be subjected to heterologous challenge despite the low impact the disease has on livestock production which might also be case in the current study.

Observations made from gGAPDH genes showed that 21.4% (3/14) were *T. b. brucei* sequences and 7.1% (1/14) was *T. congolense* (Savannah) species. These observations made on from this gGAPDH gene of *T. congolense* (Savannah) species confirms the findings made with 18S rRNA genes that indeed there are *T. congolense* (Savannah) type species circulating among livestock in Hlabisa local municipality of KwaZulu-Natal. In the case of *T. b. brucei* strains observed in this study

the BLAST results have matching sequences with *T. b. brucei* with 99% match support which covered 90% of the query sequences and also with *T. theileri* at 99% match support however, for *T. theileri* the subject sequence covered 87% of the query sequence as indicated in figure 11. The nucleotide diversity among the *T. b. brucei* strains showed significant difference and this was supported by the evolutionary divergence among sequences shown in table 7. There was only one conserved region between these sequences at positions 909 to 924 indicating that they do somehow relate to one another even though they have different genotypic makeups. A study made by Agobo *et al.* (2001), also using PCR-RPLP to measure molecular diversity within *T. brucei* subspecies indicated repeat rDNA profiles on the 5.8 S region can be used to distinguish between *T. brucei* subgroups using a unique 4 bp repeat sequence of C_3A which was not the scenario in this study as gGAPDH genes were used instead of rRNA ITS region. Additionally, limited a number of sequences were used consequently, we could not certainly conclude that indeed *T. b. brucei* species are prevalent among South African livestock and as such further analysis using different target genes are needed to confirm these findings. In contrast to the above statement the *T. b. brucei* positives were from Mtubatuba local municipality which is situated in proximity with Hluhluwe-uMfolozi game reserve that is dominated by reservoir hosts of most pathogenic trypanosome parasites therefore it might happen that the sampled livestock got infected when they were grazing near the game reserve.

In conclusion the *T. congolense* (Savannah) species obtained in this study is in agreement with the previous studies on trypanosomes in South Africa which reported that there are active *T. congolense* (Savannah and Kilifi) strains prevalent amongst South African cattle and tsetse flies (Mamabolo *et al*., 2009). However, the findings of *T. theileri* as well as *T. b. brucei* do raise some questions on whether these two strains were previously misdiagnosed or rather *T. b. brucei* is a re-emerging infection in the country. Additionally all documented *Trypanosoma* strains in the current study have great genetic diversity among one another.

CHAPTER 4

PHYLOGENETIC ANALYSIS OF SOUTH AFRICAN TRYPANOSOMES DETECTED IN KWAZULU-NATAL PROVINCE

4.1 Introduction

Phylogenetic analysis using DNA or protein sequences has been used for many years to investigate the evolutionary history of unicellular to multicellular organisms on earth (Nei and Kumar, 2000). Most of the previous phylogenies of kinetoplastids were based on analysis of variation in SSU rRNA genes (Hamilton *et al.,* 2004). The first molecular phylogenetic studies for trypanosomes were based on comparisons of genes encoding mitochondrial and nuclear ribosomal RNA (rRNA) and these studies revealed that trypanosomes are paraphyletic (Hamilton *et al.,* 2004). As noted by Hamilton *et al.* (2004), some scientists argue that previous small subunit ribosomal RNA (SSU rRNA) gene trees do not adequately confirm monophyly of trypanosomes, because they either include an inadequate number and selection of taxa, or they are rooted inappropriately. Nonetheless, recent studies which included more taxa (*Crithidia, Leptomonas, Bodo, Endotrypanum, Phytomonas, and Trypanosoma borreli*) from a broader range of host species based on rRNA genes provided support for monophyly in trypanosomes (Stevens and Gibson, 1999; Hamilton *et al.,* 2004).

The 18S rRNA gene has been the marker of choice for most studies because it is composed of conserved alternating variable domains and is easy to amplify using primers in the flanking regions (Adams *et al*., 2010). However, for the phylogenetic analysis of the 18S rRNA gene to support monophyly for trypanosomes there has to be sufficient taxa included and outgroups must be chosen correctly to produce a meaningful tree topology (Stevens and Gibson, 1999). Other molecular markers that can be used to help unravel polytomy levels in *Trypanosoma* include 28S rRNA, 9S and 12S mitochondrial rRNA genes as well as GAPDH protein-coding genes (Stevens and Gibson, 1999). Furthermore, GAPDH gene can be used to for accurate phylogenetic placement of novel trypanosomes because it is easy to align non-ambiguously without gaps and it produces phylogenetic trees with approximately the same resolution as those constructed using 18S rRNA gene (Adams *et al*., 2010).

Previous studies based on GAPDH gene have consistently revealed *Trypanosoma* to be monophyletic using few taxa therefore this might make this gene a more reliable phylogenetic marker (Stevens and Gibson, 1999). Nonetheless, GAPDH gene is an ubiquitous and essential glycolytic enzyme and this GAPDH gene has a slow rate molecular evolution making them appropriate for the studying of evolution over a large time scale (Hamilton *et al.,* 2004). According to Hamilton, *et al.* (2004), the SSU rRNA gene neither strongly support nor reject trypanosome monophyly, as different alignments give different tree topologies when tested. Furthermore Hamilton, *et al.* (2004) concluded that, all trees based on GAPDH gene support monophyly of trypanosomes and show them as a relatively late-evolving lineage within the family Trypanosomatidae, which is also monophyletic.

Vast amount of phylogenetic studies has been previously conducted to resolve and understand the *Trypanosoma* species problem and how these species relate to one another. Stevens and Gibson (1999) reviewed the phylogeny of trypanosomes and explored rRNA and protein-coding genes using maximum parsimony analysis. They came to a conclusion that trypanosomes are monophyletic, they showed that the divergence of the Salivarian clade is dated around 100 million years ago (mya), when the African continent became isolated from other continents where they observed that the *T. brucei* clade consists completely of African mammalian tsetse-transmitted trypanosome species, and they demonstrated that trypanosome species from African amphibians and reptiles (*T. maga, T. grayi* and *T. varani*) are unrelated. Additional studies by Hamilton *et al*. (2004) using gGAPDH and SSU rRNA also supported monophyly for trypanosomes whereby they used maximum likelihood analysis and maximum parsimony analysis. Results obtained from gGAPDH genes strongly support monophyly for trypanosomes however, the analysis based on SSU rRNA neither strongly support nor reject trypanosome monophyly as different alignments produced different tree topologies which made the SSU rRNA gene not to be a reliable marker for phylogenetic analysis on species level. McInnes *et al*. (2009) explored phylogenetic analysis trying to describe a new species of trypanosomes that is infectious to koala (*Phascolarctos cinereus*) in Australia. They used 18S rRNA and gGAPDH as their molecular markers to construct phylogenetic trees and came to a conclusion that trypanosome infecting koalas is proposed to be a new species

T. irwini based on observations made from biological and genetic data. Studies on trypanosomes in South Africa have never been explored to the phylogenetic analysis level therefore, the genetic relations between South African trypanosome strains and other African countries is poorly known.

Phylogenetic trees were constructed by the distance, parsimony and maximum likelihood methods (Stevens and Gibson, 1999; Hall, 2008). In the distance matrix method, a pairwise matrix of genetic distance or similarities between sequences is calculated first, the resulting matrix of distances is then used to construct a phylogenetic tree by one of many available least squares clustering methods such as neighbour-joining or unweighted pair-group method using arithmetic averages (UPGMA) (Stevens and Gibson, 1999). These distance matrix methods attempt to fit the distances to a hypothesized phylogenetic tree (Stevens and Gibson, 1999; Hall, 2008). Parsimony methods mainly focus on finding the shortest phylogenetic tree(s) to fit the data presented, which are those that require the smallest number of steps in nucleotide or amino acid substitutions (Stevens and Gibson, 1999). The advantages of parsimony include: firstly, all informative characters are considered rather than summarized by conversion to a pairwise distance; secondly, all possible solutions (most parsimonious trees) can be combined into a consensus tree; lastly, a range of related search strategies are all even for very large data sets to be analysed (Stevens and Gibson, 1999; Nei and Kumar 2000; Hall, 2008). However, the most prevailing approach to phylogenetic analysis currently available is the maximum likelihood method. This method is supported by solid statistical principles which calculate the probability of a given tree yielding the observed data (Stevens and Gibson, 1999). However, for the current study this method was not used. In chapter 3, numerous genotypes for *T. congolense* (Savannah), *T. theileri* as well as *T. b. brucei* were identified using 18S rRNA and gGAPDH genes. In this study, it was hypothesized that KwaZulu-Natal Province trypanosome species will be more related to southern and east African strains than to central and west African *Trypanosoma* strains. We also hypothesized that South African trypanosome species will form clusters with their corresponding sister species.

4.2 Objectives

1. To determine the phylogenetic position of South African *T. congolense* (Savannah) and *T. theileri* using 18S rRNA genes

2. To determine the phylogenetic position of South African *T. congolense* (Savannah) and *T. b. brucei* using GAPDH genes

4.3 Materials and methods

4.3.1 PCR sequencing

Two markers 18S rRNA gene and GAPDH gene were used and analysed to provide information on genetic identification of South African trypanosome species. ClustalW, which can detect as well as demonstrate homology between new sequences and existing families of sequences, was used in this study to align these sequences for phylogenetic analysis (Chaichoompu *et al*., 2006). Two methods were used for phylogenetic analysis namely; the distance matrix and parsimony methods.

4.3.2 Phylogenetic analysis using 18S rRNA

A total of ten sequences from uMkhanyakude district, seven for *T. congolense* (Savannah) and three for *T. theileri* with sequence lengths ranging between 680 to 720 bp were used. MEGA 5 was employed to align the sequences using a program called ClustalW (Hall, 2008). Additional sequences of *T. congolense*, *T. theileri* as well as other trypanosome species (Table 8) were downloaded from the NCBI data base to increase the number of taxa in order to produce meaningful phylogenetic trees (Stevens and Gibson, 1999). During alignment of these *Trypanosoma* species default parameters for weighing options and gap penalties were used (Tamura *et al*., 2011).

Neighbour-joining as well as maximum parsimony methods were used for phylogenetic analysis and to increase the robustness of the trees, 1000 replicates were utilized for each tree. A total of three *T. congolense* (Savannah) type sequences from Hlabisa and Big 5 False Bay local municipalities together with other *T. congolense* isolates (Table 9) obtained from the NCBI data base were used to construct the first tree. Bootstrap method was utilized to test for phylogeny of both trees and *T. vivax* was the preferred outgroup for this analysis. For phylogenetic analysis of *T. theileri*, a total of two *T. theileri* sequences from Hlabisa and Big 5 False Bay municipalities were used together with other *T. theileri* isolate sequences from the database and again *T. vivax* was used as an outgroup. *T. vivax* was the preferred outgroup as it can be passed to its host by both cyclical and mechanical transmission by tsetse flies as well as tabanid flies and in previous studies by Adams *et al.* (2009), it was clustered under the *T. brucei* clade with strong bootstrap support.

4.3.3 Phylogenetic analysis using gGAPDH gene

A total of 4 sequences from Mtubatuba and Hlabisa local municipalities, 3 for *T. b. brucei* and 1 for *T. congolense* (Savannah) with sequence lengths ranging between 920 to 940 bp were used. As mentioned above ClustalW was used to align the sequences on MEGA 5 (Hall, 2008). Additional sequences (Table 7) of *T. brucei* and *T. congolense* as well as other trypanosome species were downloaded on the NCBI data base to increase the number of taxa in order to produce meaningful phylogenetic trees (Stevens and Gibson, 1999). Default parameters for weighing options and gap penalties were used during alignment of these *Trypanosoma* species (Tamaru and Nei, 1993; Tamura *et al*., 2011).

A total of two *T. b. brucei* sequences and they were from Mtubatuba local municipality together with other *Trypanozoon* species obtained from the NCBI data base during BLAST were used in this analysis. Bootstrap method was used to test for phylogeny of both trees. For phylogenetic analysis of *T. congolense* (Savannah) type, only 1 *T. congolense* (Savannah) type strain from Hlabisa local municipality was used together with other *Nannomonas* sequences from the database and again *T. vivax* was used as an outgroup for both analysis.

Table 9: Information on *Trypanosoma* strains with their accession numbers obtained from the NCBI data base used in this study to construct phylogenetic trees in order to compare how South African *Trypanosoma* strains relate to other strains from different countries. The table includes strains which were isolated using both 18S rRNA and gGAPDH genes

Trypanosoma species	Isolate Code	Origin		Accession number	
		Host	Location	18S rRNA	gGAPDH
T. congolense Savannah	WG 81	Goat	Kenya	AJ009146	N/A
	GAM 2	Cow	Gambia	N/A	AJ620290
T. congolense Kilifi	WG 5	Goat	Kenya	AJ009144	AJ620288
T. congolense Riverine-forest	CAM 22b	Goat	Cameroon	AJ009145	N/A
	CAM 22	Goat	Cameroon	N/A	AJ620289
T. congolense Tsavo	114	Tsetse fly	Kenya	U22318	AJ620291
T. congolense	IL1180	Tsetse fly	Kenya	TCU22315	N/A
T. simiae Tsavo	AJ404608.1	Warthog	Kenya	AJ404608	N/A
	D75	Tsetse fly	Tanzania	N/A	FN190446
T. simiae	KEN 2	Tsetse fly	Gambia	AJ009162	AJ620293
T. godfreyi	KEN 7	Warthog	Gambia	AJ009155	N/A
T. b. brucei	?	?	?	AB301937	XM840454
T. b. gambiense	DAL 972	Human	West Africa	AB301938	FN554969
T. b. rhodesiense	58	Human	Zambia	AB301939	AJ620284
T. evansi	?	?	?	N/A	AF053743
T. equiperdum	STIB 818	?	?	AJ009153	N/A
T. cf. brucei	2A9	Tsetse fly	Tanzania	N/A	FM879140
T. vivax	TVU 22316	Tsetse fly	?	U22316	N/A
	?	Cow	Gambia	N/A	FN400714
T. theileri	138 clone Cl 4	Cow	USA	JX178191	N/A
	166_717	Cow	Uganda	N/A	HF545652
T. theileri	K 127	Cow	Germany	AJ009164	AJ620282
T. theileri	2095 clone Cl 9	Cow	USA	JX178163	N/A
T. theileri	ZPU 2707	Puku	Zambia	AB007814	N/A
T. cf. cervi	WTD A1 clone Cl 4	Elk	USA	JX178193	N/A
Leishmania major	MHOM/Ir/02/PIICC1	?	Brazil	AY260965	AF047497

?= No information available
N/A=Not applicable

4.4 Results

4.4.1 Phylogenetic analysis using 18S rRNA genes

To investigate the phylogenetic positioning of South African trypanosome species and whether they do support monophyly using 18S rRNA genes 6 trees were constructed, 3 by neighbour-joining and 3 by maximum parsimony analysis. The first tree is composed only of *T. congolense* and other *Nannomonas* species (Figure 12a and 12b), while the second tree is only *T. theileri* and other *Megatrypanum* species (Figure 13a and 13b) and the third tree includes both *T. congolense* and *T. theileri* from KwaZulu-Natal Province together with other trypanosomes form other countries around the world (Figure 14a and 14b). *T. vivax* was the suitable outgroup used for the first and second trees while for the third tree *Leishmania major* was the preferred outgroup.

4.4.1.1 The 18S rRNA neighbour joining trees

Figures 12a, 13a and 14a show the neighbour-joining models which consisted of nucleotide substitution model of 1000 replicates. The nucleotide substitutions included d: transitions + transversions with Gamma distributed parameters and patterns among lineages were treated as homogenous. Gaps and missing data were exposed to complete deletion by the MEGA 5 program (Hall, 2008; Tamura *et al.*, 2011).

Figure 12a, indicates three well supported monophyletic clades of the subgenus *Nannomonas* namely; the East-West African clade, the South African clade as well as the Tsavo clade. The South African clade was composed of sequences produced in this study and they appeared to be genetically related with bootstrap support of 90% and 100% respectively indicating that there was genetic exchange between strains. These South African strains are also genetically isolated from the East-West African clade due to partially low bootstrap value of 58%. However, both East-West and South African clades have some genetic similarities with the Tsavo clade by 80% bootstrap support indicating that indeed these trypanosome populations even though isolated from one another they do share the same common ancestor. The number of substitutions per site is 0.2 and the statistical test for the molecular clock where $P<0.05$ which means that these strains do not share equal evolutionary rates between one another.

Figure 13a indicates two clades of *Megatrypanum* namely; the KwaZulu-Natal clade and the non-KwaZulu-Natal clade. One *T. theileri* strain from Hlabisa local municipality in South Africa was genetically related to *T. theileri* K127 isolate from Germany with 81% bootstrap support. The two species were 100% genetically related to *T. theileri* strain from Big 5 False Bay. The non-KwaZulu-Natal clade consisted of *T. theileri* isolates from Zambia and America as well as *T. cf. cervi* also from the America with fairly strong bootstrap support of 73% and 67%. These two clades had strong bootstrap support and the tree topology supported monophyly however, statistical test for the molecular clock where $P<0.05$ did not support equal evolutionary rates between these species. However, the geographic distribution of these populations is too divergent for making any concrete conclusions on the observations made on the final makeup of the tree.

To validate the observations made in figures 12a and 13a. A tree composed of all species from KwaZulu-Natal Province, South Africa as well as other strains from the subgenus *Trypanozoon*, *Duttonella*, *Nannomonas*, *Megatrypanum* was constructed. *Leishmania major* was used as an outgroup. In figure 14a four well supported clades were observed and the KwaZulu-Natal Province strains formed clusters with their respective similar sequences from corresponding subgenera. In the *Nannomonas* clade *T. congolense* (Savannah) strains from KwaZulu-Natal formed a cluster (90% bootstrap support) with *T. congolense* (Savannah) WG 81 isolate from Kenya indicating that the South African stains are more genetically related to the East African strains than other southern or western African strains. For the subgenus *Megatrypanum,* KwaZulu-Natal Province *T. theileri* strains were partially related to one another. The *T. theileri* strain from Big 5 False Bay local municipality was slightly genetically similar to the *T. theileri* from Hlabisa local municipality by 69% bootstrap support and these two strains were genetically related to the American *T. theileri* isolate by 87% bootstrap support. However, the molecular clock analysis calculated on MEGA 5 indicated that all South African strains have equal evolutionary rates when compared to one another at $P>0.05$. The *Trypanozoon* and Tsavo clades were well supported with 99% and 95% bootstrap supports respectively indicating that the arrangements of our sequences during alignment was correct. However, all clades were partially related to one another with 50% bootstrap support. Findings attained from the

distance matrix method were confirmed by parsimonious analysis and the results obtained are discussed below.

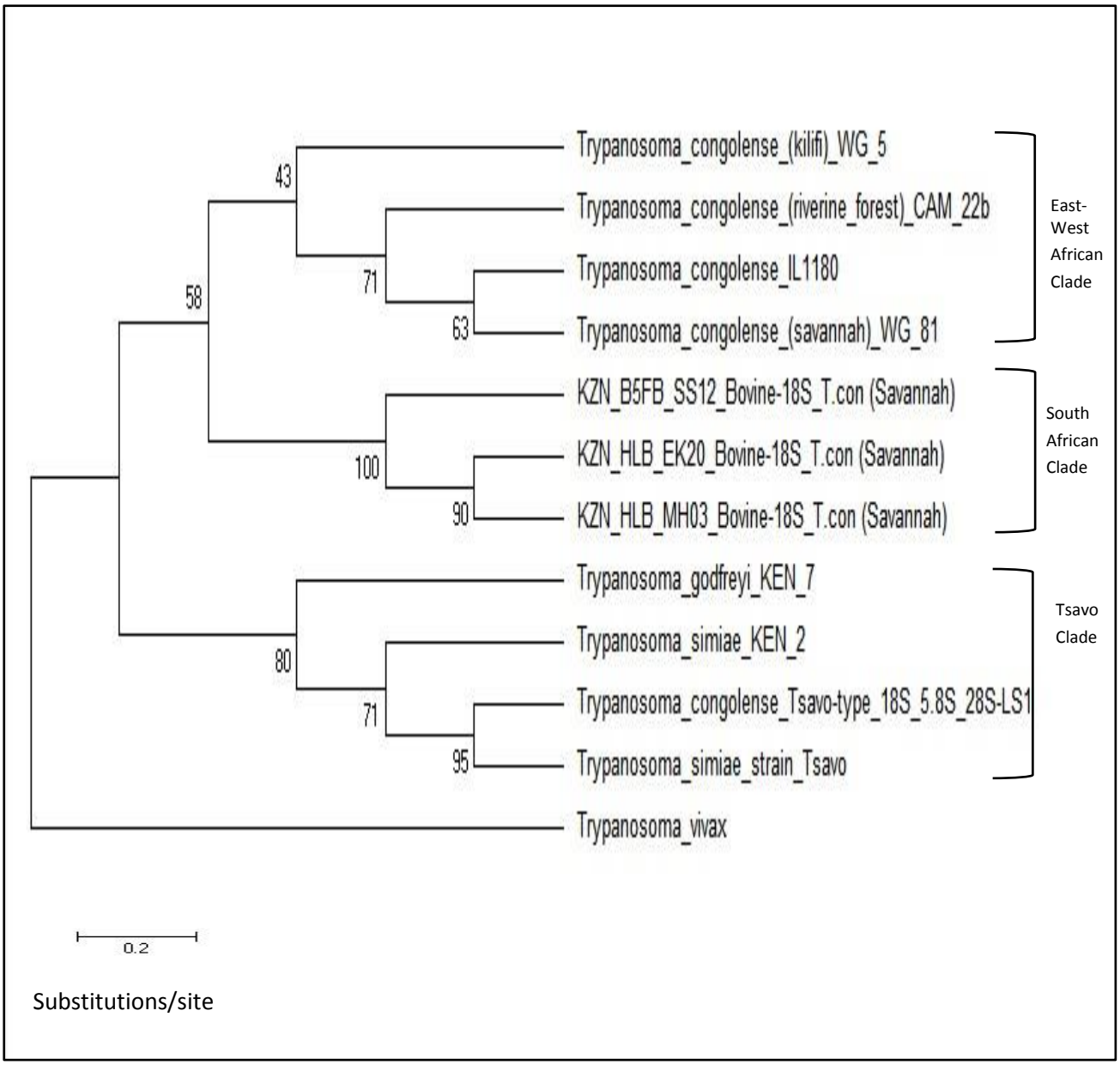


Figure 12a: Subgenus *Nanommonas* neighbour-joining 18S rRNA tree, showing relationship between South African *T. congolense* (Savannah) strains with other related species from Africa. The analysis contained 12 nucleotide sequences. Three linages (East-West African, South African and Tsavo) were identified with well supported bootstrap values. There were a total of 689 positions in the final dataset. The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model (+G) (Tamura and Nei, 1993). Differences in evolutionary rates among sites were modelled using a discrete Gamma (G) distribution, with a 4-category gamma distribution. Log L=-3311.31. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P= 1.58^{-44}). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011)

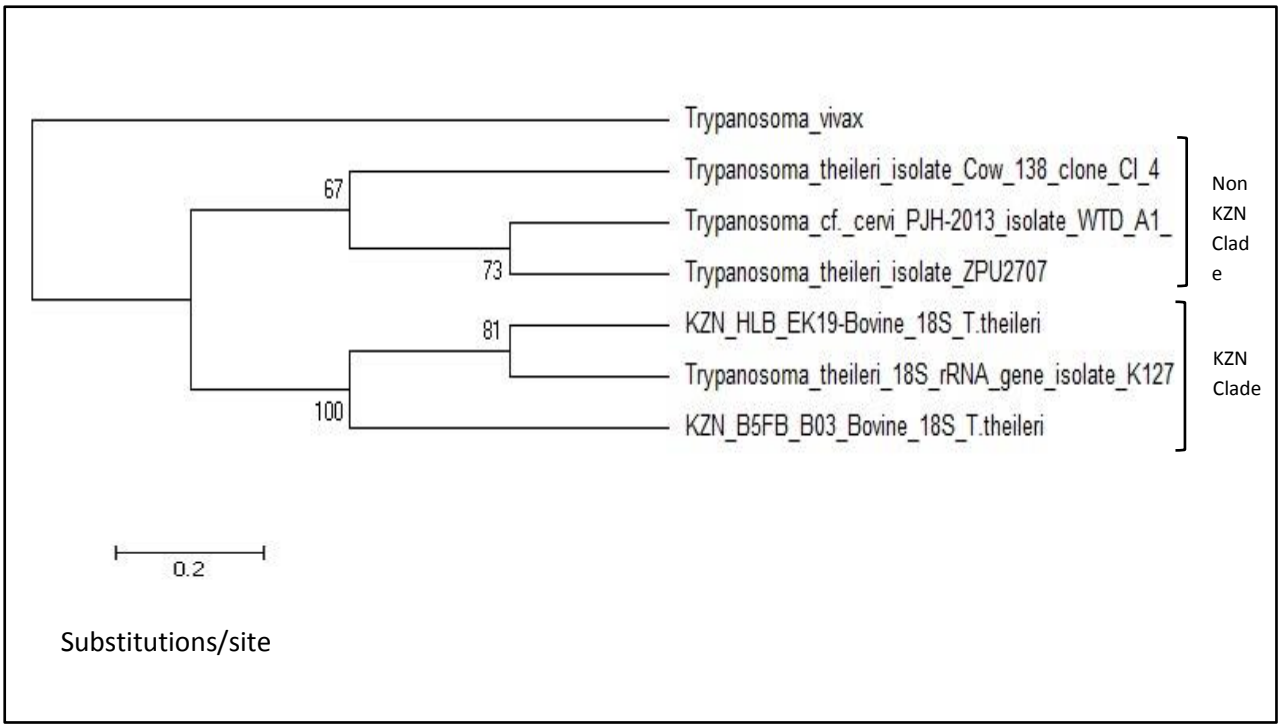


Figure13a: Subgenus *Megatrypanum* neighbour-joining 18S rRNA tree, showing the relationship between South African *T. theileri* strains with other related strains from around the world. The analysis involved 7 nucleotide sequences. Two lineages (KwaZulu-Natal and non-KwaZulu-Natal) were identified with well supported bootstrap values. There were a total of 603 positions in the final dataset. The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model (+G) (Tamura and Nei, 1993). Differences in evolutionary rates among sites were modelled using a discrete Gamma (G) distribution, with a 4-category gamma distribution. Log L=-3442.27. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (*P*= 1.50^{-166}). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

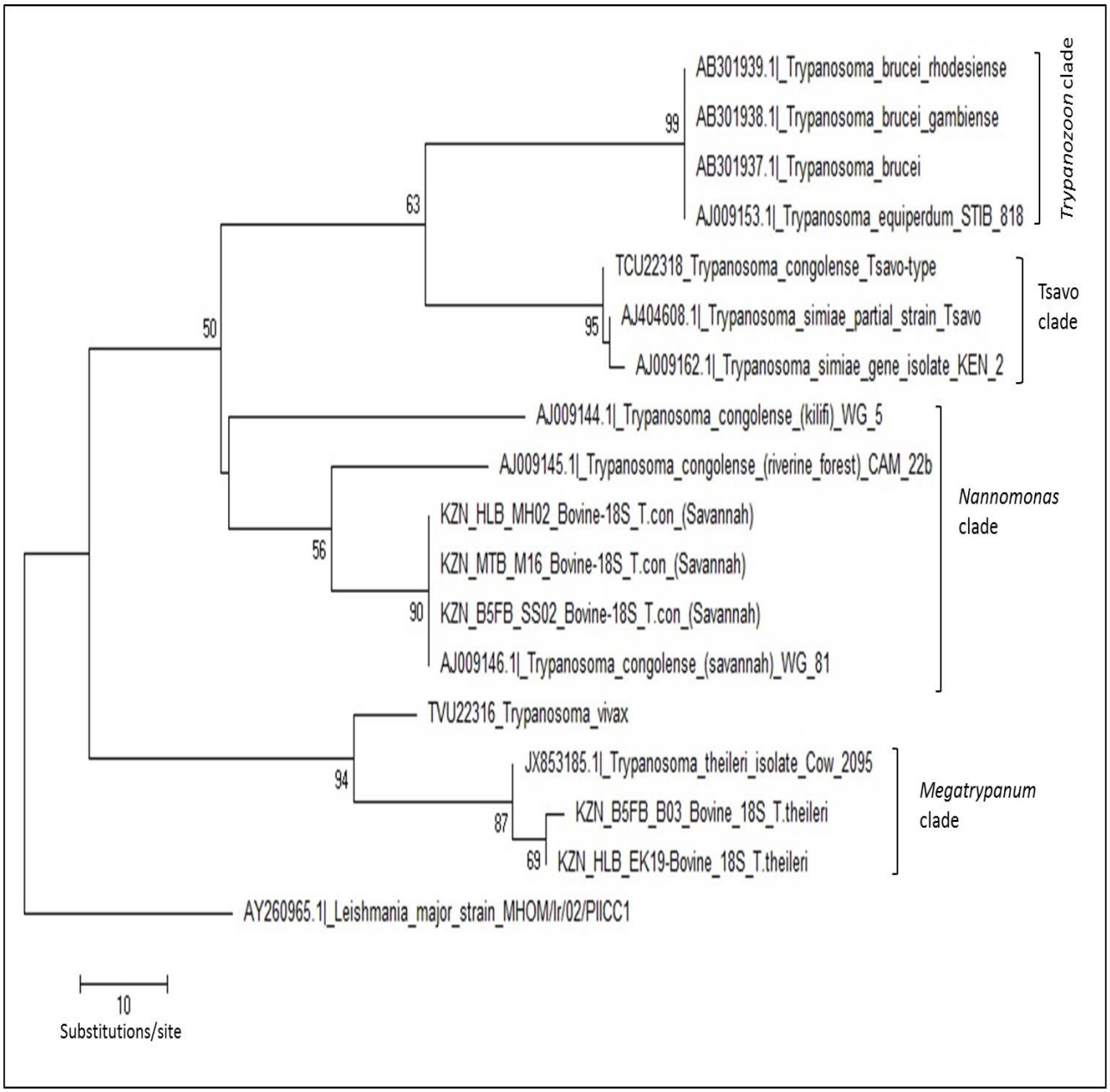


Figure 14a: 18S rRNA neighbour-joining tree composed of both South African *Trypanosoma* strains from KwaZulu-Natal as well as other trypanosomes from other countries in Africa and outside African continent. The analysis involved 19 nucleotide sequences. Four lineages (*Trypanozoon,* Tsavo*, Nannomonas* and *Megatrypanum*) were identified with well supported bootstrap values. There were a total of 698 positions in the final dataset. The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model (+G) (Tamura and Nei, 1993). Differences in evolutionary rates among sites were modelled using a discrete Gamma (G) distribution, with a 4-category gamma distribution. Log L=-6375.72. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (*P*= 0.1054). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

4.4.1.2 The 18S rRNA maximum parsimony trees

Figures 12b, 13b and 14b are evolutionary trees inferred using the maximum parsimony models, which consisted of nucleotide substitution model of 1000 replicates. The maximum parsimony trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Nucleotide substitution model was used and the codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated by complete deletion (Hall, 2008; Tamura *et al.*, 2011).

Figure 12b indicates three well supported monophyletic clades of the subgenus *Nannomonas* namely; East-West African clade, South African clade as well as the Tsavo clade. The South African clade was composed of *T. congolense* (Savannah) strains from KwaZulu-Natal. The strains from Hlabisa local municipality were 82% genetically related and both were genetically related to the *T. congolense* (Savannah) strain from Big 5 False Bay with strong bootstrap support of 100%. This showed that there was genetic exchange between the species. The South African *T. congolense* species were genetically isolated from the East-West African and Tsavo clades however, they genetically related with strong bootstrap support of 82%. These findings do correspond to what was observed with the distance matrix method and the bootstrap support values of this tree were much higher than those observed in the distance matrix method in the previous section. The East-West African clade and the Tsavo clade were also supported by high bootstrap values and monophyly in trypanosomes was supported by this evolutionary tree.

Figure 13b represents a monophyletic maximum parsimony tree from the subgenus *Megatrypanum.* As observed in the distance method two well supported clades were identified. In this tree the Zambian *T. theileri* isolate clustered with the two South African species and the American *T. theileri* species with well supported bootstrap values of 100%. Due to this the distance method and the parsimony method are in conflict because with the distance method the Zambian *T. theileri* species clustered with non-South African species. This means that South African *T. theileri* species are genetically related to other southern African *T. theileri* species. For the non-South African clade we observed that the American *T. cervi* is

genetically related to the German *T. theileri* isolate with 100% bootstrap support however, due to the geographical isolation between the two countries this might not be case in nature.

To validate the observations made in figures 12b and 13b. A tree composed of all trypanosome species from KwaZulu-Natal as well as other species from the subgenus *Trypanozoon*, *Duttonella*, *Nannomonas*, *Megatrypanum* was constructed, with *Leishmania major* as an outgroup. Figure 14b indicates four well supported clades were observed and the KwaZulu-Natal species formed clusters with their respective similar sequences from corresponding subgenera. In the *Nannomonas* clade, *T. congolense* (Savannah) species from Mtubatuba local municipality was 73% genetically related to the *T. congolense* (Savannah) species from Hlabisa local municipality. *Trypanosoma congolense* (Savannah) specie from Big 5 False Bay local municipality was 87% genetically related to *T. congolense* (Savannah) WG 81 isolate from Kenya. These observations confirmed what was observed with the distance method that the South African stains are more genetically related to the East African strains than other southern or western African strains. For the subgenus *Megatrypanum,* KwaZulu-Natal Province *T. theileri* strains were genetically related to one another. The *T. theileri* strain from Hlabisa local municipality was genetically similar to the *T. theileri* isolate from America by 93% bootstrap support and these two strains were genetically related to the KwaZulu-Natal Province *T. theileri* strain by 100% bootstrap support. All these *T. theileri* strains were genetically related to *T. vivax* by 91% bootstrap support. The *brucei* and Tsavo clades were well supported with 100% and 100% bootstrap support respectively. The molecular clock analysis calculated on MEGA 5 indicated that all our strains have equal evolutionary rates when compared to one another therefore indeed these *Trypanosoma* parasites are monophyletic and share the same common ancestor and that there is great genetic diversity within and among different trypanosome strains found in African continent.

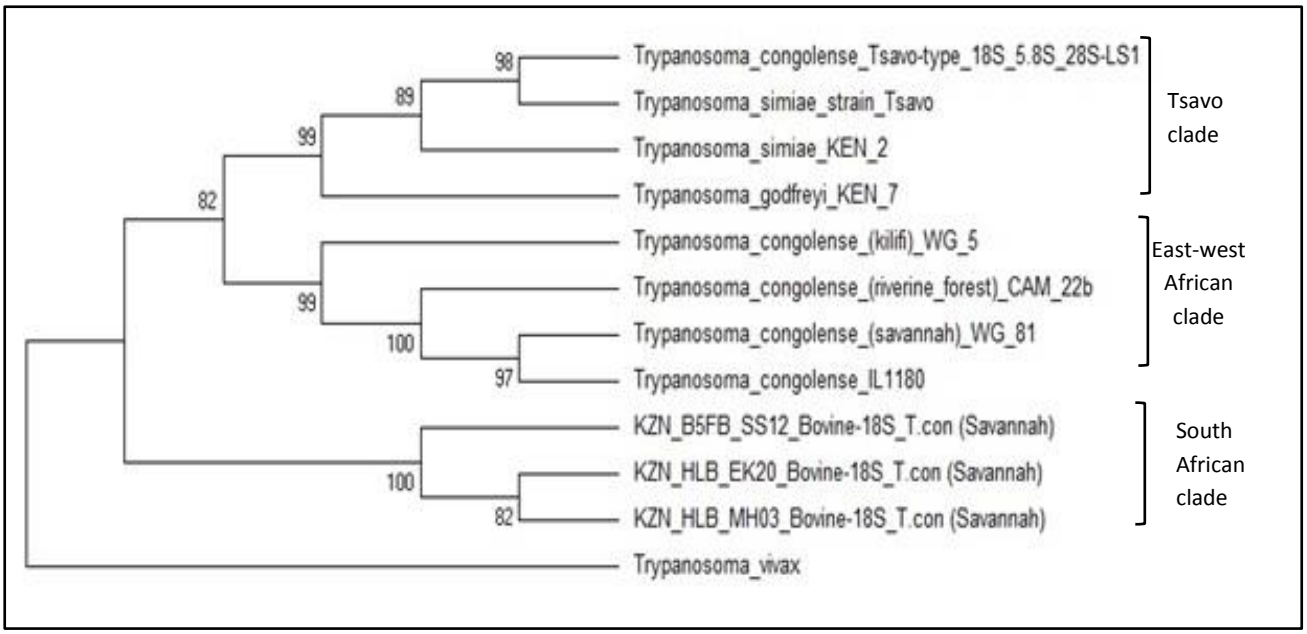


Figure 12b: Subgenus *Nannomonas* 18S rRNA maximum parsimony tree showing the relationship between KwaZulu-Natal Province *T. congolense* (Savannah) type strains with other related species from the gene bank. The analysis involved 12 nucleotide sequences. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (*P*=0.5223). The consistency index is (0.891808), the retention index is (0.936880), and the composite index is 0.840437 (0.835517) for all sites and parsimony-informative sites (in parentheses). All positions containing gaps and missing data were eliminated. There were a total of 689 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011)

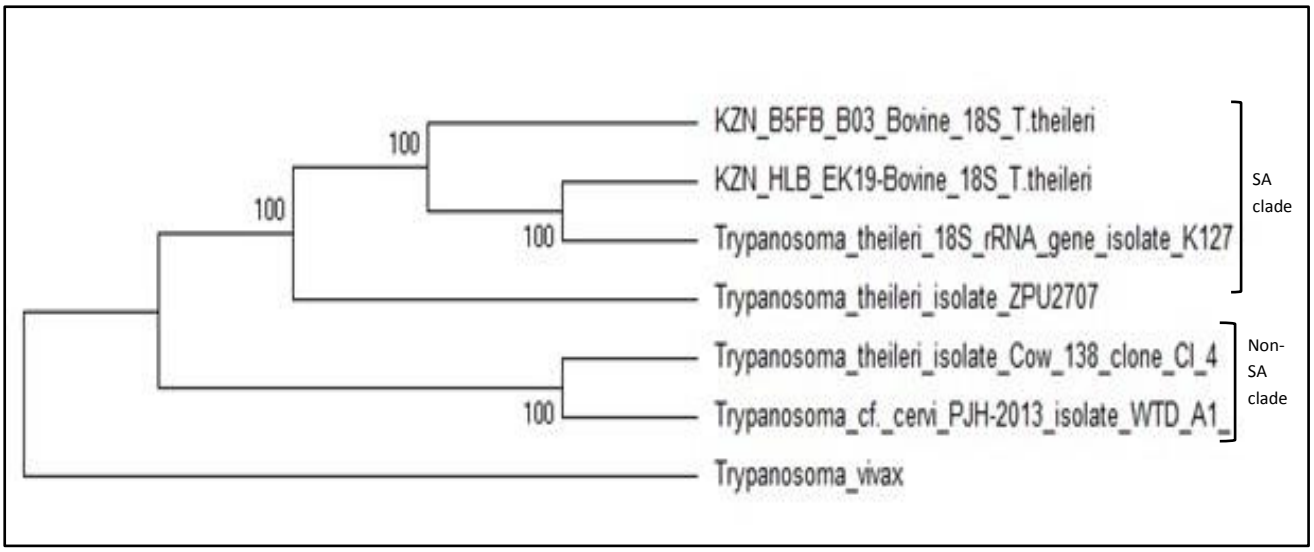


Figure 13b: Subgenus *Megatrypanum* 18S rRNA maximum parsimony tree showing the relationship between KwaZulu-Natal Province *T. theileri* strains with other related species from the gene bank. The analysis involved 7 nucleotide sequences. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P=6.17^{-130}). The consistency index is (0.961255), the retention index is (0.967085), and the composite index is 0.939491 (0.929615) for all sites and parsimony-informative sites. There were a total of 603 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, *et al*., 2011).

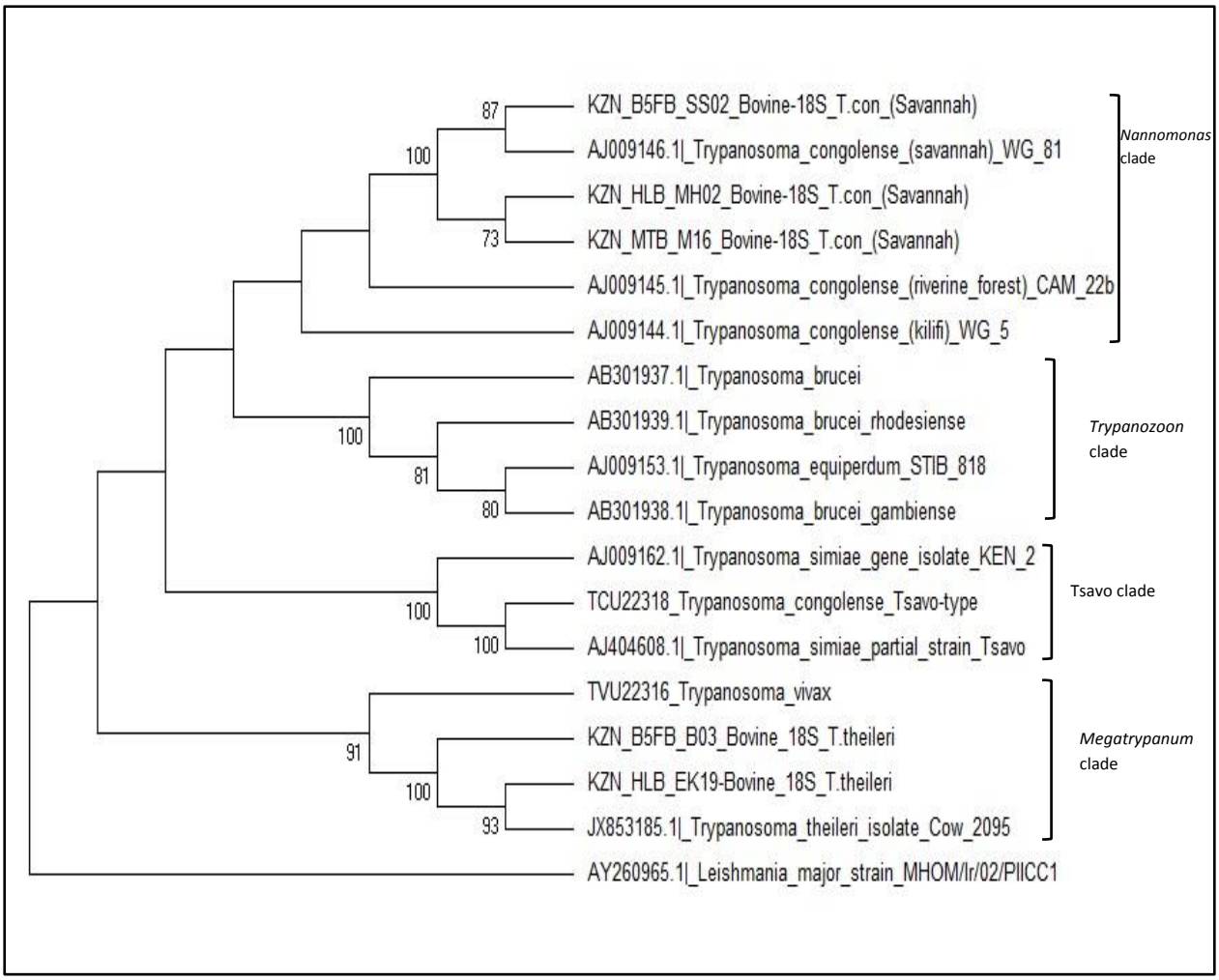


Figure14b: The 18S rRNA maximum parsimony tree composed of both South African *Trypanosoma* strains from KwaZulu-Natal as well as other trypanosomes from other countries in Africa and outside African continent. The analysis involved 19 nucleotide sequences. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (*P*= 0.7808). The consistency index is (0.634645), the retention index is (0.780473), and the composite index is 0.499204 (0.495323) for all sites and parsimony-informative sites. All positions containing gaps and missing data were eliminated. There were a total of 689 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, *et al.,* 2011).

4.4.2 Phylogenetic analysis using gGAPDH genes

To investigate the phylogenetic positioning of the South African trypanosome strains and if they do support monophyly using protein-coding gGAPDH genes 6 trees were constructed, 3 by neighbour-joining and 3 by maximum parsimony analysis. The 1st tree is composed only of *T. congolense* and other *Nannomonas* strains (Figure 15a and 15b), while the 2nd tree is only *T. brucei* and other *Trypanozoon* strains (Figure 16a and 16b) and the 3rd tree includes both *T. congolense* and *T. theileri* from KwaZulu-Natal Province, South Africa together with other trypanosomes form other countries in Africa and around the world (Figure 17a and 17b). As in the previous trees for 18S rRNA gene *T. vivax* was the suitable outgroup used for the 1st and 2nd trees while for the 3rd tree *Leishmania major* was the preferred outgroup.

4.4.2.1 The gGAPDH neighbour-joining trees

Figures 15a, 16a and 17a are the neighbour-joining models, which consisted of nucleotide substitution model of 1000 replicates. The nucleotide substitutions included d: transitions + transversions with Gamma distributed parameters and patterns among linages were treated as homogenous. Gaps and missing data were exposed to complete deletion by the MEGA 5 program (Hall, 2008; Tamura *et al.*, 2011).

In figure 15a, the 2 well supported monophyletic clades of the subgenus *Nannomonas* were observed namely the East-West-South African clade as well as the Simiae-Tsavo clade. The *T. congolense* (Savannah) strain from KwaZulu-Natal formed a clade with *T. congolense* (Kilifi) isolate from Kenya with quit low bootstrap support value (48%), while *T. congolense* (Savannah) isolate from Gambia formed a clade with *T. congolense* (forest) isolate from Cameroon with partially low bootstrap support value (50%). Both clades formed a cluster of 68% bootstrap value. Since South African *T. congolense* (Savannah) isolate produced such low bootstrap values it clearly indicates that it is divergent from the other isolates it clustered with and therefore it is a different genotype. The Simiae-Tsavo clade formed clusters of 94% and 83% bootstrap support values respectively. The molecular clock used to determine the evolutionary rate of the sequences rejected the hypothesis that these strains have the equal evolutionary rate throughout the tree at $P<0.05$.

In figure 16a only one partially supported linage was observed namely the *Trypanozoon* clade. The two KwaZulu-Natal *T. b. brucei* strains from Mtubatuba local municipality clustered together, they were 50% genetically related to one another, they were further 59% genetically related to *T. evansi* which was 81% genetically related to *T. b. rhodesiense*. Therefore all these sequences again proved that trypanosomes are monophyletic. Because the *T. b. brucei* strains obtained in this study clustered together it clearly indicates that they are different genotypes and are divergent from the other *Trypanozoon* isolates from other countries in the African continent. Statistical analysis at $P>0.05$ of the molecular clock revealed that our strains had equal evolutionary rate with other *Trypanozoon* isolates.

To confirm these observations made on the *Nannomonas* (Figure 15a) and *Trypanozoon* (Figure 16a) tree topologies were combined including all isolates to produce a third tree to prove how these isolates clustered. In figure 17a is a monophyletic tree consisting of South African strains and other trypanosome isolates from other African countries. Two lineages were observed namely the *Trypanozoon* and *Nannomonas* clades. South African *T. b. brucei* strains produced in this study clustered together and separated from all the other *Trypanozoon* isolates, indicating that these strains are genetically isolated and divergent from others. It was observed that the KwaZulu-Natal strains were 79% genetically related to one another. All *Trypanozoon* strains clustered together with fairly high bootstrap values however, *T. theileri* isolate from Uganda emerged between *T. b. brucei* and *T. cf. brucei* from Tanzania with 32% and 99% bootstrap support values. This occurrence has not been observed by previous authors who used gGAPDH as their preferred marker before and due to this topology, thus elaborative conclusions from this neighbour-joining tree has been a challenge. The *T. congolense* (Savannah, Kilifi and Forest) isolates clustered together with high bootstrap support values of 91% and 98%. The Tsavo clade also had high bootstrap support values but the *T. congolense* isolates had a very low (35%) bootstrap support when it was related to the Tsavo clade again reducing the significance of the gGAPDH neighbour-joining tree. Nonetheless, monophyly of trypanosomes was still supported by our tree topology regardless of the low bootstrap values observed and the emergence of *T. theileri* isolate in the *Trypanozoon* clade. Once again statistical analysis at $P>0.05$ of the molecular clock revealed that trypanosome strains obtained in this study had equal evolutionary rates with each other.

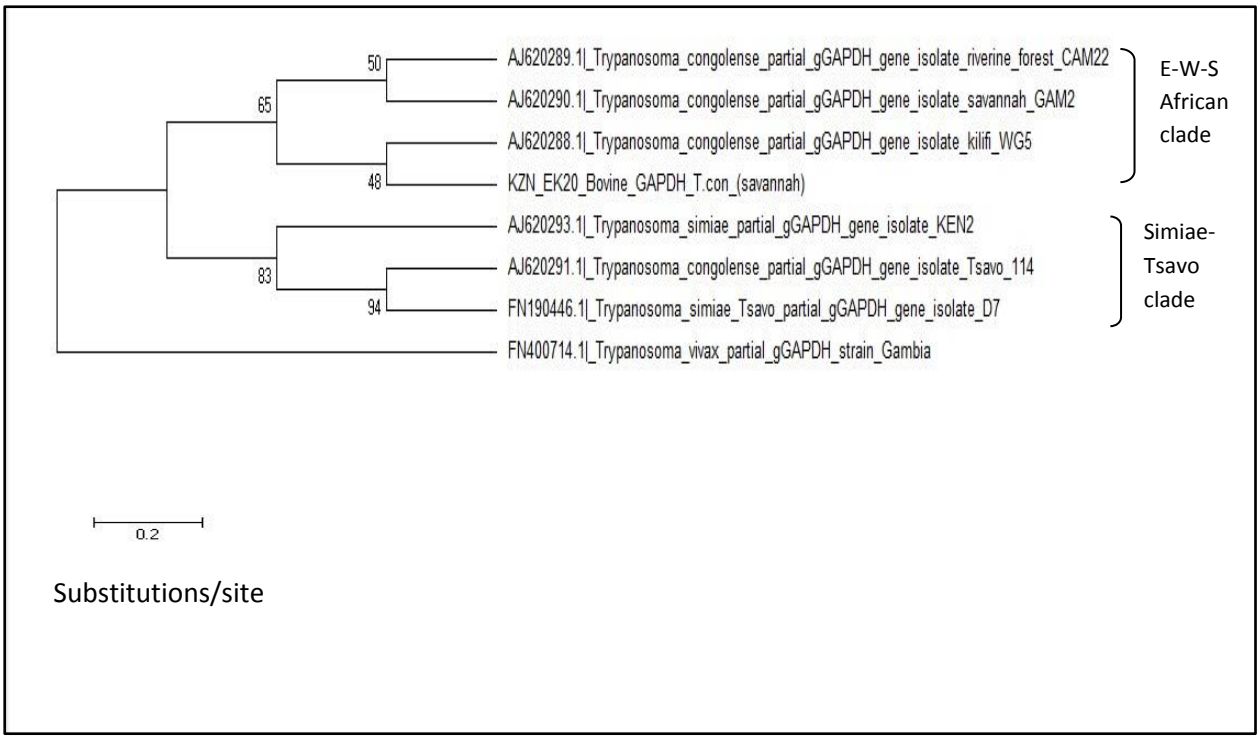


Figure 15a: Subgenus *Nannomonas* neighbour-joining gGAPDH tree, showing relationship between South African *T. congolense* (Savannah) strains with other related species from Africa. The analysis involved 9 nucleotide sequences. Two lineages (East-West-South African clade; Simiae-Tsavo clade) were identified with well supported bootstrap values. There were a total of 689 positions in the final dataset. The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model (+G) (Tamura and Nei, 1993). Differences in evolutionary rates among sites were modelled using a discrete Gamma (G) distribution, with a 4-category gamma distribution. Log L=-2525.53. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P=2.39^{-28}). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011)

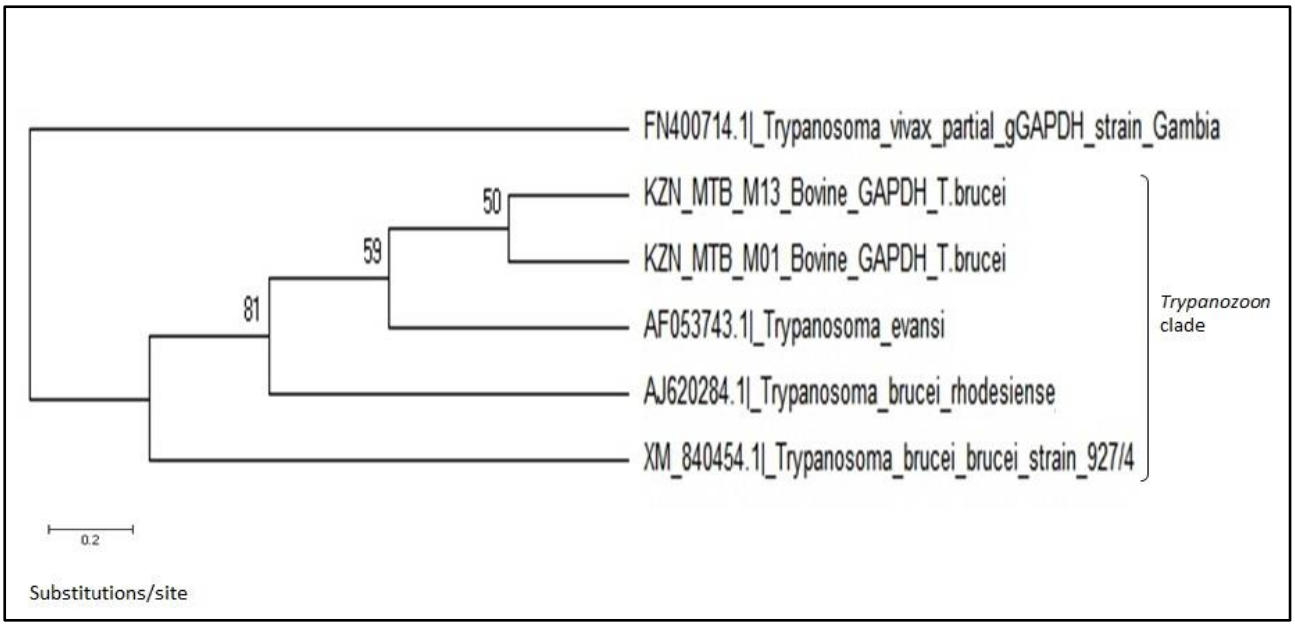


Figure 16a: Subgenus *Trypanozoon* neighbour-joining gGAPDH tree, showing the relationship between South African *T. b. brucei* strains with other related species from Africa. The analysis involved 6 nucleotide sequences. One lineage (Brucei clade) was identified with partially supported bootstrap values. There were a total of 787 positions in the final dataset. The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model (+G) (Tamura and Nei, 1993). Differences in evolutionary rates among sites were modelled using a discrete Gamma (G) distribution, with a 4-category gamma distribution. Log L=-2287.40. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (*P*=0.0689). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

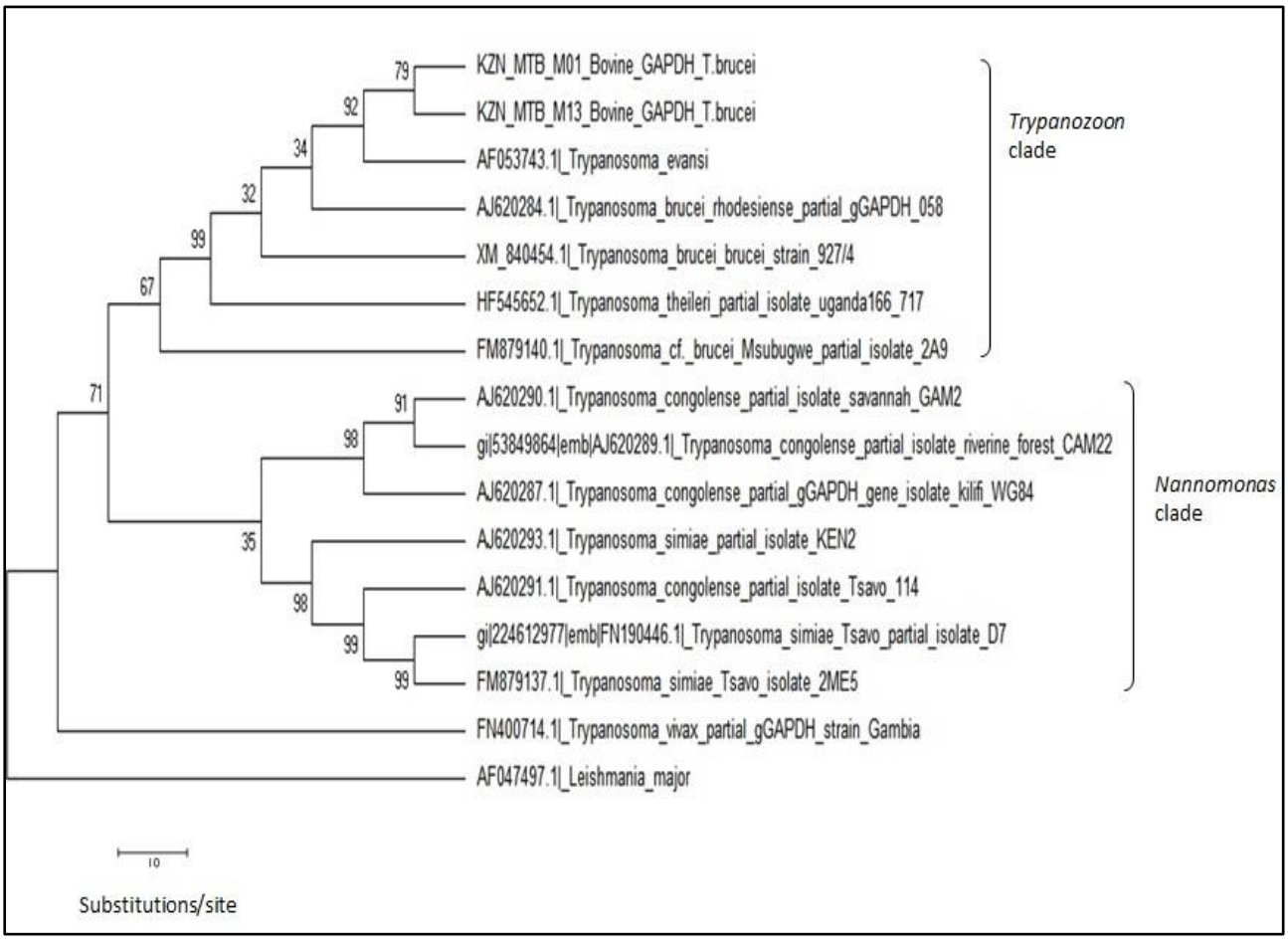


Figure 17a: gGAPDH neighbour-joining tree composed of both South African *Trypanosoma* strains from KwaZulu-Natal Province as well as other trypanosomes from other countries in Africa and outside the African continent. The analysis involved 16 nucleotide sequences. Two lineages (Brucei and congolense clades) were identified with well supported bootstrap values. There were a total of 746 positions in the final dataset. The molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under Tamura-Nei (1993) model (+G) (Tamura and Nei, 1993). Differences in evolutionary rates among sites were modelled using a discrete Gamma (G) distribution, with a 4-category gamma distribution. Log L=-2367.78. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (*P*=0.0686). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

4.2.2.2 The gGAPDH maximum parsimony trees

Figures 15b, 16b and 17b are evolutionary inferred using the maximum parsimony models, which consisted of nucleotide substitution model of 100 replicates, was composed by bootstrap method. The maximum parsimony trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Nucleotide substitution model was used, and the codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated by complete deletion (Hall, 2008; Tamura *et al.*, 2011).

In figure 15b, again two clades were observed with strong bootstrap support. The *T. congolense* (Savannah) strain appeared to be divergent and isolated from all the other *Nannomonas* isolates and it was 46% genetically related to all the other strains. This was not the case with the distance matrix method as two monophyletic clades were observed with fairly good bootstrap for *T. congolense* (Savannah) from KwaZulu-Natal. This clearly indicates that indeed our *T. congolense* (Savannah) strain is a different genotype from all the other *T. congolense* (Savannah) isolates which were previously described. The topology of this tree also showed that *T. congolense* (Savannah, Kilifi, Forest) isolates clustered together and also the *T. congolense* (Tsavo) clustered with *T. simiae* (Tsavo) and *T. simiae* with strong bootstrap support values of 97% and 99% respectively. Topology of this tree also showed these strains to be monophyletic but statistical analysis of the molecular clock revealed that these strains do not have the same evolutionary rate.

In figure 16b one lineage with two sub clades was observed with partially strong bootstrap support. This confirms the observations made from the distance matrix method. However, in this analysis one of the KwaZulu-Natal strains emerged isolated from all the other *Nannomonas* isolates, whereas the other formed a cluster with *T. b. rhodesiense* with 51% bootstrap support. These findings confirm that indeed *T. b. brucei* strains from this study are different genotypes. Monophyly of this the subgenus *Trypanozoon* is still supported by the topology of our maximum parsimony tree. There was no significance observed at $P< 0.05$ when molecular clock

analysis was tested and this indicates that the evolutionary rate of these sequences is not equal.

To confirm observations made in figures 15b and 16b tree topologies, all isolates combined to produce a third tree. The topology of this maximum parsimony tree (Figure 17b) also confirms monophyly in trypanosomes. Two lineages (*Trypanozoon* and *Nannomonas* clades) with well supported bootstrap values were observed. From the topology of this tree it was observed that in the *Trypanozoon* clade, all the other *Trypanozoon* isolates were genetically related to South African strains with 100% bootstrap support however, South African strains from this study emerged differently and were isolated from the rest. Additionally these *T. b. brucei* strains from KwaZulu-Natal were 57% genetically related to one another. Meaning that other than being different genotypes they are also genetically different from one another. Observations made in the distance matrix method showed that *T. theileri* isolate again emerged within the *Trypanozoon* clade but this time it formed a cluster with *T. evansi* which is also mechanically transmitted by non-tsetse flies with a broader geographical distribution as *T. theileri*. In the *Nannomonas* clade the *T. congolense* (Savannah, Kilifi and Forest) isolates clustered together with strong bootstrap support. The Tsavo clade also had strong bootstrap support but the *T. congolense* isolates had high (76%) bootstrap support when it was related to the Tsavo clade and within the Tsavo clade there was strong bootstrap support of 99% for all isolates. Due to the emergence of *T. theileri* isolate from Uganda as observed in the distance matrix method we cannot make any significant conclusions on the relatedness of these trypanosome species. However, statistical analysis at $P>0.05$ of the molecular clock were significant and revealed that these species had equal evolutionary rates with each other.

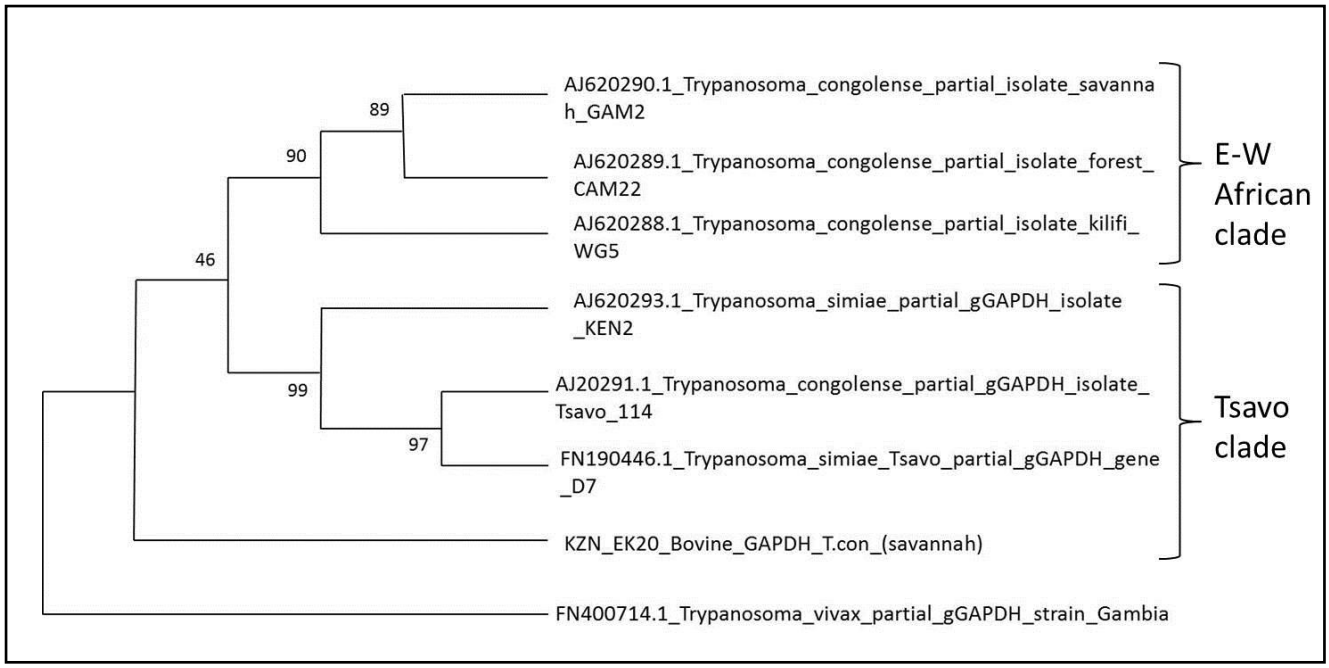


Figure 15b: Subgenus *Nannomonas* gGAPDH maximum parsimony tree showing the relationship between KwaZulu-Natal Province *T. congolense* strains with other related species from the gene bank. The analysis involved 9 nucleotide sequences. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (*P*= 4.97^{-6}). The consistency index is (0.662162), the retention index is (0.626866), and the composite index is 0.533582 (0.415087) for all sites and parsimony-informative sites. There were a total of 689 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, *et al*., 2011).

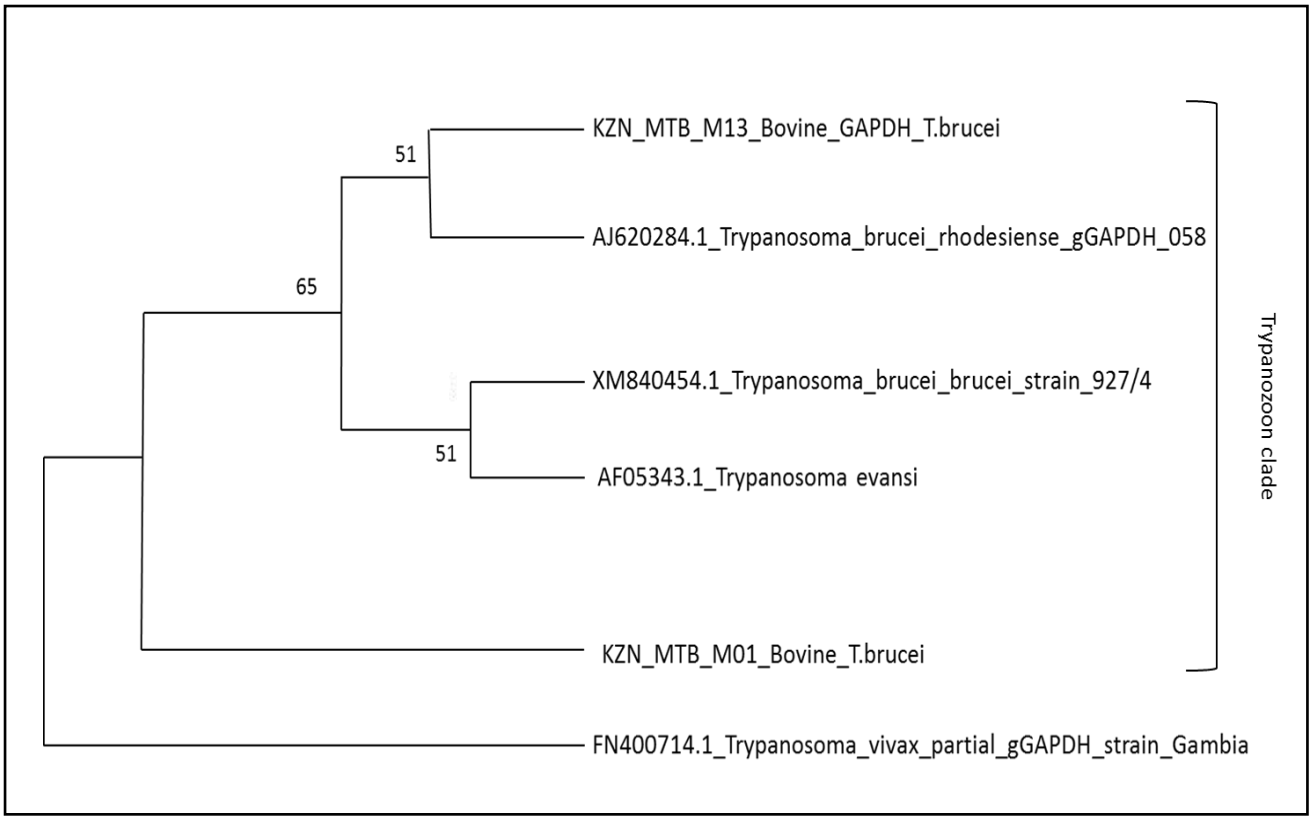


Figure 16b: Subgenus *Trypanozoon* gGAPDH maximum parsimony tree showing the relationship between KwaZulu-Natal Province *T. b. brucei* strains with other related species from the gene bank. The analysis involved 6 nucleotide sequences. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (*P*=0.0018). The consistency index is (0.692308), the retention index is (0.500000), and the composite index is 0.496491 (0.346154) for all sites and parsimony-informative sites. All positions containing gaps and missing data were eliminated. There were a total of 787 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011)

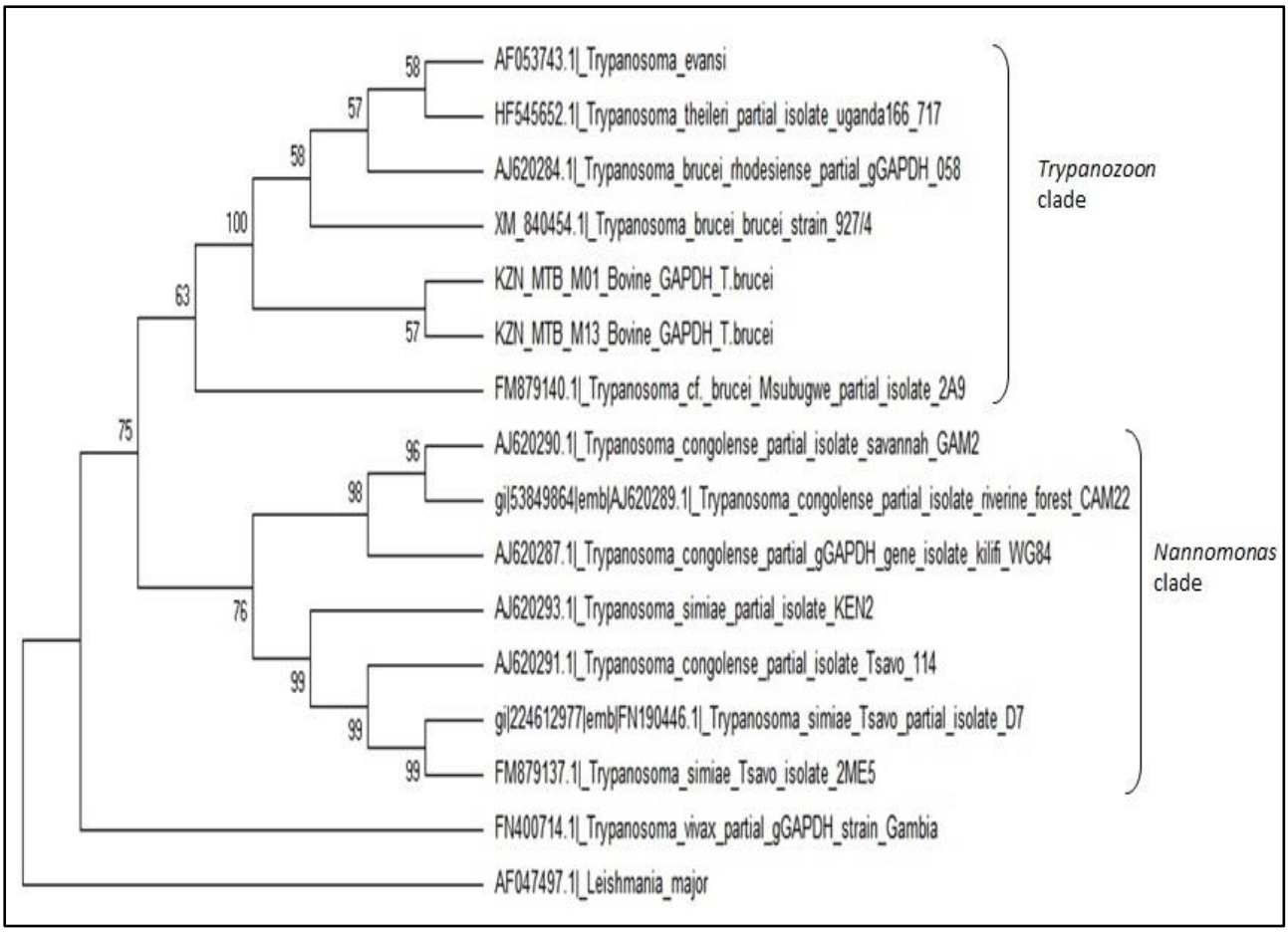


Figure 17b: The gGAPDH maximum parsimony tree composed of both South African *Trypanosoma* strains from KwaZulu-Natal as well as other trypanosomes from other countries in Africa and outside African continent. The analysis involved 16 nucleotide sequences. The null hypothesis of equal evolutionary rate throughout the tree was not rejected at a 5% significance level (*P*=0.7559). The consistency index is (0.679537), the retention index is (0.824524), and the composite index is 0.637030 (0.560295) for all sites and parsimony-informative sites. All positions containing gaps and missing data were eliminated. There were a total of 746 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, *et al.,* 2011).

4.5 Discussion

Molecular methods targeting the 18S rRNA and gGAPDH genes were used to describe and infer phylogenetic relatedness of trypanosome species found in KwaZulu-Natal Province, South Africa within species, and when compared to other related species(Hamilton *et al*., 2004; Gibson, 2007; McInnes *et al*., 2009). In chapter 3 of this study two semi-nested PCR tests were performed amplifying 18S rRNA and gGAPDH genes and the results obtained revealed that *T. congolense* (Savannah), *T. theileri* as well as *T. b. brucei* are prevalent in the uMkhanyakude district of KwaZulu-Natal and occur as different genotypes in livestock from those submitted in the NCBI data base. Therefore, two phylogenetic analysis were used namely the neighbour-joining and maximum parsimony methods.

In this study a total of 12 phylogenetic trees were constructed to understand the relatedness of South African trypanosome strains with other related strains from different countries in and outside the African continent. Twelve phylogenetic trees were constructed, six using neighbour-joining analysis whereby three trees were for 18S rRNA gene and three for gGAPDH gene. The remaining six trees were constructed using maximum parsimony analysis and three trees were made for each gene. In all the twelve phylogenetic trees two species were used as outgroups namely *T. vivax* which was used as an outgroup when constructing trees for different subgenera and *Leishmania major* when constructing larger trees composed of all trypanosome species under study for both genes. *T. vivax* was the preferred outgroup due to that previous phylogenetic studies have shown that it is on the periphery of the clade of tsetse-transmitted trypanosomes (Gibson, 2007; Adams *et al*., 2010). *Leishmania major* was the preferred outgroup because in most phylogenetic studies it branched out of the major African trypanosome clades and clusters with non-trypanosome trypanosomatids and supporting monophyly for trypanosomes (Stevens and Gibson, 1999; Hamilton *et al*., 2004; Thekisoe *et al*., 2007a). The 18S rRNA neighbour-joining phylogenetic analysis observed for the subgenus *Nannomonas* revealed that this group is monophyletic therefore supporting summaries made by Stevens and Brisse (2004), where they review work based on isoenzymes, DNA sequencing by 18S and 28S which also shown this subgenus to be monophyletic. In figure 12a the South African *T. congolense* (Savannah) strains formed a cluster which was isolated from the east-west African clade as well as the Tsavo clade with well supported bootstrap values of 90% and 100% respectively. However, it partially shared some genetic similarities with both clades with

a low bootstrap support value of 58% and due to this the molecular hypothesis of equal evolutionary rates was rejected.

Figure 13a represents the neighbour-joining tree of the subgenus *Megatrypanum*. The 18S rRNA gene sequences of this subgenus by Stevens and Brisse (2004), showed this group to cluster together with 100% bootstrap support value and also to be genetically related to *T. cyclops* a trypanosome isolated from a Malaysian primate. In the current study the South African *T. theileri* strains clustered together and were isolated from *T. theileri* strains submitted on the genebank. However, one strain in particular from Hlabisa local municipality showed to be more related to *T. theileri* isolate from a bovine in Germany with high bootstrap support value of 81%. Additionally, these two strains were then genetically similar to another *T. theileri* strain from Big 5 False Bay local municipality in KwaZulu-Natal Province by 100% bootstrap support. This indicated that there was genetic exchange between these South African strains even though they appear to be isolated and divergent from the other *T. theileri* isolates outside South African borders. Figure 14a included all South African trypanosomes strains obtained in this study together with other trypanosomes obtained from NCBI data base. In this tree the South African *T. congolense* (Savannah) and *T. theileri* strains clustered with corresponding subspecies and all had high bootstrap values of 90% and 87%. In this tree a total of 4 clades were observed which corresponds to previous studies on the phylogenetic analysis of trypanosomes. The first clade namely the *Trypanozoon* clade had 99% bootstrap support and the same observations were made previously using SSU rRNA, 18S rRNA and 28S rRNA, whereby members of the subgenus *Trypanozoon* were clustered together with high bootstrap supports ranging between 90% and 100% (Stevens and Gibson, 1999; Hamilton *et al*., 2004; Hamilton *et al.,* 2007; Auty *et al*., 2012). The second and third lineages in figure 14a include members of the subgenus *Nannomonas* whereby the Tsavo strain formed a cluster separate from the main *Nannomonas* clade with 95% bootstrap support. Additionally the spilt clustering in the subgenus *Nannomonas* between Tsavo clade and *T. congolense* species has been well documented by previous authors using SSU rRNA, 18S rRNA, 28S rRNA and GAPDH which was also case in the current study (Stevens and Gibson, 1999; Hamilton *et al*., 2004; Hamilton *et al*., 2007; Adams *et al*., 2009; Adams *et al*., 2010; Auty *et al*., 2012). The South African *T. congolense* (Savannah) formed a well-supported cluster with East African *T. congolense* (Savannah) isolate with 90% bootstrap support, indicating that indeed the South African

trypanosome species are more genetically related to Southern Eastern African species than to Western African trypanosome species. The last linage includes *T. theileri* strains from this study which also formed a well-supported clade of 87% with the American *T. theileri* strain, however observations made on this clade indicate that the *T. theileri* strain from Big 5 False Bay local municipality was partially genetically similar to the one in Hlabisa local municipality therefore this further attests to what has been suggested in the previous chapter 3 that there are different trypanosome genotypes prevalent in KwaZulu-Natal Province.

Observations made from the maximum parsimony trees in figure 12b, 13b and 14b further verifies what was noted with the neighbour-joining trees the only difference was that in the *Nannomonas* clade and also in all these trees the number of bootstrap support for all species were increased significantly. The clustering that was observed in the *Nannomonas* clade in figure 14a is now revealed in detail in figure 14b whereby, *T. congolense* (Savannah) strain from Big 5 False Bay local municipality was genetically similar to the *T. congolense* (Savannah) isolate from Kenya with 87% bootstrap support. *Trypanosoma congolense* (Savannah) strain from Hlabisa local municipality was genetically similar (73% bootstrap support) to the one in Mtubatuba local municipality and all these *T. congolense* strains were indeed related to one another with 100% bootstrap support. Therefore this clearly showed that because Hlabisa local municipality is closer to Mtubatuba local municipality as compared to Big 5 False Bay local municipality in KwaZulu-Natal there is great genetic exchange between these two local municipalities than with the Big 5 False Bay local municipality. The molecular clock hypothesis of equal evolutionary rates was accepted in both trees (Figure 14a and 14b) which included all trypanosome species from South Africa and other countries. Additionally in both trees (Figure15a and b) *T. vivax* isolate appeared at the periphery of the Salivaria clade as expected (Adams *et al.,* 2010), whereas *L. major* was positioned at the base of both trees therefore confirming observations made by other authors that the genus *Trypanosoma* is monophyletic and have the same common ancestor (Stevens and Gibson, 1999; Hamilton *et al*., 2004; Stevens and Brisse, 2004; Hamilton *et al.,* 2007; Gibson, 2007; Auty *et al*., 2012).

For phylogenetic analysis based on gGAPDH both neighbour-joining and maximum parsimony produced different analysis. In figure 16a neighbour-joining tree was used to understand the phylogenetic position of *T. congolense* (Savannah) from KwaZulu-Natal Province. In this analysis

two major lineages were observed whereby the first lineage was composed of eastern, southern and western African trypanosome strains and the second lineage was composed of isolates from east Africa namely the *T. simiae* (Tsavo), *T. congolense* (Tsavo) and *T. simiae* respectively. The first clade of east-west-south African strains had fairly low bootstrap support values. *Trypanosoma congolense* (Savannah) was clustered with *T. congolense* (Kilifi) from Kenya and these two strains were genetically similar by 48% bootstrap support, whereas *T. congolense* (Savannah) from Gambia formed a cluster with *T. congolense* (Forest) from Cameroon with 50% bootstrap support and these two clades were genetically similar by 65% bootstrap support. Nonetheless, the South African *T. congolense* (Savannah) strain clustered with an isolate from East Africa which supports the current study's hypothesis which states that South African trypanosomes will be more genetically similar to East African isolates than to Western and Central African isolates. Similar studies using gGAPDH genes were conducted by Adams *et al*. (2009), where they described new genotypes in the subgenus *Duttonella.* Their phylogenetic analysis indicated that in the clustering of the subgenus *Nannomonas*, *T. congolense* (Forest) formed a cluster with *T. congolense* (Kilifi) and they were genetically similar to *T. congolense* (Savannah) by 70% bootstrap support. In addition Adams *et al.* (2010) used a combination of 18S rRNA and gGAPDH where the authors showed the relationship of the subgenus *Nannomonas* to have equal evolutionary divergence and equal genetic similarities however, this was not the case in the current study. A similar study to Adams *et al*. (2010) was conducted by Hamilton *et al.* (2007) whereby they also combined 18S rRNA and gGAPDH genes to understand co-evolution as well as co-speciation in trypanosomes and the topology of their maximum likelihood tree under the subgenus *Nannomonas* was the same as the one observed in this study where *T. congolense* (Savannah) isolates were clustered with *T. congolense* (Forest) isolates and this cluster was isolated from *T. congolense* (Kilifi) with strong bootstrap supports of 71% and 100%.

Figures 15a and 15b are phylogenetic analyses of the subgenus *Trypanozoon*. In the analysis of neighbour-joining tree one major lineage was observed with two sub clades. *T. b. brucei* strains from KwaZulu-Natal formed a clade on their own whereby they were partially genetically similar to one another with 50% bootstrap support. The South African Brucei clade was completely isolated from the other *Trypanozoon* isolates whereby by it was similar to *T. evansi* with 59% bootstrap support. Observations made using maximum parsimony (Figure 15b) on

the other hand clearly show how *T. b. brucei* from KwaZulu-Natal is genetically similar to *T. b. rhodesiense* with 51% bootstrap support and the other strain is completely diverged and isolated from the rest of the isolates. However, it is genetically similar to the other *Trypanozoon* isolates with 65 % bootstrap support. Therefore this further confirms that indeed KwaZulu-Natal trypanosome species are different genotypes from the other species submitted on the NCBI database. Monophyly of the subgenus *Trypanozoon* is still supported by both trees even though low bootstrap support values were observed in the two trees.

Figures 17a and 17b are phylogenetic analyses of all trypanosomes obtained from gGAPDH. In figure 17a two major lineages were observed with 4 sub clades namely: the *Trypanozoon* clade, the South African clade, the *Nannomonas* clade and lastly the Tsavo clade. What was surprising about observations made from the *Trypanozoon* clade was that the *T. theileri* isolate from Uganda clustered with *T. evansi* with 58% bootstrap support and they were genetically related to *T. b. rhodesiense* isolate and *T. b. brucei* isolate with 57% and 58% bootstrap supports respectively. Such observations have never been described in previous literature before. *T. b. brucei* strains from KwaZulu-Natal Province were again isolated from the other *Trypanozoon* isolates and these two strains were 57% genetically similar however, they were 100% genetically related to other *Trypanozoon* isolates. Furthermore, *T. cf. brucei* Msubugwe emerged later in both trees with 99% bootstrap support for the neighbour-joining tree and with 63% bootstrap support for the maximum parsimony tree. These observations are further supported by the maximum likelihood tree using gGAPDH gene produced by McInnes *et al.* (2009) where they included this isolate from Tanzania in their analysis to determine the phylogenetic position of *T. irwini* in koala from Australia. In their analysis this isolate emerged separately and clustered with all the *Trypanozoon* with 98% bootstrap support (McInnes *et al*., 2009). Adams *et al*. (2010) noted that *T. cf. brucei* Msubugwe is closely related to *T. brucei sensu lato* however, it emerged separately due to its large genetic distance. Additionally, the position of the Msubugwe trypanosome in the phylogenetic analysis using gGAPDH is consistent between the subgenus *Trypanozoon* and *Nannomonas* which was the case in the current study (Adams *et al.*, 2010). In both trees the subgenus *Nannomonas* was divided into two separate clades namely the Congolense clade and the Tsavo clade with strong bootstrap support between 98% and 99%. As noted with the 18S phylogenetic trees again the tree topology in the current study confirms that genus *Trypanosoma* is monophyletic. In the analysis

of gGAPDH our low bootstrap support values and the emergence of *T. theileri* isolate within the *Trypanozoon* clade might make us have uncertainties in the reliability of our trees produced when comparing the same analysis from previous literature nonetheless, possible reasons to these observations is that maybe there were some unnoticeable errors in the alignment of the sequences, secondly most phylogenetic analysis on trypanosomes in literature use PAUP whereas in this current study MEGA5 was used. Another possible explanation could be due to different lengths of sequences used in the alignments which may either be longer or shorter than those used previous studies. Moreover most of these previous studies used maximum likelihood and in the current study neighbour-joining and maximum parsimony analysis were used to determine the phylogenetic relatedness and positioning of South African trypanosomes. This study has managed to prove that indeed South African trypanosomes are different genotypes from those submitted in the NCBI database however, in order to effectively control these parasites in livestock all different genotypes in South Africa must be identified, their virulence, epidemiology and drug resistance be understood. Therefore, the hypothesis which states that South African trypanosome species will be more genetically related to other eastern and southern African species as compared to other countries in Africa was accepted.

CHAPTER 5

DETERMINATION OF PREFERRED HOST FROM BLOOD MEAL OF *GLOSSINA BREVIPALPIS* COLLECTED IN UMKHANYAKUDE DISTRICT OF KWAZULU-NATAL PROVINCE, SOUTH AFRICA

5.1 Introduction

Tsetse flies are responsible transmitters of protozoan blood parasites from the genus *Trypanosoma* which cause a fatal human sleeping sickness in humans and nagana in domestic animals (Leak, 1999). The distribution of these flies is extensive throughout the Sub-Saharan Africa covering an area of more than 10 million km^2 (Leak, 1999; Esterhuizen *et al*., 2005; Mekata *et al.,* 2008 Steverding, 2008; Akoda *et al.*, 2009). The tsetse distribution in South Africa is restricted to the north eastern parts of KwaZulu-Natal Province (Leak, 1999; Esterhuizen *et al*., 2005; Mekata *et al.,* 2008).

South Africa had four tsetse fly species *G. pallidipes, G. morsitans morsitans, G. brevipalpis* and *G.* austeni which were responsible for transmitting parasites that cause African animal trypanosomiasis in livestock around KwaZulu-Natal Province (Kappmeier *et al*., 1998). However, during the rinderpest epizootic of 1896-1897 *G. m. morsitans* was completely eradicated in South Africa and in 1945, after the end of World War 2, DDT (dichlorodiphenyltrichloroethane) and benzene hexachloride were used as insecticides whereby active aerial and ground spraying in tsetse infested areas in KwaZulu-Natal Province. This led to a complete eradication of *G. pallidipes* by 1954 leaving only *G. brevipalpis* and *G. austeni* as isolated populations in the north eastern parts of KwaZulu-Natal Province (Kappmeier *et al*., 1998; Esterhuizen *et al*., 2005; Motloang *et al*., 2012). These two remaining species are responsible for transmitting *T. congolense* and *T. vivax* amongst livestock in these areas. Therefore for epidemiological significance and effective control measures, parasite-vector relationship as well as understanding the feeding behaviour of the vectors is of vital importance.

According to Späth (2000) knowledge of the feeding patterns of tsetse flies is essential in understanding the relationship among these strictly haematophagous vectors and their host whereby it will aid in clarifying the role tsetse flies play in disease transmission. They are also necessary in tsetse control campaigns when deciding whether traps or pour-on insecticides should be employed because a pour-on strategy will only work best if tsetse flies in that

particular area feed to a large extent on cattle, which can be treated with pour-on to reduce the fly populations (Späth, 2000). Other than species-specific preference, factors such as availability, abundance and behaviour of the host animal, which may vary considerably among habitats and seasons, are also responsible for determining the feeding behaviour of tsetse flies (Leak, 1999).

For many years *G. brevipalpis* and *G. austeni* have been considered as not an important vectors and thus have received little attention, however, this was proven not to be true as research conducted in KwaZulu-Natal based on the abundance of the flies and the spread of the disease showed that both species are important vectors of animal trypanosomiasis in the area (Kappmeier *et al*., 1998). It was also found that *G. austeni* is more competent in transmitting trypanosomes that *G. brevipalpis* despite its low abundance in KwaZulu-Natal Province (Motloang *et al*., 2012). Feeding patterns and host preferences of these too species have not been fully understood in South Africa hence the current study was conducted in order to fill the information gap on host preference of *G. brevipalpis*.

5.2 Objectives

1. To determine the mammalian blood meal of *Glossina brevipalpis* from KwaZulu-Natal Province

2. To determine the host preference of *G. brevipalpis* from KwaZulu-Natal Province

5.3 Materials and methods

5.3.1 Sampling

As described in chapter 2, a total of 376 tsetse flies were collected in Boomerang commercial farm and Charters Creek game reserve using H-traps however, for the purpose of this study only 350 tsetse flies (*Glossina brevipalpis*) were used. DNA extraction method of the tsetse flies was also described in chapter 2 and the data of the tsetse flies captured by H-traps was summarised in table 1 of chapter 2.

5.3.2 PCR using cytochrome b (cyt b) primers

Polymerase chain reaction was conducted using cytochrome b (cyt b) primers which are known to amplify orthologous regions of the cytochrome b gene located in the mitochondrial DNA of tsetse fly DNA and most several species for the detection of mammalian blood meal in the tsetse flies (Kirstein and Gray, 1996; Steuber *et al.,* 2005). The L14841 (GCC CCT CAG AAT GAT ATT TGT CCT CA) and H15149 (CCA TCC AAC ATC TCA GCA TGA TGA AA) primers were used to detect the origin of mammalian blood meal in the *Glossina brevipalpis* found in north eastern KwaZulu-Natal Province. Genomic DNA from a pig (*Sus scrofa*) was used as a positive control. For the amplification of mammalian DNA using cyt b primers the final reaction mixture was 25 µl and consisted of 2.5 µl of template DNA, 7.5 µl double distilled water, 12 µl of 2X Dream *Taq* Green PCR Master Mix (2X Dream *Taq* Green buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase) (Thermo Scientific, USA), the primer mix contained 10 µM of each oligonucleotide primer. PCR conditions for cyt b primers were set as follows: denaturation at 95°C for 10 minutes subjected to 35 cycles at 94°C for 30 seconds, annealing at 52°C annealing temperature was set for 1 minute, the first extension at 72°C for 2 minutes and final elongation at 72°C for 5 minutes with the holding temperature at 4°C (Steuber *et al.,* 2005). After completion 5 µl of amplicon was resolved by gel electrophoresis using 1% agarose gel stained with 10 µl GR – Green nucleic acid and visualized under UV (ultra violet) light. Positive PCR amplicons were also sequenced using the sequencing method mentioned in chapter 2, section 2.3.5. Retrieved sequences were subjected to BLAST to determine which mammalian mitochondrial DNA was amplified by PCR reactions.

5.4 Results

DNA fragments with bands ranging between 320 and 350 bp were observed on the agarose gel (Plate 7). A total of 54 samples tested positive for trypanosomes however, 33.33% (18/54) had positive amplification using cyt b primers. All cyt b positive samples were from traps 5, 8 and 10 respectively. From the 18 samples that tested positive 55.56% (10/18) were males and 44.44% (8/18) were females therefore, there was no significant difference observed in terms of feeding behaviour among different genders. The sequences from these positively amplified samples were subjected to BLAST and shown similarities to 6 vertebrate species. The vertebrate DNA that matched with our sequences included humans (11%), warthog (22%), red duiker (17%), crested porcupine (22%), elephant-shrew (17%), grey shrike (6%) and leaf-nosed bat (5%). A summary representing these results is given in table 10 as well as the identity match scores obtained from BLAST results with the corresponding accession numbers. Figure 18 represents the feeding patterns observed from the blood meal sequences obtained from BLAST.

Table 10: NCBI BLAST matches of >90% to blood meal sequences and their accession numbers

Class	Species	Tsetse fly gender		Identity Match	Accession No
		Male	Female		
Mammalia	*Homo sapiens* (Human)	2	0	99%	KC622272
	Hystrix cristata (Crested porcupine)	2	2	98%	FJ472578
	Cephalophus natalensis (Red duiker)	1	2	96%	AF153890
	Phacochoerus africanus (warthog)	1	3	98%	FJ785390
	Petrodromus tetradactylus (four-toed elephant-shrew)	2	1	90%	AF210659
	Hipposideros caffer (Sundeval leaf-nosed bat)	1	0	98%	JQ956448
Aves	*Lanius meridionalis pallidirostris* (grey shrike)	1	0	93%	DQ001874

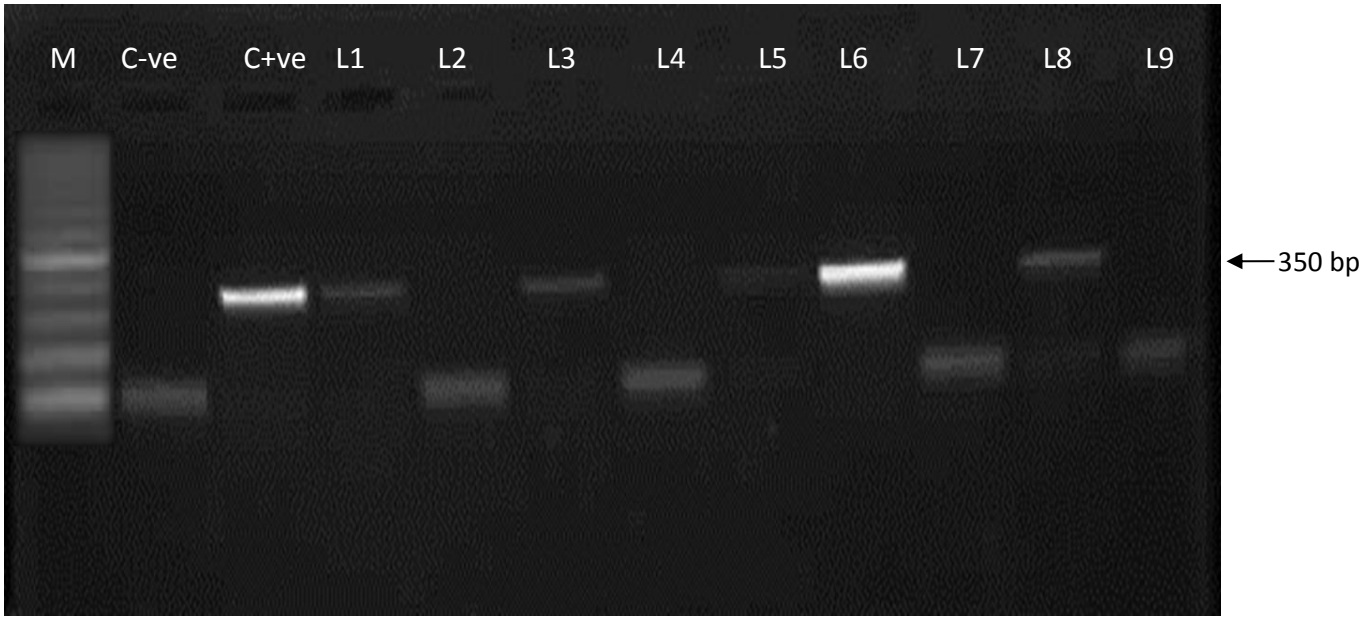


Plate 7: Agarose gel showing amplified mammalian DNA from *G. brevipalpis* blood meal by PCR test using cyt b primers. M is the molecular marker, C-ve is the negative control, C+ve is the positive control from *Sus scrofa* DNA. Lanes 1, 3, 5, 6 and 8 indicate positive amplification. Lanes 2, 4, 7 and 9 indicates amplification below detection line

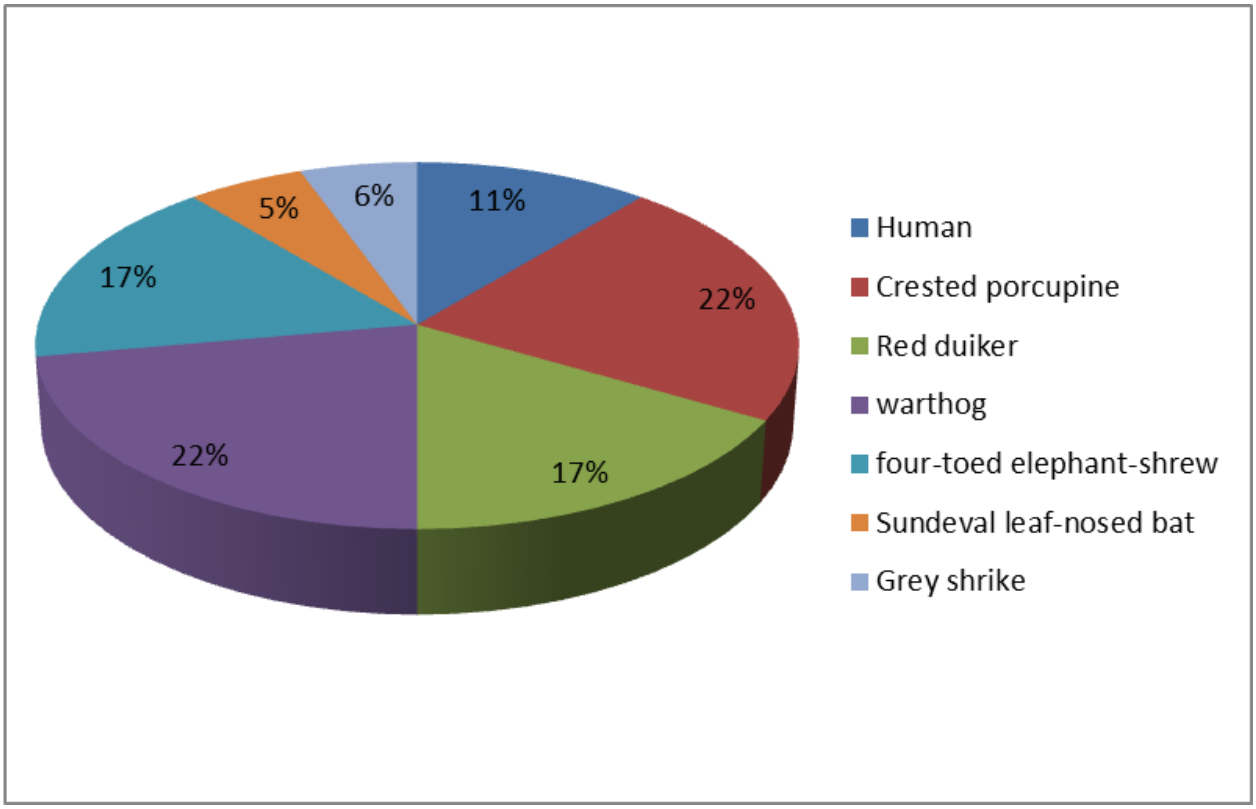


Figure 18: Feeding patterns observed from *Glossina brevipalpis* blood meal sequences when subjected to BLAST

5.5 Discussion

Blood meal and possible host preference of *G. brevipalpis* were determined using cytochrome b (cyt b) primers which amplify mammalian mitochondrial DNA in the tsetse fly DNA to identify host preference of these flies collected in KwaZulu-Natal Province of South Africa. For this study tsetse fly samples were collected in a different location from the blood samples used in the previous chapters and therefore, no domestic animal DNA was identified by the blood meal analysis. Tsetse flies are strictly haematophagous however, for blood meal analysis in this study few samples were used as most tested negative when amplified with cyt b primers. This might be due to that blood meals consumed post 36 hours are difficult to be amplified by PCR, and there are also PCR inhibitors present in the tsetse midgut (Maketa *et al*., 2008).

Blood meal analysis revealed that sequences matched with 6 different vertebrate species (5 from mammals and 1 from a bird). Small mammals such as warthog (22%) and crested porcupine (22%) were dominant species fed on as observed from blood meal analysis. These findings confirm observations made by Clausen *et al.* (1998) that most *Glossina* spp fed mainly on bush pigs (*Potamochoerus porcus*) and warthog (*Phacochoerus africanus*). Blood meals from duiker (Cepahlopinae), porcupines (Hysticidae) and humans (*Homo sapiens*) were also observed in this study. These findings have been recorded by Clausen *et al*. (1998), Späth (2000) and Farikou *et al*. (2010) from other *Glossina* species. However, it is for the first time blood meals from elephant-shrew (17%), grey shrike (6%) and leaf-nosed bat (5%) are recorded especially from feeding patterns of *G. brevipalpis* in KwaZulu-Natal Province. These new observations in feeding patterns were noticed in male *G. brevipalpis* blood meals suggesting that these males when seeking for potential females to mate with they foraged on any available host in order to obtain energy to continue with their search for mates. These observations do support statements raised by Clausen *et al*. (1998), that *G. brevipalpis* and *G. longipennis* feed on other mammals other than pigs or cattle. From their findings they reported that other than the two species mentioned above *G. brevipalpis* mainly fed on hippopotamus which was not the case in the current study even though the tsetse traps with positive samples were situated just a few kilometres from the lake St Lucia in figure 3 of chapter 2. On the other hand research has shown that *G. austeni* preferred to feed mainly on livestock such as goats and cattle, small mammals such as red duiker, hare, bush pigs and warthogs. Blood meals from humans or

reptiles such as monitor lizards have also been reported for *G. austeni* however, this variety of preferred hosts by this tsetse fly species depends on factors such as; the behaviour and availability of vertebrate hosts and the season (Clausen *et al*., 1998; Leak, 1998; Späth, 2000).

In chapter 2 it was found that positively tested *G. brevipalpis* by PCR were infected with *T. congolense* (Savannah and Kilifi) types and *T. b. brucei*. Findings of this study are supported by previous studies made by Mamabolo *et al*. (2009) and Motloang *et al*. (2012) that indeed *G. brevipalpis* species in KwaZulu-Natal Province are infected with *T. congolense* (Savannah and Kilifi) types. However, of this study disagree with concluding remarks made by Mamabolo *et al*. (2009) that *G. brevipalpis* is a poor vector of *T. b. brucei* and as such it does not occur in South Africa. Due to the observations made on the blood meal analysis it is likely that the detected vertebrate hosts from this study may act as reservoir hosts for livestock in uMkhanyakude district and as such more detailed analysis are needed to confirm these findings. If more samples from *G. austeni* were collected during sampling our findings were going to be more elaborative in terms of the feeding patterns of this fly species as well as in the parasites infecting the flies. As such, effective tsetse sampling targeting more infested areas in KwaZulu-Natal Province is needed to produce significant conclusions. Although, tsetse flies have been reported to show host preference in their feeding behaviour, this preference is also influenced by other factors such as geographical distribution of the animal population as well as the type of animal species and tsetse species present in a particular region.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 Prevalence of trypanosome parasites in livestock and tsetse flies

Polymerase chain reaction using trypanosome universal primers was conducted to determine the prevalence of African animal trypanosomiasis (AAT) as well as the *Trypanosoma* species that are responsible agents of the disease from blood samples of domestic animals (cattle, sheep, goats and dogs) and tsetse flies (*Glossina brevipalpis* and *G. austeni*) in uMkhanyakude district of KwaZulu-Natal (KZN) Province, South Africa. The current study proved that AAT still prevails in KZN and cattle were the most infected. The lethal *T. congolense* (Savannah) is the most prevalent species among the three sampled local municipalities (Big 5 False Bay, Hlabisa and Mtubatuba). *Trypanosoma theileri* which is non-pathogenic to livestock was also documented. Findings from this study do correspond to observations made by Van den Bossche (2001) and Mamabolo *et al*. (2009) where they detected *T. congolense* (Savannah and Kilifi) types in both livestock blood and tsetse flies. However, in the current study no *T. vivax* or mixed infections were observed as in previous studies. In the current study *T. theileri* and *T. b. brucei* were detected for the first time in South Africa. It is possible that they were misdiagnosed in the previous studies or this might indicate that there is a re-emergence of *T. b. brucei* in South Africa which was thought to be eradicated along with *G. m. morsitans* and *G. pallidipes* during rinderpest epizootic of 1896 and aerial and ground spraying with DDT in the 1950s (Kapmeier *et al*., 1998).

In uMkhanyakude district *G. brevipalpis* is more dominant and widespread as compared to *G. austeni* which supports findings by Van den Bossche (2001), Esterhuizen *et al*. (2005) and Motloang *et al.* (2012). Motloang *et al.* (2012) concluded that *G. austeni* was responsible for most infections in livestock despite its low dispersal and abundance. This statement could not be justified in the current study as only one *G. austeni* was collected. This fly tested negative for trypanosome infections by both microscopy and PCR. From the analysis made on tsetse flies the most dominant *Trypanosoma* species were *T. congolense* Savannah and Kilifi types followed by *T. b. brucei.* Observations made with gel electrophoresis of the amplified DNA of tsetse flies could not be supported by the sequences results following sequencing of positively amplified

samples. All sequences had no significant matches when subjected to BLAST. They matched with vector sequence used during cloning and sequencing. However, these findings remain inconclusive based on limited number of samples used in this study. In addition the method used to characterize these species is questionable due to that only one set of primers was used for the detected trypanosome species. It is suggested that more research employing adequate sample size and more sensitive molecular techniques be done to support these findings in the future before any conclusions can be drawn.

6.2 Genetic diversity in trypanosomes from livestock sampled in KwaZulu-Natal Province

Nested PCR using 18S rRNA and gGAPDH genes can be employed to determine the genotype variability among different *Trypanosoma* species detected in cattle blood samples from KwaZulu-Natal Province. The dominant *Trypanosoma* species in KwaZulu-Natal Province include *T. congolense* (Savannah) and *T. theileri* respectively and these finding were obtained from the alignment of the 18S rRNA gene sequences. There was significant genetic diversity observed within the two *Trypanosoma* species with significant nucleotide polymorphisms detected in their sequence alignments when they were compared to one another as well as to other related trypanosome sequences from the NCBI genebank. Observed variations in the genotypes of these two species might influence their virulence, epidemiology and drug resistance in their susceptible hosts. The alignment of gGAPDH also revealed similar results to the ones obtained by 18S rRNA gene. Here *T. congolense* (Savannah) and *T. b. brucei* were detected from samples collected in Mtubatuba local municipality with the exception of *T. theileri*. Nonetheless, the detection of *T. b. brucei* in the samples from Mtubatuba needs further analysis to confirm findings of this study due to limited number of samples used. Based on few sequences used to investigate the genetic diversity of trypanosomes in KwaZulu-Natal, the prevalence of *T. congolense, T. theileri* and *T. b. brucei* genotypes is still not known. These findings support observations we made in chapter 2 that in uMkhanyakude district there are three different species of trypanosomes (*T. congolense* (Savannah), *T. theileri* and *T. b. brucei*) circulating amongst livestock as well as in tsetse flies and these species occur as different genotypes with some degree of genetic diversity in their sequences which differs amongst South African trypanosome species and from other related species in other African countries.

6.3. Phylogenetic analysis of trypanosome strains from KwaZulu-Natal Province, South Africa

There was some degree of genetic diversity observed between trypanosome species found in uMkhanyakude district. The objective of this study was to determine phylogenetic position of South African trypanosome species, to compare these species with those occurring in other African countries and other countries outside Africa. Two molecular markers namely 18S rRNA and gGAPDH were used to determine the phylogenetic position of *T. congolense* (Savannah), *T. theileri* as well as *T. b. brucei* species from bovine samples in KwaZulu-Natal Province. Two phylogenetic methods used for this study comprised of the neighbour-joining and maximum parsimony methods. A total of 12 phylogenetic trees were constructed, 6 for each method used and 3 for each genetic marker for all the different species from KwaZulu-Natal Province. For the 18S rRNA gene both neighbour-joining and maximum parsimony trees confirmed monophyly in trypanosomes. These tested trypanosome species formed clusters with their related subspecies with well supported bootstrap support. It was also observed that trypanosome species from KwaZulu-Natal were more genetically related to the corresponding isolates from east African countries. Analysis of gGAPDH genes on the other hand had similar results to 18S rRNA although low bootstrap support values were frequently observed in both neighbour-joining and maximum parsimony trees however, they too confirmed that the South African trypanosome species are indeed different genotypes from one another and are more related to east African trypanosomes.

6.4 Blood meal identification and preferred host of *Glossina brevipalpis* from KwaZulu-Natal Province

In the previous chapters (Chapters 2 and 3) it was reported that in uMkhanyakude district there are three different trypanosome species (*T. congolense, T. theileri* and *T. b. brucei*) circulating in both livestock and the vector *G. brevipalpis*. This study was aimed to determine the mammalian blood meal and preferred host from *G. brevipalpis*. Blood meal origin in *G. brevipalpis* collected in Boomerang commercial farm and Charters Creek game reserve were detected using cytochrome b primers which amplified mammalian mitochondrial DNA (mtDNA) in the tsetse fly midgut. In chapter 2 it was observed that *G. brevipalpis* fly species are more abundant as compared to *G. austeni* (Esterhuizen *et al*., 2005; Motloang *et al*., 2012) and mostly were infected with *T. congolense* (Savannah and Kilifi) types and *T. b. brucei* respectively. The sequences obtained after amplification of mammalian mtDNA in the tsetse midguts were subjected to BLAST on NCBI nucleotide data base. Blood meal origins from mammals were from humans (*Homo sapiens*), red duiker (*Cephalophus natalensis*), warthog (*Phacochoerus africanus*), elephant shrew (*Petrodromus tetradactylus*) and porcupine (*Hystrix cristata*). Remarkably two blood meal origins from a leaf nosed bat (*Hipposideros caffer*) and an avian grey shrike (*Lanius meridionalis pallidirostris*) were also observed and these two blood meals were collected from male *G. brevipalpis* samples.

It is possible that these two blood meals from male *G. brevipalpis* were obtained when searching for potential mates. Feeding patterns observed in this study suggested that *G. brevipalpis* flies in Charters Creek also feed on other vertebrates such as rodents or birds in the absence of livestock or large wild ruminants. However, due to the parasites detected in these flies in chapter 2 it clearly shows that not only large wild mammals act as reservoir hosts for trypanosomes small mammals as well as birds are possible reservoir host of these parasites (Auty *et al*., 2012). Therefore, if sufficient samples of both tsetse flies species were collected in the same areas where blood samples were collected the blood meal results obtained would have more value in the epidemiology of trypanosome parasites in uMkhanyakude district

6.5 Conclusions

Different PCR techniques (conventional and nested PCR) were used to confirm the prevalence of African animal trypanosomiasis in the uMkhanyakude district of KwaZulu-Natal Province, South Africa. Results from this study do correspond to previous findings on trypanosomes in South Africa that indeed *T. congolense* (Savannah and Kilifi) types are circulating amongst livestock and tsetse flies in KwaZulu-Natal Province. It was proven that *G. brevipalpis* is more abundant than *G. austeni*. Moreover, this study was able to detect two more trypanosome species *T. theileri* and *T. b. brucei* which have not been reported in previous studies. This study has confirmed that there is great genetic diversity within these trypanosome species prevailing in uMkhanyakude district municipality. These different trypanosome species are more genetically related to east African trypanosome species than to central and western African species. However, the epidemiology, virulence and drug resistance of these species still needs to be further investigated due to the difference observed in their genotypes. The feeding patterns observed in this study demonstrated that *G. brevipalpis* feeds mainly on small mammals in the absence of livestock and also on human when they are in contact with them. The detection of non-pathogenic *T. theileri* which is said to be mechanically transmitted by tabanid flies highlights the importance of accurate diagnosis methods which can differentiate between different trypanosome species.

6.6 Recommendations

It is said that in order to effectively control trypanosomes and the diseases they cause to humans and domestic animals, control measures should be focused on controlling the vector flies. Therefore, in South Africa to achieve this, effective entomological survey of the distribution of the tsetse flies and comprehensive sampling of infected livestock with AAT in KwaZulu-Natal Province is required. Other haematophagous arthropods must be investigated to determine if they are also infected with *Trypanosoma* parasites or whether they can transmit these parasites to susceptible hosts. The employment of H-traps and targets treated with insecticides should be practised in areas that are heavily infested with tsetse flies to reduce the numbers of the wild tsetse populations. Tsetse fly populations in game reserves and private parks should be monitored and reduced to few populations which will be isolated from the rest of the communities and their livestock. Farmers and livestock owners in areas that are

heavily infested with tsetse flies should spray or dip their animals in insecticides to eliminate tsetse flies that were not captured by the H-traps or targets. This should be done in an organized manner so that the tsetse flies do not develop resistance against the insecticides used. Infected livestock or animals that show symptoms of animal trypanosomiasis should be treated with trypanocidal drugs but, this should also be practiced in a controlled manner by the affected farmers so that the trypanosome parasites do not develop immunity against the drugs. The trypanosome species detected in this study have different genotypes and therefore further studies are needed to determine their pathogenicity on livestock.

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