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AEGP	Addo Elephant Game Park
BLAST	Basic local alignment search tool
Cox III	Cytochrome oxidase subunit III
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
FRET	Fluorescence resonance energy transfer
GLTP	Greater Limpopo Transfronteir Park
HIP	Hluhluwe iMfolozi Game Park
ITS	Internal transcribed spacer
KNP	Kruger National Park
LAMP	Loop mediated isothermal amplification
$MgCl_2$	Magnesium chloride
ml	Milliliter
µl	Microliter
OVI	Onderstepoort Veterinary Institute
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction

RFLP	Restriction fragment length polymorphism
RLB	Reverse line blot
rRNA	Ribosomal ribonucleic acid
T_m	Melting temperature

THESIS SUMMARY

In South Africa, the diagnosis of *Theileria parva* in cattle and buffalo has been complicated by the presence of mildly pathogenic and non-pathogenic *Theileria* spp. This can lead to inaccurate diagnostic results and confuse the epidemiology of theileriosis. The aims of this study were to identify and characterize the 18S rRNA genes of novel *Theileria* spp. of the African buffalo, as well as to test new gene targets that will allow for the development of more accurate diagnostic tests for the identification of *T. parva* infections in cattle and buffalo.

Buffalo blood samples originating from different geographical regions in South Africa and from Mozambique were screened for the presence of *Theileria* spp. by the reverse line blot (RLB) hybridization assay. A total of six *Theileria* spp., namely *T. parva*, *Theileria* sp. (buffalo), *Theileria mutans*, *Theileria velifera* and *Theileria buffeli*, were identified from the buffalo samples. These occurred mainly as mixed infections. Some of the samples hybridized only with the *Theileria*/*Babesia* genus specific probe that is used in the RLB assay, and not with any of the species-specific probes used, suggesting the presence of novel genotypes or species.

The full-length 18S rRNA genes of parasites from selected samples were characterized by cloning and sequencing. In addition to the identification of 18S rRNA gene sequences that were similar to published *Theileria* spp. of cattle and buffalo, we identified *Theileria* sp. (bougasvlei), and novel 18S rRNA gene variants of *T. mutans*, *T. velifera*, *T. buffeli*. This variation explained why the RLB hybridization assay failed to detect these species in some of the analysed samples. As extensive variation was observed within the *T. mutans* group, specific RLB oligonucleotide probes were designed from the V4 hypervariable region of the *T. mutans*-like 1 and 2/3 18S rRNA gene sequences. Unfortunately these cross-hybridized with *T. mutans* target DNA and could not be used to screen buffalo samples to determine the occurrence of these genotypes in buffalo in South Africa. This problem could be solved by designing probes from a more variable area of the 18S rRNA gene of the *T. mutans* groups. Alternatively, a quantitative real-time PCR (qPCR) assay could be used for differentiation of these genotypes as it is more sensitive than the RLB assay.

Despite the variation observed in the full-length *T. parva* 18S rRNA gene sequences, the area in the V4 hypervariable region where the *T. parva* RLB and real-time PCR hybridization probes were developed was relatively conserved between sequences obtained in this study. The existing *T. parva*-specific qPCR assay was able to successfully detect all *T. parva* variants identified in this study and, although amplicons were obtained from *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) DNA, these species were not detected by the *T. parva*-specific hybridization probes. The sequences of the other *Theileria* spp. and the novel genotypes identified in this study under the probes were also different from that of *T. parva* and therefore these species do not compromise the specificity of the *T. parva* 18S qPCR assay.

In order to determine the sequence variation and phylogenetic positions of *T. buffeli* spp. of the African buffalo, we cloned and sequenced their 18S rRNA gene and complete internal transcribed spacer (ITS). We identified novel *T. buffeli*-like and *T. sinensis*-like 18S rRNA and ITS genotypes from buffalo originating from two different geographical regions in South Africa. There was extensive sequence variation between these novel South African genotypes and known *T. buffeli*-like and *T. sinensis*-like genotypes. The presence of organisms with *T. buffeli*-like and *T. sinensis*-like genotypes in the African buffalo is of significant importance, particularly to the cattle industry in South Africa as these animals might act as sources of infections to naïve cattle.

Recently, a qPCR assay based on the cox III gene was developed for the diagnosis of *Theileria* spp. in cattle. This test detects and differentiates six *Theileria* spp. in cattle. We evaluated the use of this assay for the detection of *Theileria* spp. in buffalo. The results of the cox III qPCR were compared to those of the RLB and 18S qPCR for the simultaneous detection and differentiation of *Theileria* spp. of the African buffalo, and for the specific detection of *T. parva*, respectively. The cox III genes from selected samples with non-specific melting peaks were characterized by cloning and sequencing. Extensive sequence variation in the cox III gene was observed between and within species. The *T. mutans* group was the most variable. The qPCR assay could be further improved by designing new primers and probes using all known cox III gene sequences of *Theileria* spp. of buffalo and cattle.

This study highlights the complexity of the diagnosis of *T. parva* in cattle and buffalo in South Africa. It provides invaluable information towards the development of an improved molecular diagnostic assay for *T. parva* and co-infecting species in cattle and buffalo in South Africa which will assist the veterinary regulatory authorities in the control of Corridor disease in South Africa.

CHAPTER 1

General Introduction

1.1 Background

Corridor disease, caused by *Theileria parva*, is a controlled disease in South Africa and strict measures are applied to control outbreaks and prevent the spread of the parasite. It is the form of theileriosis that persists in South Africa after the eradication of East Coast fever (ECF) and it poses a threat to the cattle farming industry in this country (Stoltsz, 1989). *Theileria parva* is transmitted transstadially by the three-host ticks, *Rhipicephalus appendiculatus, Rhipicephalus. zambeziensis* and *Rhipicephalus duttoni* (Lawrence et al., 1983; Norval et al., 1992). The main vector, *R. appendiculatus*, is wide spread in South Africa (Horak et al., 1995; 2007; 2009). The African buffalo (syn. Cape buffalo) (*Syncerus caffer*) is the natural reservoir host of *T. parva* and infections in buffalo are usually asymptomatic but are acute, and usually fatal in cattle (Lawrence et al., 1994). Cattle get infected by sharing grazing land with infected buffalo in the presence of the tick vectors (Uilenberg, 1999).

Infection by *T. parva* limits the movement of cattle between countries and can result in production losses and high mortality in infected animals (Allsopp et al., 1993). After foot-and-mouth disease, Corridor disease is the most important disease transmitted from the African buffalo to cattle and is therefore a major constraint to the introduction of buffalo in cattle-rearing areas (Lawrence et al., 1994). It is also a constraint on the importation of new cattle breeds and improved stock as it is most severe in recently introduced, naïve animals (CFSPH, 2009). In South Africa, infected populations of buffalo exist in the Kruger National Park and in the KwaZulu-Natal parks (Collins et al., 2002; Mashishi, 2002). These have been declared as Corridor disease endemic areas and sporadic outbreaks occur in these areas when susceptible cattle share grazing land with infected buffalo (Stoltsz, 1989). Movement of buffalo outside of these areas is strictly controlled by the veterinary authorities in South Africa.

1.1.1 The importance of proper diagnosis and characterization of *Theileria* infections of buffalo in South Africa

African buffalo also play an important role in the epidemiology of several other livestock diseases in South Africa, including foot-and-mouth disease, bovine brucellosis and bovine tuberculosis (Collins et al., 2002; Mashishi, 2002). In order to limit the spread of infection and protect susceptible cattle from infection, infected buffalo are isolated by use of approved fences in national and provincial game parks as well as in a limited number of buffalo breeding projects. Farming of buffalo and cattle on the same farm is not allowed. The major reason for imposing these restrictions on the movement of buffalo is the threat of "transformation" of buffalo-associated to cattle-associated theileriosis, resulting in the re-emergenge of ECF (Stoltsz, 1989) which is a readily transmitted and more virulent form of theileriosis.

In South Africa, buffalo are important for eco-tourism and this has led to an increasing demand for buffalo (Collins et al., 2002). There are several breeding projects in South Africa, regulated by the veterinary authorities, that breed "disease-free" buffalo and it is compulsory to test all buffalo for *T. parva* and other infectious diseases prior to translocation in order to protect susceptible cattle and buffalo from infection (Collins et al., 2002).

Control measures rely on the ability to detect *T. parva* in infected cattle and buffalo. These animals undergo a series of parasitological, serological and molecular diagnostic tests before they are certified "disease-free" and fit for translocation (Collins et al., 2002). The tests have to be sensitive and specific for accurate detection of the parasite. As *T. parva* usually co-occurs with non-pathogenic and mildly pathogenic *Theileria* species in infected animals (Stoltsz, 1989), it is important to characterize and differentiate between the different species and variants of a species so that it is possible to develop highly specific and sensitive diagnostic tests for the diagnosis of *T. parva*.

Earlier diagnosis of *T. parva* involved microscopic and serological methods. The former is based on morphological differences between the different stages of the parasite, and the latter detects serum antibodies to parasite schizont or sporozoite antigens (Katende et al., 1998; Ogden et al., 2003; Oura et al., 2004; Billiouw et al., 2005). However, these tests lack specificity and sensitivity as *T. parva* cannot be easily distinguished from co-infecting *Theileria* spp. Furthermore, the morphology of the parasites can vary during the course of infection and this renders morphological methods unreliable (Norval et al., 1992). Other disadvantages of the two methods include the inability to

detect carrier animals, cross reactivity and lack of standardization of the assays (Allsopp et al., 1993; Katende et al., 1998; Billiouw et al., 2005).

Advances in molecular biology have resulted in the development of more sensitive and specific diagnostic tests based on the detection of minute amounts of the parasite DNA. This has decreased the subjectivity that usually occurs in the interpretation of diagnostic results, and allows for inter- and intra-specific detection and characterization of the different species (Zarlenga and Higgins, 2001; Monis et al., 2005). The development of a highly sensitive and specific assay for the detection of *T. parva* requires prior identification and characterization of known and novel *Theileria* species in buffalo (Zweygarth et al., 2009).

1.2 Problem Statement

The identification of species-specific regions in the parasite 18S rRNA gene has improved the specificity of diagnostic tests and has allowed for discrimination between *Theileria* species. A sensitive, *T. parva*-specific quantitative real-time PCR (qPCR) assay based on this gene (Sibeko et al., 2008) has superseded the assay of Allsopp et al. (1993) in the diagnosis of *T. parva* infections in cattle and buffalo in South Africa. The South African buffalo is known to harbour different *Theileria* spp. (Stoltz, 1989; Young et al., 1978), but no comprehensive molecular characterization studies of these species had been done when this study was initiated, although Mans et al. (2011) recently reported on sequence variation in the hypervariable (V4) region of the 18S rRNA gene. Preliminary sequencing studies of the full-length 18S rRNA gene in our laboratory have revealed sequence differences within the 18S rRNA gene among *Theileria* sp. (buffalo) isolates. However, the extent of this variation is unknown and if strains exist with sequences even more similar to *T. parva* than those currently known, this may compromise the specificity of the assay. In addition, hitherto uncharacterized *Theileria* species could exist which could compromise the specificity of the currently used qPCR assay. This could lead to inaccurate diagnostic results.

1.3 Objectives of the study

In view of the above, the following were the main objectives of the study:

- Identification of pathogenic, mildly pathogenic and benign *Theileria* spp. of the African buffalo from different localities in South Africa.
- Characterization (by cloning and sequencing) of partial and full-length sequences of the 18S rRNA genes of *Theileria* spp. of the African buffalo.
- Evaluation of a recently developed quantitative real-time PCR assay based on the cytochrome c oxidase III (cox III) gene for the simultaneous identification and differentiation of *Theileria* spp. in buffalo.

1.4 Thesis overview

Chapter 1 - provides a general introduction and outlines the rationale and objectives of the study.

Chapter 2 - provides a detailed review of the literature on *T. parva* and other *Theileria* parasites of buffalo and cattle in South Africa.

Chapter 3 - The aim of this chapter was to characterise the 18S rRNA genes of *T. parva* and *Theileria* sp. (buffalo), and to determine whether all identified genotypes can be correctly detected by the qPCR assay. The reverse line blot (RLB) hybridization assay was used to screen buffalo blood samples collected from different geographical regions of South Africa, and in Mozambique. Based on the RLB results, the 18S rRNA genes of selected samples that were positive for *T. parva* and/or *Theileria* sp. (buffalo) were cloned and sequenced, and the resulting clones were analysed.

Chapter 4 - The mildly pathogenic *Theileria mutans* and non-pathogenic *Theileria velifera* often co-occur with *T. parva* in infected cattle and buffalo. In order to determine whether these species, or their variants can compromise the specificity of the 18S rRNA qPCR in the detection of *T. parva*, we cloned and sequenced their full-length 18S rRNA genes. New oligonucleotide probes were developed from the novel *T. mutans* genotypes identified for inclusion in the RLB hybridization assay. We also report on the presence of *Theileria* sp. (strain MSD) in the African buffalo. This species was first identified from a naturally infected bovine in Pretoria, South Africa (Chae et al., 1999) but no further attempt has been made to clarify its identity.

Chapter 5 - We characterized the full-length 18S rRNA gene and complete internal transcribed spacer (ITS1-5.8S-ITS2) region of *Theileria buffeli* of the South African buffalo in order to determine their genetic diversity and establish their phylogeny based on these sequences.

Chapter 6 - This chapter is a continuation of the studies on the characterization of the 18S rRNA gene of *Theileria* species of buffalo in South Africa in an attempt to identify novel genotypes that might interfere with the diagnosis of *T. parva* infections in cattle and buffalo in when using the 18S qPCR assay. In view of the genetic diversity that was observed in the previous chapters, we cloned and sequenced the V4 hypervariable region of the 18S rRNA gene from additional buffalo and cattle samples in order to determine the extent of sequence variation in this area of the gene. The results obtained were compared with those of a recent similar study by Mans et al. (2011).

Chapter 7 - The limitations of the 18S rRNA qPCR assay in the specific diagnosis of *T. parva* have been outlined in the previous chapters. In order to improve the diagnosis of *T. parva* in cattle and

buffalo in South Africa, alternative assays based on molecular markers that can effectively differentiate between *T. parva* and co-infecting species are needed. A qPCR assay based on the cytochrome oxidase III (cox III) gene was recently developed and evaluated for the simultaneous detection and differentiation of *Theileria* species of cattle (Janssens, 2009). We evaluated this assay for the simultaneous detection and differentiation of *Theileria* spp. of the African buffalo. The results obtained were compared with those of the RLB assay for simultaneous detection and differentiation of *Theileria* spp. in buffalo, and with those of the 18S rRNA qPCR assay for the specific detection of *T. parva*.

Chapter 8 - provides a general discussion, conclusions and recommendations emanating from this study.

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CHAPTER 2

Literature Review

The genus *Theileria* is a group of obligate, intracellular, tick-transmitted, apicomplexan parasites that infect wild and domestic ruminants throughout the world (Allsopp et al., 1993). Cattle and buffalo in Africa are usually co-infected with pathogenic, mildly pathogenic and non-pathogenic *Theileria* spp. These species are transmitted by different tick vectors and their geographical distribution depends on the distribution of their tick vectors.

2.1 Classification of *Theileria* spp.

Theileria parasites belong to the super-group Chromalveolata; phylum Apicomplexa (Adl et al., 2005). Apicomplexan parasites are single celled eukaryotes with an apical complex in some of the life-cycle stages. Members of the family Theileridae (*Theileria* and *Babesia*) have schizont stages in lymphocytes. In *Theileria*, the piroplasm stages in erythrocytes lack a pigment (Irvin, 1987).

Classification: (Adl et al., 2005)

Supergroup: Chromalveolata

Superphylum: Alveolata

Phylum: Apicomplexa

Class: Aconoidasida

Order: Piroplasmida

Family: Theileriidae

Genus: *Theileria*

2.2 Life cycle of *Theileria* in cattle and tick vector

The life cycle of *Theileria parva* is a typical apicomplexan life cycle (Figure 2.1) with an alternation of sexual and asexual stages that are found in the mammalian and tick host. Sporozoites are inoculated into the bovine host by the tick during a blood-meal. These enter lymphocytes and develop into schizonts. The lymphocytes are transformed and immortalized by the parasite. Schizonts stimulate the host cells to divide, and as cell divides, the schizont also divides, resulting in infection of the daughter cells. This synchronization of the division of host cells and schizonts results in the invasion of various host tissues by infected cells, causing a severe and sometimes fatal disease (Kaba et al., 2005). Some of the schizonts develop into merozoites, which are then released into the bloodstream where they invade erythrocytes and transform into piroplasms which are the stages that are infective to the tick.

Inside the gut of the tick, the piroplasms differentiate into male (micro-) and female (macro-) gametes which then fuse to form a zygote. The zygote then enters gut epithelial cells and develops into a kinete. Kinetes then emerge from the epithelial cells and migrate to the salivary glands of the tick where they transform into sporoblasts, each of which produces thousands of sporozoites. The cycle is then continued by inoculation of the sporozoites into the mammalian host by the tick.

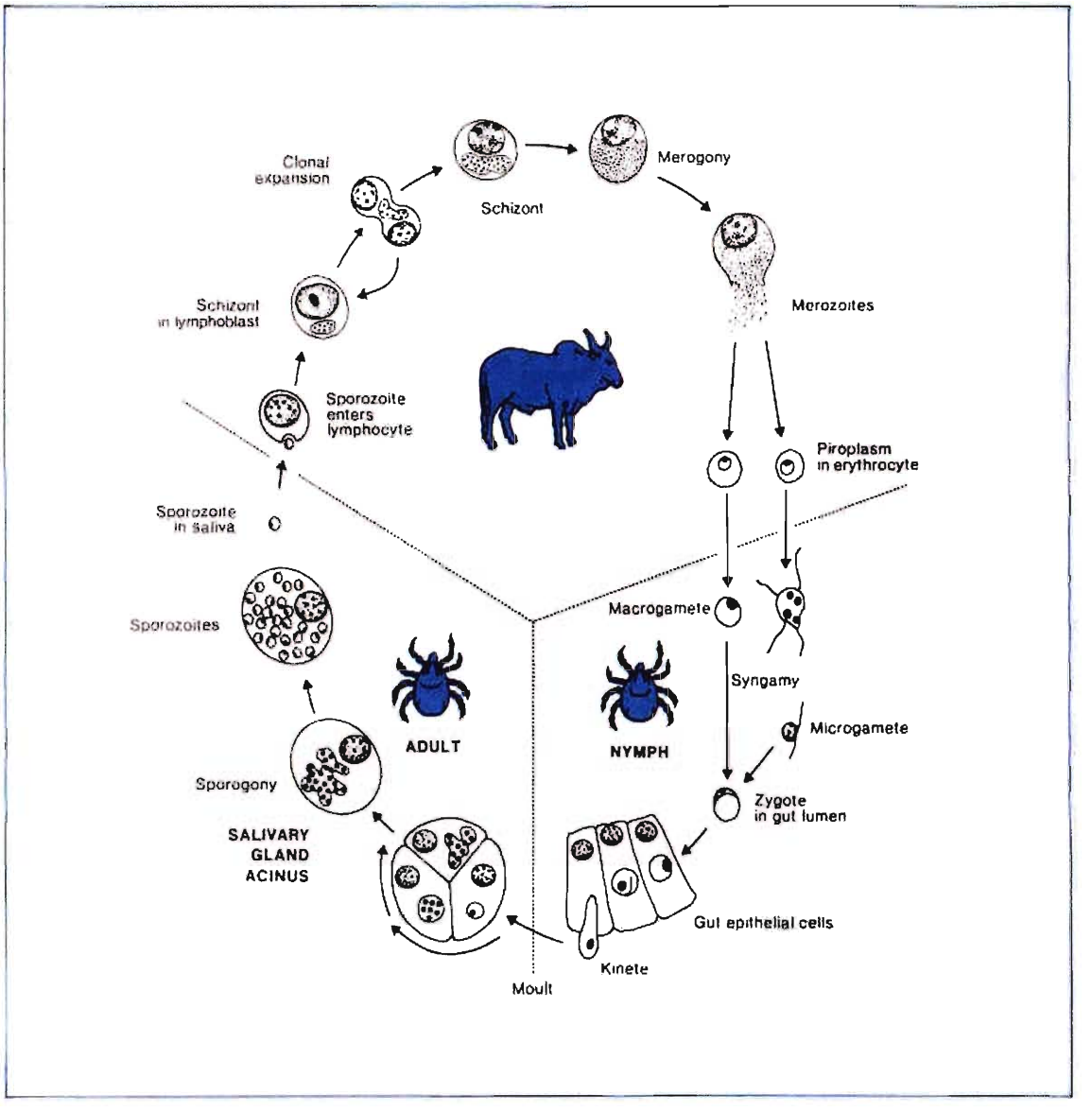

Figure 2.1: The life-cyle of *T. parva* (ILRAD, 1990)

2.3 *Theileria* species of buffalo and cattle in Africa

The most pathogenic (malignant) species of cattle are *T. parva* and *Theileria annulata*. These *Theileria* parasites are of major economic importance to the cattle industry due to high mortality and morbidity, cost of control and treatment, as well as loss in production by infected animals (Allsopp et al., 1993; ILRAD, 1990; OIE 2000; McKeever, 2001; Schnittger et al., 2002).

Theileria parva infects cattle and buffalo in eastern, central and southern Africa and is the causative agent of East Coast fever (ECF), January disease and Corridor disease in cattle (ILRAD, 1990; Gubbels et al., 1999). *Theileria parva* is transmitted transstadially by the three-host ticks, *Rhipicephalus appendiculatus, Rhipicephalus zambeziensis* and *Rhipicephalus duttoni* (Young et al., 1978b; Lawrence et al., 1983; Norval et al., 1992). The African buffalo (*Syncerus caffer*) is the natural reservoir host of *T. parva* and infections in buffalo are usually asymptomatic, but are acute and usually fatal in cattle (Lawrence et al., 1994c). The African buffalo is an indigenous bovine of sub-Saharan Africa and has lived in harmony with *T. parva* and its vector *R. appendiculatus* long before cattle were introduced into the region (Grootenhuis, 1988). Other reservoir hosts of *T. parva* are water buffalo (*Bubalus bubalis*) and waterbuck (*Kobus defassa*) (Stagg et al., 1994; CFSPH, 2009).

Theileria annulata infects cattle, yak, water buffalo and camels in northern Africa, southern Europe, the Middle East and Central Asia where it causes tropical theileriosis (Sergent et al., 1935)

Mildly pathogenic and benign species of *Theileria* that infect cattle and buffalo in Africa are *Theileria mutans*, *Theileria velifera, Theileria buffeli, Theileria taurotragi* and *Theileria* sp. (buffalo) (Allsopp et al., 1993; Gubbels et al., 1999; Oura et al., 2004). *Theileria* parasites usually occur as mixed infections in infected animals (Georges et al., 2001) and although the benign and mildly pathogenic forms do not have any significant economic importance, they can interfere with the diagnosis of the pathogenic forms and therefore confuse their epidemiology (Lawrence et al., 1994a).

2.4 *Theileria parva* (Theiler, 1904)

Theileria parva is transmitted by the three-host ixodid ticks, *R. appendiculatus*, *R. zambeziensis* and *R. duttoni* (Jongejan et al., 1980; Lawrence et al., 1983). It occurs in 11 countries in Africa, extending from southern Sudan to northern KwaZulu-Natal in South Africa (Figure 2.2). The African buffalo (*Syncerus caffer*) is the natural host of this parasite and infected buffalo usually remain long-term, asymptomatic carriers (ILRAD, 1990; Uilenberg, 1999). The parasite is the causative agent of ECF, January disease and Corridor disease in eastern, central and southern Africa (Collins et al., 2002). These disease syndromes differ in their clinical symptoms, pathogenicity, epidemiology and host (cattle or buffalo) (Allsopp et al., 1993).

Due to these differences, *T. parva* was initially classified into three sub-species, namely *T. parva parva*, *T. parva bovis* and *T. parva lawrencei*. However, this classification was abandoned due to lack of molecular evidence as these sub-species are genetically similar (Norval et al., 1992; Allsopp et al., 1993). It has since been recommended that the different isolates should rather be classified as cattle- or buffalo-associated depending on the original host (Anon, 1989).

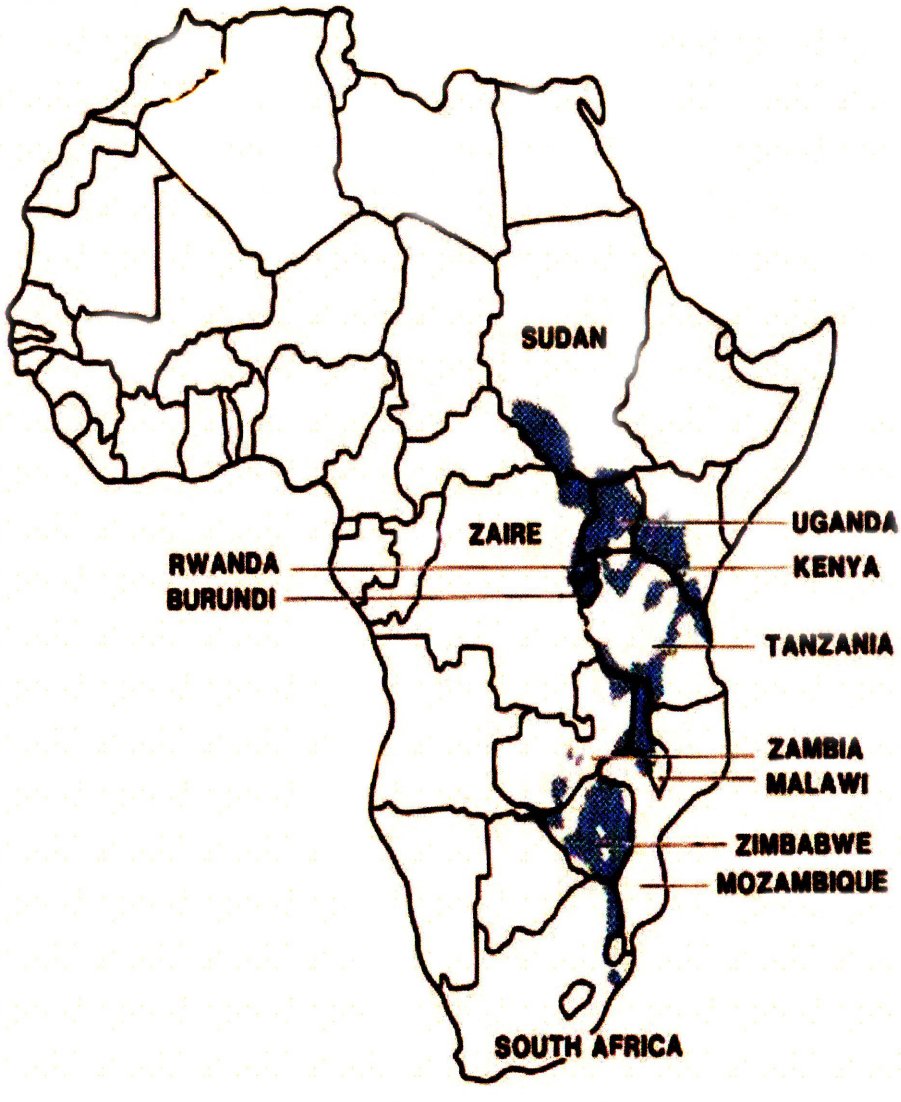

Figure 2.2: The distribution of *T. parva* in Africa (ILRAD, 1990)

2.4.1 East Coast fever (ECF)

ECF is a fatal disease of cattle caused by cattle-associated *T. parva* (previously known as *T. parva parva*) (Lawrence et al., 1994b). It is a major constraint to livestock production in Africa. Total annual losses due to ECF on the continent are estimated at around one million cattle and $168 million loss in revenue (Mukhebi et al., 1992; Norval et al., 1992). Twenty-four million cattle are at risk of infection (Norval et al., 1992). The pathogenicity of *T. parva* is mainly due to transformation and proliferation of the host T-lymphocytes that is induced by the parasite during schizogony, resulting in lymphocytolysis, and usually death of the animal (Ebel et al., 1997; Nene et al., 2000). The clinical signs of ECF include fever, anorexia, decreased milk productions and nasal discharge (CFSPH, 2009). If untreated, death occurs within three to four weeks of infection (ILRAD, 1990). Naïve animals develop an acute infection, and if they survive, are able to mount an immune response that results in a carrier state with low levels of parasitaemia and disease (Beck et al., 2009). These animals usually become asymptomatic carries and are therefore responsible for most of the transmission.

2.4.1.1. The epidemiology of ECF in southern Africa

The disease was introduced into southern Africa in early 1900 by importation of cattle from the East coast of Africa after the rinderpest epidemic (Norval et al., 1992; Uilenberg, 1999). It was first reported in Zimbabwe in 1902 where it was introduced through a consignment of cattle brought from East Africa through Mozambique. The disease later spread to southern Zimbabwe and then southwards to Swaziland and neighbouring south-eastern Transvaal and Natal (now Mpumalanga and KwaZulu-Natal) provinces of South Africa until it reached the Cape of Good Hope (Cape Town) in 1910 (Norval et al., 1992).

An estimated 5.5 million cattle died in South Africa due to ECF (Stoltsz, 1989). Due to its devastating effects in cattle, massive control strategies were implemented. These included clearing of infected pastures by removal of healthy cattle, intensive dipping and surveillance programmes, and mass slaughtering of infected cattle. This led to the total eradication of the disease from South Africa by 1955 (Stoltsz, 1989; Lawrence et al., 1994b; Uilenberg 1999). Although the disease has been totally eradicated, cattle in South Africa are still at risk of infection because the tick vector is still present in some parts of the country (Stoltsz, 1989; ILRAD, 1990).

2.4.2 January disease

January disease (also known as Zimbabwean theileriosis or malignant Rhodesian theileriosis) is a milder form of cattle-associated theileriosis that emerged in Zimbabwe after the eradication of classical ECF (Uilenberg, 1999). The symptoms of January disease are similar to but milder than those of ECF, and the two diseases can only be distinguished by seasonality. January disease is highly seasonal (occurs between December and March), with high mortalities in January which coincide with the availability of the tick host (Uilenberg, 1999). The causative agent was previously known as *T. parva bovis*. There is no evidence of the occurrence of January disease in South Africa (Stoltsz, 1989).

2.4.3 Corridor disease

Corridor disease is the buffalo-associated form of the disease which still persists in most southern African countries, including South Africa (the causative agent was previously known as *T. parva lawrencei*) (Lawrence, 1992). The parasite does not cause disease in the African buffalo reservoir host, but it can be transmitted from buffalo to cattle by infected ticks. It is thought that this form of the parasite is not transmitted between cattle, as infected cattle usually die before piroplasms appear or are too few to infect new ticks (Uilenberg, 1999). The clinical symptoms are similar to those of ECF, except that death usually occurs within a short time after the onset of the first symptoms (Lawrence et al., 1994c). It is therefore regarded as a self-limiting disease in cattle (Norval et al., 1992). However, cattle infected by buffalo-derived *T. parva* can recover from infection after treatment by chemotherapy and become carriers of the parasite which are capable of infecting susceptible cattle (Potgieter et al., 1988).

2.4.3.1 The epidemiology of Corridor disease in South Africa

Corridor disease has become the most important form of theileriosis in South Africa after the eradication of ECF as it poses a threat to the cattle farming industry in this country (Stoltsz, 1989).The disease was first recognized in the 'corridor' between the then Hluhluwe and Umfolozi (now Hluhluwe-iMfolozi) game reserves in the KwaZulu-Natal province of South Africa (Neitz et al., 1955; Lawrence et al., 1994a) and is currently endemic in the Kruger National Park (KNP) and in the Hluhluwe-iMfolozi game park, as well as in adjacent farms where cattle and buffalo are in close contact (Collins et al, 2002; Mashishi, 2002).

Measures used to control Corridor disease in South Africa include the prevention of contact between cattle and buffalo, regular dipping and spraying of all cattle in disease-infected areas and

testing of buffalo for theileriosis (and other controlled diseases) before translocation (South African Animal Disease Act 35 of 1984). However, despite all these strict control measures, sporadic outbreaks of the disease still occur in the country. In 1994, an outbreak of Corridor disease occurred in Warmbaths, Limpopo province, as a result of an illegal translocation of buffalo from an endemic area near the Kruger National Park (Collins et al., 2002). More recently, Thompson et al. (2008) reported an outbreak of theileriosis on a farm near Ladysmith in the KwaZulu-Natal province which is outside the declared Corridor disease endemic area. Infected buffalo from a neighbouring farm were suspected as the source of infection to cattle.

The phenomenon of 'transformation' of buffalo-associated *T. parva* to cattle-associated *T. parva* after serial tick passages in cattle has been described in East Africa by Barnett and Brocklesby (1966) and Young and Purnell (1973). However, experiments aimed at determining whether transformation can occur in South African *T. parva* isolates were unsuccessful (Potgieter et al., 1988; Norval et al., 1991).

2.4.4 Control of *T. parva*

2.4.4.1. Chemical control of ticks

Tick control is one of the most important and effective ways of controlling theileriosis, and it was largely through tick control by use of acaricides that ECF was totally eradicated in southern African (Norval, 1989, Norval et al., 1992). Acaricides are usually applied by dipping or spraying. Other methods that have been used include the slow release of the chemicals from impregnated ear-tags and the pour-on method which is applied to the back of the animal and then spread over the entire body (Norval, 1989). Acaricides that have been used include the arsenical forms, organochlorines, organophosphates, carbamates, amides and synthetic pyrenoids (Norval, 1989). Although highly effective, chemical control is not sustainable due to the high cost of acaricides, development of resistance by ticks, cross-resistance between some of the acaricides, and the fact that acaricides are not environmentally friendly (Norval, 1989; Minjauw et al., 1998). Due to these disadvantages, other control measures had to be devised.

2.4.4.2 Immunization of cattle

The history of immunization of cattle against theileriosis in South Africa has been reviewed by Uilenberg (1999). Initial immunization trials involved using blood from sick or recovered cattle and later with material from the spleen and lymph nodes of infected animals. None of these methods were effective, as many animals died from fatal ECF or various bacterial infections. Subsequent

experiments resulted in the use of the `infection and treatment' method that is currently used in the immunization of cattle against theileriosis in some African countries (Bishop et al., 2001).

The technique involves inoculation of the animal with an infective dose of sporozoites and simultaneous treatment with an antibiotic (e.g. oxytetracycline) to decrease the severity of infection (Cunningham et al., 1974; Brown et al., 1977). Although this provides life-long immunity to cattle, the technique has major limitations as it does not provide cross-immunity against all field strains; vaccination provides immunity to homologous strains of the parasite while animals remain susceptible to infection by heterologous strains (Uilenberg, 1999). This problem has been partially overcome by using `cocktails' such as the Muguga cocktail which is composed of three *T. parva* stocks (Bishop et al., 2001). Other limitations include the fact that immunized animals remain carriers and therefore become reservoirs of infection for the tick; the infective dose is potentially fatal and preservation of the live parasites requires liquid nitrogen and cold storage, facilities that are usually lacking in developing countries where the disease is endemic (Figueroa and Buening, 1995; Uilenberg, 1999; Bishop et al., 2001; McKeever, 2001).

2.4.4.3 Subunit vaccines

The problems mentioned above have resulted in the need for the development of other vaccination alternatives and research is focused on the identification of parasite proteins/genes, which could be used as subunit vaccines. Monoclonal antibodies against *T. parva* and *T. annulata* have been generated and are able to neutralize entry of sporozoites into host cells. The antibodies detect surface antigens such as SPAG-1 and *Ta*SP (for *T. annulata*) and p67, p104, p150 and PIM genes (for *T. parva*) (Schnittger et al., 2002). Some of these have been used in animal experiments as candiate vaccine against theileriosis (Schnittger et al., 2002; Kaba et al., 2005; Musoke et al., 2005; Akoolo et al., 2008; Janssens, 2009).

2.4.4.4 Chemotherapy

Tetracylines were the first compounds to be used in the control of theileriosis. Their efficacy was limited though, as they have a suppressive effect only in the early stages of *Theileria* infection. Further research led to the discovery of the naphthoquinone compound, menotone, which demonstrated theileriacidal activity but could not be further developed, as it was too expensive to synthesize. Subsequently, more effective derivatives of this compound were developed. Hydroxynaphthoquinone compounds such as parvaquone and buparvaquone have all been effectively used in the treatment of *T. parva* infections, particularly against ECF (McHardy et al.,

1983). However, in South Africa, the treatment of Corridor disease has been discontinued due to the carrier state that these drugs induce in cattle (Potgieter et al., 1988).

2.4.5 Diagnosis of *T. parva*

Diagnosis of *T. parva* is a crucial step in the control and treatment of the disease. Early detection of the parasite in the mammalian host also allows for proper treatment before clinical symptoms appear. The techniques used should therefore be specific and sensitive to low infections.

2.4.5.1 Conventional parasitological techniques

Conventional parasitological techniques include the microscopic examination of Giemsa-stained blood smears for the presence of piroplasms and examination of lymph node biopsies for schizonts in clinically suspected animals (Ogden et al., 2003; Oura et al., 2004). Although inexpensive and easy to perform, these techniques lack sensitivity and specificity as the piroplasms of *T. parva* cannot be differentiated from those of the non-pathogenic forms which commonly co-exist with *T. parva* in infected animals (Almeria et al., 2001).

2.4.5.2 Serological techniques

Serological assays detect serum antibodies to parasite schizont or sporozoite antigens (Katende et al., 1998; Billiouw et al., 2005). The most widely used serological method for the detection of *T. parva* infections is the indirect fluorescent antibody (IFA) test (Collins et al., 2002). However, the test has several disadvantages which include lack of specificity and sensitivity, cross reactivity, difficulty in standardization, subjectivity in interpretation of the results, inability to detect carrier animals, and diagnosed infections might not necessarily be active infections as the animal remains seropositive long after the infection has been cleared (Allsopp et al., 1993; Katende et al., 1998; Billiouw et al., 2005). Enzyme-linked immunosorbent assay (ELISA) has also been used for the detection of *T. parva* (Muraguri et al., 1999) antibodies and was found to be more sensitive than the IFA (Katende et al., 1998).

2.4.5.3 Molecular biology techniques

Advances in molecular biology have led to the development of more sensitive and specific diagnostic tests based on the detection and discrimination of parasite nucleic acid (DNA or RNA) sequences, and have decreased the subjectivity that usually occurs in interpreting results (Zarlenga and Higgins, 2001). *Theileria* parasites usually occur at low levels in infected animals, and molecular biology techniques are able to detect these low levels of infections. The identification of

carrier animals is important for the assessment of infection risk, since these animals serve as reservoirs of disease (Dolan, 1989). Inter- and intraspecific detection and characterization of parasites has become possible using molecular biology techniques (Monis et al., 2005).

I. Conventional Polymerase Chain Reaction (PCR)

A widely used technique is the Polymerase Chain Reaction (PCR). The conventional PCR involves the use of a thermostable DNA polymerase enzyme to amplify target DNA, and a pair of oligonucleotides (primers) that are complimentary to the two strands of the target DNA (Mullis, 1990). Post PCR analysis involves electrophoresis of the PCR-product using an agarose gel and visualization (using a suitable stain) under UV light. The agarose gel separates DNA molecules according to their size and therefore the size of the separated molecules can be determined by comparison to DNA molecules of known length.

PCR is more specific and sensitive than conventional parasitological and serological techniques and has been widely used in epidemiological studies of bovine theileriosis (Katzer et al., 1998; Almeria et al., 2001; Ogden et al., 2003). However, despite all these advantages, cross-contamination and false-positives are often encountered (Zarlenga and Higgins, 2001).

II. PCR-based hybridization assays

Polymerase chain reaction, coupled with hybridization, has increased the sensitivity and specificity of molecular diagnosis (Collins et al., 2002). DNA probes can be developed from variable regions of the gene target and used for simultaneous detection of related parasites from hosts and vectors (Figueroa and Buening, 1995). Primers corresponding to conserved sequences of a gene can be designed for PCR amplification and a variable region of the gene used to develop species-specific oligonucleotide probes. These species-specific probes can then be used in epidemiological studies and diagnosis of the parasites in mixed infections in hosts and vectors. Allsopp et al. (1993) used PCR amplification and species-specific probes to differentiate between the variable regions of the 18S rRNA gene of six *Theileria* species. This technique led to the identification of a novel *Theileria* sp. in buffalo which was designated *Theileria* sp. (buffalo).

Gubbels et al. (1999) developed a reverse line blot (RLB) hybridization method that combines both PCR and hybridization, and can simultaneously detect and differentiate different *Theileria* and *Babesia* species in infected hosts and vectors. PCR products are hybridized on a membrane on which species-specific probes are covalently linked. The products are visualized with chemiluminescence after a series of washes of the membrane with specific buffers. This assay has

since been used extensively in epidemiological surveys of theileriosis and babesiosis, and in many cases has resulted in the identification of novel *Theileri*a and *Babesia* genotypes (Georges et al., 2001; Almeria et al., 2001; Oura et al.. 2004; Nijhof et al., 2003; Salih et al., 2007; Matjila et al., 2008; Oosthuizen et al., 2008, 2009; Bhoora et al., 2009; Bosman et al., 2010; Chaisi et al., 2011).

The RLB assay is cost-effective as the membrane can be used several times if stored properly, it is reproducible and cross-reactions do not usually occur between the different species (Gubbels et al., 1999). However, the preparation of the membrane, hybridization step and the several post-hybridization washes involved make the assay laborious and time-consuming.

III. PCR-based RFLPs

Several PCR-based restriction fragment length polymorphism (RFLP) assays have been used in the identification and characterization of *T. parva* isolates based on their unique polymorphic profiles after restriction digestion. PCR-RLFP assays based on the 18S rRNA, p104, polymorphic immunodominant molecule (PIM), p150 and cox III genes have been used to either differentiate between different *T. parva* stocks or between different *Theileria* spp. in mixed infections (Geysen et al., 1999; Bishop et al., 2001; Bazarusanga et al., 2007; Janssens, 2009, Sibeko et al., 2010). Although such assays are easy to perform, they require well equipped laboratories, and restriction enzyme digestion requires an overnight incubation which makes RFLP assays laborious and time-consuming (Sibeko et al., 2010).

IV. PCR-based LAMP assays

Loop-mediated isothermal amplification (LAMP) is a simple, rapid and highly sensitive method for the amplification of DNA under isothermal conditions (Notomi et al., 2000; Nagamine et al., 2002). The test requires the use of four specific primers and DNA polymerase and is easy to perform as it requires the use of a regular laboratory heat block or water bath for the reaction (Notomi et al., 2000). Several LAMP assays have recently been developed for the detection of different *Theileria* and *Babesia* spp. Liu et al. (2008) developed a LAMP assay for diagnosis of ovine theileriosis in China and Thekisoe et al. (2010) developed two LAMP assays that target the PIM and p150 genes of *T. parva* in cattle and buffalo.The LAMP assays by He et al. (2009) and Wang et al. (2010b) target the 18S rRNA and 33-kDa major piroplasm surface protein (p33) genes of *Babesia orientalis* and *Theileria sergenti* respectively.

V. Quantitative real-time PCR (qPCR) assays

Real-time PCR has greatly improved molecular detection and differential diagnosis of closely related organisms. The assay simultaneously amplifies, detects and quantitatively analyzes target DNA sequences in real-time (Zarlenga and Higgins 2001; Monis et al., 2005). Post-PCR analysis is therefore not required and this reduces the risk of contamination, loss of the PCR product, and allows for rapid attainment of results (Bell and Ranford-Cartwright, 2002). Contamination is also minimized and the cycling instrument can be automated for large-scale processing of samples, thereby reducing both the time and labour required for analysis.

Real-time PCR using flourescence resonance energy transfer (FRET) technology has been used for the detection and differentiation of *Theileria* and *Babesia* species in mixed infections by melting curve analysis. FRET involves the use of sequence-specific oligonucleotide (hybridization) probes that are labelled with fluorescence dyes (Reuter et al., 2005). Hybridization probes provide a simple way of analysing sequence variations using a single reaction and one set of probes as both amplification and hybridization occur in the same reaction (Caplin et al., 1999). Sibeko et al. (2008) developed a quantitive real-time PCR (qPCR) assay for the detection of *T. parva* in cattle and buffalo based on the 18S rRNA gene. This assay is currently used as the official test for the detection of *T. parva* in these animals in South Africa. In addition to the identification of *T. parva*, the assay can simultaneously detect *T. taurotragi* and *T. annulata* when the *Theileria* and *Babesia* genus-specific primer set is used. Recently, Papli et al. (2011) developed another qPCR assay for the detection of *T. parva* in cattle and buffalo in South Africa. The latter also targets the parasite 18S rRNA gene but is based on TaqMan probe chemistry. Comparison of the two *T. parva* qPCR assays indicated a good correlation in their ability to detect the parasite in infected animals (Papli et al., 2011). More recently, Pienaar et al. (2011) developed another qPCR, designated the Hybrid II assay, for the specific diagnosis of *T. parva*. The assay uses a single primer set to amplify both *Theileria* sp. (buffalo) and *T. parva*, and two distinct melting peaks are obtained for these species.

Other 18S rRNA gene qPCR assays for *Theileria* and *Babesia* spp. were developed by Criado-Fornelio et al. (2009) and Wang et al. (2010a). The former is used for simultaneous identification of *Babesia bovis*, *B. divergens*, *B. major* or *B. bigemina*, *Theileria annae* and an unidentified *Theileria* sp. in bovines, and the latter differentiates between *B. gibsoni*, *B. canis canis*/*B. canis vogeli* and *B. canis rossi* in canines. A nested qPCR assay based on the cytochrome oxidase subunit III (cox III) gene was described by Janssens (2009) for simultaneous detection and differentiation of *T. parva* and five co-infecting *Theileria* spp., in cattle.

2.5 Benign and mildly pathogenic *Theileria* species of cattle and buffalo in South Africa

Several benign and mildly-pathogenic *Theileria* species frequently co-exist with *T. parva* in infected animals. They are usually carried asymtomatically, but under conditions of stress, malnutrition and immune-deficiency, some can also cause disease, loss of production and may increase the severity of theileriosis in infected animals (Noval et al., 1992; CFSPH, 2009). Although schizogony still occurs in the benign species, host cell transformation does not occur (non-lymphoproliferative) in this case and the pathology is mainly due to multiplication of piroplasms in the host red blood cells, resulting in anaemia, a condition that rarely occurs with *T. parva* infections (Nene et al., 2000). These parasites are transmitted by different tick species and therefore their geographic distribution coincides with the distribution of their tick vectors. *Theileria* spp. can be differentiated from each other based on their serological, morphological, epidemiological and molecular characteristics.

2.5.1 *Theileria mutans* (Theiler, 1906)

Theileria mutans is a parasite of buffalo, it is infective to cattle and can cause latent infections in sheep (Paling et al 1981; Allsopp et al 1993). It is transmitted by *Amblyomma* ticks (Uilenberg et al., 1976, 1982; Paling et al., 1981). Previously, *T. mutans* was implicated in all benign bovine *Theileria* infections worldwide (Gill, 2004). However, transmission, serology and phylogenetic studies have indicated that it is an African species and is different from benign *Theileria* species that were isolated from cattle in other parts of the world, namely, *T. orientalis* and *T. buffeli* (Uilenberg et al., 1977; Chae et al., 1999; Gill, 2004). Although generally considered as a benign species in buffalo, some strains of *T. mutans* have been associated with severe disease in cattle (Young et al., 1978a; b; Paling et al., 1981).

2.5.2 *Theileria* sp. (strain MSD)

This species was first identified from a naturally infected bovine at the Merck, Sharp & Dome (MSD) experimental centre at Hartebeespoort, Pretoria, South Africa (Chae et al., 1999). It was initially suspected to be a variant of *T. velifera,* but sequence and phylogenetic analyses based on 18S rRNA gene sequences indicated that it is most closely related to *T. mutans* (Chae et al., 1999; Martins et al., 2010; Chaisi et al., 2011; Mans et al., 2011). Although no attempts have been made to clarify the identity of *Theileria* sp. (strain MSD) after its first description by Chae et al. (1999), the identification of similar sequences in buffalo and cattle indicates that this genotype is circulating

in some buffalo and cattle populations in southern Africa (Martins et al., 2010; Mans et al., 2011).

2.5.3 *Theileria* sp. (buffalo)

Conrad et al. (1987) reported on the presence of antigenically distinct *Theileria* parasites from the African buffalo in Kenya. The unknown parasite was thought to be buffalo-derived *T. parva* as it occurred several times among stocks that were isolated from buffalo and were characterized using monoclonal antibodies (Conrad et al., 1987). Subsequent sequencing of the 18S rRNA gene of the unknown parasite by Allsopp et al. (1993) indicated that it is a new species, as the 18S rRNA gene sequence was different from both cattle- and buffalo-derived *T. parva*. The new species was designated as *Theileria* sp. (buffalo). To date *Theileria* sp. (buffalo) has only ever been identified in buffalo, and is genetically closely related to *T. parva* and other pathogenic *Theileria* spp. (Chaisi et al., 2011; Mans et al., 2011). Recently, Zweygarth et al. (2009) established a macroschizont-infected lymphoblastoid cell line from an African buffalo infected with *Theileria* sp. (buffalo), suggesting that it is able to transform lymphocytes. However, it does not appear to infect cattle and its vector is unknown.

2.5.4 *Theileria buffeli/sergenti/orientalis* (Neveu-Lemaire, 1912; Yakimov and Dekhterven, 1930; Yakimov and Sudachenkov, 1931)

Theileria buffeli/sergenti/orientalis is a group of closely related benign parasites of cattle and buffalo with a cosmopolitan distribution. They infect cattle and buffalo in Africa, Australia, Asia, Europe and the United States of America (USA) (Chae et al., 1998; Chansiri et al, 1999; Cossio-Bayugar et al., 2002; Aktas et al., 2007; Altay et al., 2008; M'ghirbi et al., 2008; Gimenez et al., 2009; Chaisi et al., 2011; Mans et al., 2011). Ticks of *Haemaphysalis* spp. act as vectors in Australia, Asia and Europe, but the vectors in Africa and the USA are still unknown (M'ghirbi et al., 2008).

Theileria sergenti and *T. orientalis* were first described from eastern Siberia in the early 1930s by Yakimov and Dekhterev, and Yakimov and Soudatschenkov, respectively, while *T. buffeli* was first described from the Asian water buffalo (*Bubalus bubalis*) in 1908 by Schein (reviewed by Fujisaki et al., 1994). Their classification is confusing and it is still unclear if they represent the same species or different species. Biological differences such as the occurrence of macroschizonts and piroplasm morphology have been observed among isolates of the *T. buffeli/sergenti/orientalis* complex (Uilenberg et al., 1985). Uilenberg (2011) indicated that although the term "*T. sergenti*" has traditionally been used for this species, *T. sergenti* actually refers to a sheep parasite and it was incorrectly termed as a parasite of cattle and buffalo.

Due to all this confusion, Uilenberg et al. (1985) suggested that the benign species (*T. buffeli*/*T. orientalis*) should be classified as *T. orientalis*. The term *T. buffeli* is preferred over *T. orientalis* on the basis of molecular and biological data, as well as the fact that all characterized isolates are infective for buffalo (Steward et al., 1996; Gubbels et al., 2002; Gill, 2004). Moreover, the name *T. orientalis* is misleading as it implies that the parasite occurs only in the Far East, whereas it is known to occur all over the world. Gubbels et al. (2002) proposed that these organisms should be referred to as *T. buffeli* until more biological data becomes available for further classification, and the names *T. orientalis* and *T. sergenti* should only refer to isolates that have been previously described under these names.

2.5.5 *Theileria taurotragi* (Martin and Brocklesby, 1960)

Theileria taurotragi is a parasite of eland (*Taurotragus oryx*) and was first described from these animals in Kenya by Martin and Brocklesby (1960). However, fatal infections by this parasite in these animals have been never reported. It also infects cattle, sheep and goats (Uilenberg et al., 1982; Stagg et al., 1983). Like *T. parva*, it is transmitted by *R. appendiculatus* and *R. zambeziensis* (Uilenberg et al., 1982; Lawrence et al., 1983). It has been isolated from cattle together with *T. parva*, *T. annulata*, *T. mutans*, *T. velifera* and *T. buffeli*, from different parts of eastern and southern Africa (De Vos and Roos, 1981a; Oura et al., 2004; Bazarusanga et al., 2007; Salih et al., 2007; Sibeko et al., 2008). Infection in cattle is characterized by a transient fever and small numbers of microschizonts and piroplasms (De Vos and Roos, 1981b). In South Africa *T. taurotragi* infection has been associated with bovine cerebral theileriosis and Tzaneen disease (De Vos and Roos, 1981b; Stoltsz 1989). There are no reports of the occurrence of *T. taurotragi* from the African buffalo.

2.5.6 *Theileria velifera* (Uilenberg, 1964)

Theileria velifera was first described from cattle by Uilenberg (1964). It is a mild pathogen of the African buffalo and cattle (Noval et al., 1992; Oura et al., 2005) and is transmitted by ticks of the genus *Amblyomma* (Norval et al., 1992).

2.6 Molecular characterization and phylogeny of *Theileria* spp.

Initial studies on the characterization of *T. parva* and *T. annulata* involved the use of isoenzyme electrophoresis and monoclonal antibodies (Minami et al., 1983, Shiels et al., 1986) and RFLP analysis (Bishop et al., 1993; Geysen et al., 1999). These studies indicated that many isolates contained more than one genotype, and that different isolates have distinct phenotypic and genotypic profiles.

Molecular characterization using DNA and protein sequences has surpassed antigenic and phenotypic characterization in phylogenetic studies. DNA is suitable for studying phylogenetic relationships between organisms as it is passed down ancestral lineages and therefore reliably reflects ancestry.

The most commonly used marker in the characterization of *Theileria* spp. is the small subunit ribosomal RNA (18S rRNA) gene. This gene is highly conserved between all organisms but has variable regions which differ between species. Primers can therefore be designed in the conserved areas, and these will amplify a part of the gene from all related species, and species-specific probes can be designed from the variable regions in order to differentiate between the different species. This concept has been utilized in *Theileria* research for the development of several different *Theileria* species-specific diagnostic assays (Allsopp et al., 1993; Gubbels et al., 1999; Sibeko et al., 2008; Bhoora et al., 2009; Criado-Fornelio et al., 2009; Wang et al., 2010b; Papli et al., 2010), in the identification of new species and species variants (Nijhof et al., 2003; 2005; Altay et al., 2007; Oosthuizen et al., 2008; 2009; Bosman et al., 2010; Mans et al., 2011) and in determining phylogenetic relationships between species (Chae et al., 1998; Gubbels et al., 2002).

Other genetic markers that have been used in the characterization and phylogeny of *Theileria* spp. include the large subunit rRNA (28S rRNA), 5.8S rRNA and S5 genes (Bishop et al., 1995; 2000; Mans et al., 2011), internal transcribed spacers (ITS) (Collins and Allsopp, 1999; Bosman et al., 2010; Kamau et al., 2011), polymorphic immunodominant molecule (PIM) and p150 genes (Geysen et al., 2004; Sibeko et al., 2011); p67 gene (Musoke et al., 2005; Sibeko et al., 2010), major piroplasm surface protein (MPSP) genes (Kawazu et al., 1999; Gubbels et al., 2002), and the cytochrome c oxidase gene (Kairo et al., 1994; Hikosaka et al., 2010).

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CHAPTER 3

Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African buffalo (*Syncerus caffer*) in southern Africa

3.1 Abstract

Theileria parva is the causative agent of Corridor disease in cattle in South Africa. The African buffalo (*Syncerus caffer*) is the reservoir host, and, as these animals are important for eco-tourism in South Africa, it is compulsory to test and certify them disease free prior to translocation. A *T. parva*-specific real-time polymerase chain reaction (PCR) test based on the small subunit ribosomal RNA (18S rRNA) gene is one of the tests used for the diagnosis of the parasite in buffalo and cattle in South Africa. However, because of the high similarity between the 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo), the latter is also amplified by the real-time PCR primers, although it is not detected by the *T. parva*-specific hybridization probes. Preliminary sequencing studies have revealed a small number of sequence differences within the 18S rRNA gene in both species but the extent of this sequence variation is unknown.

The aim of the current study was to sequence the 18S rRNA genes of *T. parva* and *Theileria* sp. (buffalo), and to determine whether all identified genotypes can be correctly detected by the real-time PCR assay. The reverse line blot (RLB) hybridization assay was used to identify *T. parva* and *Theileria* sp. (buffalo) positive samples from buffalo blood samples originating from the Kruger National Park, Hluhluwe-iMfolozi Park, the Greater Limpopo Transfrontier Park, and a private game ranch in the Hoedspruit area. *Theileria parva* and *Theileria* sp. (buffalo) were identified in 42% and 28%, respectively, of 252 samples, mainly as mixed infections. The full-length 18S rRNA gene of selected samples was amplified, cloned and sequenced. From a total of 20 sequences obtained, 10 grouped with previously published *T. parva* sequences from GenBank while 10 sequences grouped with a previously published *Theileria* sp. (buffalo) sequence. All these formed a monophyletic group with known pathogenic *Theileria* species. Our phylogenetic analyses confirm the distinction between *Theileria* sp. (buffalo) and *T. parva* and indicate the existence of a single group of *T. parva* and two *Theileria* sp. (buffalo) 18S rRNA gene variants in the African buffalo.

Despite the observed variation in the full-length parasite 18S rRNA gene sequences, the area in the V4 hypervariable region where the RLB and real-time PCR hybridization probes were developed was relatively conserved. The *T. parva* specific real-time PCR assay was able to successfully detect the *T. parva* variants and, although amplicons were obtained from *Theileria* sp. (buffalo) DNA, none of the *Theileria* sp. (buffalo) 18S rRNA sequence variants were detected by the *T. parva*-specific hybridization probes.

3.2. Introduction

The haemoprotozoan parasite, *Theileria parva*, transmitted mainly by the ticks *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis*, is the causative agent of Corridor disease in cattle in South Africa, and East Coast fever (ECF) in eastern and central Africa (Norval et al., 1991; Uilenberg, 1999). ECF was introduced into South Africa in the early 1900s and eventually eradicated in the 1950s, but a different form of theileriosis, Corridor disease, persists (Lawrence et al., 1994). The African buffalo (*Syncerus caffer*) is the reservoir host; infections are asymptomatic in buffalo but potentially fatal in cattle. It is thought that Corridor disease is transmitted mainly from buffalo to cattle but not between cattle, as infected bovines usually die before piroplasms appear or piroplasms are too few to infect new ticks (Lawrence et al., 1994; Uilenberg, 1999). Cattle that survive an acute ECF infection are able to mount an immune response that results in an asymptomatic carrier state and therefore become sources of infection for tick vectors (Potgieter et al., 1988; Norval et al., 1991; Altay et al., 2008, Beck et al., 2009). The strict control measures that were put in place to control theileriosis in South Africa after the eradication of ECF were designed to prevent the creation of carrier animals and the subsequent spread of the disease in the cattle population.

In South Africa, *T. parva* is endemic in buffalo in the Kruger National Park (KNP) and the Hluhluwe-iMfolozi Park, and Corridor disease occurs in neighbouring farms and game parks where cattle and buffalo are in close contact in the presence of vector ticks (Collins, 1997; Mashishi, 2002). In addition to *T. parva*, the African buffalo is the natural host of the relatively benign *Theileria mutans* and the apathogenic *Theileria velifera*, both of which are transmitted by *Amblyomma hebraeum* (Norval et al., 1992). *Theileria buffeli* and the hitherto uncharacterized *Theileria* sp. (buffalo) have also been identified in some buffalo populations in South Africa (Stoltsz, 1996; Zweygarth et al., 2009) but the local tick vectors of these parasites are unknown. *Theileria* sp. (buffalo) was first reported in 1993 from a buffalo in Kenya (Allsopp et al., 1993), and very little is known about this parasite.

Buffalo play an important role in the epidemiology of several other livestock diseases in South Africa, including foot-and-mouth disease, bovine brucellosis and bovine tuberculosis. Infected buffalo are isolated by approved fences in national and provincial game parks as well as in a limited number of buffalo breeding projects, under veterinary supervision. Farming of buffalo and cattle on the same farm is not allowed. In South Africa, buffalo are important for eco-tourism and this has led to an increasing demand for buffalo (Collins et al., 2002).

3.3. Materials and Methods

3.3.1 Blood samples and DNA extraction

Ninety-eight blood samples spotted on filter-paper, collected from buffalo in the Kruger National Park (KNP), and 100 whole blood samples (in EDTA) collected from buffalo in the Hluhluwe-iMfolozi Park (HIP), South Africa, were investigated. Five buffalo blood samples from a private game ranch located in the Hoedspruit area (H) bordering the KNP and 49 samples from the Greater Limpopo Transfrontier Park (GLTP, Mozambique) close to the KNP border were also included in the study. Genomic DNA was extracted from the filter paper blood spots using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany) and from whole blood using the High Pure Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' protocols. DNA was eluted in 100 µl elution buffer and stored at -20 °C pending further analysis.

3.3.2 PCR amplification and reverse line blot (RLB) assay

The V4 hypervariable region of the piroplasm 18S rRNA gene was amplified using the *Theileria* and *Babesia* genus-specific primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof et al., 2003; 2005). Platinum Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group, South Africa) was used to prepare PCRs according to the manufacturer's instructions. Each reaction contained 2.5 µl genomic DNA (~75 ng), 0.1 µM of each primer, 3 mM $MgCl_2$, 200 µM each of dGTP, dATP and dCTP, 400 µM dUTP, 0.75 U Platinum® Taq DNA polymerase, 0.5 U uracil deoxy-glycosylase (UDG) and nuclease-free water to a total volume of 25 µl. Amplification was done using a touchdown PCR programme as previously described (Nijhof et al., 2003). DNA from a *T. parva* positive buffalo, KNP 102 (Sibeko et al., 2008) and water were used as positive and negative controls respectively and were included in all amplifications. Amplicons were analysed on 2% ethidium bromide stained agarose gels and then screened by the RLB hybridization assay as previously described (Gubbels et al., 1999; Nijhof et al., 2005). The *Theileria* and *Babesia* genus- and species-specific oligonucleotide probes that were used are shown in Table 3.1.

Table 3.1: *Theileria* and *Babesia* oligonucleotide probe sequences used in this study. The degenerate position R denotes either A or G, W denotes either A or T, and Y denotes either C or T.

Oligonucleotide probe	Sequence (5' – 3')	Reference
Theileria/Babesia genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels et al., 1999
Theileria genus-specific	ATT AGA GTG TTT CAA GCA GAC	Nijhof[a] (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof et al., 2003
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al., 1999
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al., 1999
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al., 2008
Babesia canis canis	TGC GTT GAC GGT TTG AC	Matjila et al., 2004
Babesia canis rossi	CGG TTT GTT GCC TTT GTG	Matjila et al., 2004
Babesia canis vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al., 2004
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Nijhof et al, 2003
Babesia felis	TTA TGC TTT TCC GAC TGG C	Bosman et al., 2007
Babesia major	TCC GAC TTT GGT TGG TGT	Georges et al., 2001
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof et al., 2003
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges et al., 2001
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al., 2003
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels et al., 2000
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler et al., 2008
Theileria lestoquardi	CTT GTG TCC CTC CGG G	Schnittger et al., 2004
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels et al., 1999
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof et al., 2003
Theileria separata	GGT CGT GGT TTT CCT CGT	Schnittger et al., 2004
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T	Oura et al., 2004
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof et al., 2005
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof et al., 2005
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al., 1999
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels et al., 1999

[a] Dr. Ard M. Nijhof, (Faculty of Veterinary Medicine, UtrechtUniversity, Utrecht, The Netherlands)

3.3.3 Amplification, cloning and sequencing of the 18S rRNA gene

Thirteen selected samples that tested positive for *T. parva* and/or *Theileria* sp. (buffalo) on RLB were characterized by cloning and sequencing of the near full-length 18S rRNA gene. The full length (~1 700 bp) genes were amplified by conventional PCR using forward primer Nbab-1F (AAG CCA TGC ATG TCT AAG TAT AAG CTT TT) and reverse primer TB 18S-Rev (GAA TAA TTC ACC GGA TCA CTC G) (Oosthuizen et al., 2008; Bhoora et al., 2009). The High Fidelity PCR Master System (Roche Diagnostics, Mannheim, Germany) was used to prepare PCRs according to the manufacturer's instructions. Each reaction contained 2.5 µl (~75 ng) genomic DNA, 0.1 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTPs, High Fidelity Enzyme blend (concentration unknown) and nuclease-free water to a total volume of 25 µl. Amplification was performed using an initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 30 sec, 60°C for 45 sec and 72°C for 1 min. Final extension was at 72°C for 7 min. For each sample, four PCR reactions were done and the resulting amplicons were pooled as previously reported (Oosthuizen et al., 2008; 2009). The QIAquick PCR Purification Kit (Qiagen, Southern Cross Biotechnologies) was used for PCR product purification. Purified products were then ligated into the pGEM-T Easy Vector and transformed into JM109 High Efficiency Competent cells (Promega, Madison, WI). At least 5 white colonies were selected per sample and screened by colony PCR using primers RLB-F2 and RLB-R2. Recombinant plasmid DNA was extracted from overnight bacterial cultures using the High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

Sequencing was performed using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), 350 ng plasmid DNA and 3.2 pmol of primer RLB-F2 as an initial screen. The sequences obtained were subjected to a BLASTn homology search (Altschul et al., 1990). The full-length 18S rRNA gene of those recombinants with sequences closely related to the published 18S rRNA gene sequences of *T. parva* or *Theileria* sp. (buffalo) were sequenced using the vector primers SP6 (5'-TTA TAC GAC TCA CTA TAG GG-3') and T7 (5'-TAT TTA GGT GAC ACT ATA-3') (http://www.promega.com), and internal sequencing primers, RLB-F2, RLB-R2, Nbab_1F,18SRev-TB, BT18S_2F (GGG TTC GAT TCC GGA GAG GG), BT18S_3F (GGG CAT TCG TAT TTA ACT GTC AGA GG), BT18S_4F (CGG CTT AAT TTG ACT CAA CAC GGG), BT18S_4R (CCC TCT CCG GAA TCG AAC CC) (Oosthuizen et al., 2008; Bhoora et al., 2009). Purified sequencing reactions were analyzed on an ABI3100 genetic analyzer at the ARC-OVI (South Africa) sequencing facility.

3.3.4 Phylogenetic analysis

Sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield et al., 1995; Staden et al., 2000) and aligned with sequences of related genera from Genbank using the MAFFT v5 multiple sequence alignment programme (Katoh et al., 2005). The alignment was truncated to the size of the smallest sequence using BioEdit v7 (Hall, 1999). The TrN + I +G model was determined to be the best-fit for the data by using the Modeltest v3.7 software package (Posada and Crandall, 1998) and was subsequently used in the construction of the phylogenetic trees. A total of 44 sequences with 1590 characters were analysed. Phylogenetic trees were constructed by the neighbor-joining (Saitou and Nei, 1987), maximum parsimony and maximum likelihood methods using PAUP* v4b10 (Swofford, 2003). Distance and parsimony methods were done in combination with the bootstrap method (Felsenstein, 1985) using 1 000 replicates/tree for each method. Bayesian analysis was done using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). In all instances, the 18S rRNA sequences of *Sarcocystis muris* (M64244), *Prorocentrum micans* (M14649) and *Toxoplasma gondii* (X68523) were included as outgroups to root the phylogenetic trees. All consensus trees were edited using the MEGA 4 (Tamura et al., 2007) software package.

3.3.5 Real-time PCR

The thirteen selected field samples as well as the *Theileria parva* clones (H/240/b, H/240/c, H/240/e, H/241/a, H/241/e, KNP/B15/d, H/B15/e, GLTP/6/c, GLTP/13/j, KNP/Q15/b) and *Theileria* sp. (buffalo) clones (HIP/A21/a, KNP/G2/d, HIP/H22/c, HIP/H22/e, KNP/K1/c, GLTP/20/a, KNP/Q4/c, KNP/Q15/c, KNP/Q15/e,KNP/V8/b) were subjected to the *T. parva* specific real-time PCR assay as previously described (Sibeko et al., 2008). The amplification mixture contained 2x LightCycler-FastStart DNA Master^{Plus} Hybridization Probes mix (Roche Diagnostics, Mannheim, Germany), 0.5 µM of the *T. parva* specific forward primer (5'-CTG CAT CGC TGT GTC CCT T-3'), 0.5 µM of the reverse primer (5'-ACC AAC AAA ATA GAA CCA AAG TC-3'), 0.1 µM of each hybridization probe (*T. parva* anchor: 5'-GGG TCT CTG CAT GTG GCT TAT--FL); *T. parva* sensor: (5'-LCRed640-TCG GAC GGA GTT CGC T--PH); *Theileria* genus anchor: (5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT--FL); *Theileria* genus sensor: 5'-LCRed705-(5'-GCC TTG AAT AGT TTA GCA TGG AAT--PH), 1U UDG (Roche Diagnostics, Mannheim, Germany) and 2.5 µl (~ 0.15 ng) of DNA in a final volume of 20 µl. Temperature cycling was performed in a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany). The UDG was activated at 40°C for 10 min before the FastStart *Taq* DNA polymerase activation step of 10 min at 95°C.

The amplification programme included 45 cycles of 95°C for 10 sec, 58°C for 10 sec, and 72°C for 15 sec. Following amplification, a melting curve analysis was performed by heating the samples from 40°C to 95°C with a heating rate of 0.2°C/sec. Fluorescence values were measured at 640 and 705 nm.

3.3.6 Nucleotide sequence accession numbers

The 18S rRNA gene sequences of the sequences identified in this study have been submitted to GenBank with accession numbers HQ895968– HQ895987.

3.4 Results

3.4.1 RLB Results

The RLB results obtained from the buffalo samples from the four study areas demonstrated the presence of *T. parva*, *T. mutans*, *T. velifera*, *T. buffeli* and *Theileria* sp. (buffalo) in 174 samples (69.0%), either as single or as mixed infections. The most commonly occurring *Theileria* spp. present in buffalo from the KNP were *T. mutans* (52.0%), *T. parva* (45.9%) and *T. velifera* (29.6%) (Figure 3.1). In the HIP, *T. buffeli* (55.0%) was the most prevalent *Theileria* spp. followed by *Theileria* sp. (buffalo) (38.0%) and *T. parva* (29.0%). *Theileria parva* (59.2%) and *T. mutans* (34.7%) were the most common species in the Greater Limpopo Transfrontier Park. *Theileria buffeli* was not detected in buffalo from the KNP and the Greater Limpopo Transfrontier Park. In 17.1% of the samples (n = 43), the PCR products hybridized only with the *Theileria* and/or the *Babesia/Theileria* genus-specific probes and not with any of the *Babesia* or *Theileria* species-specific probes. Thirty-five samples (13.9%) were negative (or below detection limit of the test) for the presence of *Theileria* species (Table 3.2).

The results indicated that 26.2% (n = 66) of the samples had single infections, while multiple infections with two or more species were found in 42.9% (n = 108) of the samples (Table 3.2). Details of the multiple *Theileria* spp. infections in buffalo in KNP, HIP, the GLTP, and private game ranch in Hoedspruit are shown in Figure 3.2. A total of 18 different combinations of multiple infections were found from the four localities. Double and triple infections with *T. velifera* and *T. mutans*, and *T. velifera*, *T. mutans*, *T. parva* were the most common combinations (Figure 3.2).

The RLB results obtained from the samples collected from buffalo from the game ranch near Hoedspruit revealed a single infection of *T. parva* in one sample, and mixed *Theileria* spp. infections in the other four specimens (Figure 3.2). None of these five samples tested positive for the presence of *T. mutans* or *T. buffeli* DNA.

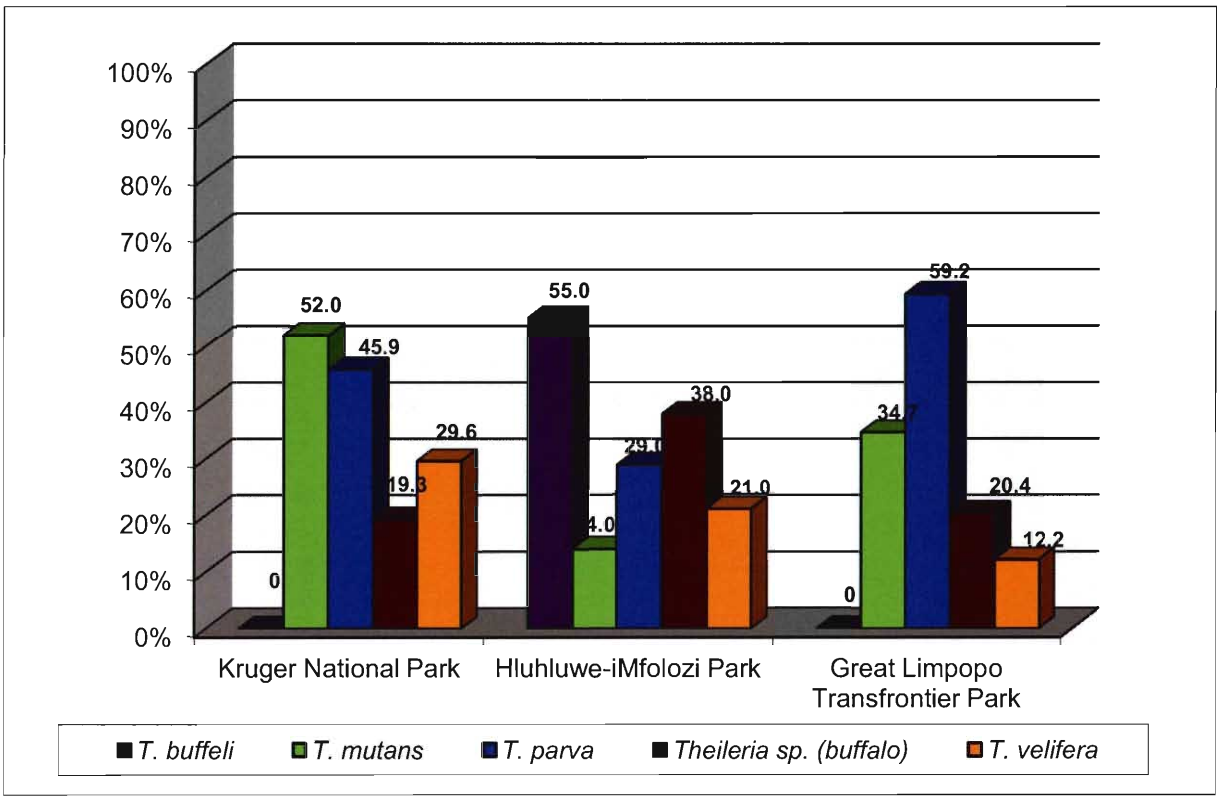


Figure 3.1: Occurence of *Theileria* spp. infections in buffalo blood samples from the Kruger National Park (KNP), Hluhluwe-iMfolozi Game Park (HIP) and the Greater Limpopo Transfronteir Park (GLTP), South Africa, as determined by the Reverse Line Blot hybridization assay.

Table 3.2: The occurrence of *Theileria* species infections in buffalo blood samples from four localities in South Africa and Mozambique as determined by the RLB hybridization assay.

	Kruger National Park (n = 98)	Hluhluwe-iMfolozi Park (n = 100)	Private game ranch Hoedspruit (n = 5)	Greater Limpopo Transfrontier Park (n = 49)	TOTAL (n= 252)
Single infections:	23 (23.4%)	21 (21%)	1 (20%)	21 (42.9%)	**66** (26.2%)
T. buffeli	0	17 (17.0%)	0	0	**17** (6.8%)
T. mutans	10 (10.2%)	0	0	4 (8.2%)	**14** (5.6%)
T. parva	10 (10.2%)	2 (2.0%)	1 (20.0%)	13 (26.5%)	**26** (10.3%)
Theileria sp. (buffalo)	2 (2.0%)	2 (2.0%)	0	4 (8.2%)	**8** (3.2%)
T. velifera	1 (1.0%)	0	0	0	**1** (0.4%)
Mixed *Theileria* spp. infection:	47 (47.9%)	40 (40.0%)	4 (80.0%)	17 (34.7%)	**108**(42.9%)
T. buffeli	0	38 (38%)	0	0	**38** (15.1%)
T. mutans	41 (41.8%)	14 (14%)	0	13 (26.5%)	**68** (30.0%)
T. parva	35 (35.7%)	27 (27%)	1 (20.0%)	16 (32.7%)	**79** (31.3%)
Theileria sp. (buffalo)	17 (17.3%)	36 (36%)	3 (60.0%)	6 (12.2%)	**62** (24.6%)
T. velifera	28 (28.6%)	21 (21%)	4 (80%)	6 (12.2%)	**59** (23.4%)
Theileria/Babesia genus-specific only	22 (22.5%)	21 (21.0%)	0	0	**43** (17.1%)
Negative/below detection limit	6 (6.1%)	18 (18.0%)	0	11 (22.5%)	**35**(13.9%)

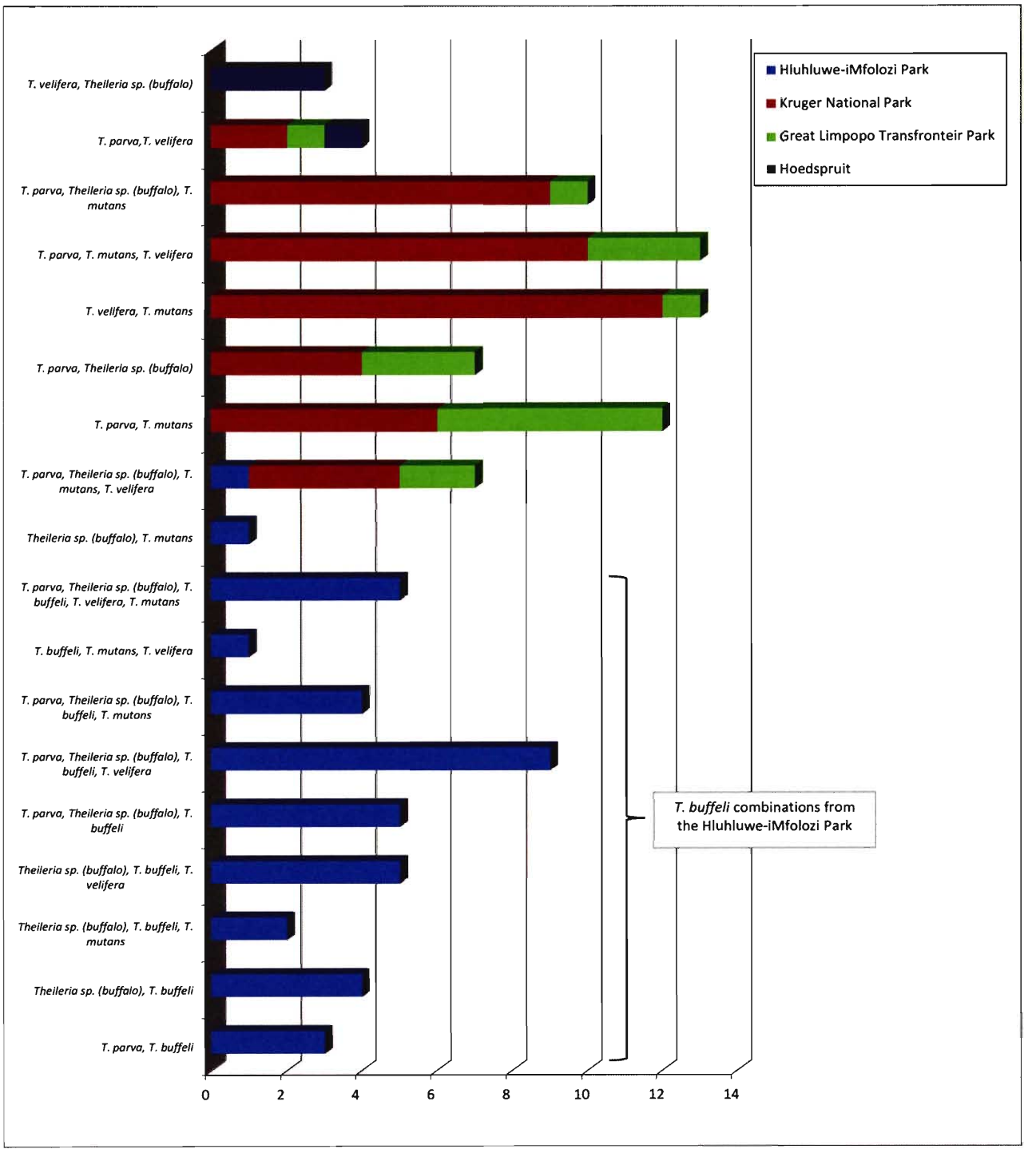


Figure 3.2: Composition of mixed *Theileria* spp. infections, as determined using the RLB hybridization assay, in buffalo blood samples from the Kruger National Park, Hluhluwe-iMfolozi Park, Greater Limpopo Transfrontier Park and a private game farm in Hoedspruit.

3.4.2 Sequencing and phylogenetic results

Near full-length parasite 18S rRNA genes from 13 selected samples that were positive for *T. parva* and/or *Theileria* sp. (buffalo) on RLB were amplified and cloned, and of 54 clones sequenced, 23 *T. parva* and *Theileria* sp. (buffalo) clones were identified. The RLB results of these 13 samples (Table 3.3) indicated that all the samples had single or mixed *Theileria* spp. infections. Since recombinant DNA molecules can be generated during the PCR when multiple templates containing homologous regions are present (Wang and Wang, 1997; Thompson et al., 2002), the sequences were compared across their full-length to identify possible PCR artifacts and these were eliminated from the dataset. The sequences from three clones, GLTP/6/g, KNP/V8/a and KNP/G8/b were shown to be recombinants between *T. parva* and *T. mutans*; *T. parva*, *Theileria* sp. (buffalo) and *T. mutans*; and *Theileria* sp. (buffalo) and *T. mutans* respectively. BLASTn homology searches indicated that the remaining 20 clones were closely related (99.9% identity) to published 18S rRNA gene sequences of cattle-derived *T. parva* [Accession numbers: L02366 (Allsopp et al., 1993), L28999 (Kibe et al., 1994)], buffalo-derived *T. parva* [Accession numbers: AF013418 (Collins and Allsopp, 1999) and HQ684067 from buffalo 102 (Sibeko et al., 2008)], and *Theileria* sp. (buffalo) [Accession no. DQ641260 (Sibeko et al., 2008)].

Table 3.3: Origin and results of buffalo blood samples selected for 18S rRNA gene characterization

Sample nr	Place of Origin (Locality)	RLB results	*T. parva* real-time PCR results	Clone	Sequence length (bp)	Phylogenetic classification
KNP/B15	KNP[a] (Masorini)	*T. parva*	+	d	1582	*T. parva*
				e	1583	*T. parva*
KNP/G2	KNP (Mahubyeni)	*T. parva*, *T. mutans*	+	d	1581	*Theileria* sp. (bougasvlei)[e]
KNP/K1	KNP (Graspan Dam)	*T. parva*, *T. mutans*, *T. velifera*	+	c	1581	*Theileria* sp. (bougasvlei)[e]
KNP/Q4	KNP (Shikokola)	*T. parva*, *Theileria* sp. (buffalo), *T. mutans*	+	c	1583	*Theileria* sp. (buffalo)
KNP/Q15	KNP (Shikokola)	*T. parva*, *Theileria* sp. (buffalo)	+	b	1583	*T. parva*
				c	1581	*Theileria* sp. (bougasvlei)[e]
				e	1581	*Theileria* sp. (bougasvlei)[e]
KNP/V8	KNP (Manqeva)	*T. parva*, *Theileria* sp. (buffalo), *T. mutans*	+	b	1583	*Theileria* sp. (buffalo)
HI/A21	HI[b]	*Theileria* sp. (buffalo) (faint signal)	-	a	1583	*Theileria* sp. (buffalo)
HI/H22	HI	*T. parva*, *T. buffeli*	+	c	1583	*Theileria* sp. (buffalo)
				e	1583	*Theileria* sp. (buffalo)
Hoed/240	PGR[c], Hoedspruit	*T. parva*	+	b	1583	*T. parva*
				c	1583	*T. parva*
				e	1583	*T. parva*
Hoed/241	PGR, Hoedspruit	*T. parva*, *Theileria* sp. (buffalo), *T. velifera*	+	a	1583	*T. parva*
				e	1583	*T. parva*
Mz/6	GLTP[d], Mozambique	*T. parva*	+	c	1583	*T. parva*
Mz/13	GLTP,	*T. parva*	+	j	1581	*T. parva*
Mz20	GLTP,	*T. parva*, *Theileria* sp. (buffalo)	+	a	1583	*Theileria* sp. (buffalo)

[a]KNP = Kruger National Park [b] HIP = Hluhluwe-iMfolozi Park [c] H, Hoedspruit = Private game ranch, Hoedspruit [d] GLTP = Greater Limpopo Transfrontier Park
[e]*Theileria* sp. (bougasvlei) 18S rRNA sequences differed from the published *Theileria* sp. (buffalo) sequence (DQ641260) by one nucleotide substitution and one deletion in the region under the RLB probe, explaining why *Theileria* sp. (buffalo) was not detected in some of these samples using RLB.

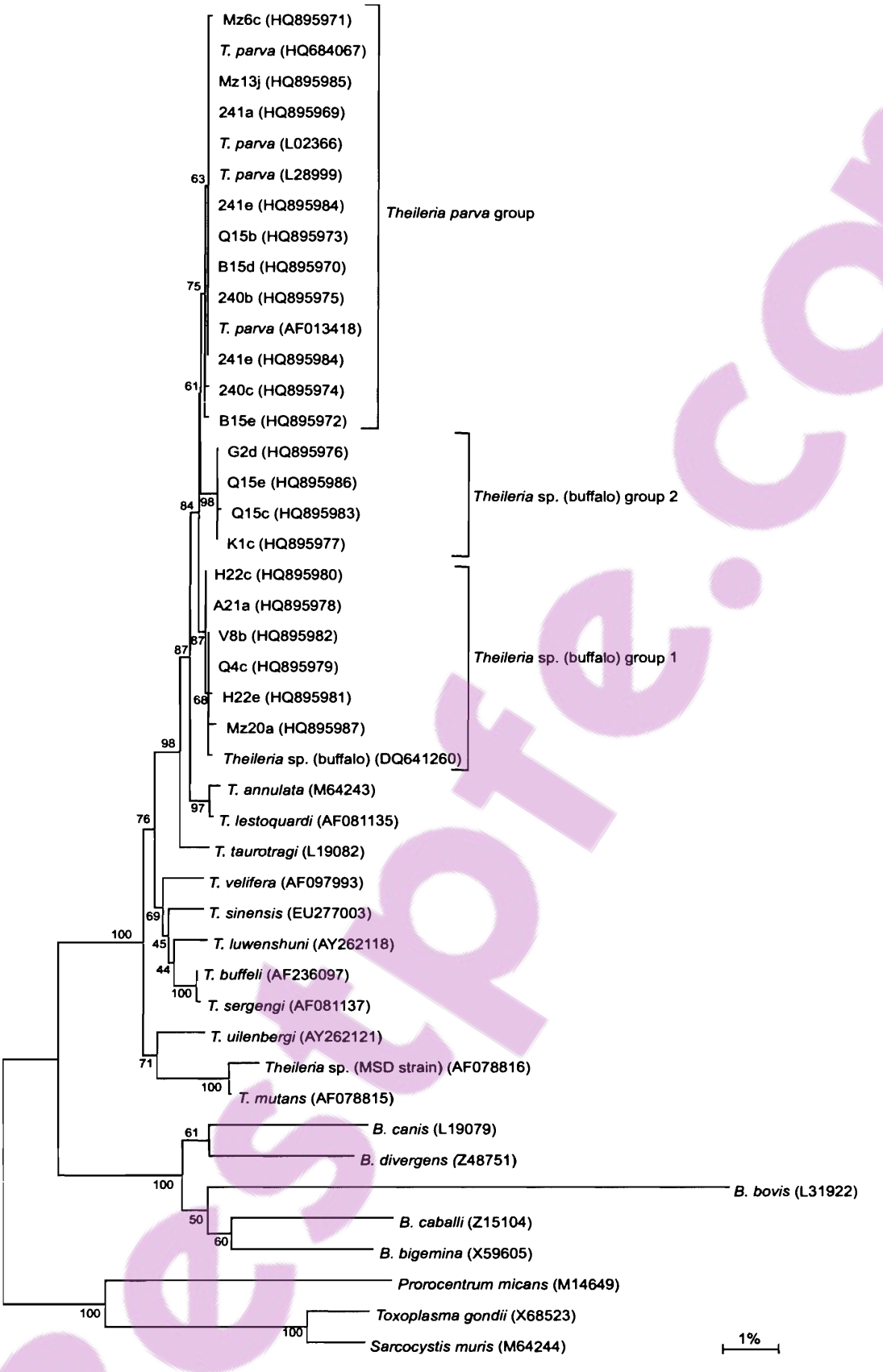
The previously published 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo) differ by 11 bp (Table 3.4). Estimated evolutionary divergences between the *T. parva* and *Theileria* sp. (buffalo) sequences obtained in this study were compared by determining the number of base differences per sequence. Some sequences were identical, whereas others differed from each other by between 1 and 15 bp (Table 3.4).The sequences of clones H/240/e, H/241/a, H/241/e and KNP/B15/d were identical to the published *T. parva* 18S rRNA gene sequences, AF013418, L02366 and L28999. The sequences of clones KNP/Q15/b and H/240/b were identical to the South African buffalo-derived *T. parva* 18S rRNA gene sequence, HQ684067, which differs by 1 base pair from the other published *T. parva* sequences. The sequences of the remaining four *T. parva* clones differed from the published sequences by 1 to 3 nucleotides.

None of the *Theileria* sp. (buffalo) sequences were identical to the published *Theileria* sp. (buffalo) sequence, DQ641260, although six of the sequences (HIP/A21/a, HIP/H22/c, HIP/H22/e, GLTP/20/a, KNP/Q4/c and KNP/V8/b) differed by only 1 to 3 bp. The remaining four *Theileria* sp. (buffalo) sequences (KNP/G2/b, KNP/K1/c, KNP/Q15/c and KNP/Q15/e) differed from DQ641260 by 9 or 10 nucleotides. The V4 hypervariable region of the 18S rRNA sequence from these clones is identical to the recently published partial sequence (GU570997) of *Theileria* sp. (bougasvlei) (Mans et al., 2011) (Chapter 6).

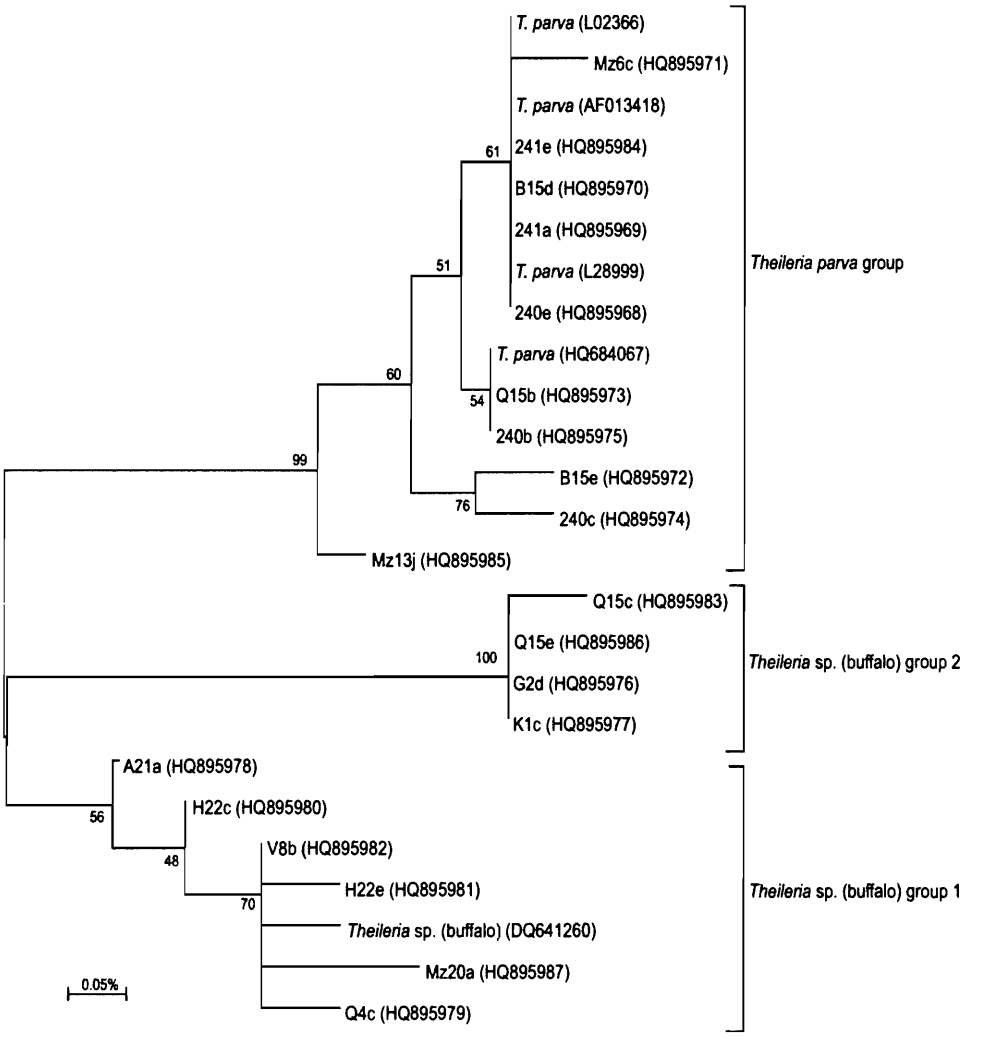
Table 3.4: Estimates of evolutionary divergence between the *T. parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequences. The number of base differences per sequence from analysis between sequences is shown. All results are based on the pairwise analysis of 25 sequences. Analyses were conducted in MEGA4 (Tamura et al., 2007). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1505 positions in the final dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	17	19	20	21	22	23	24	25
1. *T. parva* (L02366)																									
2. *T. parva* (L28999)	0																								
3. *T. parva* (AF013418)	0	0																							
4. *T. parva* (HQ684067)	1	1	1																						
5.KNP/B15d	0	0	0	1																					
6. H/240e	0	0	0	1	0																				
7. H/241e	0	0	0	1	0	0																			
8.H/241a	0	0	0	1	0	0	0																		
9. GLTP/6c	1	1	1	2	1	1	1	1																	
10. KNP/Q15b	1	1	1	0	1	1	1	1	2																
11. H/240b	1	1	1	0	1	1	1	1	2	0															
12. KNP/B15e	3	3	3	3	3	3	3	3	4	3	3														
13.H/240c	3	3	3	3	3	3	3	3	4	3	3	2													
14. GLTP/13j	3	3	3	2	3	3	3	3	4	2	2	5	5												
15. GLTP/20a	12	12	12	12	12	12	12	12	13	12	12	11	11	10											
16. KNP/Q4c	11	11	11	11	11	11	11	11	12	11	11	10	10	9	3										
17. *Theileria* sp. buffalo (DQ641260)	11	11	11	11	11	11	11	11	12	11	11	10	10	9	3	2									
18. HIP/H22e	11	11	11	11	11	11	11	11	12	11	11	10	10	9	3	2	2								
19.KNP/V8b	10	10	10	10	10	10	10	10	11	10	10	9	9	8	2	1	1	1							
20. HIP//H22c	9	9	9	9	9	9	9	9	10	9	9	8	8	7	3	2	2	2	1						
21.HIP/A21a	8	8	8	8	8	8	8	8	9	8	8	7	7	6	4	3	3	3	2	1					
22. KNP/Q15c	14	14	14	14	14	14	14	14	15	14	14	13	13	12	11	10	10	10	9	9	10				
23.KNP/G2d	13	13	13	13	13	13	13	13	14	13	13	12	12	11	10	9	9	9	8	8	9	1			
24.KNP/K1c	13	13	13	13	13	13	13	13	14	13	13	12	12	11	10	9	9	9	8	8	9	1	0		
25. KNP/Q15e	13	13	13	13	13	13	13	13	14	13	13	12	12	11	10	9	9	9	8	8	9	1	0	0	

The observed sequences similarities were confirmed by phylogenetic analyses. Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian phylogenetic analyses were used to reveal the relationships between the *T. parva* and *Theileria* sp. (buffalo) 18S rRNA gene variants and other related *Theileria* and *Babesia* species. No significant differences in the topology of the trees obtained using different algorithms, or in the bootstrap values, were found. A representative tree obtained by the neighbor-joining method is shown in Figure 3.3a. The in depth phylogenetic relationships amongst the *T. parva* and *Theileria* sp. (buffalo) variants as indicated by neighbour-joining analysis is shown in Figure 3.3b. The new *T. parva* and *Theileria* sp. (buffalo) sequences formed a monophyletic group with *Theileria taurotragi*, and the pathogenic *Theileria annulata* and *Theileria lestoquardi*. Ten sequences grouped with the previously published *T. parva* sequences and 10 sequences grouped together with the previously published *Theileria* sp. (buffalo) 18S rRNA sequence. The *T. parva* sequences formed a monophyletic group, whereas the *Theileria* sp. (buffalo) group formed two distinct clusters.



(a)



(b)

Figure 3.3: Phylogenetic tree showing (a) relationship of the *T. parva* and *Theileria* sp. (buffalo) variants identified in this study with other *Theileria* and *Babesia* species and (b) relationships amongst the *T. parva* and *Theileria* sp. (buffalo) variants based on the near full-length 18S rRNA gene sequences as indicated by neighbour-joining analysis. Values at the nodes are bootstrap values indicating the degree of support for each cluster. Vertical lengths in each tree are not significant and are merely set for clarity. GenBank accession numbers are indicated in parentheses. The tree was rooted using *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii*.

A sequence alignment of the area in which the RLB and real-time PCR probes were designed indicated that the *T. parva* real-time PCR anchor probe region was identical in all sequences (Figure 3.4). All *T. parva* sequences were identical under the *T. parva* real-time PCR sensor probe and under the *T. parva* RLB probe. In the sensor probe region, six of the *Theileria* sp. (buffalo) clones (HIP/A21/a, HIP/H22/e, HIP/H22/c, GLTP/20/a, KNP/V8/b and KNP/Q4/e) were identical in sequence to the available *Theileria* sp. (buffalo) gene sequence on GenBank (DQ641260) (Figure 3.4). It has previously been shown that this *Theileria* sp. (buffalo) sequence is not detected by the *T. parva*-specific hybridization probes because of these nucleotide differences (Sibeko et al., 2008). The remaining four *Theileria* sp. (buffalo) clones (KNP/G2/d, KNP/K1/c, KNP/Q15/c andKNP/Q15/d) had more sequence difference in this specific region (three nucleotide differences and one deletion) and are therefore also unlikely to be detected by the *T. parva*-specific hybridization probes. In addition, these four sequences differed from the *Theileria* sp. (buffalo)-specific RLB probe by one substitution and one deletion. Samples KNP/G2, KNP/K1 and GLTP/6 were negative for *Theileria* sp. (buffalo) using RLB (Table 3.3), indicating that the *Theileria* sp. (buffalo)-specific RLB probe probably does not detect this 18S rRNA sequence variant. Although the RLB results indicated that sample KNP/Q15 was *Theileria* sp. (buffalo)-positive (Table 3.3), only *Theileria* sp. (buffalo) variant sequences were identified in this sample. It is possible that RLB-detectable *Theileria* sp. (buffalo) sequences would have been identified from this sample if more clones had been examined.

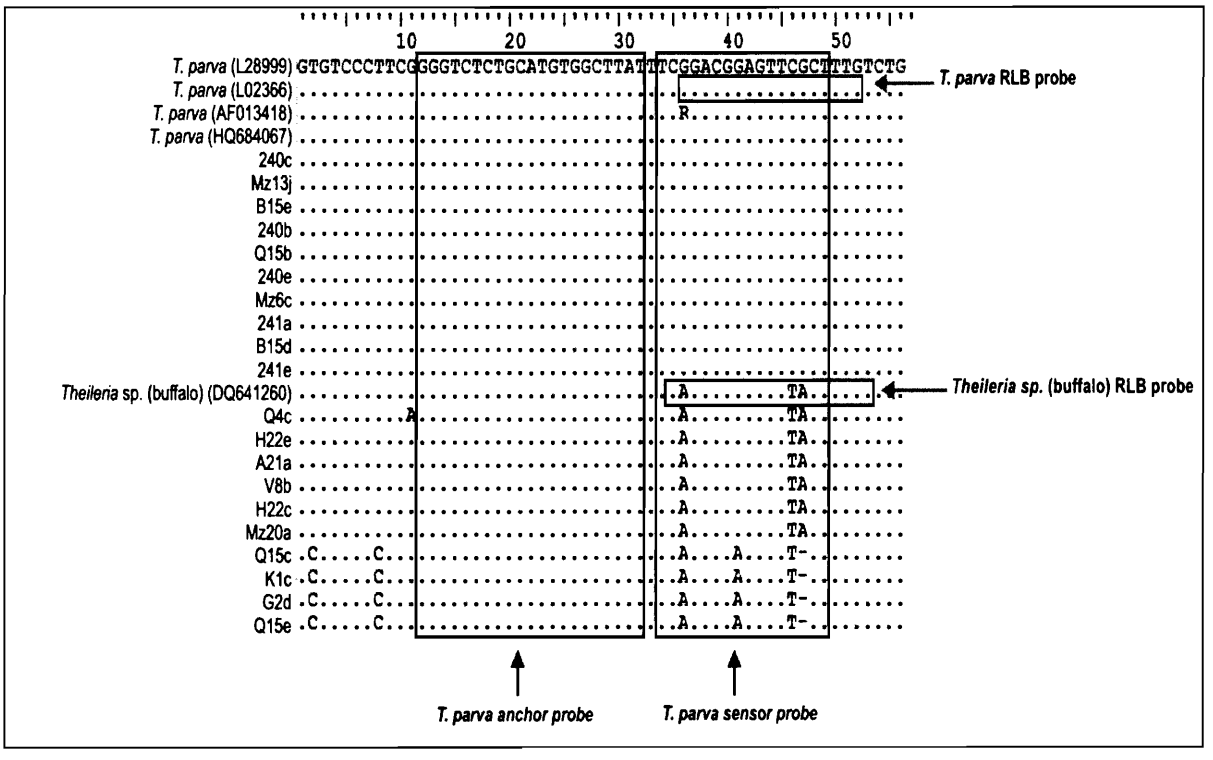
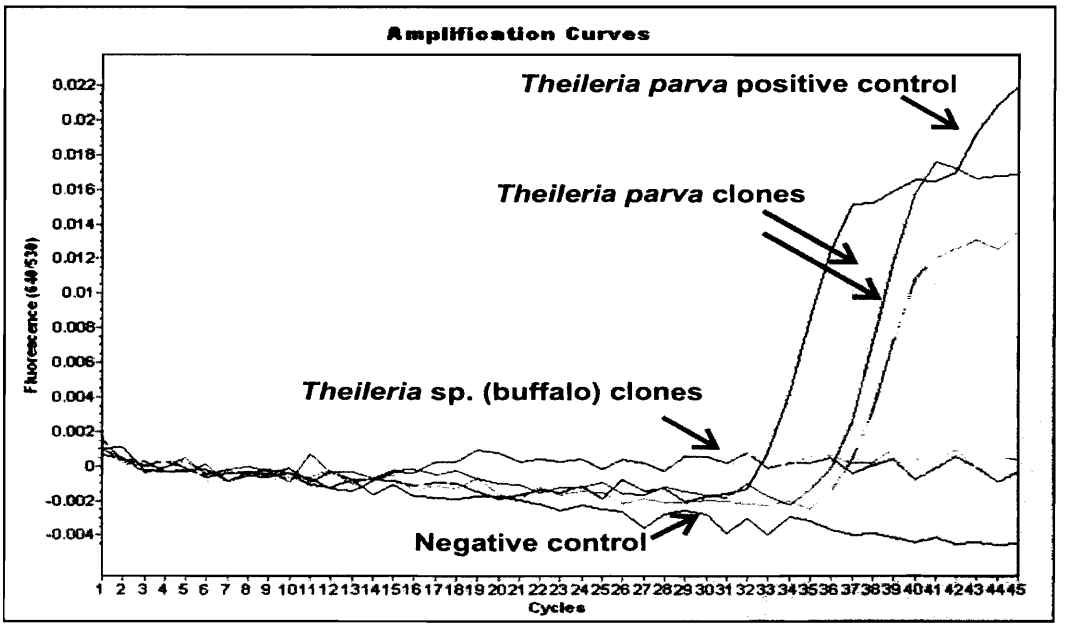


Figure 3.4: Nucleotide alignment of a 56 bp region of the V4 hypervariable region of the published 18S rRNA gene of *T. parva* and *Theileria* sp. (buffalo), as well as the variants identified in the study. Regions where the *T. parva* real-time PCR and RLB probes were designed are indicated with boxes.

3.4.3 *T. parva* real-time results

All 13 selected field samples (Table 3.3), as well as the *T. parva* and *Theileria* sp. (buffalo) clones were subsequently subjected to the *T. parva* real-time PCR assay as described by Sibeko et al. (2008). The *T. parva*-specific melting peak at 63°C was observed in the *T. parva* positive control DNA samples and in the *T. parva* clones (Figures 3.5a and b; Table 3.3). Although amplicons were obtained from *Theileria* sp. (buffalo) DNA, the *Theileria* sp. (buffalo) positive control DNA and all 10 clones identified as *Theileria* sp. (buffalo) were not detected by the *T. parva*-specific hybridization probes.

(a)



(b)

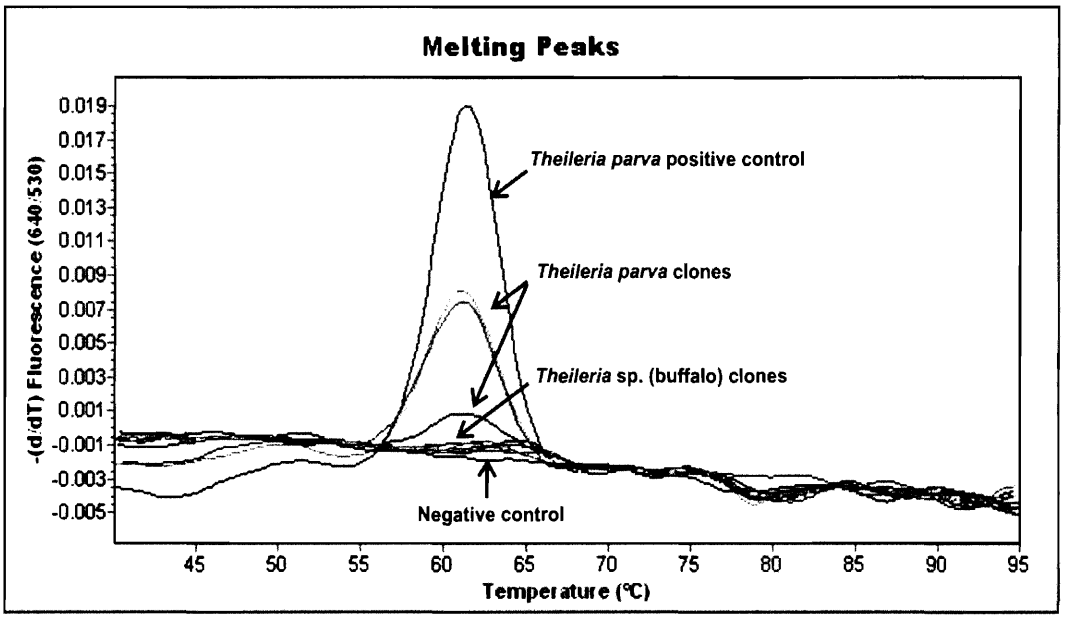


Figure 3.5: *Theileria parva*-specific real-time PCR results obtained with the cloned *T. parva* and *Theileria* sp. (buffalo) variants obtained in this study. (a) Amplification curves showing increase in fluorescence at 640 nm for both *T. parva* and *Theileria* sp. (buffalo) clones. (b) Melting curve analysis at 640 nm, showing the *T. parva*-specific melting peak at 63°C only for *T. parva* positive control and clones.

3.5. Discussion

Simultaneous detection and differentiation of *Theileria* and *Babesia* spp. by the reverse line blot hybridization assay was initially described by Gubbels and colleagues in 1999. This assay has since been used by several authors to detect these organisms in infected hosts and vectors (Gubbels et al., 2000; Georges et al., 2001; Nijhof et al., 2003, 2005; Schnittger et al., 2003; Brigido et al., 2004; Nagore et al., 2004; Oosthuizen et al., 2008, 2009; Oura et al., 2011). In our study, the RLB results demonstrated the presence of *T. parva*, *T. mutans*, *T. velifera*, *T. buffeli* and *Theileria* sp. (buffalo), either as single or as mixed infections, in the buffalo blood samples collected in the Kruger National Park, Hluhluwe-iMfolozi Park, Greater Limpopo Transfrontier Park and from a game ranch near Hoedspruit. This was not an unexpected finding as it is well-known that the African buffalo is the natural reservoir host of both pathogenic and non-pathogenic *Theileria* species (Young et al., 1978; Uilenberg, 1995). In our study, a total of 18 different combinations of mixed infections by these parasites were observed. Salih et al. (2007) reported 17 combinations of these species (together with *T. taurotragi*, *B. bovis*, *B. bigemina*) from cattle in Southern Sudan. In both studies co-infection by *T. parva*, *T. mutans* and *T. velifera* was the most common combination. Oura et al. (2011) identified these *Theileria* species in buffalo samples originating from four geographically different national parks in Uganda, with prevalences of 0% - 95% for *T. parva*, 92 - 100% for *T. mutans*, 80 - 100% for *T. velifera*, and 0 - 100% for *T. buffeli* and *Theileria* sp. (buffalo).

Theileria mutans and *T. parva* were the most prevalent *Theileria* spp. present in buffalo from the KNP and the Greater Limpopo Transfrontier Park, but *T. buffeli* was not detected in these buffalo, or from five buffalo from the game ranch near Hoedspruit. In contrast, *T. buffeli* was the most prevalent *Theileria* sp. in the Hluhluwe-iMfolozi Park, followed by *Theileria* sp. (buffalo) and *T. parva*. We have also detected *T. buffeli* DNA in buffalo from the Addo Elephant National Park in the Eastern Cape Province (data not shown). In Australia, Asia and Europe, *T. buffeli* is transmitted by ticks of the genus *Haemaphysalis* but the vector is still unknown in America and Africa (Uilenberg, 1995; M'ghirbi et al., 2008). Our results suggest that the tick vector of *T. buffeli* is not present in the KNP, the Greater Limpopo Transfrontier Park and the game ranch near Hoedspruit but is present in the Hluhluwe-iMfolozi region of KwaZulu-Natal and in the Eastern Cape. Although detailed transmission studies are needed to elucidate the vector of *T. buffeli* in South Africa, *Haemaphysalis silacea* ticks could be a possible vector. This tick has a wide host range (Horak et al., 1983) and it has been recovered from nyalas in Umfolozi (now Hluhluwe-iMfolozi), Mkuzi and Ndumu game parks in north-eastern KwaZulu-Natal, and from kudu in the Addo

Elephant National Park and Andries Vosloo Kudu Reserve in the Eastern Cape, but not from animals in the KNP (Horak et al., 1992, 1995).

The RLB hybridization assay was not able to detect any *Theileria* and/or *Babesia* spp. in 13.9% of the samples.These could be either true negative results or due to a very low *Theileria* spp. parasitaemia which was below the detection limit of the test. The RLB hybridization assay has previously been shown to be able to detect *Theileria* and *Babesia* spp. at a parasitaemia of $1x10^{-6}$%, enabling detection of the carrier state of most parasites (Gubbels et al., 1999). Bhoora et al. (2010) demonstrated that real-time PCR is more sensitive than the RLB hybridization assay and that *Theileria* and *Babesia* spp. infections can be detected by real-time PCR in samples that test negative by the RLB hybridization assay. The occurence of *Theileria* sp. (buffalo) as indicated by RLB is under-represented in our study, and in all previous RLB studies, since the *Theileria* sp. (buffalo)-specific RLB probe does not detect the novel *Theileria* sp. (buffalo) variant identified here. This highlights an inherent limitation of the RLB: a novel species or variant will not be detected by RLB in a mixed infection containing parasites for which species-specific probes are incorporated in the assay.

The 18S rRNA sequences identified in this study were shown to be highly similar to the published *T. parva* and *Theileria* sp. (buffalo) sequences and intraspecific variation in the 18S rRNA genes of both species was revealed. Although the published *T. parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequences are highly similar (11 bp difference over a 1501 bp region), Zweygarth et al. (2009) indicated that *Theileria* sp. (buffalo) seems not to be merely a region-specific genetic variant of *T. parva* circulating in South Africa. Our phylogenetic analyses confirm the distinction between *Theileria* sp. (buffalo) and *T. parva* and further indicate the existence of a single group of *T. parva* and two *Theileria* sp. (buffalo) 18S rRNA gene variants in the African buffalo. In a recent study, Mans et al. (2011) cloned and sequenced the V4 hypervariable region of the *Theileria* 18S rRNA gene from 62 buffalo and 49 cattle samples and identified, in addition to the *T. parva* genotypes identified in our study, these authors identified two additional *T. parva* variants from buffalo and five from cattle.

Although variation in the 18S rRNA gene sequence has been widely used to characterize and classify previously unknown *Theileria* and *Babesia* parasites (Birkenheuer et al., 2004; Gubbels et al., 2000; Nijhof et al., 2003; 2005; Schnittger et al., 2003; Oosthuizen et al., 2008), there is no universally used criterion for classifying organisms to species level based on this variation (Chae et al., 1999).

It remains difficult to establish how much 18S rRNA gene sequence variation must exist for the source organism to be considered a different species or to be considered merely a variant/genotype of a species (Allsopp and Allsopp, 2006; Chae et al., 1999). Based on this, as well as the fact that we do not have any data on the morphology of the parasites, their possible vectors or their role in clinical disease, we suggest that these variants/genotypes cannot be classified as new *Theileria* species, but rather as variants of *T. parva* and *Theileria* sp. (buffalo).

Our phylogenetic analyses showed that the *T. parva* and *Theileria* sp. (buffalo) variants grouped together with other pathogenic *Theileria* spp., namely, *T. annulata* and *T. lestoquardi*. The observed relationship between these parasites has been previously reported (Allsopp et al., 1994; Katzer et al. 1998; Chae et al., 1999; Brigido et al., 2004; Nijhof et al., 2005), and is considered as an indication of a common ancestry (Allsopp et al., 1994; Katzer et al. 1998). Zweygarth et al. (2009) established a macroschizont-infected lymphoblastoid cell line from an African buffalo infected with *Theileria* sp. (buffalo), suggesting that *Theileria* sp. (buffalo) is able to transform lymphocytes. However, to date this species has only ever been identified in buffalo and not in cattle and is not pathogenic in these animals.

Despite the variation found in the full-length parasite 18S rRNA gene sequences, the area in the V4 hypervariable region where the RLB and real-time PCR hybridization probes were developed is relatively conserved. The *T. parva* specific real-time PCR assay was able to successfully detect all *T. parva* variants identified in this study and although amplicons were obtained from *Theileria* sp. (buffalo) DNA, this species and its variants were not detected by the *T. parva*-specific hybridization probes. Although the specificity of the existing *T. parva*-specific real-time PCR test does not seem to be compromised by the presence of 18S rRNA gene sequence variants as indicated in this study and by Mans et al. (2011) the close similarity between the 18S rRNA of the two species still poses a challenge to the sensitivity of the test because of competition for primers in cattle and buffalo samples which contain mixed *Theileria* sp. (buffalo) and *T. parva* infections (Sibeko et al., 2008). It is possible that other as yet unidentified *T. parva* 18S rRNA gene variants may exist. In order to develop a more sensitive diagnostic test for *T. parva*, it will be necessary to identify an alternative genetic marker that is both specific for and highly conserved in *T. parva*.

3.6 References

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CHAPTER 4

Sequence variation in the 18S rRNA gene of *Theileria mutans* and *Theileria velifera* isolated from the African buffalo (*Syncerus caffer*)

4.1 Abstract

The African buffalo (*Syncerus caffer*) is the natural reservoir host of both pathogenic and non-pathogenic *Theileria* species. These usually occur as mixed infections in infected animals. Although the benign and mildly pathogenic forms do not have any significant economic importance, their presence complicates the interpretation of results obtained from the diagnosis of the pathogenic *T. parva* in cattle and buffalo in South Africa. Although the 18S rRNA gene has been used as the target in a quantitative real-time PCR (qPCR) assay for the specific detection of *T. parva* in these animals, the extent of sequence variation within this gene in non-pathogenic *Theileria* spp. of the Africa buffalo is currently unknown. Hence the aim of this study was to characterize the 18S rRNA genes of *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera.*

We selected samples which either tested positive for several different *Theileria* spp., or which hybridized only with the *Babesia/Theileria* genus-specific probe and not with any of the *Babesia* or *Theileria* species-specific probes used in the reverse line blot (RLB) hybridization assay. The full-length 18S rRNA genes of 14 samples, originating from 13 buffalo and one bovine from different localities in South Africa, were amplified, cloned and resulting recombinants sequenced. We identified sequence variations in the 18S rRNA genes of *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera*. The variation possibly explained why the RLB hybridization assay failed to detect *T. mutans* and *T. velifera* in some of the analysed samples. As extensive variation was observed within the *T. mutans* group, two RLB oligonucleotide probes were designed to detect the *T. mutans*-like genotypes. Unfortunately these cross-hybridized with *T. mutans* DNA and could not be used to screen buffalo samples to determine the occurrence of these genotypes in buffalo in South Africa. None of the *T. mutans* or *T. velifera* genotypes identified in this study compromise the specificity of the real-time PCR test currently used to detect *T. parva* infections in South Africa.

4.2 Introduction

Theileriosis is a widespread disease of wild and domestic ruminants caused by tick-transmitted apicomplexan parasites of the genus *Theileria* (Mehlhorn and Schein, 1984). The disease is recognized as a major threat to the livestock industry as some members of the genus may cause severe disease and mortality, and others mild or subclinical infections in their respective hosts (Mukhebi et al., 1992; Uilenberg, 1999). The most economically important *Theileria* species are *Theileria annulata* and *Theileria parva. Theileria annulata* causes tropical theileriosis in cattle in the tropical and subtropical regions of southern Europe, northern Africa, the Middle East and Central Asia, and is transmitted by ticks of the genus *Hyalomma* (Uilenberg, 1981; Dolan, 1989; Brown, 1997). *Theileria parva*, which appears to have evolved in the African buffalo (*Syncerus caffer*) (Norval et al., 1992), is by far the most pathogenic and is of significant economic importance in eastern, central and southern Africa, where it causes East Coast fever (ECF), January disease and Corridor diseasein cattle (Lawrence, 1979; Uilenberg et al., 1982; Perry et al., 1991; Mukhebi et al., 1992). Although tick-transmission experiments have implicated ten *Rhipicephalus* and three *Hyalomma* tick species as possible vectors of *T. parva*, only three species, namely, *Rhipicephalus appendiculatus*, *Rhipicephalus zambesiensis* and *Rhipicephalus duttoni* have been shown to be definite vectors (Lawrence et al., 1994; Uilenberg, 1999).

In South Africa, Corridor disease occurs when *T. parva* is transmitted from the African buffalo to cattle by tick vectors. It is an acute, usually fatal disease in cattle and is a controlled disease in South Africa. African buffalo are also thought to be the original hosts of other *Theileria* spp., namely, *Theileria mutans*, *Theileria velifera*, *Theileria buffeli,* and *Theileria* sp. (buffalo) (Norval et al., 1992; Allsopp et al., 1993; Stoltsz, 1996) and therefore play an important role in the epidemiology of theileriosis in South Africa.

Theileria mutans (Theiler, 1906) is a parasite of buffalo, it is infective to cattle and has been shown to cause latent infections in sheep (Young et al., 1978; Paling et al., 1981). It occurs in some parts of sub-Saharan Africa and is transmitted by different species of *Amblyomma* ticks (Uilenberg et al., 1976, 1982; Perie et al., 1979; de Vos and Roos, 1981; Paling et al., 1981; Musisi et al., 1984). Previously, *T. mutans* was implicated in all benign bovine *Theileria* infections worldwide (Gill, 2004). However, transmission, serology and phylogenetic studies have indicated that this parasite is an African species and is different from benign *Theileria* species isolated from cattle in other parts of the world, namely, *Theileria orientalis* and *T .buffeli* (Uilenberg et al., 1977; Chae et al., 1999; Gill, 2004). Although generally considered a benign species in buffalo, some strains of *T. mutans*

have been associated with disease in cattle (Irvin et al., 1972; Young et al., 1978; Paling et al., 1981; Saidu, 1981; Uilenberg, 1981).

Theileria sp. (strain MSD) was first identified from a naturally infected bovine at the Merck, Sharp & Dome (MSD) experimental centre at Hartebeespoort, Pretoria, South Africa (Chae et al., 1999). It was initially suspected to be a variant of *T. velifera,* but sequence and phylogenetic analyses based on 18S rRNA gene sequences indicated that it is most closely related to *T. mutans* (Chae et al., 1999; Martins et al., 2010; Chaisi et al., 2011; Mans et al., 2011). *Theileria velifera* was first described from cattle by Uilenberg (1964). It is a mild pathogen of the African buffalo and cattle and is transmitted by ticks of the genus *Amblyomma* (Norval et al., 1992).

A quantitative real-time PCR (qPCR) test that is based on the 18S rRNA gene has been developed (Sibeko et al., 2008) and is currently used, together with other tests, for the diagnosis of *T. parva* in cattle and buffalo in South Africa. 18S rRNA gene sequence variants in both *T. parva* and *Theileria* sp. (buffalo) have recently been identified (Chapter 3; Chaisi et al., 2011, Mans et al., 2011). Although these sequence variants do not compromise the specificity of the qPCR test, the specificity could be compromised if there are strains of other *Theileria* spp. with 18S rRNA sequences similar to *T. parva* in the qPCR probe region. The aim of the current study was to determine the sequence variation in the 18S rRNA of *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera*. The results of a more detailed study on the characterization of *T. buffeli* are provided in Chapter 5.

4.3 Materials and Methods

4.3.1 Blood samples

Thirteen buffalo blood samples originating from the Kruger National Park (KNP), Hluhluwe-iMfolozi Game Park (HIP), and a private game ranch in the Hoedspruit area (H) in Mpumalanga (Table 4.1) were analysed. The samples were part of a previous study (Chapter 3) and had either tested positive for different *Theileria* spp. using the reverse line blot (RLB) hybridization assay or only hybridized with the *Theileria* and/or *Babesia* genus-specific probe and not with any of the *Theileria* and/or *Babesia* species-specific probes used in the RLB assay (Table 4.1). Additionally, a bovine (KZN/bov) blood sample from a farm in KwaZulu-Natal, was included in the study. Genomic DNA was extracted from the blood samples using the High Pure Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. DNA was eluted in 100 µl elution buffer and stored at -20°C pending further analysis.

4.3.2 PCR amplification and reverse line blot (RLB) hybridization assay

The V4 hypervariable region of the piroplasm 18S rRNA gene was amplified using the *Theileria* and *Babesia* genus-specific primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and biotin-labelled RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof et al., 2003). The reaction conditions were as outlined in Chapter 3 and a touchdown PCR programme (Nijhof et al., 2003) was used for amplification. The amplicons were then screened by the RLB hybridization assay (Gubbels et al., 1999) using *Theileria* and *Babesia* genus- and species-specific oligonucleotide probes as described in Chapter 3.

4.3.3 PCR amplification, cloning and sequencing of the full-length 18S rRNA gene

A fragment of approximately 1700 bp of the 18S rRNA gene of *Theileria* spp. was amplified using the forward primer Nbab-1F and reverse primer TB 18S-Rev (Oosthuizen et al., 2008; Bhoora et al., 2009). The PCR reaction conditions were as reported in Chapter 3. Purified amplicons were ligated into the pGEM-T Easy Vector and transformed into JM109 High Efficiency cells (Promega, Madison, WI). At least 5 white colonies per sample were selected and screened by colony PCR using primers RLB-F2 and RLB-R2. The colony PCR mixture and cycling conditions were as described for the RLB hybridization assay (Chapter 3), except that colony DNA was used as template. Recombinant plasmid DNA was extracted from overnight bacterial cultures using the High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The full-length 18S rRNA gene of selected clones was sequenced using both vector primers (SP6 and T7) (Chapter 3) and internal primers (RLB-F2, RLB-R2, Nbab_1F,

18SRev-TB, BT18S_2F, BT18S_3F, BT18S_4F, BT18S_4R) (Chapter 3; Oosthuizen et al., 2008; Bhoora et al., 2009). Purified products were analyzed on an ABI3100 genetic analyzer at the ARC-OVI (South Africa) sequencing facility.

4.3.4 Sequence and phylogenetic analyses

The sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield et al., 1995; Staden et al., 2000). Full-length 18S rRNA gene consensus sequences were obtained from 16 clones and were submitted to GenBank with accession numbers FJ213585, FJ213586 and JN572692 – JN572705. A search for homologous sequences was performed using the Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990). Novel sequences were aligned with published sequences from GenBank using the MAFFT (version 5) multiple sequence alignment programme employing the FFT-NS-i algorithm (Katoh et al., 2005). The alignment was manually edited and truncated to the size of the shortest sequence using BioEdit (version 7) (Hall, 1999). The sequences were compared across their full-lengths to identify possible PCR or sequencing artifacts, and sequences with such artifacts were eliminated from the final dataset. The genetic distances between the sequences were estimated by determining the number of base differences between sequences using MEGA4 (Tamura et al., 2007).

Phylogenetic trees were constructed from a final alignment of 39 sequences with 1575 characters using MEGA4 for distance-based (neighbor-joining) trees (Saitou and Nei, 1987), and PAUP* v4b10 (Swofford, 2003) for character-based (maximum likelihood and maximum parsimony) trees. Bootstrap analysis was done using 1000 replicates/tree. Phylogenetic trees were also constructed by Bayesian inference using MrBayes (v3.1.2) (Ronquist and Huelsenbeck, 2003), accessed via the Computational Biology Service Unit, Cornell University. In all cases, the trees were rooted by the 18S rRNA gene sequences of *Sarcocystis muris* (M64244), *Prorocentrum micans* (M14649) and *Toxoplasma gondii* (X68523) and the consensus trees were edited using MEGA4.

4.3.5 RLB probe design

Oligonucleotide probes for two novel *T. mutans*-like 18S rRNA genotypes identified in this study were designed in the V4 hypervariable region of the 18S rRNA gene and synthesized containing an N-terminal *N*-(trifluoracetamidohexylcyanoethyl, *N*,*N*-diisopropyl phosphoramidite)-C6 amino linker (Southern Cross Biotechologies, South Africa). The two new probes were designated *T. mutans*-like 1 (5'-CTT GCG ATG CCG AAT GTT-3') and *T. mutans*-like 2/3 (5'-TTG CGT GCA TCT CCG AAT GTT-3'). These were incorporated into the RLB assay and used to screen 75 South African buffalo blood samples.

4.4 Results

4.4.1 RLB results

The RLB results of the 14 samples selected for characterization (Table 4.1) indicated that ten had mixed *Theileria* spp. infections, three had single infections, and the PCR amplicon of one sample (KNP/C21) did not hybridize with any of the *Theileria* and/or *Babesia* species-specific probes present on the blot, but hybridized only with the *Theileria/Babesia* genus-specific probe. With the exceptions of KNP/C21 and KZN/bov, all of the samples selected for *Theileria* 18S rRNA gene characterization tested positive for *T. parva* and/or *Theileria* sp. (buffalo) when analysed by the RLB assay (Table 4.1).

Table 4.1: RLB hybridization assay, cloning and sequencing results of buffalo blood samples selected for 18S rRNA gene sequence analysis. The samples originated from the Kruger National Park (KNP), Hluhluwe-iMfolozi game park (HIP), a private game range near Hoedspruit area (H), and a farm in the KwaZulu-Natal province (KZN).

Sample	RLB results	Clone (size in bp)	Phylogenetic classification
KNP/B15	*T. parva*	a (1580)	*Theileria* sp. (strain MSD)-like
KNP/B22	*T. parva*, *Theileria* sp. (buffalo), *T. velifera*, *T. mutans*	a (1579)	*T. mutans*-like 1
KNP/C11	*T. parva*	b (1579)	*T. mutans*-like 3
KNP/C21	*Theileria/Babesia* genus-specific probe only	a (1638)	*T. mutans*-like 1
		b (1576)	*T. mutans*-like 1
KNP/G8	*T. parva*, *Theileria* sp. (buffalo), *T. velifera*, *T. mutans*	a (1582)	*T. mutans*-like 2
KNP/Q15	*T. parva*, *Theileria* sp. (buffalo)	a (1590)	*T. velifera*-like
		d (1582)	*T. mutans*-like 2
KNP/V8	*T. parva*, *Theileria* sp. (buffalo)	c (1579) *	*T. mutans*-like 1
HIP/A4	*T. parva*, *Theileria* sp. (buffalo), *T. buffeli*, *T. velifera*	d (1558)	*T. mutans*-like 1
		d (1593)	*T. velifera*
HIP/A21	*Theileria* sp. (buffalo) (faint signal)	e (1579)	*T. mutans*-like 1
HIP/C5	*T. parva*, *Theileria* sp. (buffalo), *T. buffeli*, *T. velifera*	c (1599) *	*T. velifera*-like
HIP/H4	*T. parva*, *T. buffeli*	a (1587)	*T. velifera*-like
HIP/H22	*T. parva*, *T. buffeli*	b (1579)	*T. mutans*-like 3
		d (1593)	*T. velifera*
H/241	*Theileria* sp. (buffalo), *T. velifera*	b (1582)	*T. mutans*-like 2
KZN/bov	*Theileria* sp. (sable), *T. mutans*, *T. taurotragi*, *T. velifera*	d (1588)	*T. velifera*

* Excluded from the final dataset due to the presence of PCR or sequencing-induced artifacts.

4.4.2 18S rRNA gene sequence analysis

Sample KNP/C21 originated from a buffalo captured in the Olifants area of the KNP during a tuberculosis survey done in 2003 (Dr Roy Bengis, personal communication). The RLB PCR amplicon from this sample did not hybridize with any of the *Theileria* and/or *Babesia* species-specific probes present on the blot, but hybridized only with the *Theileria/Babesia* genus-specific probe. The near full-length 18S rRNA gene of the parasites was amplified from the sample, cloned and sequenced. The resulting sequences (KNP/C21/a and KNP/C21/b) were identical, indicating a single infection. BLASTn search results revealed no identical sequences in the public databases. The most closely related sequences were *Theileria mutans* Intona (AF078815) with 98% identity and *Theileria* sp. (strain MSD) (AF078816) with 97% identity. *Theileria uilenbergi* (AY262121), a parasite of sheep and goats in China known to be transmitted by *Haemaphysalis qinghaisensis* ticks (Schnittger et al., 2003; Yin et al., 2007) showed 95% sequence identity.

Subsequently, twelve more buffalo samples (KNP/B15, KNP/B22, KNP/C11, KNP/G8, KNP/Q15, KNP/V8, HIP/A4, HIP/A21, HIP/C5, HIP/H4, HIP/H22, H/241) and a bovine sample (KZN/bov) (Table 4.1) were selected for characterization of their 18S rRNA genes. With the exception of KZN/bov, these samples had tested positive for *T. parva* and/or *Theileria* sp. (buffalo) when analysed by the RLB assay. In addition to the identification of sequences that were similar or identical to the 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo) (Chapter 3), we identified sequences that were similar to the 18S rRNA sequences of *T. mutans* Intona (AF078815), *Theileria* sp. (strain MSD) (AF078815) and/or *T. velifera* (AF0978993) (Table 4.1). Although a BLASTn search indicated that sequences KNP/V8/c and HIP/C5/c were closely similar to the 18S rRNA gene sequences of *T. mutans* and *T. velifera*, respectively, examination of the full-length sequences of these sequences revealed that they were recombinant/cross-over sequences and they were eliminated from further analyses.

The 18S rRNA gene sequence obtained from sample KNP/B15 (clone KNP/B15/a) which originated from a buffalo captured in the Shikokola area of the KNP, showed highest sequence identity to the 18S rRNA sequence of *Theileria* sp. (strain MSD) (AF078816) (99%), *T. mutans* Intona (AF078815) (98%) and *T. uilenbergi* (AY262121) (95%). Seven sequences (HIP/A21/e, KNP/B22/a, KNP/C11/b, HIP/H22/b, H/241/b, KNP/G8/a, KNP/V8/d) showed highest sequence identity of 98%, 97% and 95% to *T. mutans* Intona, *Theileria* sp. (strain MSD), and *T. uilenbergi*, respectively. Sequence KNP/Q15/d was 98% similar to both *T. mutans* and *Theileria* sp. (strain MSD), and 96% similar to *T. uilenbergi*.

Comparisons of the novel sequences to *T. mutans* and *Theileria* sp. (strain MSD) over a region of 1562 bp (Table 4.2) indicated that the *Theileria* sp. (strain MSD)-like sequence (KNP/B15/a) differed from those of *Theileria* sp. (strain MSD) and *T. mutans* by 7 and 17 nucleotides, respectively. The ten *T. mutans*-like sequences differed from the *T. mutans* and *Theileria* sp. (strain MSD) sequences by 18-23 and 25-30 base positions, respectively. There were up to 29 nucleotide differences within the *T. mutans*-like sequences (Table 4.2). Sequences obtained from clones KNP/V8/d (originating from a KNP buffalo captured at Manqeva) and KNP/C21/a (captured at Olifants in the KNP) were identical, as were KNP/Q15/d and KNP/G8/a (originating from KNP buffalo captured at Mahubyeni and Shikokola, respectively).

BLASTn sequence homology search indicated that sequences of five clones (HIP/A4/d, HIP/H4/a, HIP/H22/d, KNP/Q15/a and KZN/bov/d) were 99% and 97% similar to the 18S rRNA gene sequences of *T. velifera* (AF097993) and *Theileria* sp. North Texas (AY735137), respectively. Micro-heterogeniety of 1–7 bp (Table 4.2) mismatches was observed between the *T. velifera* sequences.

Table 4.2: Estimates of evolutionary divergence between *Theileria* spp. sequences by pairwise sequences as indicated by MEGA4 (Tamura et al., 2007). The numbers are base differences between sequences. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). A total of 1562 [for *T. mutans* and *Theileria* sp. (strain MSD)] and 1587 (for *T. velifera*) were compared.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. ***T. mutans* AF078815**																			
2. **T. sp. MSD AF078816**	18																		
3. KNP/B15/a	17	7																	
4. KNP/V8/d	18	25	20																
5. HIP/A21/e	18	25	20	2															
6. KNP/B22/a	19	26	21	1	3														
7. KNP/C21/a	18	25	20	0	2	1													
8. KNP/C21/b	21	28	23	3	5	4	3												
9. KNP/C11/b	21	29	24	18	19	19	18	21											
10. HIP/H22/b	22	30	25	18	19	19	18	21	4										
11. H/241/b	22	27	22	26	26	27	26	29	22	21									
12. KNP/Q15/d	23	26	19	23	23	24	23	26	18	18	3								
13. KNP/G8/a	23	26	19	23	23	24	23	26	18	18	3	0							
14. ***T. velifera* AF0978993**																			
15. HIP/H4/a														3					
16. KNP/Q15/a														4	3				
17. HIP/H22/d														1	4	5			
18. HIP/A4/d														3	6	7	4		
19. KZN/bov/d														0	3	4	1	3	

4.4.3 Phylogenetic analysis

Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian phylogenetic analyses were used to reveal the relationships between the *T. mutans, Theileria* sp. (strain MSD) and *T. velifera* 18S rRNA gene variants and related *Theileria* and *Babesia* species. There were no differences in the groupings of the *T. mutans* variants but the branching of the variants differed in the different trees (Appendix B). A representative tree generated by maximum parsimony is shown in Figure 4.1. Sequence KNP/B15/a grouped closely with *Theileria* sp. (strain MSD). Although the ten *T. mutans*-like sequences grouped with *T. mutans* (Intona) and *Theileria* sp. (strain MSD), they formed three distinct clades (1, 2, 3), designated as *T. mutans*-like 1, 2 and 3 (Figure 4.1).

The *T. velifera* sequences formed two clades. Clade 1 consisted of sequences originating from parasites from both bovine (*T. velifera* AF097993, KZN/bov/d) and buffalo (HIP/H22/d, HIP/A4/d), while clade 2 consisted of only buffalo-derived (HIP/H4/a and KNP/Q15/a) sequences (Figures 4.1). This grouping is further confirmed by the sequence alignment; in the RLB probe region, clade 1 sequences (HIP/A4/d, KZN/bov/d, HIP/H22/d) are identical to the *T. velifera* RLB probe sequence while clade 2 sequences (HIP/H4/a and KNP/Q15/a) differ from the *T. velifera* probe sequence at 2 nucleotide positions (Figure 4.2).

4.4.4 Development of RLB probes for detection of the *T. mutans*-like sequences

The V4 hypervariable region of the 18S rRNA gene sequences of *T. mutans* (AF078815), *Theileria* sp. (strain MSD) (AF078816) and the novel sequences were aligned. A 100 bp region of the alignment including the area from which the RLB oligonucleotides were developed is shown in Figure 4.2. Two RLB oligonucleotide probes, each with 3 bp differences from the *T. mutans* probe, were designed in this area for the specific detection of *T. mutans*-like sequences (Figure 4.2). One probe was designed to detect the *T. mutans*-like 1 sequences and the second probe was designed to detect the *T. mutans*-like 2 and 3 sequences. Although there were sequence differences along the full-length of the 18S rRNA genes of *T. mutans*-like 2 and 3 sequences, their sequences in the chosen RLB probe region were identical (Figure 4.2) and therefore both genotypes should be detected by the *T. mutans*-like 2/3 probe. The new probes were used to screen KNP buffalo blood samples (n = 75) in an attempt to determine the occurrence of these genotypes in the KNP buffalo population. Although both probes were shown to bind to their respective target sequences, both cross-hybridized with the *T. mutans* target sequence. An attempt to increase the stringency of the assay failed to distinguish between these genotypes.

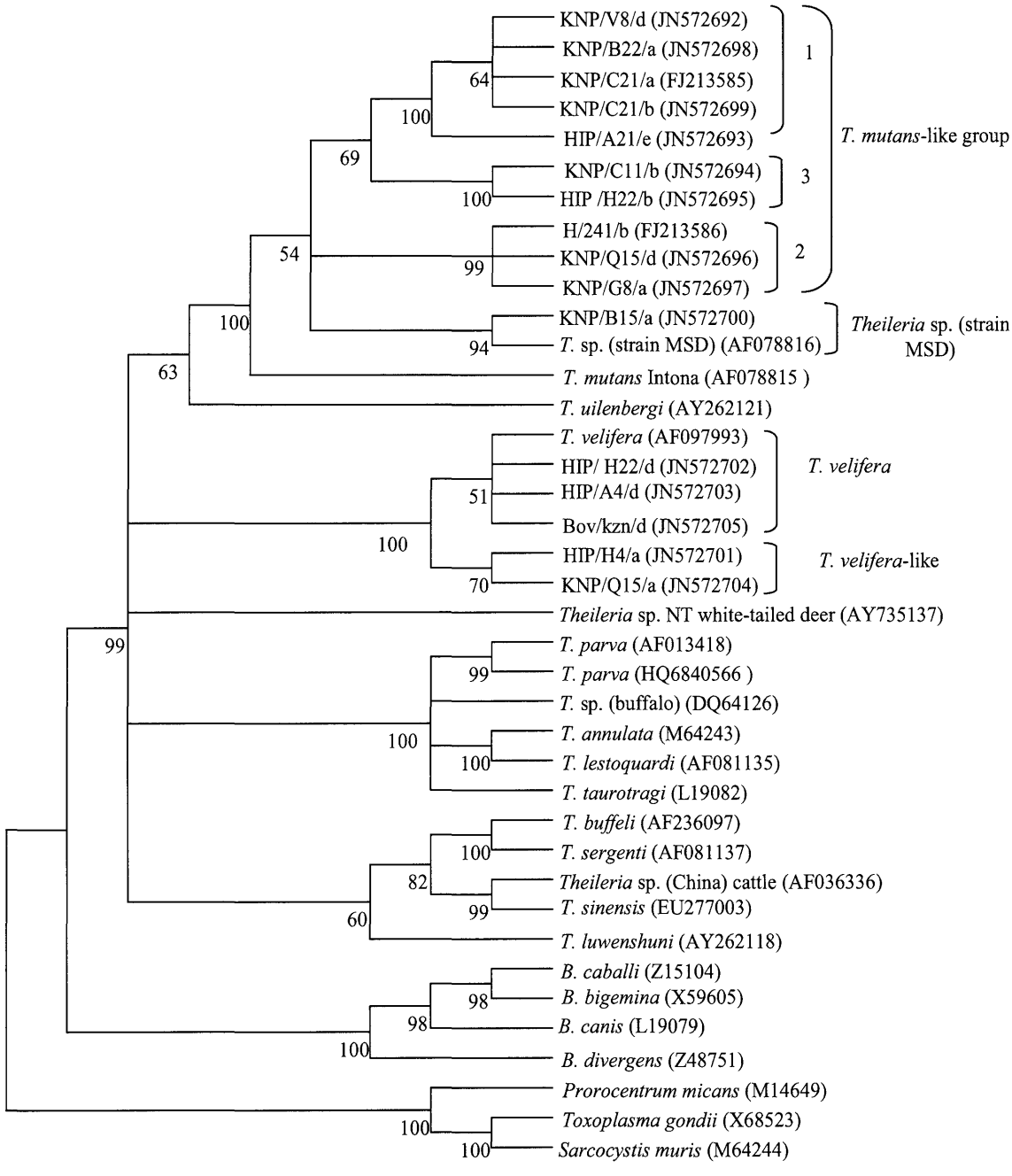

Figure 4.1: Phylogenetic tree showing the relationship of the *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera* 18S rRNA gene sequence variants identified in this study with other *Theileria* and *Babesia* species as indicated by maximum parsimony analysis. Bootstrap values indicate the degree of support for each cluster. The tree was outgroup rooted using *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii*.

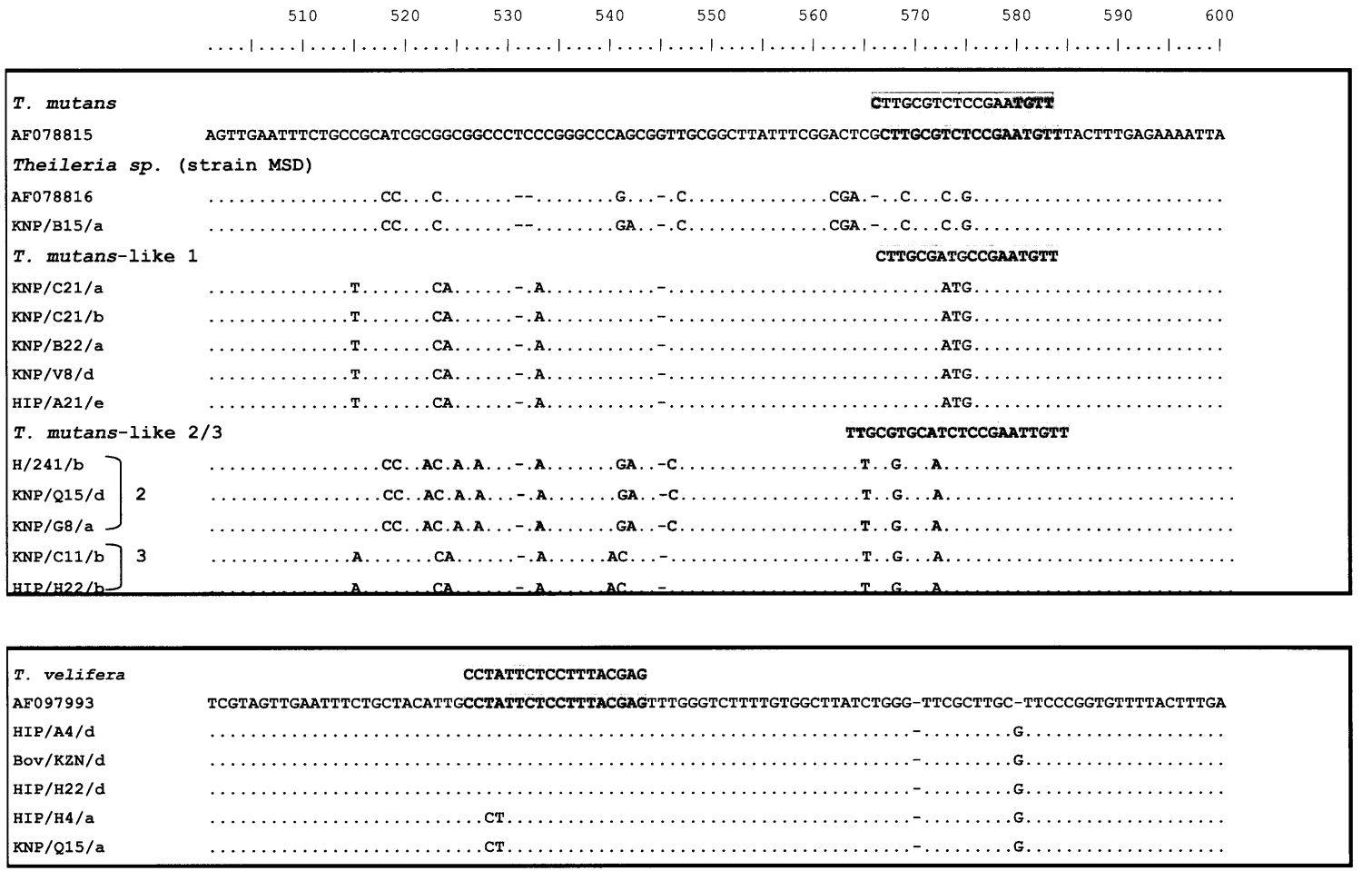

Figure 4.2: Nucleotide alignment of a 100 bp region of the V4 hypervariable region of the published 18S rRNA gene of *T. mutans* and *T. velifera*, as well as the variants identified in the study. Regions where the RLB probes were designed are indicated in grey.

4.5 Discussion

We observed extensive heterogeneity in the 18S rRNA gene of *T. mutans* in the South African buffalo population. Recently, Mans et al. (2011) reported on the occurrence of similar *T. mutans*-like genotypes identified in buffalo originating from different geographical regions in South Africa, although their study was based only on the V4 hypervariable region of the gene. Although *T. mutans* was identified by the RLB hybridization assay in some of the samples in our study, none of the new sequences were identical to the *T. mutans* Intona (AF078815) sequence from which the RLB probe was designed. We identified *T. mutans*-like variants from these *T. mutans*-positive samples as well as from several *T. mutans*-negative samples, suggesting that the parasitaemia of *T. mutans*-like parasites was higher than that of *T. mutans* in the *T. mutans*-positive samples. *Theileria mutans* was not detected from samples containing *T. mutans*-like genotypes due to mis-matches in the *T. mutans*-specific RLB probe sequence.

Sequence variation within the 18S rRNA gene has previously been reported in *Theileria equi* (Nagore et al., 2004; Bhoora et al., 2009), *Babesia caballi* (Bhoora et al., 2009), *Theileria buffeli* (Gubbels et al., 2000, 2002) and *Babesia bigemina* (Martins et al., 2010). However, there is currently no consensus on how much variation in the 18S rRNA gene is required for a sequence variant to be classified as a different species or subspecies, and additional molecular and biological data (morphology, transmission, pathogenicity) are required for such classification (Chae et al., 1999; Schnittger et al., 2003). We therefore designated the novel sequences as *T. mutans*-like genotypes. It is possible that some of the micro-heterogeneity observed within conserved areas of the 18S rRNA gene was due to amplification and sequencing errors. The presence of identical *T. mutans*-like sequences (e.g. KNP/C21/a and KNP/V8/d) in different localities within the KNP could be expected in national parks where animals move freely from one area to another.

Although no attempts have been made to clarify the identity of *Theileria* sp. (strain MSD) after its first description by Chae et al. (1999), the identification of similar sequences in buffalo and cattle in our study and by other studies (Martins et al., 2010; Mans et al., 2011) indicates that this genotype is circulating in some buffalo and cattle populations in southern Africa. *Theileria* sp. (strain MSD) was not identified in any of the samples that we analysed by the RLB hybridization assay as there is currently no probe to detect it. The availability of a specific RLB probe in future studies would enable the detection of this genotype in cattle and buffalo.

The *Theileria* and *Babesia* genus-specific probes are included in the RLB hybridization assay as internal controls and to ensure that new species or variants of species are detected (Nagore et al., 2004; Schnittger et al., 2004; Salih et al., 2007). In our study, the presence of this probe allowed for the identification of *T. mutans*-like sequences in sample KNP/C21. However, novel *T. mutans*-like and *T. velifera*-like sequences were also identified from clones of samples which tested negative for these species by RLB hybridization assay, but were positive for other *Theileria* spp. In the case of sample RLB/241, the *T. parva* specific real-time PCR detected *T. parva* DNA in the sample (Chapter 3). RLB results, however, indicated that *T. velifera* and *Theileria* sp. (buffalo) DNA were present; *T. parva* was not detected. The parasitemia of *T. parva* infection in this sample was probably lower than $1x10^{-6}$%, which has been indicated as the detection limit of the RLB assay (Gubbels et al., 1999). In an attempt to confirm these findings, the 18S rRNA gene was amplified, cloned and sequenced. Of the five clones sequenced, two were identified as *Theileria* sp. (buffalo) and two as *T. velifera*, but no *T. parva* clones were detected. If the level of *T. parva* infection in this buffalo was indeed low compared to the other species present, we would expect to obtain relatively more amplicon from species present at highest concentrations and therefore most clones would be representative of these species. It would therefore probably be necessary to sequence more than five clones in order to identify *T. parva* in this sample. However, of particular interest was the detection of a novel genotype closely related to *T. mutans* Intona. This illustrates that new species or variants of species cannot be detected by the RLB assay when dealing with mixed infections containing both known species and novel variants. As the occurrence of mixed *Theileria* spp. in cattle and buffalo is common (Lawrence, 1979; Oura et al., 2004, 2011a; b; Salih et al., 2007), it is possible that sequence analysis of more *Theileria* spp. positive samples would identify yet more previously unknown genotypes.

Despite the three bp difference between the *T. mutans* and *T. mutans*-like 1 and 2/3 probes, the probes cross-hybridized with *T. mutans* amplicons. Although three nucleotide differences has been indicated as sufficient to prevent hybridization of the probe and the PCR amplicon (Molano et al., 2004; Martins et al., 2010), in our study, the RLB assay failed to distinguish between the different genotypes despite attempts made to increase the stringency of the assay. This problem could be overcome by designing new RLB probes in the region of about 20 bp downstream of the current probe area as there are more nucleotide differences between the different genotypes in the proposed region. A single RLB probe that detects all known *T. mutans* variants could also be designed for use in future studies. Alternatively, real-time PCR could be used to distinguish between these variants, since it is possible to use melting curve analysis to distinguish between PCR products containing

just one or two nucleotide differences, as demonstrated by Criado-Fornelio et al. (2009) and Wang et al. (2010).

Theileria velifera is a non-pathogenic species of cattle and buffalo and like *T. mutans*, it is transmitted by *Amblyomma* ticks (Norval et al., 1992) and therefore the co-occurrence of these species in buffalo was expected. In comparison to the *T. mutans* group, there seems to be less sequence variation in the 18S rRNA gene sequences within this group, although this may be merely due to the smaller number of sequences analysed. Mans et al. (2011) identified partial 18S rRNA sequences from buffalo in South Africa, designated as *T. velifera* B, that were similar (based on analysis of the V4 hypervariable region; Chapter 6) to the *T. velifera*-like sequences identified in our study. Additionally, these authors identified another *T. velifera*-like genotype from cattle which they designated as *T. velifera* A.

The 2 nucleotide differences in the RLB probe area between the *T. velifera* and *T. velifera*-like sequence possibly prevented annealing of the RLB probe to the PCR amplicon, resulting in failure of the RLB assay to identify *T. velifera* in positive samples. In contrast, the sequence of the probe area of sequences HIP/H22/d, HIP/A4/d and KZN/bov/d was identical to that of *T. velifera*, and *T. velifera* was identified from these samples using the RLB assay.

The samples containing novel *T. mutans* and *T. velifera* variants were tested using the *T. parva* specific qPCR as part of Chapter 3 and none of these were positive for *T. parva* as their sequences are different from those of the *T. parva* primers and probes. They would therefore not interfere with the diagnosis of *T. parva* infections in cattle and buffalo in South Africa.

The phylogenetic position of *Theileria* sp. (strain MSD), and the *T. mutans* and *T. velifera* variant is consistent with the results of Mans et al. (2011).

4.6 Summary

We identified extensive sequence variation in the 18S rRNA genes of *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera* in the buffalo population in South Africa. This variation could explain why the RLB hybridization assay failed to detect *T. mutans* and *T. velifera* in some positive samples. Our study supports the recommendation that a thorough survey should always be carried out prior to the development of molecular-based diagnostic tests (Bhoora et al., 2009). Although there is extensive variation within the 18S rRNA gene of the *T. mutans* group, we can only classify these novel genotypes as *T. mutans* variants, and not as new *Theileria* species as we do not have additional molecular and biological data on these novel genotypes (Chae et al., 1999; Schnittger et al., 2003).

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CHAPTER 5

Sequence variation and molecular phylogeny of novel *Theileria buffeli*-like and *Theileria sinensis*-like genotypes of the African buffalo (*Syncerus caffer*) based on their 18S rRNA gene and internal transcribed spacer (ITS) sequences

5.1 Abstract

In a previous study, we identified *T. buffeli* from samples originating from the African buffalo (*Syncerus caffer*) in South Africa. The aim of this study was to characterize these *T. buffeli* genotypes, and to establish their phylogenetic positions based on their 18S rRNA gene and internal transcribed spacer (ITS) sequences. The 18S rRNA gene and the complete ITS (ITS1-5.8S-ITS2) region was amplified from DNA extracted from blood samples originating from buffalo in the Hluhluwe-iMfolozi Game Park (HIP) and the Addo Elephant Game Park (AEGP), and cloned and sequenced. We identified novel *T. buffeli*-like 18S rRNA gene and ITS genotypes from buffalo in the AEGP, and novel *T. sinensis*-like 18S rRNA genotypes from buffalo in the HIP. Phylogenetic analyses indicated that the *T. buffeli*-like sequences are similar to *T. buffeli*-like sequences from cattle and buffalo in China and India, and the *T. sinensis*-like sequences are similar to *T. sinensis* 18S rRNA sequences of cattle and yak in China. There was extensive sequence variation between the novel *T. buffeli* genotypes of the African buffalo and previously described *T. buffeli* and *T. sinensis* genotypes. The presence of organisms with *T. buffeli*-like and *T. sinensis*-like genotypes in the African buffalo is of significant importance, particularly to the cattle industry in South Africa as these animals might act as sources of infections to naïve cattle. This is the first report on the characterization of the full-length 18S rRNA gene and ITS region of *T. buffeli*-like and *T. sinensis*-like genotypes in South Africa. Our study provides invaluable information towards the classification of the complex *T. buffeli*/*T. sergenti*/*T. orientalis* group of benign and mildly pathogenic species.

5.2 Introduction

Theileria buffeli/Theileria sergenti/Theileria orientalis is a group of closely related parasites of cattle and buffalo with a cosmopolitan distribution. They infect cattle and buffalo in Africa, Australia, Asia, Europe and the United States of America (USA) (Chae et al., 1998; Chansiri et al., 1999; Cossio-Bayugar et al., 2002; Sarataphan et al., 2003; Aktas et al., 2007; Altay et al., 2008; M'ghirbi et al., 2008; Gimenez et al., 2009, Liu et al., 2010a, Wang et al., 2010). Ticks of *Haemaphysalis* spp. act as vectors in Australia, Asia and Europe, but the vectors in Africa and the USA are still unknown (Yin et al., 2004; Bendele, 2005; M'ghirbi et al., 2008). Benign isolates from Britain, Australia and the USA were initially designated as *T. mutans* as their pathology was similar to that of *T. mutans* (Chae et al., 1999c). However, further studies indicated that *T. mutans* is an African species and is serologically and genetically distinct from other benign *Theileria* spp. (Morzaria et al., 1977, Chae et al., 1999c).

Theileria sergenti and *T. orientalis* were first described from eastern Siberia in the early 1930s by, respectively,Yakimoff and Dekhtereff, and Yakimoff and Soudatschenkoff, while *T. buffeli* was first described from the Asian water buffalo (*Bubalus bubalis*) in 1908 by Schein (reviewed by Fujisaki et al., 1994). The classification of these benign parasites is still confusing and is complicated by their similar morphology, serology, vector transmission, geographical distribution, difficulties in obtaining pure isolates and incomplete life-cycles (Uilenberg et al., 1985; Chae et al., 1999c; Chansiri et al., 1999; Yin et al., 2004; M'ghirbi et al., 2008, Uilenberg, 2011). It is still unclear if these organisms represent the same species or different species. *Theileria sergenti* is pathogenic to cattle and yak, and is regarded as a separate species from the benign *T. buffeli/T. orientalis* (Kawazu et al., 1999). Other authors (Fuujisaki, 1992; Chae et al., 1999a; Uilenberg, 2011) indicated that although the term "*T. sergenti*" has traditionally been used for this species, *T. sergenti* actually refers to a sheep parasite and it was incorrectly termed as a parasite of cattle and buffalo. Due to all this confusion, Uilenberg et al. (1985) suggested that the benign species (*T. buffeli*/*T. orientalis*) should be classified as *T. orientalis*. However, the term *T. buffeli* is preferred over *T. orientalis* on the basis of molecular data, as well as the fact that all characterized isolates are infective for buffalo (Steward et al., 1996).

Gubbels et al. (2000) proposed that these organisms should be referred to as *T. buffeli* until more biological data becomes available for further classification, and the names *T. orientalis* and *T. sergenti* should only refer to isolates that have been previously described under these names. Another closely related species, *Theileria sinensis*, was recently described in China and is also

regarded as a cause of bovine theileriosis in that country (Bai et al., 2002a; b, cited by Yin et al., 2004).

Molecular biology studies based on the 18S ribosomal RNA (rRNA) gene, internal transcribed spacers (ITS), major piroplasm surface protein (MPSP) gene and other genetic markers have provided useful information on the epidemiology, diagnosis, taxonomy and phylogeny of these benign *Theileria* spp. (Allsopp et al., 1994; Chae et al., 1998, Chansiri et al., 1999; Gubbels et al., 2000, 2002; Sarataphan et al., 2003; M'ghirbi et al., 2008; Liu et al., 2010a; b; Wang et al., 2010; Kamau et al., 2011). However, there is no molecular data on these species in the African buffalo (*Syncerus caffer*) in South Africa although *T. buffeli* has been identified in some buffalo populations in this country (Chapter 3). The aims of this study were to: (1) sequence the 18S rRNA gene and complete ITS (ITS1-5.8S-ITS2) region of *T. buffeli* of the South African buffalo; (2) determine the level of genetic variation between novel *T. buffeli*-like and *T. sinensis*-like genotypes of the African buffalo with known *T. buffeli* and *T. sinensis* genotypes; and (3) to establish their phylogenetic positions based on their 18S rRNA gene and ITS sequences.

5.3 Materials and Methods

5.3.1 DNA samples

A molecular epidemiological survey based on the 18S rRNA gene was previously carried out to determine the prevalence of *Theileria* spp. from the African buffalo in different geographic areas in South Africa and Mozambique using the reverse line blot (RLB) hybridization assay (Chapter 3). *Theileria buffeli* was identified from buffalo blood samples originating from the Hluhluwe-iMfolozi Game Park (HIP) (Chapter 3). From these results, four samples (HIP/A2, HIP/A4, HIP/C5, HIP/C23) were selected for the characterization of their full-length 18S rRNA genes, and the complete ITS (ITS1-5.8S-ITS2) region was characterized from ten samples (HIP/A36, HIP/B62, HIP/C11, HIP/C13, HIP/C15, HIP/C18, HIP/C19, HIP/C23, HIP/C25, HIP/C27). *Theileria buffeli* is also known to occur in buffalo in the Addo Elephant Game Park (AEGP), Eastern Cape Province, South Africa. The parasite 18S rRNA gene and ITS region were also characterized from seven samples originating from buffalo in the AEGP (AEGP/65, AEGP/66, AEGP/69, AEGP/70, AEGP/73, AEGP/74, AEGP/76). These samples were analysed by the RLB hybridization assay in a separate study.

5.3.2 Amplification, cloning and sequencing of the full-length 18S rRNA gene

The 18S rRNA gene and ITS from all samples were amplified, cloned and sequenced separately as we were unable to amplify the approximately 3 kb rDNA fragment which spans both regions. For this reason, in the samples originating from HIP, cloned 18S rRNA gene and ITS sequences from the same sample could not be directly compared due to mixed infections.

The full length 18S rRNA genes of 11 samples (4 from HIP and 7 from AEGP) were amplified by conventional PCR using forward primer Nbab-1F and reverse primer 18SRev-TB (Chapter 3).The reaction mixture and cycling conditions were as described in Chapter 3. The resulting amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Southern Cross Biotechnologies). Amplicons of four reactions per sample were pooled to avoid *Taq* polymerase induced errors.

For the HIP samples, which all contained mixed *Theileria* spp. infections and therefore could not be directly sequenced, purified amplicons were ligated into the pGEM-T Easy Vector and transformed into *E. coli* JM109 High Efficiency Competent cells (Promega, Madison, WI). At least 5 positive white colonies were selected per sample. Recombinant plasmid DNA was extracted from overnight bacterial cultures using the High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany). The plasmids were initially screened by sequencing using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), 350 ng plasmid DNA

and 3.2 pmol of primer RLB-F2. The obtained sequences were subjected to a BLASTn (Altschul et al., 1990) similarity search. The full-length 18S rRNA genes of recombinants with sequences that were closely similar to the published 18S rRNA gene sequences of *T. buffeli* or *T. sinensis* were subsequently sequenced using primers Nbab-1F, 18SRev-TB, RLB-R2, BT18S-2F, BT18S-3F, BT18S-4F, BT18S-4R, SP6, T7 (Chapter 3). For samples from the AEGP, which had single *Theileria* species infections, full-length 18S rRNA genes were directly sequenced using ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), ~40 ng of PCR product and 3.2 pmol of each primer. Sequencing was done on an ABI3100 genetic analyzer at the sequencing facility of the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI), South Africa.

5.3.3. Amplification, cloning and sequencing of the ITS (ITS1-5.8S-ITS2) region

A nested PCR protocol was used to amplify the complete parasite ITS region. The primary reaction contained 2.5 µl (~75 ng) genomic DNA, 0.1 µM each of primer 1055F (5'- GGT GGT GCA TGG CCG-3') and LSUR300 (5'-T(A/T)G CGC TTC AAT CCC-3') (Holman et al., 2003; Aktas et al., 2007), 1.5 mM $MgCl_2$, 200 µM dNTPs, High Fidelity Enzyme blend (concentration unknown) and nuclease-free water to a total volume of 25 µl. The thermal cycling programme was done at an initial denaturation at 96°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s; annealing at 50°C for 30 s; extension at 72°C for 3 min; a final extension at 72°C for 7 min and then hold at 4°C. Primers ITSF (5'-GAG AAG TCG TAA CAA GGT TTC CG-3') and LSUR50 (5'-GCT TCA CTC GCC GTT ACT AGG-3') (Holman et al., 2003) were used for the nested PCR. The reaction mixture was as above, except that 1 µl (~ 30ng) of the primary PCR product was used as template. The cycling conditions were also as above, except that annealing was done at 60°C for 30 s and extension was done at 72°C for 2 min.

Amplicons of the correct size (approximately 1200 bp) were excised from ethidium-bromide stained gels and purified using the Qiaquick Gel Extraction Kit (Qiagen, Southern Cross Biotechnologies), after which they were directly ligated into the pGEM-T Easy Vector and transformed into *E. coli* JM109 High Efficiency Competent cells (Promega, Madison, WI). Sequencing reactions were done using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems), ~300 ng of plasmid DNA and 2 pmol each of primers ITSF, SP6 and T7. The reactions were purified by the Zymo research sequencing clean-up kit (Inqaba Biotechnogies, South Africa) according to the manufacturer's protocol, and analysed with an ABI 3500XL genetic analyzer. Sequencing was done at Inqaba Biotechnologies, South Africa.

5.3.4 Sequence and phylogenetic analyses

The sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield et al., 1995; Staden et al., 2000). A BLASTn homology search of GenBank was done using the full length consensus sequences. These were then aligned with 18S rRNA gene sequences (Table 5.2) or ITS sequences of related genera from GenBank using the MAFFT (multiple sequence alignment programme) v6 employing the FFT-NS-1 algorithm (Katoh et al., 2005). The alignments were manually examined and edited across their full-lengths, and then truncated to the size of the smallest sequence using BioEdit v7 (Hall, 1999). Sequences with PCR or sequencing-induced artifacts (Thompson et al., 2002) were eliminated from the alignments. A total of 58 (new and known) 18S rRNA gene sequences (1514 characters), and 30 ITS sequences (1510 characters) for the complete ITS region, were analysed. Estimated evolutionary divergence was calculated by determining the number of nucleotide differences between similar sequences over a region of 1499 and 1215 nucleotides for the 18S rRNA gene and ITS sequences, respectively. Nucleotide differences were also determined in the V4 hypervariable regions of the 18S rRNA sequences, and in the ITS1, 5.8S gene and ITS2 regions of the ITS sequences.

Phylogenetic trees were inferred from the alignments by the neighbor-joining method (Saitou and Nei, 1987), maximum parsimony and maximum likelihood methods using PAUP* v4b10 (Swofford, 2003). These were done in combination with bootstrapping (1000 replicates). Bayesian inference was done using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003), accessible via the Computational Biology Service Unit, Cornell University. For comparison of the phylogenetic trees, 18S rRNA gene and ITS sequences of the same species or isolate were included, where possible. The 18S rRNA gene and ITS sequences of *Babesia canis*, *B. caballi* and *B. orientalis* were included as outgroups to root the phylogenetic trees. All consensus trees were edited using MEGA4 (Tamura et al., 2007).

5.3.5 GenBank Accession numbers

The near full-length 18S rRNA gene sequences have been deposited in GenBank under accession numbers JQ037779 – JQ037790: The complete ITS sequences have been deposited in GenBank under accession numbers JQ0377791 – JQ037795.

Table 5.1: 18S rRNA gene sequences of *T. buffeli*-like isolates and field samples used in the construction of phylogenetic trees.

	Accession number	Country of origin	Reference
***Theileria* sp. type A**	U97047	USA, Korea, Japan	Chae et al., 1998
T. sergenti	AF081137	China	Gubbels et al., 2000
T. sergenti	EU083802	China	Not published
T. buffeli	AF236097	China	Gubbels et al., 2000
T. buffeli Marula	Z15106	Kenya	Allsopp et al., 1994
***Theileria* sp. type B**	U97048	USA, Korea, Japan	Chae et al., 1998
T. sergenti (Ikeda)	AY661515	Japan	Atkas et al., 2007
***Theileria* sp. type B1**	U97049	USA, Korea	Chae et al., 1998
T. sergenti (Ikeda)	AB000271	Japan	Chansiri et a., 1999
Type C	U97051	Korea	Chae et al., 1998
T. buffeli	EU407240	Tunisia*	M'ghirbi et al., 2008
T. buffeli	AJ616716	Portugal*	Brigido et al., 2004
Theileria sp. Macheng	DQ256380	China	Liu et al., 2010a
Theileria sp. Xiaogan	DQ256381	China	Liu et al., 2010a
Type D	U97052	USA, South Korea	Chae et al., 1998
T. sinensis	EU277003	China	Not published
T. sinensis	EU274472	China	Not published
Theileria sp China (cattle)	AF036336	China	Gubbels et al., 2000
Theileria sp. Thung Song	AB000270	Thailand	Chansiri et al., 1999
Type E	U97053	Korea	Chae et al., 1998
Theileria sp. Ipoh	AB000273	Malaysia	Chansiri et al., 1999
Theileria sp. Hongan	DQ286801	China	Liu et al., 2010a
T. buffeli Warwick	AB000272	Australia	Chansiri et al., 1999
T. buffeli	FJ225391	Spain	Gimenez et al., 2009
T. buffeli (cow)	DQ289795	Spain	Criado et al., 2006
Theilera sp. Hubei	DQ104610	China	Liu et al., 2010a
T. buffeli	AF236094	Australia	Gubbels et al., 2000
T. buffeli China	DQ104611	China	Liu et al., 2010a
T. buffeli	HM538212	China	Not published
T. buffeli Indian	EF126184	India	Not published
AEGP/65/18S	JQ037779	South Africa	This study
AEGP/66/18S	JQ037780	South Africa	This study
AEGP/69/18S	JQ037781	South Africa	This study
AEGP/70/18S	JQ037782	South Africa	This study
AEGP/73/18S	JQ037785	South Africa	This study
AEGP/74/18S	JQ037783	South Africa	This study
AEGP/76/18S	JQ037784	South Africa	This study
HIP/A2/a	JQ037790	South Africa	This study
HIP/A4/c	JQ037786	South Africa	This study
HIP/A4/e	JQ037787	South Africa	This study
HIP/C23/a	JQ037788	South Africa	This study
HIP/C23/b	JQ037789	South Africa	This study
Type H	U97050	Korea	Chae et al., 1998
Theileria sp. Medan	AB000274	Indonesia	Chansiri et al., 1999

* Partial sequences were not included in the phylogenetic trees.

5.4 Results

5.4.1 Identification of *T. buffeli*-like 18S rRNA gene sequences

Samples HIP/A2, HIP/A4 and HIP/C5 had mixed *Theileria* spp. infections (as identified by the RLB hybridization assay) (Chapter 3; Appendix A). Sample HIP/C23, together with the AEGP samples had single *T. buffeli* infections (Appendix A). Single bands of approximately 1700 bp, as viewed on a 2% ethidium bromide stained agarose gel, were obtained after amplification (Figure 5.1a). These were cloned and sequenced. Five near full-length 18S rRNA sequences (1582 – 1592 bp) were obtained from the HIP samples (Table 5.2). A BLASTn homology search did not reveal any identical sequences in GenBank, the closest homology (98% and 99%) was found with 18S rRNA gene sequences of *Theileria* sp. (Thung Song) (AB000272), *Theileria* sp. type D (U97052), *T. sinenses* (EU277003), *T. sinensis* (EU27442) and *Theileria* sp. China (cattle) (AF036336) (Table 5.3).

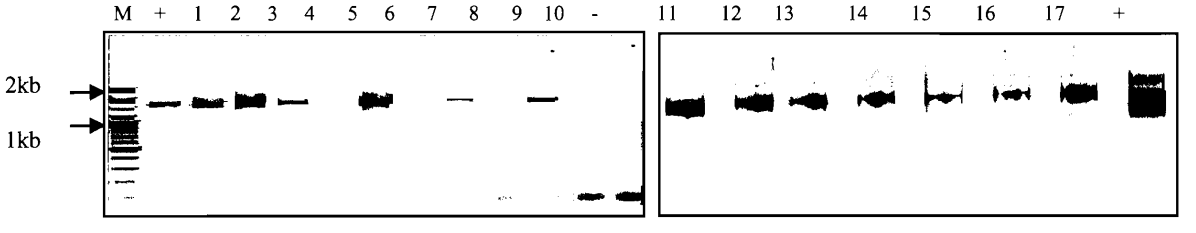

Figure 5.1: Agarose gel eletrophoresis analyis of amplicons of the full-length 18S rRNA gene (1-10) and ITS region (11-17) of *Theileria* spp. of buffalo from field samples. M = 100 bp plus marker, + = *T. parva* positive control, - = water negative control.

Examination of the alignment of the novel sequences with other 18S rRNA sequences suggested that sequence HIP/C5/e was a cross-over sequence (Thompson et al., 2002) between *T. parva* and *T. buffeli*, it was therefore excluded from further analyses. Seven 18S rRNA sequences (AEGP/65/18S, AEGP/66/18S, AEGP/69/18S, AEGP/70/18S, AEGP/73/18S, AEGP/74/18S, AEGP/76/18S) with lengths of 1587 – 1588 bp, were obtained from the samples originating from the Addo Elephant Game Park. BLASTn similarity searches of these sequences did not reveal any identical sequences, but they were most similar (99%) to *T. buffeli* 18S rRNA gene sequences from China (DQ104611 and HM538212) and India (EF126184).

Table 5.2: Highest percentage identity BLASTn hits of novel *Theileria spp.* 18S rRNA gene sequences obtained from samples from the Hluhluwe-iMfolozi Game Park (HIP).

Sequence (length: bp)	**GenBank match (Accession number)**				
	Theileria sp Thung Song (AB000270)	*Theileria* sp type D (U97052)	*T. sinensis* (EU274472)	*Theileria* sp China (cattle) (AF036336)	*T. sinensis* (EU2770073)
HIP/A2/a (1589)	99%	98%	99%	98%	98%
HIP/A4/c (1592)	98%	98%	98%	98%	98%
HIP/A4/e (1582)	99%	99%	99%	99%	98%
HIP/C23/a (1588)	99%	99%	99%	99%	98%
HIP/C23/a (1579)	99%	99%	99%	99%	98%

5.4.2 Sequence and phylogenetic analyses of the 18S rRNA genes

Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian inference were used to determine the relationships of the novel 18S rRNA *T. buffeli* sequences with known *T. buffeli* sequences isolated from cattle and buffalo in different countries. The *T. buffeli* sequences formed 9 distinct clusters, and were clearly separated from other *Theileria* spp. (Figure 5.2). The clustering was similar in all trees, but there were differences in the branching of the clusters in some trees. Figure 5.2 is a representative tree generated by neighbor-joining analysis. Six of the nine *T. buffeli* genotypes (designated Types A, B/Ikeda, C/Medan, D, E/H/Ipoh, Warwick) (Figure 5.2) are previously identified *T. buffeli* 18S rRNA genotypes (Chae et al., 1998; 1999a; Chansiri et al., 1999; Gubbels et al., 2000; Yin et al., 2004). Types F and G are *T. cervi* 18S rRNA sequences from the elk and white-tailed deer in the USA and Canada (Chae et al., 1999c) and are distantly related to the *T. buffeli* genotypes (Figure 5.2).

Sequence and phylogenetic analyses indicated the presence of one more known, but unclassified *T. buffeli* genotype from China (Liu et al., 2010a) and India (unpublished), and two novel *T. buffeli*-like genotypes from South Africa which we designated as types SA1 and SA2 (Figure 5.2). Genotype SA1 is composed of 18S rRNA gene sequences originating from the AEGP, and the sequences of this group are closely related to those of the unclassified group (DQ104611, HM538212, EF126184). Genotype SA2 is composed of 18S rRNA gene sequences from the HIP, and is closely related to the *T. buffeli* type D/*T. sinensis* group (Figure 5.2).

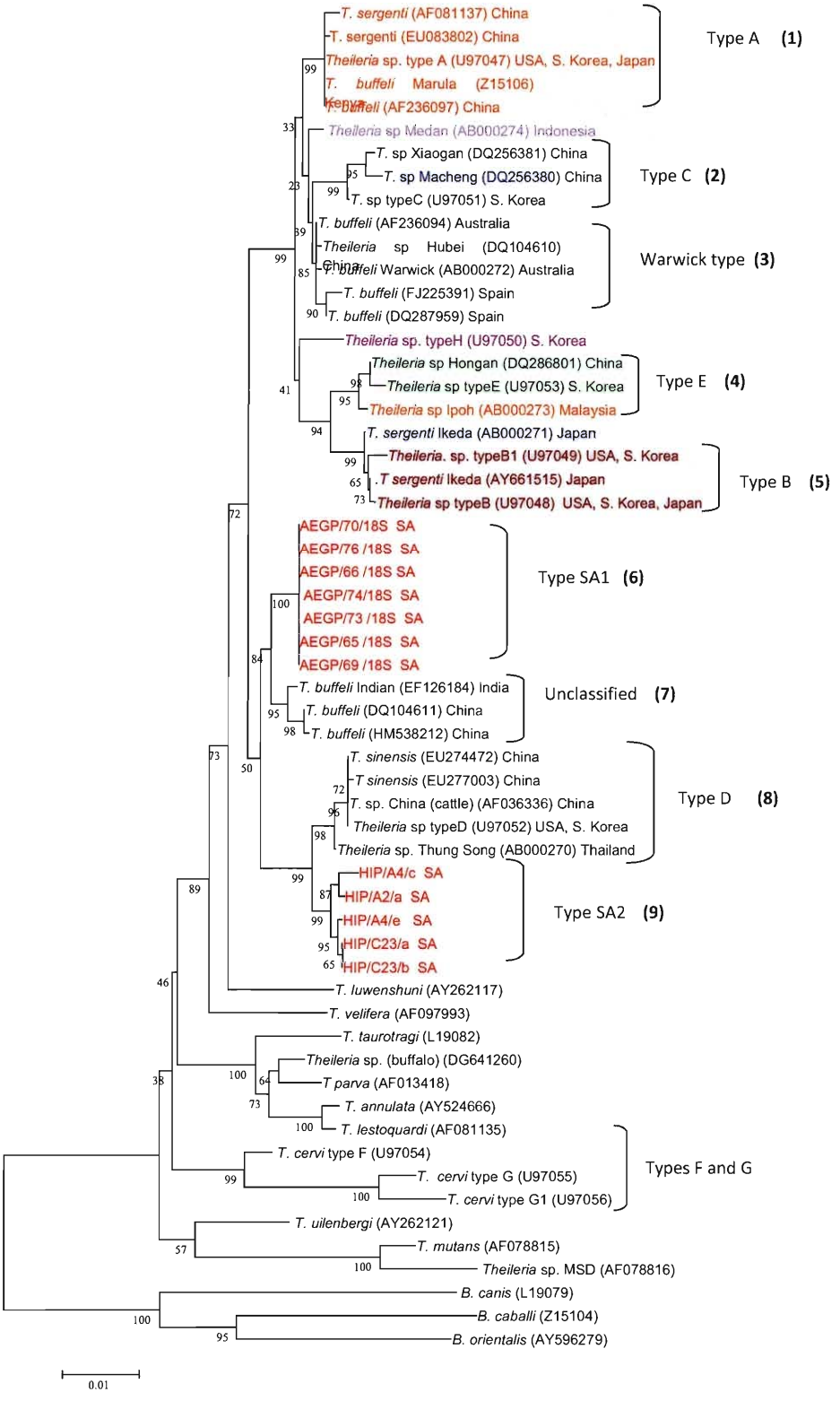

Figure 5.2: Phylogenetic relationships of novel *T. buffeli*-like and *T. sinensis*-like 18S rRNA gene sequences from South Africa (red) with known *T. buffeli* sequences as inferred by the Neighbor-joining method. GenBank accession numbers are indicated in parenthesis. Numbers in brackets are designated cluster numbers. Bootstrap values are indicated at the nodes.

In order to estimate the genetic distance between the *T. buffeli*-like sequences, the novel genotypes were aligned with 17 known *T. buffeli* 18S rRNA gene sequences (representing the different genotypes), and compared along a region of 1499 bp. Sequence variation was observed both within and between the different *T. buffeli* genotypes. All seven novel sequences from AEGP were identical within this region and along their full lengths (results not shown). These sequences differed from those of the closely related genotype (unclassified) by 9 – 12 bp, and from the novel sequences from HIP by 21 – 23 bp (Table 5.3). The HIP sequences differed from the *T. buffeli* type D/*T. sinensis* sequences by 11 - 16 bp. Sequences HIP/C23/a and HIP/C23/b were identical, while there was a 7 bp difference between sequences HIP/A4/c and HIP/A4/e. The other novel sequences from HIP differed from each other by 2 – 7 bp. The greatest variation (~ 45 bp) was observed between sequence HIP/A4/c and *Theileria* sp. type E, which is from cattle isolates in the USA and South Korea (Chae et al., 1998).

Table 5.3: Estimates of evolutionary divergence between novel *T. buffeli*-like (SA1 – red) and *T. sinensis*-like (SA2 – green) 18S rRNA genotypes of the African buffalo, and known *T. buffeli* (black and blue) and *T. sinensis* (orange) genotypes of cattle and buffalo. The number of base differences per sequence from analysis between sequences is shown. The results are based on the pairwise analysis of 29 sequences. Analyses were conducted using MEGA4 (Tamura et al., 2007). There were a total of 1499 positions in the final dataset.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	23	25	26	27	28	29
1 U97047 - A																													
2 AB000272	8																												
3 AB000274	9	5																											
4 AB000271	20	18	13																										
5 U97048 - B	22	20	15	2																									
6 U97049 - B1	25	23	28	5	5																								
7 U97053 - E	24	22	27	9	11	14																							
8 U97051 - C	12	9	12	17	19	22	22																						
9 U97050 - H	17	11	14	19	19	18	23	15																					
10 EF126184	21	22	19	29	31	34	33	26	28																				
11 DQ104611	22	23	20	30	32	35	34	27	29	5																			
12 HM538212	23	24	21	31	33	36	35	28	30	6	1																		
13 AEGP/65	22	23	20	30	32	33	34	27	27	9	11	12																	
14 AEGP/66	22	23	20	30	32	33	34	27	27	9	11	12	0																
15 AEGP/69	22	23	20	30	32	33	34	27	27	9	11	12	0	0															
16 AEGP/70	22	23	20	30	32	33	34	27	27	9	11	12	0	0	0														
17 AEGP/74	22	23	20	30	32	33	34	27	27	9	11	12	0	0	0	0													
18 AEGP/76	22	23	20	30	32	33	34	27	27	9	11	12	0	0	0	0	0												
19 AEGP/73	22	23	20	30	32	33	34	27	27	9	11	12	0	0	0	0	0	0											
20 AB000270	27	30	28	37	39	41	41	32	35	20	19	20	22	22	22	22	22	22	22										
21 AF036336	29	32	31	40	42	44	44	34	37	23	22	23	25	25	25	25	25	25	25	3									
22 EU274472	29	32	31	40	42	44	44	34	37	23	22	23	25	25	25	25	25	25	25	3	0								
23 U97052	29	32	31	40	42	44	44	34	37	23	22	23	25	25	25	25	25	25	25	3	0	0							
24 EU277003	30	33	32	40	42	45	45	35	38	24	23	24	26	26	26	26	26	26	26	4	1	1	1						
25 HIP/A4/c	33	35	34	41	43	45	45	37	38	23	24	25	23	23	23	23	23	23	23	14	15	15	15	16					
26 HIP/A4/e	30	32	31	38	43	42	42	34	35	21	22	23	21	21	21	21	21	21	21	11	12	12	12	13	7				
27 HIP/C23/a	30	32	31	38	40	42	42	34	35	21	22	23	21	21	21	21	21	21	21	11	12	12	12	13	7	2			
28 HIP/C23/b	30	32	31	38	40	42	42	34	35	21	22	23	21	21	21	21	21	21	21	11	12	12	12	13	7	2	0		
29 HIP/A2/a	31	33	32	39	41	43	43	35	36	21	22	23	21	21	21	21	21	21	21	12	13	13	13	14	4	5	5	5	

5.4.3 Analysis of the V4 hypervariable region of the 18S rRNA gene

Most of the variation between *T. buffeli*-like genotypes occurred in the V1 variable region (positions 70 – 140) and V4 hypervariable region (positions 490 – 560) of the gene (Figure 5.3A and B). The *T. buffeli* probe (Figure 5.3B) that we used in the RLB hybridization assay (Chapter 3) was designed for the detection of all *T. buffeli* 18S rRNA gene sequences (Gubbels et al., 1999). Subsequently, Gubbels et al. (2000) designed more probes for the specific detection of the different *T. buffeli* genotypes (Figure 5.3B). Gubbels et al. (2000) showed that the Type A and Type D probes detected, respectively, all *T. buffeli* type A and type D 18S rRNA sequences that were known at the time, and the non-D type detected all sequences that were not classified as type D (in black). However, we have identified three additional non-D genotypes (in green, blue and brown) with at least 4 nucleotide differences from the sequence of the non-D probe (Figure 5.3).

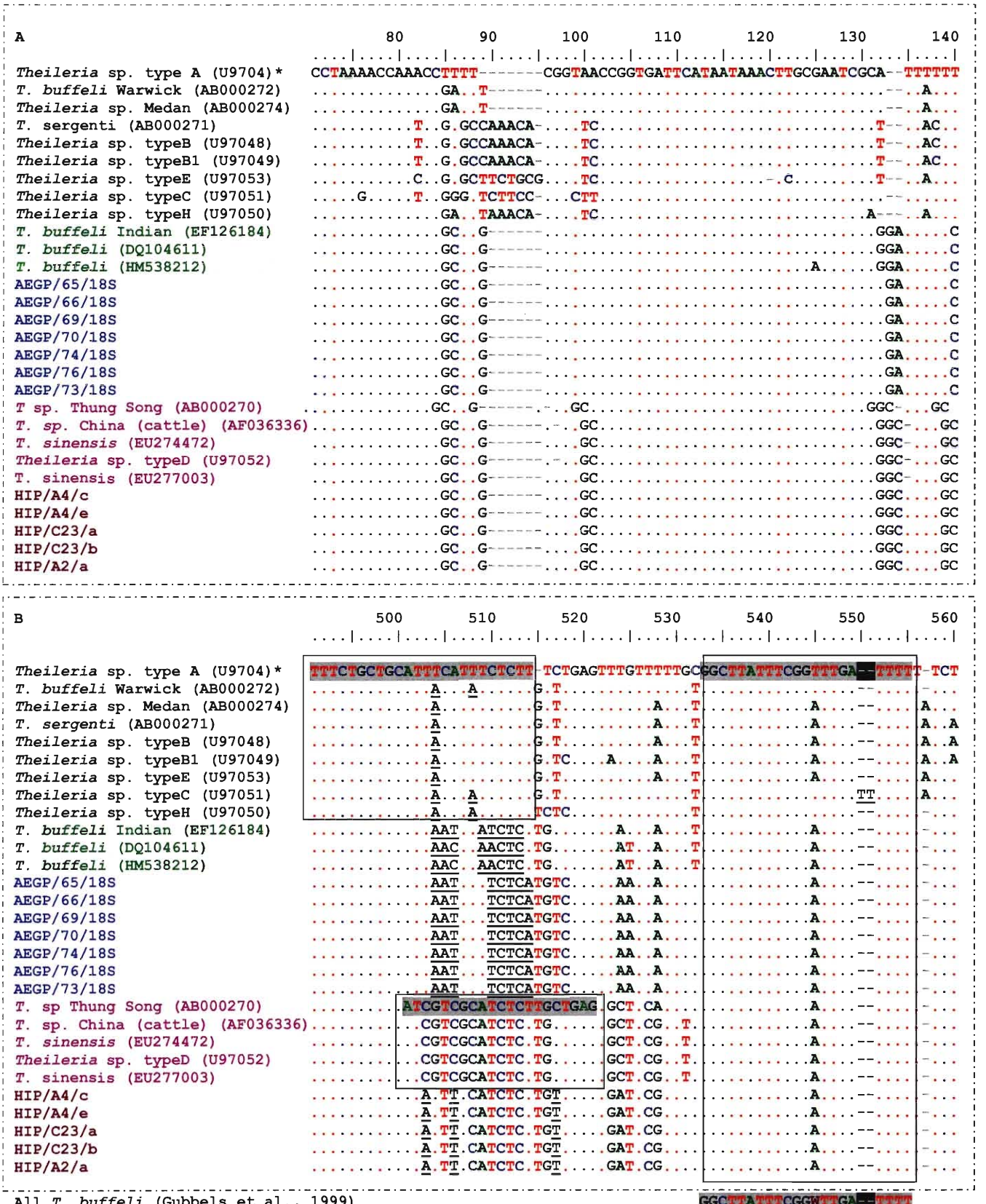

Figure 5.3: Alignment of novel *T. buffeli*-like (blue) and *T. sinensis* (brown) 18S rRNA gene sequences with known *T. buffeli* sequences (black, green and pink) showing V1 variable (A) and V4 hypervariable (B) regions of the gene. RLB probes were designed from the V4 hypervariable region (B). Blocks indicate sequences from which the RLB probes were designed. Nucleotide differences in the probe sequences within the different genotypes are underlined.

5.4.4 Sequence and phylogenetic analyses of the novel ITS sequences

All of the 17 samples (section 5.3.1) selected for characterization of the ITS region tested positive for single *T. buffeli* infections by the RLB hybridization assay (results not shown). Amplicons of approximately 1200 bp, as viewed on a 2% agarose gel (Figure 5.1b), were observed from the nested PCR products. Since *T. buffeli* is the only *Theileria* spp. known to infect buffalo in the AEGP, the amplicons obtained from samples from this game park were directly sequenced. However, the samples from the HIP were cloned prior to sequencing as mixed *Theileria* spp. infections are common in buffalo from this game park (Chapter 3). Amplicons from five out of seven samples from AEGP were successfully sequenced. ITS sequences were obtained from 11 clones obtained from the 10 samples from HIP. The new sequences were subjected to a BLASTn analysis and then aligned with similar sequences from GenBank. Examination of the alignments indicated that two HIP ITS sequences were closely similar to published *T. buffeli* ITS sequences, and five HIP ITS sequences were more closely similar to published *T. parva* and *T. mutans* ITS sequences. This result was confirmed by phylogenetic analysis of the complete ITS, 5.8S gene and ITS2 sequences. The other 4 ITS sequences from HIP were eliminated from the analysis as they were very different from each other and from any of the *Theileria* sequences.

The sequence alignment of the AEGP ITS sequences (AEGP/65/ITS, AEGP/66/ITS, AEGP/69/ITS, AEGP/73/ITS, AEPG/73/ITS) is indicated in Figure 5.4. The sequences differed in the lengths of their complete ITS region (952 – 1173 bp), as well as in the ITS1 (458 – 642 bp) and ITS2 (297 – 344 bp) regions. The 5.8S gene was conserved amongst the sequences and shorter (187 bp) than the ITS1 and ITS2 regions. Unlike the novel 18S rRNA gene sequences which were identical, there was polymorphism among the novel ITS sequences, with most of the variation occurring in the ITS1 region. This was mainly due to insertions or deletions of blocks of sequences as seen with the sequences of the ITS2 region (Figure 5.4). Three sequences (AEPG/65/ITS, AEPG/66/ITS, AEPG/69/ITS) were identical, while sequences AEPG/73/ITS and AEPG/73/ITS differed from each

other at 4 positions, and differed from the other samples by 134 bp. There was extensive variation between these sequences and the homologous *T. buffeli* ITS sequences from China (107 – 164 bp), USA and Japan (159 - 200 bp), and *T. sinensis* ITS sequences (144 – 178 bp). These differences were in concordance with those obtained from analyses of similar genotypes/species of the 18S rRNA gene.

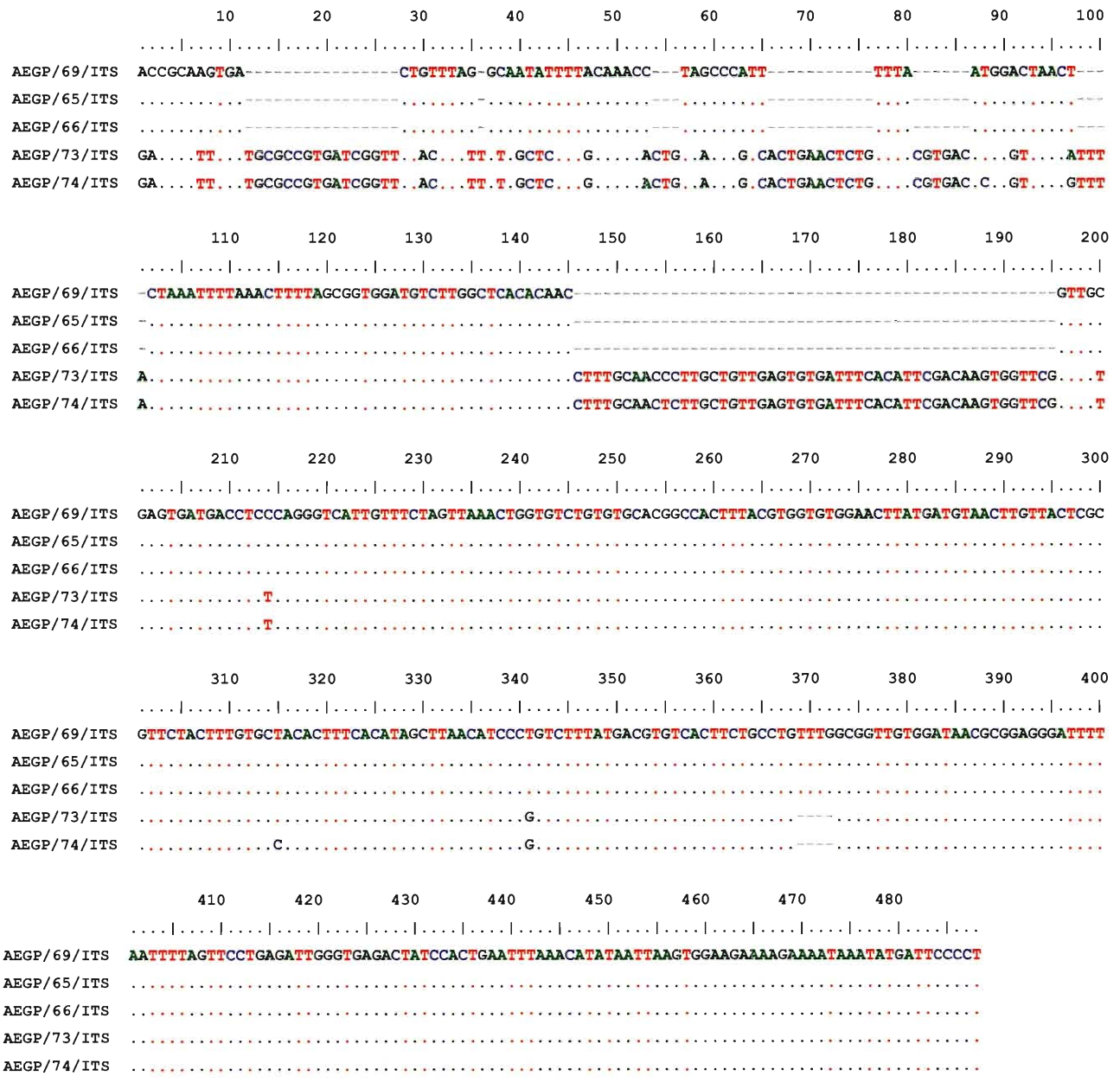

Figure 5.4: Sequence alignment of the complete ITS2 region of novel *Theileria* spp. from the Addo Elephant Game Park. The dots indicate conserved nucleotides; gaps (-) indicate missing nucleotides and were introduced to maintain homology.

Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian inference were used to determine the phylogenetic relationships of the novel ITS sequences with closely related *T. buffeli*/*T. sergenti* and *T. sinensis* sequences. A representative tree generated by Bayesian inference is shown in Figure 5.5. Three distinct clusters of the *T. buffeli* sequences were observed in all the trees. Cluster 1 was composed of the *T. sergenti* and *T. buffeli* sequences from the USA and Japan (Chitose). These two groups share identical 18S rRNA sequences (Figure 5.2, Chae et al., 1998, 1999a; Aktas et al., 2007) but different ITS (Figure 5.5; Aktas et al., 2007) and MPSP (Gubbels et al., 2000) sequences.

Cluster 2 was composed of the novel ITS sequences from AEGP and the *T. buffeli* ITS sequences from China. The latter clade is probably synonymous to that of the unclassified 18S rRNA sequences which also grouped together with AEGP 18S rRNA sequences (Figure 5.2). Cluster 3 was that of the *T. sinensis* ITS sequences (Figure 5.5). The ITS sequences of *T. cervi*, *T. uilenbergi* and *T. luwenshuni* always grouped together, while the 18S rRNA gene sequences of these species grouped separately. The 18S rRNA *T. cervi* sequences (Types F and G) grouped together, the *T. uilenbergi* 18S rRNA sequence grouped together with *T. mutans*, and the 18S rRNA sequence of *T. luwenshini* grouped together with the *T. velifera* sequence (Figure 5.2). Unlike with the 18S rRNA sequences, the *T. mutans* ITS sequence grouped together with the *T. parva* and *T. annulata* sequences (Figure 5.5; Aktas et al., 2007).

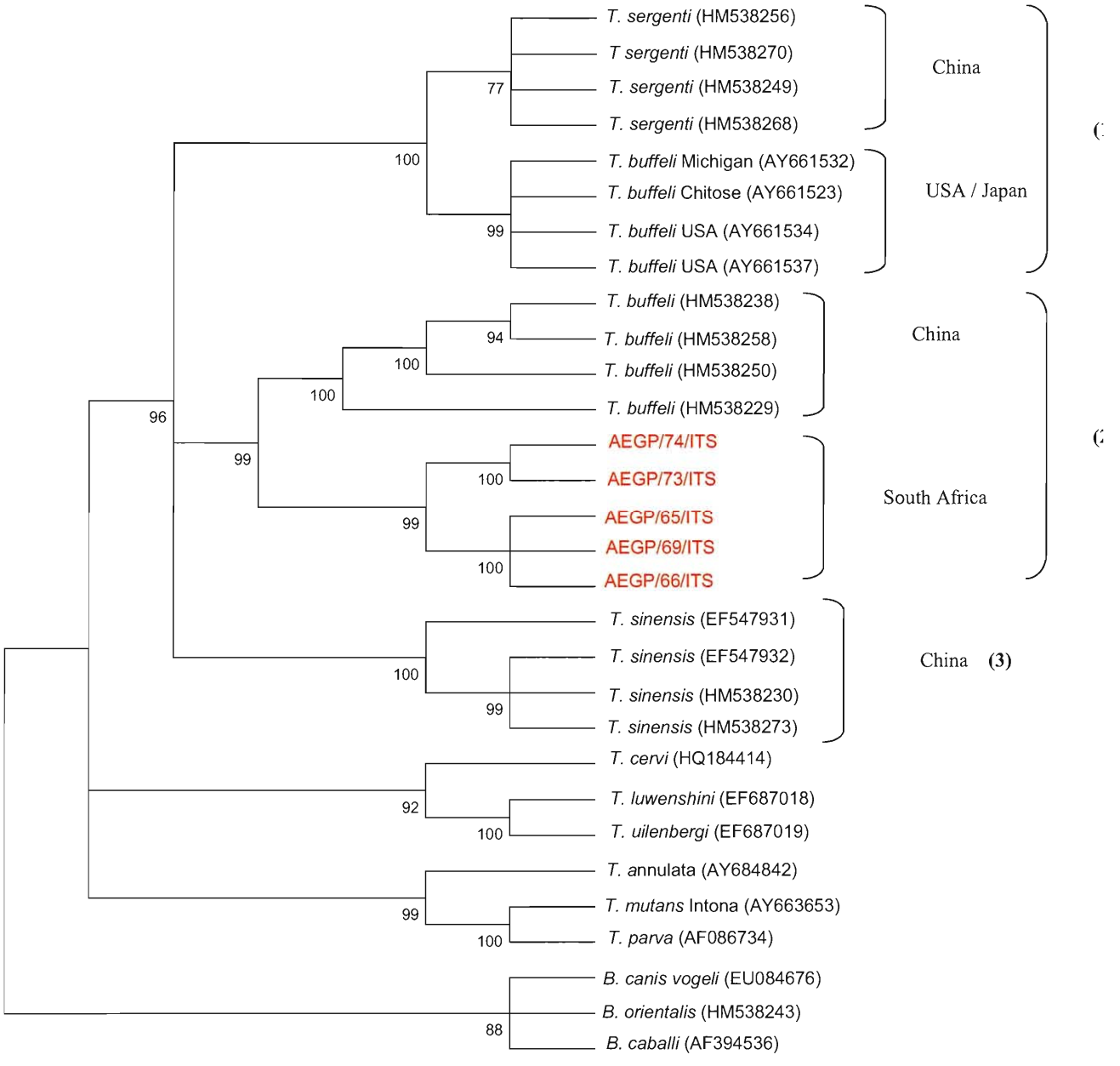

Figure 5.5: Phylogenetic relationships of novel *T. buffeli*-like ITS sequences from South Africa (red) with known *T. buffeli*-like ITS sequences from Genbank (accession numbers in parenthesis) as determined by Bayesian inference. The numbers in brackets are designated cluster numbers. Posterior probabilities are indicated at the nodes of the tree.

5.5 Discussion

5.5.1 Identification of *T. buffeli*-like and *T. sinensis*-like 18S rRNA genotypes

We identified *T. buffeli* from buffalo samples originating from the Hluhluwe-iMfolozi Game Park (HIP) (Chapter 3), and from the Addo Elephant Game Park (AEGP) (Appendix A). It was the most commonly occurring species in buffalo in the HIP, mainly co-occurring with other *Theileria* spp., and was the only *Theileria* sp. infecting buffalo in the AEGP. In contrast, *T. buffeli* was not identified from buffalo samples from the Kruger National Park (KNP), Greater Limpopo Transfrontier Park (GLTP) and a private game farm bordering the KNP (Chapter 3; Chaisi et al., 2011). This species was also identified as the most common *Theileria* spp. infecting cattle in Tunisia, and was more frequently identified in the sub-humid zone of the country than from the other climatic zones (M'ghirbi et al., 2008).

The cosmopolitan distribution of the *T. buffeli*/*T. sergenti*/*T. orientalis* species has been attributed to the global movement of cattle (and buffalo) without any regard to infection, and therefore their distribution mainly depends on the availability of a suitable tick vector (Chae et al., 1999c; Cossio-Bayugar et al., 2002). *Haemaphysalis* ticks act as vectors for this species in Australia, Asia and Europe, but the vectors in the USA and Africa are still unknown (Uilenberg, 1995; Yin et al., 2004; M'ghirbi et al., 2008). We speculate that *Haemaphysalis silacea* is the possible vector of *T. buffeli* in South Africa. This tick has a wide host range (Horak et al., 1983) and has been identified from nyalas from game parks in north-eastern KwaZulu-Natal (including the HIP), and from kudu originating from the AEGP, but not from animals in the KNP and surrounding game parks (Horak et al., 1992; 1995). Tick transmission studies are needed to confirm this speculation.

Our study has revealed that extensive variation exists between the 18S rRNA gene sequences of *T. buffeli* of the African buffalo and homologous sequences of Asian buffalo (*Bubalus bubalis*) and cattle. More variation would probably have been observed if more samples were analysed. The distribution of the different *T. buffeli* 18S rRNA genotypes (A, B, C, D, E, H) in buffalo and cattle has previously been reported (Chae et al., 1998; 1999a, Chansiri et al., 1999, Gubbels et al., 2000; 2002). Although the initial classification of these genotypes was based on a 200 bp fragment of the V4 hypervariable region of the 18S rRNA gene (Chae et al., 1998), it is applicable to the full-length of the sequences. *Theileria buffeli* type D was indicated as the most divergent genotype (Gubbels et al., 2000), and Type A as the most cosmopolitan genotype (Chae et al., 1999a). Although we did not include all known *T. buffeli* 18S rRNA sequences from GenBank in the analyses, our results

indicated that both types might be equally cosmopolitan as each type has been identified, mainly from cattle, from at least five different countries (Table 5.2). Type A and D-like organisms have been associated with bovine theileriosis in Missouri (Stockham et al., 2000), Texas (Chae et al., 1999b) and Michigan (Cossio-Bayugar et al., 2002).

There was extensive variation between *T. buffeli* type D sequences and those of the other *T. buffeli* types. Analyses of the 18S rRNA gene and MSPS gene sequences in other studies (Chansiri et al., 1999; Gubbels et al., 2000; Yin et al. 2004; Liu et al., 2010b) indicated that *T. buffeli* type D organisms may be genetically intermediate between the well-characterized pathogenic *Theileria* spp. (*T. annulata, T. parva, T. lestoquardi, T. uilenbergi, T. luwensuni)* and the benign *T. buffeli*/*T. orientalis* spp. Maximum likelihood and parsimony analysis of 18S rRNA gene sequences of *Theileira* spp. by Chanisiri et al. (1999) grouped the *T. buffeli* type D sequences with the pathogenic *Theileria* spp., whereas distance methods grouped them with those of the other *T. buffeli* sequences. Additionally, randomly amplified polymorphic DNA (RAPD) profiles generated from *Theileria* sp. Thung Song (a type D sequence), were different from those of the other benign *T. buffeli*-like species (Chansiri et al., 1999). For these reasons, these authors indicated that the classification of *T. buffeli* type D species is questionable and should be investigated. Bai et al. (1995) identified a sequence from cattle in China that was similar to the 18S rRNA sequences of *T. buffeli* type D and *Theileria* sp. Thung Song. After studying the morphology, vector and phylogenetic relationship of this novel genotype with other *Theileria* spp., this genotype was found to be a distinct species and was designated as *T. sinensis* (Bai et al., 2002a, b).

Further investigations indicated that *T. sinensis* is transmitted by *Haemaphysalis qinghaiensis* ticks and is pathogenic to cattle, yak (Yin et al., 2002; 2004; Sun et al., 2008) and probably water buffalo (Lan He – personal communication) in China. Together with *T. annulata*, *T. sinensis* and *T. sergenti* are the causative agents of bovine theiloriosis in China (Liu et al., 2010b). Morphologically, *T. sinensis* and *T. sergenti* are indistinguishable (Yin et al., 2002), but they have different tick vectors as *T. sergenti* is transmitted by *Haemaphysalis longicornis* (Liu et al., 2010b). Liu et al. (2010b) developed a PCR assay, based on MPSP gene sequences, for the detection and discrimination of these two species from cattle and yak.

The presence of organisms with *T. sinensis*-like 18S rRNA gene sequences in the African buffalo is of significant importance, particularly to the cattle industry in South Africa as these animals might act as a source of infection, via infected ticks, to naïve cattle. However, there are currently no reported cases of theileriosis that have been attributed to *T. buffeli* in South Africa.The vectors of

both *T. buffeli*-like and *T. sinensis*-like genotyes of the South African buffalo are unknown, and should be investigated.

5.5.2 Identification of novel 18S rRNA gene sequences by the RLB hybridization assay

The *T. buffeli* RLB hybridization assay probe that was used in Chapter 3 was designed by Gubbels et al. (1999) and it has been shown to detect rDNA of all known *T. buffeli*-like genotypes. All characterized samples tested positive for *T. buffeli* by this assay. Subsequently, Gubbels et al. (2000) designed additional RLB probes for the specific detection of Type D, non-type D, and Type A genotypes, and another probe that detected all the other known *T. buffeli* 18S rRNA genotypes (Ikeda, B, C, E, H, Warwick).

The novel *T. buffeli* genotype that we identified from buffalo in South Africa, and the unclassified genotype that was identified from buffalo and cattle in China and India, are all non-D genotypes but they will not be detected by the non-D probe due to the nucleotide differences (4 – 7 bp) in the RLB probe sequence. A new non-D probe can be designed in a different area to include the detection of these novel variants from buffalo in South Africa. Additional probes can also be designed for the specific detection of type SA1 and SA2 genotypes in cattle and buffalo in South Africa.

5.5.3 *Theileri buffeli*-like ITS genotypes

The ribosomal ITS region in eukaryotes is located between the small (18S) and large (28S) subunits of the ribosomal RNA gene, and spans the two ribosomal RNA transcribed spacers and the 5.8S gene (ITS1-5.8S-ITS2) (Aktas et al., 2007). Unlike the 18S rRNA gene which is highly conserved between closely related species, the spacer regions (ITS1 and ITS2) are subjected to higher evolutionary rates and are therefore more variable in their lengths and nucleotide composition (Hillis and Dixon, 1991). These regions have therefore been used for the discrimination of closely related species and subspecies, and in the description of new species (Zahler et al., 1998; Holman et al., 2003; Lew et al., 2003; Aktas et al., 2007; de Rojas et al., 2007; Hilpertshauser et al., 2007; Saito-Ito et al., 2008; Niu et al., 2009; Bosman et al., 2010).

Theileria parva, *T. annulata*, *T. mutans*, *T. ovis*, *T.sergenti* and *T. buffeli/orientalis* have previously been studied at this locus (Collins and Allsopp, 1999; Bendele, 2005; Aktas et al., 2007; Kamau et al., 2011). As observed in our study, the two spacer regions (ITS1 and ITS2) were highly polymorphic in both length and nucleotide composition, and the 5.8S region is highly conserved between sequences of related species and is shorter than the spacer regions. Our results also indicate a closer evolutionary relationship between *T. mutans* and *T. parva* at this locus as previously

indicated by Aktas et al., 2007. Minor polymorphism occurring in a single sequence is possibly due to *Taq* polymerase error but nucleotide differences occurring in more than one sequence are regarded as real (Zahler et al., 1998; Aktas et al., 2007). We therefore regard the variations that we observed as real as the variations were observed in more than one sequence.

Aktas et al. (2007) indicated that there were more variations in the ITS sequences of the pathogenic *T. annulata*, than in the mildly pathogenic *T. mutans* and *T. sergenti,* or benign *T. buffeli*/*orientalis*. The genetic variation in pathogenic species may be due to the presence of mixed parasite populations within isolates or to the ingestion of greater numbers of organisms by ticks during the acute phase of the disease, leading to a greater chance of recombination during gametogenesis (Collins and Allsopp, 1999; Aktas et al., 2007). We could not make a comparison of the variation between the different *Theileria* species as our study was based only on the *T. buffeli*/*orientalis* group.

As was the case with the 18S gene sequences from HIP clones, we expected the cloned ITS sequences from samples from this park to group together with the *T. sinesis* ITS sequences from China. However, this was not the case as five of the ITS sequences grouped together with *T. parva* and *T. mutans* sequences and two ITS sequences were more similar to the *T. buffeli*-like ITS sequences from AEGP. It is therefore possible that buffalo in HIP harbour both *T. buffeli* and *T. sinensis*-like ITS genotypes, however, a lot more sequence data is required to verify this speculation. The occurrence of *T. parva* and/or *T. mutans* sequences in samples that had tested negative for these species by RLB probably indicated parasitemia that was below the detection limit of the assay. This also confirms the complexity of identification of *Theileria* spp. in mixed infections in buffalo as we reported in Chapters 3 and 4.

5.5.4 Classification of the novel *Theileria* spp. genotypes

Based on the phylogenetic positions, nucleotide differences (with known sequences) in the full-length sequences and the hypervariable (V4) region of the 18S rRNA gene and ITS region, it is possible that the two novel *Theileria* spp. genotypes from the South African buffalo represent distinct species. However, additional molecular and biological data are required for such classification. Moreover, there is currently no consensus in the classification of novel genotypes as new species based on the number of nucleotide differences. It is also not clear if the genetic distances within the 18S rRNA gene sequences of Types B, C, E, H, Ikeda, Ipoh and Medan represent heterogeneity within the same or different *Theileria* spp. (Chae et al., 1999a). The 18S

rRNA sequences of these genotypes form a monophyletic group that is separated from the other *T. buffeli* genotypes (Figure 5.2).

5.6 Conclusion

We established the phylogenetic position, based on 18S rRNA gene and ITS sequences, of *T. buffeli*-like organisms occurring in buffalo in the AEGP. We also established, by 18S rRNA gene sequence analysis, that the novel *Theileria* spp. of buffalo in the HIP is more closely related to the *T. sinensis*-like genotype of cattle and yak from China. This study has confirmed that *T. buffeli* is a highly diverse and cosmopolitan species. The role of buffalo and other wildlife as reservoir hosts of these species should be investigated as buffalo are known to be sources of many infectious diseases of cattle in South Africa (Mashishi, 2002). Future studies should focus on animal transmission studies in order to determine the tick vectors of the *T. buffeli*-like and *T. sinensis*-like genotypes, and on epidemiological studies using new probes that specifically detect and differentiate these novel genotypes in hosts and tick vectors in South Africa. Our study provides useful genetic information towards the proper classification of this very complex group.

5.7 References

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CHAPTER 6

Sequence variation in the V4 hypervariable region of the 18S rRNA gene of *Theileria* spp. of the African buffalo (*Syncerus caffer*) and cattle: Implications for the diagnosis of *Theileria parva* infections in cattle and buffalo in South Africa

6.1 Abstract

This study is a continuation of our previous studies on the characterization of the 18S rRNA gene of *Theileria* species of buffalo in South Africa in an attempt to identify novel genotypes that might interfere with the diagnosis of *T. parva* infections in cattle and buffalo in South Africa when using the quantitative real-time PCR (qPCR) assay. In this study, we characterized and analysed the V4 hypervariable region of the 18S rRNA gene from fifty-seven clones obtained from 31 buffalo (n=28) and cattle (n=3) samples originating from different geographic regions in South Africa. A total of thirteen genotypes of these *Theileria* spp., namely, *Theileria* sp. (buffalo), *Theileria* sp. (bougasvlei), *T. parva*, *T. velifera*, *T. velifera* B, *T. taurotragi*, *T. buffeli*-like, *T. sinensis*-like, *T. mutans*, *T. mutans*-like 1, 2, 3, and *Theileria* sp. (strain MSD), were identified. Only *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) had sequences that were similar to the *T. parva* specific qPCR forward primer sequence. However, the *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) sequences were different in the region of the *T. parva*-specific qPCR probe. This implies that although *Theileria* sp. (buffalo) and *Theileria* sp. (bougaslvei) could be co-amplified with *T. parva* in mixed infections, only *T. parva* amplicons would hybridize with the sensor and anchor probes resulting in characteristic melting peaks that are used to identify *T. parva* infections.

These results are consistent with those obtained recently by Mans et al. (2011) who characterized the V4 hypervariable region of the 18S rRNA gene of *Theileria* spp. of cattle and buffalo in South Africa. In addition to the sequences identified in our study, these authors identified 13 more *Theileria* genotypes/species variants from cattle and buffalo samples from South Africa.

6.2 Introduction

The 18S rRNA gene is part of the ribosomal unit of eukaryotes. It is present in all extant species and cells and can therefore be easily amplified and sequenced (Smit et al., 2007). It is also one of the most frequently used genes in phylogenetic studies as it consists of regions that are highly conserved between species, allowing for the design of universal primers, as well as variable regions from which species-specific primers and probes can be designed. The gene is tandemly repeated within eukaryotic genomes and therefore provides excess amounts of template DNA for PCR amplication (Meyer et al., 2010). Although the variable regions between species differ in both number and nucleotide composition, the V4 region is the most variable (hypervariable) region in almost all species. Sequence variations within this region of the 18S rRNA gene have enabled the development of specific and sensitive diagnostic assays for several different pathogenic *Theileria* and *Babesia* spp. (Kim et al., 2008; Sibeko et al., 2008; Criado-Fornelio et al., 2009; Bhoora et al., 2010; Wang et al., 2010; Papli et al., 2011). The specificity of a diagnostic assay for the identification of pathogenic species based on the variable regions of the 18S rRNA gene depends on the knowledge of all genotypes in the specific locality.

In the previous three chapters, we characterized the full-length sequences of the 18S rRNA gene of the pathogenic, mildly pathogenic and non-pathogenic *Theileria* spp. of buffalo in order to determine the sequence variation within this group and whether the specificity of the 18S rRNA gene quantitative real-time PCR (qPCR) assay for the diagnosis of *Theileria parva* could be compromised by the presence of unknown 18S rRNA sequences of other *Theileria* spp. in cattle and buffalo that are similar to those of *T. parva*. The qPCR primers and probes are located in and around the V4 hypervariable region of the 18S rRNA gene (Sibeko et al., 2008).

In this chapter, we characterized and analysed additional partial 18S rRNA sequences encompassing the V4 hypervariable region of all *Theileria* spp. of the African buffalo and cattle in order to determine if there are yet unidentified 18S rRNA genotypes. In addition, all of our results were compared with those of a recent study by Mans et al. (2011) which involved the identification of *Theileria* spp. variants of buffalo and cattle in South Africa. The analyses in this chapter included the new partial 18S rRNA gene sequences and those obtained from Chapters 3, 4 and 5, as well as the partial 18S rRNA gene sequences identified by Mans et al. (2011).

6.3. Materials and Methods

6.3.1 Blood samples and DNA extraction

Twenty-eight buffalo and three cattle blood samples were analysed. The buffalo samples originated from the Kruger National Park (KNP), Hluhluwe-iMfolozi Game Park (HIP), Addo Elephant Game Park (AEGP), a private game ranch in the Hoedspruit (H) area in Mpumalanga, and the Greater Limpopo Transfrontier Park (GLTP). These samples were part of a previous study (Chapter 3) and had either tested positive for different *Theileria* spp., when analysed with the reverse line blot (RLB) hybridization assay, or only hybridized with the *Theileria* and/or *Babesia* genus-specific probe and not with any of the *Theileria* or *Babesia* species-specific probes used in the assay. The cattle samples were obtained from a farm in KwaZulu-Natal (KZN/bov) and from the Onderstepoort Veterinary Research Institute (OVI/778 and OVI/779). Genomic DNA was extracted from the blood samples using the High Pure Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. DNA was eluted in 100 µl elution buffer and stored at -20°C pending further analysis.

6.3.2 PCR amplification, cloning and sequencing of the hypervariable V4 region of the 18S rRNA gene

Partial 18S rRNA gene sequences were obtained by amplification of the V4 hypervariable region of the gene using primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (without biotin) (5'-CTA AGA ATT TCA CCT CTG ACA GT-3') (Nijhof et al., 2003). DNA extracted from buffalo KNP102 (Sibeko et al., 2008) and nuclease-free water were used as positive and negative controls, respectively. The reaction mixture and cycling conditions were as outlined for the RLB hybridization assay (Chapter 3; Chaisi et al., 2011). Purified amplicons (~500 bp) were ligated into the pGEM-T Easy Vector and transformed into JM109 High Efficiency cells (Promega, Madison, WI). At least 5 white colonies per sample were selected and screened by colony PCR (Chapter 4). Recombinant plasmid DNA was extracted from overnight bacterial cultures using the High Pure Plasmid Isolation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The recombinants were sequenced using the RLB-F2 and RLB-R2 primers, or vector primers, SP6 and T7 (Chapter 3; Chaisi et al., 2011). Purified products were analyzed on an ABI3100 genetic analyzer at the ARC-OVI (South Africa) sequencing facility.

6.3.3 Sequence and phylogenetic analyses

The sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Bonfield et al., 1995; Staden et al., 2000). The corresponding partial sequences from the full-length 18S rRNA gene sequences obtained in previous studies (Chapters 3, 4. 5), were included in further analyses. A search for homologous sequences was performed using the Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990). The sequences were then aligned with known sequences from GenBank using the MAFFT (version 5) multiple sequence alignment programme employing the FFT-NS-i algorithm (Katoh et al., 2005), and manually edited using BioEdit (version 7) (Hall, 1999). The GenBank sequences included recently identified novel 18S rRNA sequences of *Theileria* spp. of cattle and buffalo in South Africa (Mans et al., 2011). The genetic distances between the sequences were estimated by determining the number of base differences between sequences using MEGA4 (Tamura et al., 2007). Phylogenetic trees were constructed from a final alignment of 102 sequences with 312 characters using MEGA4 for distance-based (neighbor-joining) trees, and PAUP* v4b10 (Saitou and Nei, 1987; Swofford, 2003) for character-based (maximum likelihood and maximum parsimony) trees. Bootstrap analysis was done using 1000 replicates/tree. Phylogenetic trees were also constructed by Bayesian inference using MrBayes (v3.1.2) (Ronquist and Huelsenbeck, 2003), accessed via the Computational Biology Service Unit, Cornell University (http://mafft.cbsuapps.tc.cornell.edu/mrbayes.aspx). In all cases, the trees were rooted by the 18S rRNA sequences of *Sarcocystis muris* (M64244), *Prorocentrum micans* (M14649) and *Toxoplasma gondii* (X68523) and the consensus trees were edited using MEGA4.

6.4. Results

A total of 58 18S rRNA gene sequences (partial = 8, and full-length = 50) were obtained from 31 samples. All full-length sequences, with the exception of the *T. taurotragi* sequences (KZN/bov/a and KNZ/bov/d) (Appendix A), were obtained as part of Chapters 3, 4, 5. The lengths of the partial sequences varied from 401 to 800 bp.

We identified a total of 13 *Theileria* spp. genotypes: *T. buffeli*-like (n = 7), *T. sinensis*-like (n = 5), *T. velifera* (n = 3), *T. velifera*-like/B (n = 2), *Theileria* sp. (buffalo) (n = 6), *Theileria* sp. (buffalo)-like/*Theileria* sp. (bougaslvei) (n = 6), *T. parva* (n = 10), *Theileria taurotragi* (n = 2), *Theileria mutans* (n = 1), *T. mutans*-like 1 (n = 8), *T. mutans*-like 2 (n = 3), *Theileria mutans*-like 3 (n = 2), *Theileria* sp. MSD (n = 3) (Figure 6.1). Seven of these genotypes were recently reported as novel/unique (Chaisi et al., 2011; Mans et al., 2011; Chapters 3, 4, 5) (Figure 6.1) and six were known *Theileria* spp. A maximum of 4 and 2 genotypes were identified from buffalo and cattle samples, respectively.

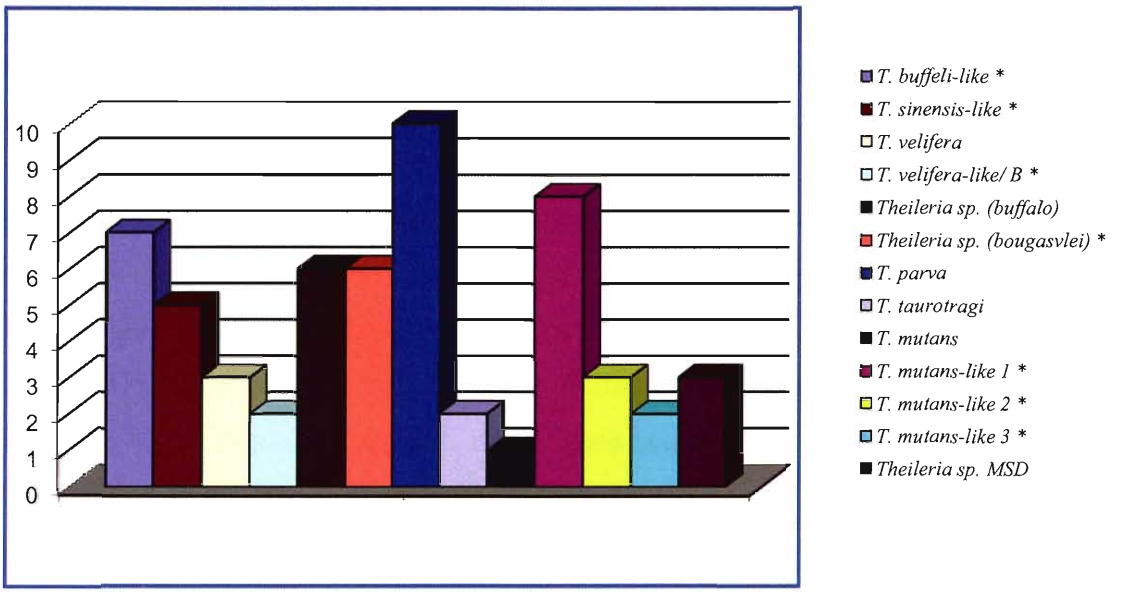

Figure 6.1: The frequency of known and novel (*) 18S rRNA gene sequences (n = 57) of *Theileria* spp. identified from buffalo and cattle samples (n = 31) from different geographical regions in South Africa, and from the GLTP in Mozambique.

The 13 genotypes grouped together into four distinct clades as indicated by Neighbor-joining, maximum parsimony, maximum likelihood and Bayesian analysis. A representative tree generated by Neighbor-joining analysis is shown in Figure 6.2. Two novel genotypes, *T. buffeli*-like and *T. sinensis*-like, were included in Clade 1. Within this clade, AEGP sequences, identical to *Theileria* cf. *buffeli*C (GU733373) (Mans et al., 2011) grouped closely with the *T. buffeli* sequences from India and China. The partial sequences from HIP were identical to the *Theileria* cf. *sinensis* (GU733372) sequence (Mans et al., 2011) and grouped closely with *T. sinensis*, *Theileria* sp. type D and *Theileria* sp. Thung Song. The two genotypes differed from each other by eleven nucleotides over a 312 bp region (Table 6.1)

Clade 2 was the *T. velifera* group. The buffalo (HIP/A4/d) and cattle (KZN/bov/d) *T. velifera* sequences grouped together with *T. velifera* sequences from cattle in Tanzania (AF097993) (Gubbels et al., 1999) and Mozambique (FJ869896 and FJ869897) (Martins et al., 2010).

The second genotype included novel *T. velifera*-like buffalo sequences (KNP/Q15/a and HIP/H4/a), and *T. velifera* B (GU733376) (Mans et al., 2011). The partial 18S rRNA gene sequences of the two genotypes differed from each other by three nucleotide differences (Table 6.1).

Twenty-three sequences, consisting of four genotypes, grouped together in Clade 3. We identified a single *T. parva* genotype from buffalo (Figure 6.2). These were identical to published *T. parva* sequences from buffalo (HQ6840566) and cattle (L02366), and differed from the cattle *T. parva* sequence AF013418 by one nucleotide. The *Theileria* sp. (buffalo) sequences were identical to the published *Theileria* sp. (buffalo) 18S rRNA gene sequence (DG641260), with the exception of a single nucleotide difference in sequence KNP/Q4/c (Figure 6.3). The novel *Theileria* sp. buffalo-like sequences that we previously identified (Chapter 3; Chaisi et al., 2011) grouped together with the partial 18S rRNA gene sequence of *Theileria* sp. (bougaslvei) (GU570997) (Mans et al., 2011), obtained from buffalo from the Bougaslvei farm, Limpopo province, South Africa (Zweygarth et al., 2009). Two cattle-derived sequences (KZN/bov/a and KZN/bov/c) were identical to the published 18S rRNA gene sequence of *T. taurotragi* (L19802). Heterogeneity between the partial 18S rRNA gene sequences of this clade differed by 3 (*T. parva* and *Theileria* sp. (buffalo)) to 11 (*T. parva* and *T. taurotragi*) nucleotides (Table 6.1).

Clade 4 consisted of five genotypes which included the *T. mutans*, *T. mutans*-like 1, 2, 3 and *Theileria* sp. (strain MSD) 18S rRNA gene sequences (Figure 6.2). One sequence (OVI/779/a) was identical to published cattle *T. mutans* sequences from Kenya (AF078815) (Chae et al., 1999), Mozambique (FJ869898 and FJ869899) (Martins et al., 2010), and Sudan (EF469605) (Salih et al., 2007). *Theileria mutans*-like sequences 1, 2, 3 were identified from 8, 3 and 2 buffalo samples, respectively (Figure 6.2). The *T. mutans*-like 3 sequences were identical to the*Theileria* cf. *mutans* (GU733377) sequence, also from buffalo in South Africa (Mans et al., 2011). Lastly, three sequences grouped together with published *Theileria* sp. (strain MSD) sequences from cattle in South Africa (AF078816) (Chae et al., 1999) and Mozambique (FJ869895) (Martins et al., 2010). The published sequences were identical to the cattle-derived sequences (OVI/778/a and OVI/778/e), and differed from the buffalo sequence (KNP/B15/a) by one nucleotide. Polymorphism within and between clades is indicated in Table 6.1. *Theileria* sp. MSD sequences had the greatest variation (up to 39 bp) with the other *Theileria* spp. sequences.

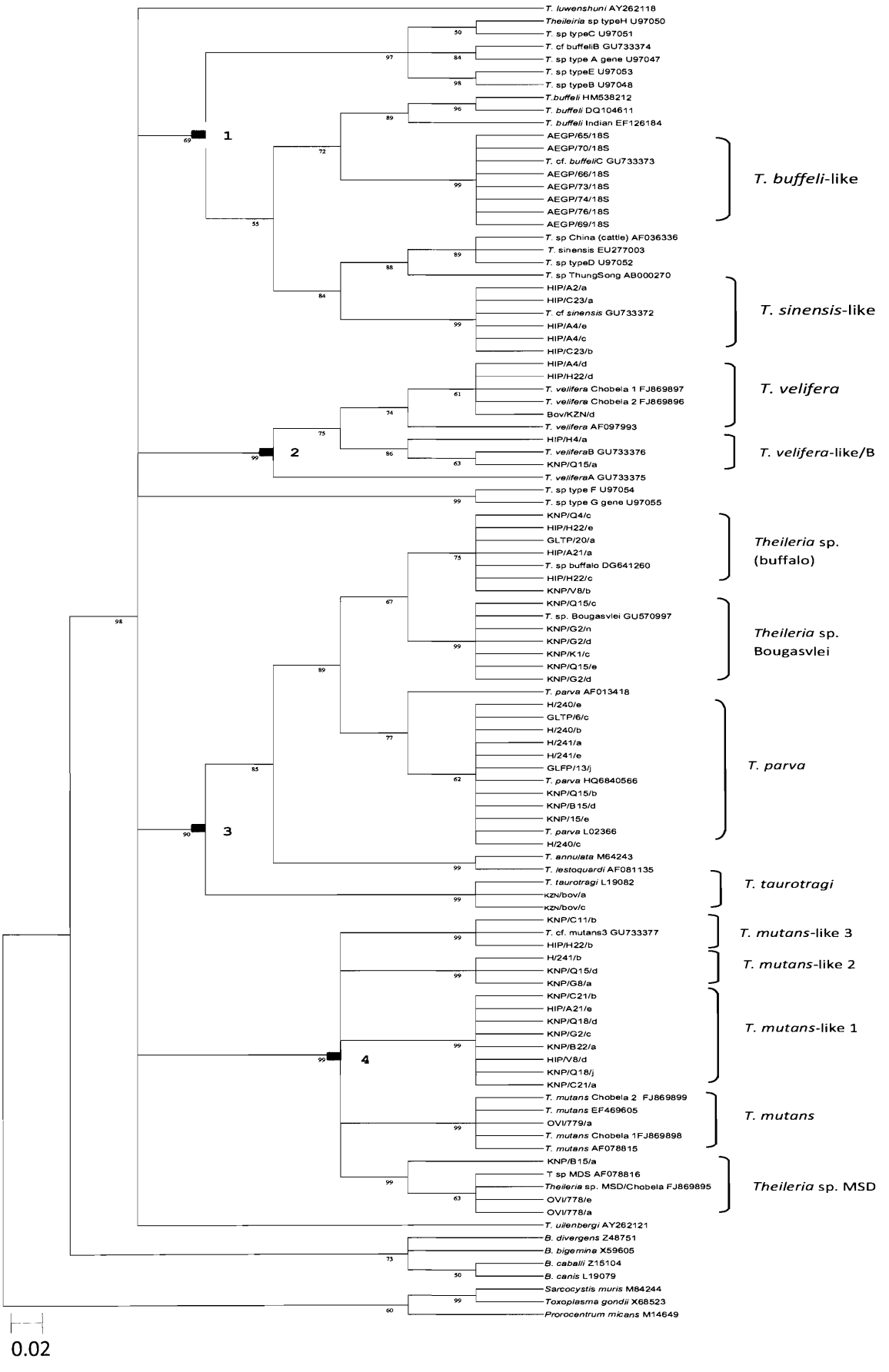

Figure 6.2: Phylogenetic tree showing the relationships between *Theileria* spp. variants identified in this study with known *Theileria* and *Babesia* species as indicated by Neighbour-joining analysis. Bootstrap values indicate the degree of support for each cluster. The tree was rooted using *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii*.

Table 6.1: Estimates of the evolutionary divergence between *Theileria* spp. genotypes identified in this study as indicated by MEGA4 (Tamura et al., 2007). The numbers are base difference between sequences. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). A total of 255 positions were compared.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1.*T. buffeli*-like													
2. *T. sinensis*-like	11												
3. *T. velifera*	17	24											
4.*T. velifera*-like/B	18	25	3										
5. *Theileria* sp. (buffalo)	21	22	25	24									
6. *Theileria* sp. (bougaslvei)	21	25	27	28	4								
7. *T. parva*	24	22	24	25	3	7							
8. *T. taurotragi*	24	22	27	27	10	9	11						
9.*T. mutans*	30	30	36	37	22	22	22	26					
10. *T. mutans*-like 1	30	29	36	38	25	22	25	28	7				
11. *T. mutans*-like 2	31	30	37	38	25	25	25	26	10	8			
12. *T. mutans*-like 3	30	27	36	37	22	22	22	28	6	15	11		
13. *Theileria* sp. MSD	38	36	39	39	27	27	27	28	11	13	13	10	

Figure 6.3 shows an alignment of part of the V4 hypervariable region of *Theileria* spp. and their variants. The sequences of the *T. parva* forward primer and probes (anchor and sensor) of the 18S rRNA qPCR assay that is used for the diagnosis of *T. parva* infections in cattle and buffalo in South Africa are indicated in blocks. All the *T. parva* sequences are identical in this region, indicating the presence of a single genotype. The *Theileria* sp. (buffalo) sequences were identical to the *T. parva* sequences in the primer and anchor probe areas, and differ by three nucleotide differences in the sensor probe area. There are one and four base differences between the *Theileria* sp. (bougasvlei) and *T. parva* sequences in the primer and sensor probe areas, respectively. There are more than three base pair differences between the sequences of the other genotypes and the *T. parva* forward primer, anchor and sensor sequences.

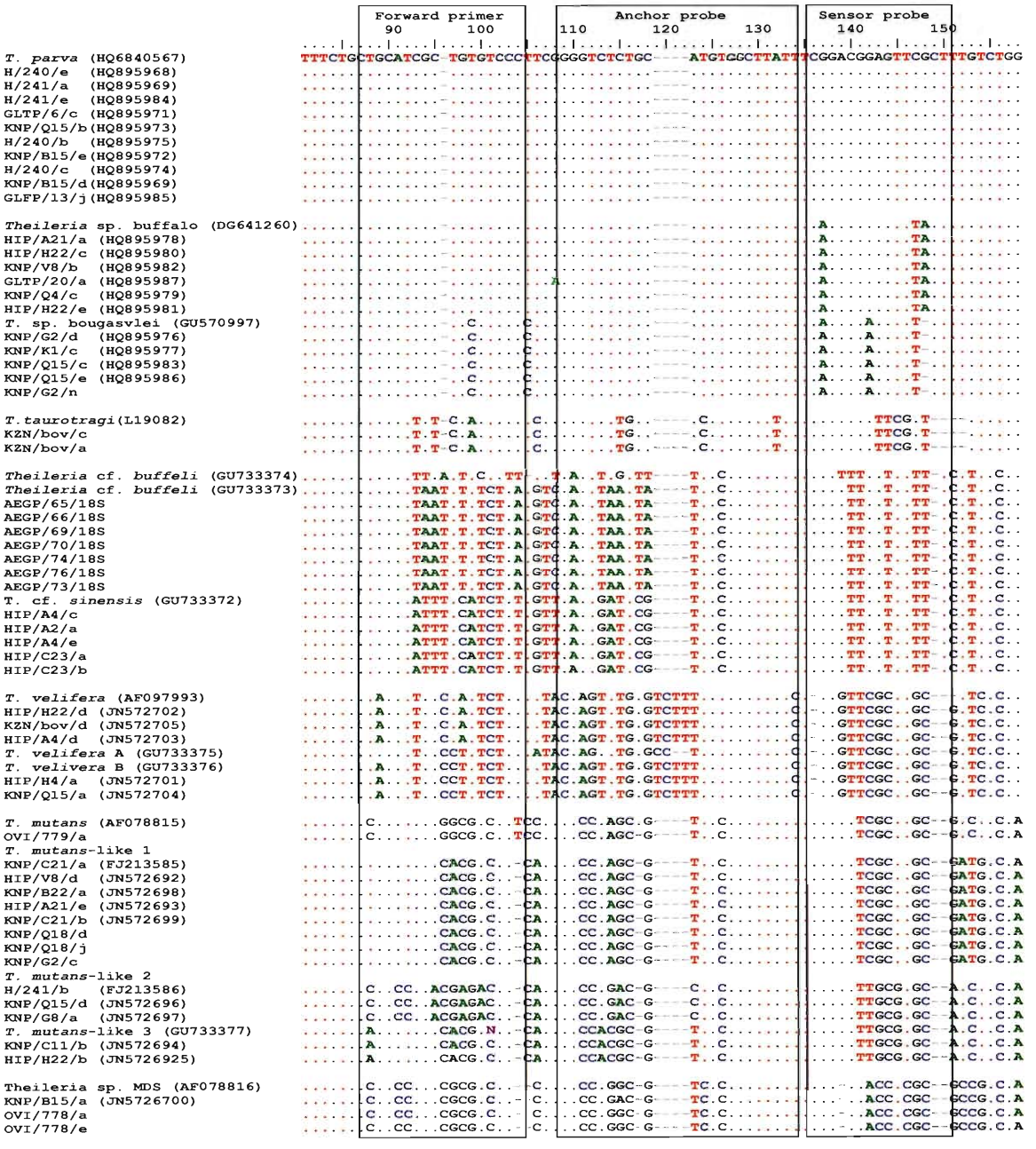

Figure 6.3: Nucleotide alignment of a region of 78 bp of the V4 hypervariable region of published 18S rRNA gene sequences of *Theileria* spp. of cattle and buffalo and their variants identified in this study. Dots represent nucleotides that are identical to those of the *T. parva* (HQ680566) sequence and dashes represent nucleotide deletions. Areas where the *T. parva* real-time qPCR forward primer and probes sequences were designed are indicated with boxes.

6.5. Discussion

The RLB hybridization assay of Gubbels et al. (1999) was developed for the simultaneous identification and discrimination of *Babesia* and *Theileria* spp. in infected hosts based on variations within the V4 hypervariable region of their 18S rRNA genes. The concurrent use of genus-specific and species-specific probes in this assay has resulted in the identification and description of novel *Theileria* and *Babesia* species and species variants (Nijhof et al., 2003; Nagore et al., 2004; Criado et al., 2006; Matjila et al., 2008; Oosthuizen et al., 2008; Bosman et al., 2010). It is possible to use RLB to identify novel species or genotypes in animals that harbor single *Theileria* and/or *Babesia* species, but this is usually not the case in hosts which harbor multiple *Theileria* species (as is the case with cattle and buffalo), as the presence of positive signals for known species may lead to the conclusion that there are no novel genotypes (Chaisi et al., 2011, Mans et al., 2011; Chapters 3, 4, 5). This limitation can be overcome by random sequencing of 18S rRNA genes amplified from samples that test positive for multiple *Theileria* species (Mans et al., 2011).

We previously reported on the sequence variations within the full-length 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo) (Chapter 3), *T. mutans* and *T. velifera* (Chapter 4), and *T. buffeli* (Chapter 5). The genotypes identified from partial 18S rRNA gene sequences in the present study were similar to those that we identified from the full-length sequences. In all cases, the novel genotypes were derived from multiple samples and can therefore be regarded as true species variants. However, single nucleotide polymorphisms occurring in only one sequence (as is the case with sequences KNP/Q4/c and KNP/B15/a) are likely to be due to PCR or sequencing errors (Zahler et al., 1998).

Our study describes the occurrence of at least 13 distinct 18S rRNA *Theileria* genotypes of the African buffalo based on the V4 hypervariable region of the 18S rRNA gene. Seven of these genotypes differed from known or previously reported sequences and were recently reported as novel (unique) by Mans et al. (2011). Our study was limited by a small sample size, while Mans et al. (2011) analysed more samples from both buffalo and cattle. In addition to the *T. parva* genotype that we identified Mans et al. (2011) identified seven additional novel 18S rRNA variants of *T. parva*, five of which were identified only in cattle and two were identified in both cattle and buffalo. Our results indicated that the sequence of the novel genotype that we previously designated as *Theileria* sp. (buffalo)-like (Chapter 3; Chaisi et al., 2011), is identical to *Theileria* sp. (bougasvlei), as a novel species of the Africa buffalo (Zweygarth et al., 2009; Mans et al., 2011).

Additionally, Mans et al. (2011) described two more novel variants of *Theileria* sp. (buffalo).

The four *T. mutans* genotypes that we identified from buffalo consist of one known, and three novel genotypes. The novel genotypes have thus far only been detected in buffalo, while the former has been identified in both cattle and buffalo. These results are consistent with those of Mans et al. (2011). In addition to the known *T. velifera* and novel *T. velifera*-like/B genotypes, Mans et al. (2011) identified an additional genotype, designated as *T. velifera* A, from cattle and buffalo samples. *T. velifera*-like/B has thus far been identified from buffalo samples only. Our results of the novel *T. buffeli*-like and *T. sinensis*-like genotypes (Chapter 5) are similar to those of Mans et al. (2011). Similarly, the identification of *Theileria* sp. strain MSD from cattle and buffalo samples was reported from both studies.

In the present study, *T. parva* sequences were the most frequently identified. This is because we mainly selected samples that were positive for *T. parva* and/or *Theileria* sp. (buffalo) for characterization. Although we identified *Theileria* sp. (bougaslvei) sequences only from buffalo samples originating from the KNP, this genotype is not limited to this geographical region as the first description of this genotype was from buffalo originating from a farm in the Limpopo province (Zweygarth et al., 2009). The geographical distribution of *Theileria* sp. (bougasvlei), and of the other novel genotypes, in South Africa can only be determined by epidemiological studies using newly designed genotype-specific RLB probes.

In both studies, *T. taurotragi* was identified only from cattle samples, *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) were identified from buffalo only, while *T. parva*, *T. velifera* and *Theileria* sp. MSD were identified from cattle and buffalo samples. *Theileria taurotragi* is a parasite of eland (*Taurotragus oryx*) (Martin and Brocklesby, 1960). It also infects cattle, sheep and goats and is transmitted by *Rhipicephalus appendiculatus* and *Rhipicephalus zambeziensis* (Uilenberg et al., 1982; Stagg et al., 1983). Although we identified the 18S rRNA gene sequence of *T. mutans* from a single cattle sample, this genotype was identified from both cattle and buffalo by Mans et al. (2011). Although we previously identified this species in buffalo samples using the RLB hybridization assay (Chapter 3; Chaisi et al., 2011), identification of *T. mutans* sequences from buffalo in our study was probably limited by low parasitaemia in the blood samples and/or the small number of samples characterized, since our sample set was biased towards *T. parva* samples and samples with a genus-specific signal only and we did not characterize samples that were positive for *T. mutans* on the RLB assay.

In conclusion, our results confirm the findings of Zweygarth et al. (2009) and Mans et al. (2011) that although extensive sequence variation does occur within *Theileria* spp. of buffalo and cattle, only *T. parva* and its variants (as identified by Mans et al., 2011), *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) will be amplified by the primers used in the 18S rRNA qPCR assay. Differences in the *T. parva*–specific anchor and sensor probe sequences prevent their annealing to PCR amplicons of *Theileria* sp. (buffalo) or *Theileria* sp. (bougasvlei) and therefore characteristic melting peaks (Sibeko et al., 2011; Chaisi et al., 2011) will only be observed in *T. parva* positive samples. However, the diagnosis of *T. parva* in animals that are co-infected with *Theileria* sp. (buffalo) and/or *Theileria* sp. (bougasvlei), and in which the parasitemia of the latter genotypes is greater than that of *T. parva*, might still pose a challenge due to competition of primers (Pienaar et al., 2011). This necessitates the identification of new gene targets which sufficiently differentiate between these species. Such gene targets can be used to develop diagnostic assays that either replace or complement existing assays.

There are currently no guidelines on the definition of new protozoan species based on their molecular phylogeny, and the use of multiple genes in inferring phylogenetic trees as evidence in the determination of new species is encouraged (Taylor et al., 1999, Naumov et al., 2000; Meyer et al., 2010). We will have to sequence more genes in order to designate the novel genotypes as new species.

6.6 References

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CHAPTER 7

Evaluation of a "pan" FRET real-time PCR test for the discrimination of *Theileria* species in the African buffalo (*Syncerus caffer*)

7.1 Abstract

A quantitative fluorescence resonance energy transfer (FRET)-real-time PCR (qPCR) assay based on the cox III gene was developed for the simultaneous detection and discrimination of *Theileria* species in cattle using melting curve analysis. The cox III qPCR assay can detect *Theileria parva*, *Theileria mutans*, *Theileria velifera*, *Theileria annulata*, *Theileria taurotragi* and *Theileria buffeli* in infected cattle using a nested qPCR assay with a single set of probes. The assay was modified, by the development of a new set of primers and probes, to include the detection of *Theileria* sp. (buffalo) from buffalo samples. In the current study, we evaluated the modified cox III qPCR assay for the identification and discrimination of *Theileria* spp. of the African buffalo.

The results of the cox III qPCR assay were compared with the reverse line blot (RLB) hybridization assay for the simultaneous detection and differentiation of *Theileria* spp. in 220 buffalo and 4 cattle blood samples from South Africa and Mozambique. The following species were detected using the cox III qPCR assay: *T. parva* (83.5%), *Theileria* sp. (buffalo) (55.8%); *T. taurotragi* (1.8%), *T. buffeli* (5.8%) and *T. mutans* (2.2%). Seventeen percent of the samples had non-specific melting peaks and 4.5% of the samples were negative or below the detection limit of the assay. *Theileria velifera* was not detected from any of the samples analysed by the cox III qPCR. The assay detected more *T. parva* and *Theileria* sp. (buffalo) infections but fewer *T. mutans* and *T. buffeli* infections than the RLB assay.The cox III qPCR test identified *Theileria* spp. infections in samples that were negative or below detection limit of the RLB assay and therefore seems to be the more sensitive test. However, the identification of *T. taurotragi* by the cox III qPCR assay in some buffalo samples was unexpected. To our knowledge *T. taurotragi* has never been isolated from the African buffalo. Because of the discrepancies between *Theileria* species identified by the RLB and cox III qPCR assays, the identification of *T. taurotragi* in buffalo samples, and the non-specific peaks observed in the cox III qPCR assay, cox III qPCR products were cloned and the resulting clones were

sequenced. Sequencing and phylogenetic analysis indicated extensive sequence differences, in both the number of genotypes and co-infecting genotypes, in buffalo and explained the apparently low occurrence of benign *Theileria* spp. as detected by the cox III qPCR assay. The cox III qPCR was subsequently also compared with the 18S qPCR assay for the specific detection of *T. parva* in 206 samples. *Theileria parva* was detected in 51.9%, 79.2% and 86.4% of the 206 samples analysed by the RLB, 18S qPCR and cox III qPCR assays, respectively

7.2 Introduction

Theileria parva is the causative agent of Corridor disease (theileriosis) in cattle in South Africa and the African buffalo (*Syncerus caffer*) is the reservoir host. As theileriosis and other diseases carried by buffalo are a threat to farming communities in the endemic areas of the country (Uilenberg, 1999), interaction between cattle and buffalo is limited. Buffalo must test negative for *T. parva* before translocation, and this has resulted in an increased demand and cost of disease-free animals (Collins et al., 2002). The tests used for the diagnosis of *T. parva* should therefore be sensitive and specific for accurate diagnosis. However, benign and non-pathogenic *Theileria* species that co-exist with *T. parva* in infected animals can compromise the specificity and sensitivity of the tests used.

Polymerase chain reaction (PCR) assays are more sensitive and specific than microscopy and serological methods, and usually limit the subjectivity that occurs in the interpretation of results obtained from these methods (Figueroa and Buening, 1995; Zarlenga and Higgis, 2001). Real-time PCR is easy to perform, less prone to contamination and reduces the time and labour required for attainment of results (Bell and Ranford-Cartwright, 2002; Jaton et al., 2006). A quantitative real-time PCR (qPCR) assay based on the 18S rRNA gene developed by Sibeko et al. (2008) is currently used, together with other diagnostic tests, for the diagnosis of *T. parva* infections in cattle and buffalo in South Africa. However, the sensitivity of this test in diagnosing *T. parva* from buffalo that are co-infected with *Theileria* sp. (buffalo) is compromised as the 18S rRNA genes of both species are amplified by the *T. parva*-specific primer set (Sibeko et al., 2008; Chaisi et al., 2011; Mans et al., 2011; Pienaar et al., 2011). Alternative assays, based on more informative molecular markers, are needed to accurately detect and differentiate between pathogenic and non-pathogenic *Theileria* species in cattle and buffalo. Since PCR performance is proportional to the number of amplification targets present in a sample, targeting a repeated gene should increase the sensitivity of PCR assays (Criado et al., 2006).

A nested qPCR based on the cytochrome oxidase subunit (cox) III gene was developed for simultaneous detection and differentiation of six *Theileria* spp. in cattle samples by melting curve analysis (Janssens, 2009). This assay was further modified to include the detection of *Theileria* sp. (buffalo) from buffalo (results not published). Cox is the terminal component of the mitochondrial respiratory chain, it catalyzes the reduction of oxygen to water, producing ATP via oxidative phosphorylation and therefore allows the cell to utilise energy and oxygen (Cannino et al., 2004; Lienard et al., 2006). It is composed of 13 sub-units, and cox III is one of its three mitochondrial components (Schmidt et al., 1997).

The qPCR assays by Sibeko et al. (2008) and Janssens et al. (2009) are both based on fluorescence resonance energy transfer (FRET) technology. This involves the use of sequence-specific oligonucleotide (hybridization) probes that are labelled with fluorescence dyes (Reuter et al., 2005). Hybridization probes provide a simple way of analysing sequence variations using a single reaction and one set of probes as both amplification and hybridization occur in the same reaction (Caplin et al., 1999). When both probes have hybridized to the PCR product, and are heated by slowly raising the temperature, the donor (sensor) probe absorbs light and transfers the resonance energy to the acceptor (anchor). The temperature (T_m) at which the hybridization probes are melted off the DNA strand can then be quantitatively measured in real-time by melting curve analysis (Caplin et al., 1999; Reuter et al., 2005). The sensor probe covers the variable target sequence, and therefore determines the T_m, and the anchor probe produces the fluorescent signal. If the sensor probe is designed to perfectly fit the DNA strand, then it melts off at a higher temperature than when there is a mismatch (Reuter et al., 2005). Generally, a T_m difference of more than 1.5ºC from the standard curve is an indication of the presence of a mutation (Reuter et al., 2005), and a single mismatch between the sensor probe and mutant will reduce the T_m by about 5-8ºC (Landt and Nitsche, 1999). To improve the discriminative power of the sensor probe, the T_m of the sensor probe should be lower than that of the anchor probe, and this can be achieved by: (i) shortening the sequence of the sensor probe, (ii) increasing the G/C content of the anchor probe, or (iii) modifying the sensor probe with locked nucleic acids (LNAs) on the polymorphic position (Caplin et al., 1999; Op den Buijsch et al., 2005).

The aim of the current study was to evaluate the modified cox III qPCR assay for use in the detection and differentiation of *Theileria* spp. in mixed infections in buffalo. The blood samples analysed originated from buffalo from South Africa and Mozambique. The results obtained were compared to those obtained by the reverse line blot (RLB) hybridization assay (Gubbels et al., 1999) which is also used to simultaneously detect and differentiate *Theileria* spp. in mixed infections. The results of the cox III qPCR assay were also compared to those of the 18S rRNA gene qPCR (Sibeko et al., 2008) for the specific detection of *T. parva*.

7.3 Materials and methods

7.3.1 Samples and DNA extraction

A total of 224 samples (buffalo=220: cattle= 4), collected either in EDTA tubes or on filter paper, were analysed. The buffalo samples originated from African buffalo in different game parks in South Africa and Mozambique (Table 7.1). Four cattle samples were obtained from the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI). DNA was extracted using the QIAamp® DNA Extraction Kit (QIAGEN, Southern Cross Biotechnologies), or the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' protocols, and stored at -20°C until further analysis.

Table 7.1: Origin and no. of samples analysed by the modified cox III qPCR assay

Place of origin	**Country (Province)**	**No. of samples**
Kruger National Park (KNP)	SA* (Mpumalanga and Limpopo)	54
Hluhluwe-iMfolozi Game Park (HIP)	SA (KwaZulu-Natal)	100
Agricultural Research Council - Onderstepoort VeterinaryInstitute (ARC-OVI)	SA (Gauteng)	4
Greater Limpopo Transfrontier Game Park (GLTP)	Mozambique	33
Others:	SA	
Addo Elephant Game Park (AEGP)	(Eastern Cape)	13
MarakeleNational Park	(Limpopo)	5
IthalaNational Park	(KwaZulu-Natal)	8
VaalbosNational Park	(Northern Cape)	6
Kwanare Game Park	(Mpumalanga)	1
TOTAL		**224**

* SA – South Africa

7.3.2 Polymerase Chain Reaction

A nested PCR protocol was used for the amplification of a fragment of the cox III gene of the parasite. Forward primer F3Cox (5'-AAG ATG AAT CCG ATT TGA TGA-3') and reverse primer MJCox (5'-AAA TGG ACT ATG TAA GTT AAC CTAT-3') were used in a primary conventional hot start PCR reaction. The reaction mixture contained 1 µl yellow sub (GENEO BioProductions, Hamburg, Germany), 5 µl of 1X Go Taq buffer (Promega), 1.65 mM $MgCl_2$, 2.5 mM dNTPs, 0.4 µM of each primer, 0.5 U *Taq* Polymerase (Promega), 5 µl (~ 100 ng) DNA and PCR grade water to a total volume of 25 µl. The cycling conditions included an initial hold at 84^{0}C for 10 s, initial denaturation at 92^{0}C for 4 min, amplification of 25 cycles each of denaturation at 92^{0}C for 30 s, annealing at 56^{0}C for 45 s and extension at 72^{0}C for 60 s, and a final extension at 72^{0}C for 10 min. All primary PCR products were analysed by a nested cox III qPCR assay protocol using the Rotor Gene 3000 (Corbett Research, Australia). Each reaction contained 0.5 µl of the primary PCR product, 1X Go Taq buffer (Promega), 1.65 mM $MgCl_2$, 2.5 mM dNTPs, 0.66 µM of primer Fcox(5'-CAA CAT TGT TAA AGC TAT CCA A-3') and 0.13 µM of primer nRCox(5'-TTA TAG TAC AGG ATT AGA TAC-3'), together with 0.5 µM each of the modified Anchor probe Cox1-6FAM (5'-ATT GGa tga cat taT AtT tct ata ttt taa CaG GAc-3') and Sensor probe Cox1-Cy5 (5'-Att caT tac acG Tat gtg Ctg gaa g-3'), 5U *Taq* polymerase and water to a total volume of 25 µl. Capital letters in the anchor and sensor probe sequences represent locked nucleic acids (LNAs). The programme included a hold at 95^{0}C for 15 min, 40 cycles each of denaturation at 95^{0}C for 30 s, annealing at 56^{0}C for 45 s, extension at 72^{0}C for 60 s. Melting curves were generated by heating the samples from 33^{0}C to 99^{0}C with a heating rate of 1^{0}C/min. Fluorescence was measured at 640 nm.

Plasmid DNA from cox III clones (obtained from the Department of Animal Health, Institute of Tropical Medicine, Antwerp, Belgium) of the following species were used as positive controls: *Theileria* sp. (buffalo) clone 1.5 originated from a buffalo isolate from South Africa; *T. parva* Katete clone 1.5 was obtained from a bovine isolate (used as a vaccine strain) from the Eastern province of Zambia; *T. taurotragi* N355 clone 2.7 was obtained from a bovine isolate from the Eastern province of Zambia; *T. buffeli* M2138 clone 538 was from an imported bovine in Butare, Rwanda; *T. velifera* C914 clone 2.8 and *T. mutans* C914 clone 2.2 were from a mixed infection sample obtained from a bovine in the Eastern province of Zambia. Molecular grade water was used as a negative control.

7.3.3 Cloning and sequencing

Based on the qPCR results, 17 samples were selected for further characterization. Primary PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The samples originating from the AEGP (n = 7) had single *T. buffeli* infections and their cox III genes were amplified and PCR products were directly sequenced. The PCR product from the sample from buffalo KNP102 (Sibeko et al., 2008) was directly sequenced and also cloned before sequencing, and PCR products from the remaining nine samples were cloned prior to sequencing. Ligations and transformations were done using the pCR2.1TOPO cloning vector (Invitrogen, Carlsbad, USA) as recommended by the manufacturer. Ninety-three recombinants obtained from these samples were screened using the cox III qPCR assay as described above. Based on the qPCR results, desired clones were then cultured overnight in LB-broth, after which they were re-grown on fresh agar plates and sent for sequencing. Plasmid extraction and sequencing were done at the Genetic Service Facility, University of Antwerp, Belgium and at Inqaba Biotechnologies, South Africa. In South Africa, sequencing reactions were done using the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA) using ~350 ng of plasmid DNA and 2 pmol each of primers F3 Cox, MJCox3 and nRcox.

7.3.4 Sequence and phylogenetic analysis

The cox III sequences were assembled and edited using GAP4 of the Staden software package (version 1.6.0 for Windows) (Staden et al., 2000). MAFFT version 5 (Katoh et al., 2005) was used to align the new sequences with cox III gene sequences of the control clones, and with published *Theileria* cox III gene sequences from GenBank (*T. parva* Z23263, *T. parva* AB499089, *T. orientalis* AB499090, *T. annulata* U32225). The alignment was manually edited using BioEdit (version 7) (Hall, 1999). A BLASTn homology search of GenBank was done using the consensus sequences. The genetic distances between the sequences were estimated by determining the number of base differences between sequences using MEGA4 (Tamura et al., 2007). Phylogenetic trees were constructed using MEGA4 for neighbor-joining analysis with 1000 bootstrap replicates (Felsenstein, 1985); PAUP* (v4b10) (Swofford, 2003) for maximum-parsmony and maximum likelihood methods, and MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian inference, accessed via the Computational Biology Service Unit, Cornell University (http://mafft.cbsuapps.tc.cornell.edu/mrbayes.aspx). The TrN + I + G substitution method was determined as the best fit model by Modeltest v.3.7 (Posada et al., 1998) and used in the likelihood

and Bayesian analyses.The trees were rooted using the cox III gene sequence of *Theileria annulata* (U32225) and consensus trees were edited using MEGA 4.

7.3.5 Comparison of the cox III qPCR assay with the RLB assay and the 18S rRNA qPCR

The samples (n = 224) were analysed by the RLB hybridization assay as described by Gubbels et al. (1999), for the simultaneous detection and differentiation of *Theileria* spp. The *Theileria* and *Babesia* species- and genus-specific probes used were as previously described (Chapter 3; Chaisi et al., 2011). Additionally, 206 samples were analysed for the specific detection of *T. parva* using the 18S rRNA qPCR assay, as described by Sibeko et al. (2008). The occurrence of *T. parva* in these samples was compared to that of the coxIII pPCR and RLB hybridization assays. For the 18S rRNA qPCR assay, the amplification mixture consisted of 4 µl of 10X LightCycler-FastStart DNA Master^{Plus} Hybridization Probes mix (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each of *T. parva* specific forward primer (5'-CTG CAT CGC TGT GTC CCT T-3') and *Theileria* genus-specific reverse primer (5'-ACC AAC AAA ATA GAA CCA AAG TC-3'), 0.1 µM of each hybridization probe (*T. parva* anchor (5'-GGG TCT CTG CAT GTG GCT TAT-FL), *T. parva* sensor (5'-LCRed640-TCG GAC GGA GTT CGC T-PH), *Theileria* genus anchor (5'-AGA AAA TTA GAG TGC TCA AAG CAG GCT TT-FL) and *Theileria* genus sensor (5'-LCRed705-GCC TTG AAT AGT TTA GCA GCA TGG AAT-PH), 1 U uracil deoxy-glycosylase (UDG) and 2.5 µl (~ 37.5 ng) of template DNA in a final volume of 20 µl (Sibeko et al., 2008). Cycling was done using a LightCycler® v2 (Roche Diagnostics, Mannheim, Germany). The cycles include UDG activation at 40°C for 10 min, *Taq* DNA polymerase activation at 95°C for 10 min, 45 cycles of three steps each of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 15 s. Melting curve analysis was performed by heating the samples from 40°C to 95°C with a heating rate of 0.2°C/s, and fluorescence values were measured at 640 and 705 nm.

7.4 Results

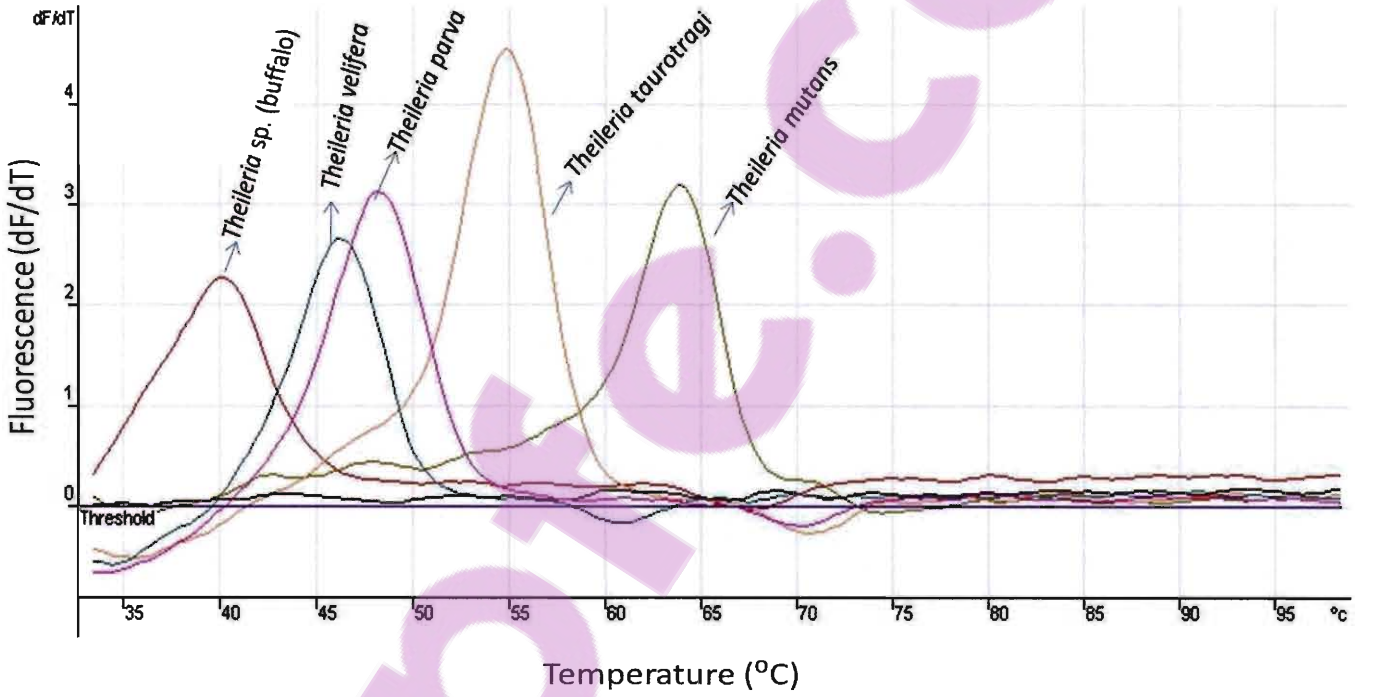
7.4.1 cox III qPCR results

Amplicons of approximately 1000 bp were obtained from primary PCR amplification of the cox III gene of *Theileria* spp. The hybridization probes used in the cox III real-time PCR assay allow for the detection and discrimination of the different species based on differences in their melting temperatures (T_m). The sequences of the modified anchor and sensor probes and *Theileria* spp. controls are indicated in Figure 7.1(a). Melting peak analysis was used to identify the different species, based on the comparison of their melting peaks with those of the control clones as illustrated in Figure 7.1(b). As the melting peaks can shift slightly from run to run, the mean and standard deviation of the T_m of the control plasmids and analysed samples were determined.

Theileria parva and *Theileria* sp. (buffalo) were the most commonly detected species in the field samples from buffalo and cattle, with prevalences of 83.5% and 55.8%, respectively (Figure 7.2). *Theileria taurotragi*, *T. buffeli* and *T. mutans* were identified in 1.8%, 5.8% and 2.2% of samples, respectively (Figure 7.2). *Theileria velifera* was not identified in any of the samples, and 4.5% of the samples were negative or below the detection limit of the assay. Additionally, 17% of the samples had non-specific peaks which were located between those of *Theileria* sp. (buffalo) and *T. velifera* or *T. taurotragi* and *T. mutans* (Figure 7.1c).

```
                    MOD-anchor probe                    MOD-sensor probe
                    ATTGGatgacattaTAtTtctatattttaaCaGGAc  AttcaTtacacGTatgtgCtggaag
ZAM/C914/2.2        acaattattggatgacattgtatttctatattttaacaggacttcattcattacacgtatgtgttggaagtgtatttgtacttat  T. mutans
ZAM/N355/2.7        .t.....................a.....t.t.....................g..t.......c....g..a.c.....gtag..  T. taurotragi
SA/buffalo/1.5      .t.....................a..c..t.t.....................c.gt.t.............g..a.t......t.a..  Theileria sp.buffalo)
ZAM/Kat/1.1         .t.....................a..c..t.t.....................g.....t.......cc...g.ca.t........a..  T. parva
ZAM/C914/2.8        .t..c.....................c.a..c..t.....t.....c.t.........a.a.........c......t.a..  T. velifera
RWA/M2138/538       .tg.....a.....a.....t.....t...t.a.....t.....t.....a...t.....tctt.a..  T. buffeli
```

(a)



(b)

(c)

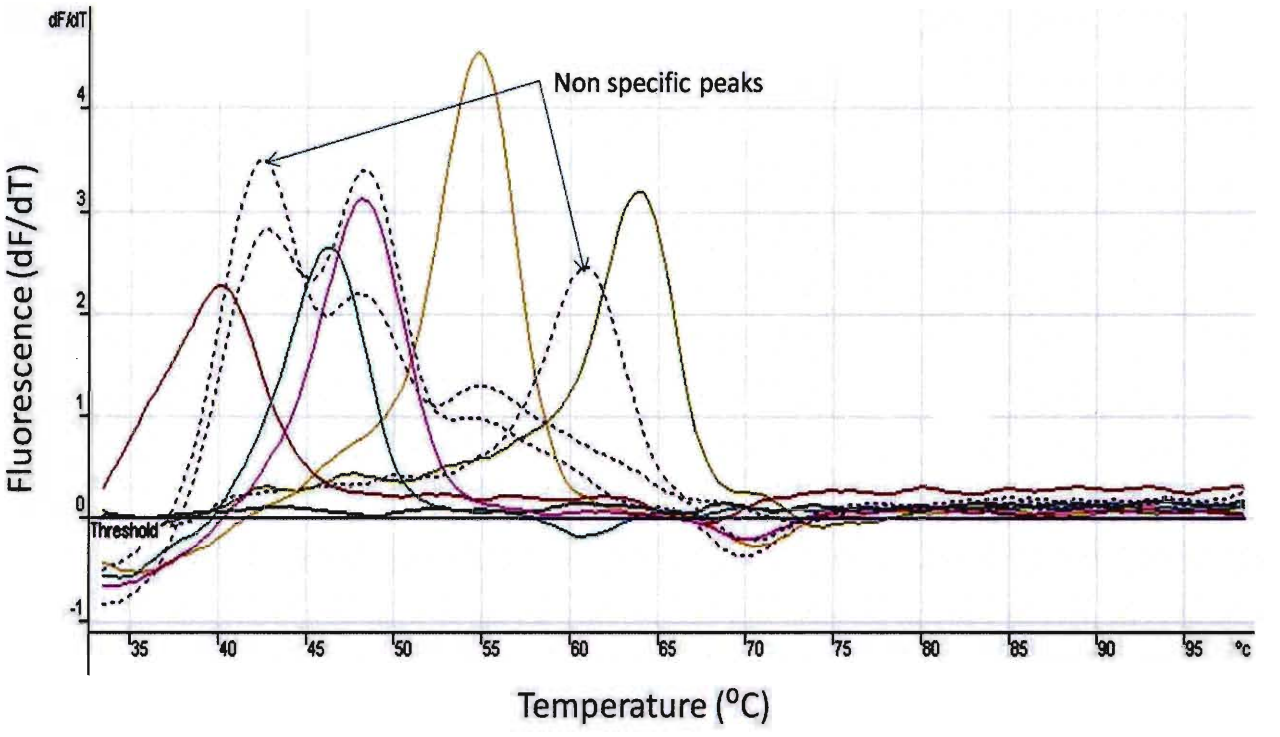


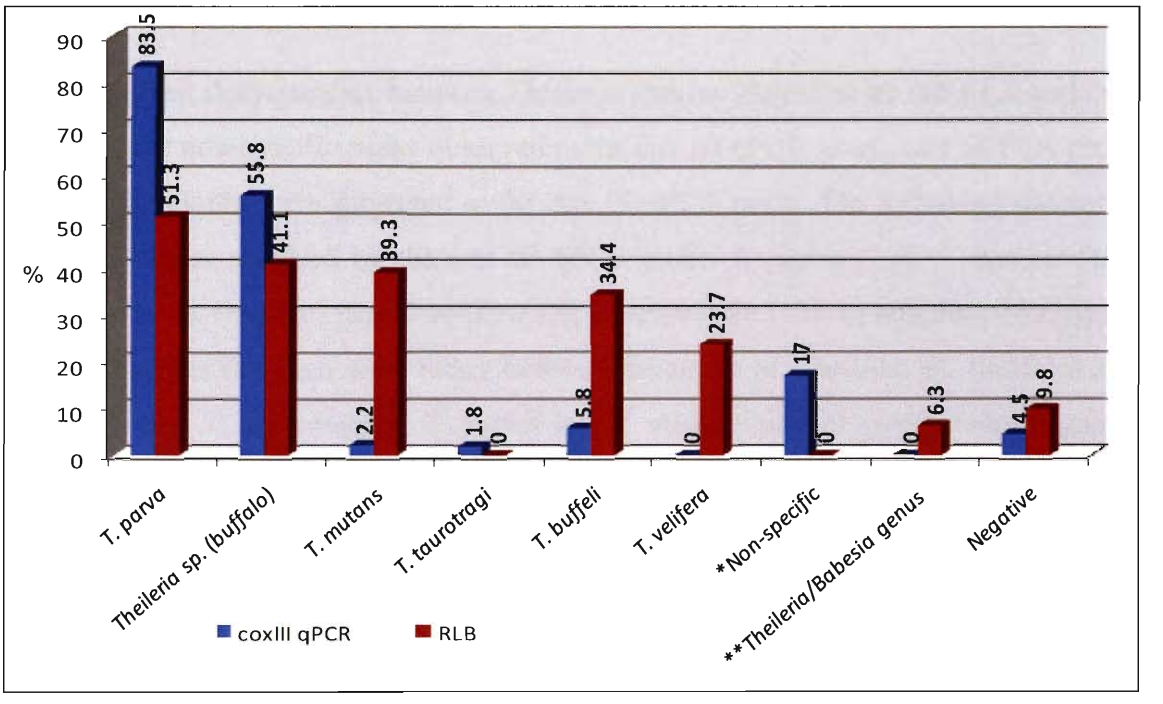
Figure 7.1: (a) Sequence alignment showing the number of mismatches in the modified FRET anchor and sensor probe areas in the different species. (b) Melting curve analysis of the cox III gene plasmid controls of *Theileria* spp. as determined by the cox III qPCR assay. Melting peaks shown are for *Theileria* sp. (buffalo) (39.7 ± 0.5^{0}C), *T. velifera* (46.0 ± 0.4^{0}C), *T. parva* (48.4 ± 0.3^{0}C), *T. taurotragi* (54.7 ± 0.8^{0}C), and *T. mutans* (63.9 ± 0.4^{0}C). The melting peak of *T. buffeli* (53.7 ± 0.1^{0}C) is not shown. (c) Non-specific peaks (arrows) were observed from some samples. No flourescence was detected from the water control.

7.4.2 Comparison of the cox III qPCR assay with the 18S qPCR and RLB hybridization assays

The cox III qPCR results were compared to those obtained by the RLB hybridization assay for the simultaneous detection and differentiation of *Theileria* spp. in buffalo and cattle (n = 224). *Theileria parva* and *Theileria* sp. (buffalo) were also identified as the most commonly occurring species by the RLB assay (Figure 7.2). However, the RLB assay detected more infections of *T. mutans* and *T. buffeli* than the cox III qPCR assay, 23.7% of samples were positive for *T. velifera* and no *T. taurotragi* infections were detected by the RLB assay. Of the 14 samples that only had a *Theileria/Babesia* genus-specific signal on the RLB assay, 11 showed mixed *Theileria* spp. infections and 3 were negative on the cox III qPCR assay (Figure 7.2a).

The cox III qPCR assay results were also compared with those of the RLB and 18S qPCR assays (n = 206) for the specific detection of *T. parva*. This species was detected in 107 (51.9%), 157 (79.2%) and 178 (86.4%) of the 206 samples analysed by the RLB, 18S qPCR and cox III qPCR assays, respectively (Figure 7.2b).

(a)



(b)

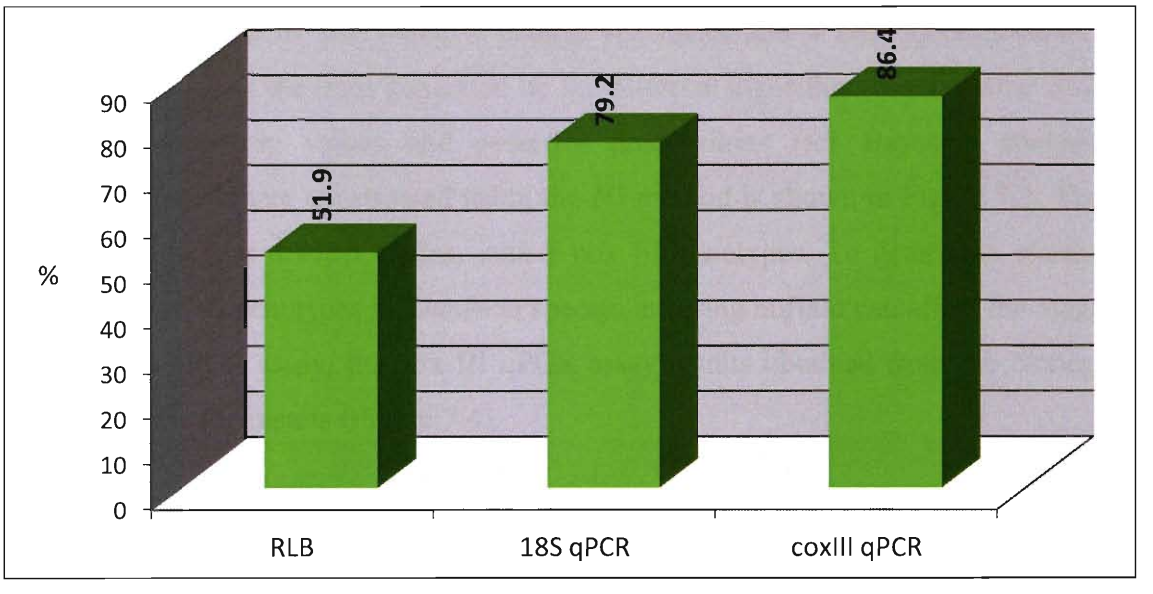


Figure 7.2: (a) The occurrence of *Theileria* species infections in buffalo and cattle samples from South Africa and Mozambique as determined by the RLB and cox III qPCR assays (n = 224). The number of samples with non-specific melting temperatures on the cox III qPCR assay (*) and those that hybridized only with the *Theileria/Babesia* genus-specific probes using the RLB assay (**) are shown, (b) Comparison of the RLB, 18S qPCR and cox III qPCR assays for detection of *T. parva* (n = 206).

7.4.3 Sequencing and phylogenetic results

Because of the marked discrepancies between *Theileria* species identified by the RLB and cox III qPCR assays, and the non-specific peaks observed in the cox III qPCR assay, cox III PCR products were cloned and the clones were subjected to the cox III qPCR assay. The following species were detected from 93 clones analysed by the cox III qPCR assay: *T. parva* (13); *T. mutans* (10); *T. buffeli* (7); *Theileria* sp. (buffalo) (9); *T. velifera* (2); *T. taurotragi* (17). In addition, 14 clones had non-specific melting peaks which were either between the peaks of *Theileria* sp. (buffalo) and *T. velifera*, *T. buffeli* and *T. taurotragi* or *T. buffeli* and *T. mutans,* and 21 clones were negative or below detection limit. Based on these results, selected clones were sequenced.

A total of 26 new cox III gene sequences were obtained from 17 samples that were selected for sequencing. A BLASTn homology search revealed the closest homology with cox III gene sequences of *T. parva* (Z23263) (81 -100%), *T. parva* (AB499089) (79 - 100%) and *T. orientalis* (AB499090) (78 - 88%). Phylogenetic trees were constructed from a total of 36 cox III sequences (26 new sequences obtained in this study, 6 control sequences and 4 sequences obtained from GenBank). The groupings in the trees generated by the different algorithms were similar and were supported by high bootstrap values and posterior probabilities (for Bayesian analysis). A representative phylogenetic tree constructed using the NJ method is shown in Figure 7.3. The new sequences grouped into four distinct clades, with 9 cox III genotypes. To determine whether the presence of diverse cox III genotypes in *Theileria* species infecting buffalo can affect the diagnostic results of the cox III qPCR assay, the cox III qPCR assay results obtained from the clones were compared to the sequencing results (Figure 7.4).

The *T. mutans* group (clade A) contained four genotypes and was the most polymorphic group. Sequence KNP/C8/9.1 from a buffalo sample, was identical to the *T. mutans* control cox III sequence ZAM/914/2.2 from a bovine sample. Clone KNP/C8/9.1 tested positive for *T. mutans* by the cox III qPCR assay. The original sample, KNP/C8, tested positive for *T. mutans* and *T. velifera* when analysed by the RLB hybridization assay (Figure 7.4), but no *T. velifera* cox III clones were obtained from this sample. The three *T. mutans*-like cox III genotypes were designated *T. mutans*-like A – C (Figure 7.3). All of the clones from which these sequences were obtained had non-specific melting peaks that were between those of *T. taurotragi* and *T. mutans* when analysed by the cox III qPCR assay (Figure 7.4).

The sequencing results correlated with the results of the RLB assay as these samples tested positive for *T. mutans*, although the signal in sample HIP/A2 was very weak. The four *T. mutans*-like A sequences were all derived from buffalo samples. The two *T. mutans*-like B sequences from clones KNP/K4/3.8 and OVI/8227/4.10, were derived from a buffalo sample and a bovine sample respectively, and the *T. mutans*-like C sequences (4) were buffalo-derived. Nucleotide differences of 1-3 bp in the modified sensor probe between *T. mutans* and *T. mutans*-like cox III genotypes resulted in a shift in T_m of up to 8°C (Figure 7.4), and as a result, clones with *T. mutans*-like cox III sequences yielded non-specific melting peaks when analysed by the cox III qPCR assay.

Clade B consisted of cox III gene sequences of the pathogenic *Theileria* spp. (*T. taurotragi* and *T. parva*) as well as those of *Theileria* sp. (buffalo). None of the new sequences grouped with the *T. taurotragi* cox III control sequence (ZAM/N355/2.7) obtained from a bovine sample from Zambia. Although sequence OVI/8227/4.7, obtained from a bovine at the OVI, was most closely related to the *T. taurotragi* sequence, its identity could not be established and it was therefore indicated as inclusive. This clone had a melting peak between *Theileria* sp. (buffalo) and *T. velifera* when tested using the cox III qPCR assay, and the field sample was positive for *T. mutans* by the RLB assay. The sequence from clone HIP/A2/1.9 was identical to the cox III sequence of the *Theileria* sp. (buffalo) control clone (SA/buffalo/1.5). The *T. parva* cox III sequences (KNP/102 and KNP/102/6.2) from buffalo 102 were identical to the *T. parva* (Muguga stock) cox III sequences from cattle, Z23263 (Kairo et al., 1994) and AB499089 (Hikosaka et al., 2010), and to the cox III sequence (ZAM/Kat/1.1) of the *T. parva* control used in this study (Figure 7.3). All three clones tested positive for *T. parva* by the cox III PCR and *T. parva* was identified in the original field samples using the RLB assay.

Clade C contained the *T. velifera* cox III sequences from cattle (ZAM/C914/2.8) and buffalo (HIP/A2/1.1), and *T. velifera*-like cox III sequences (KNP/K8/2.2, KNP/E2/8.5, HIP/A2/1.7) which were all derived from buffalo. In contrast, *T. velifera* was only identified (using the RLB assay) in two of the three field samples from which these clones were obtained (Figure 7.4). The clones of the three *T. velifera*-like cox III sequences had peaks between *T. taurotragi* and *T. mutans* when analysed by the cox III qPCR assay.

The last clade (D) was the *T. buffeli* group. The cox III sequences of the seven samples from the AEGP were identical and differed from the published cox III gene sequence of *T. orientalis* (AB499090) (Hikosaka et al., 2010) and the sequence of the *T. buffeli* control clone (RWA/M2138/538). These sequences were therefore designated as *T. buffeli*-like cox III sequences. Although there are three bp differences in the sensor probe areas of RWA/M2138/538 and the AEGP sequences, their melting temperature was almost identical (0.3°C difference). All the field samples from which these sequences were derived tested positive for *T. buffeli* using the RLB assay.

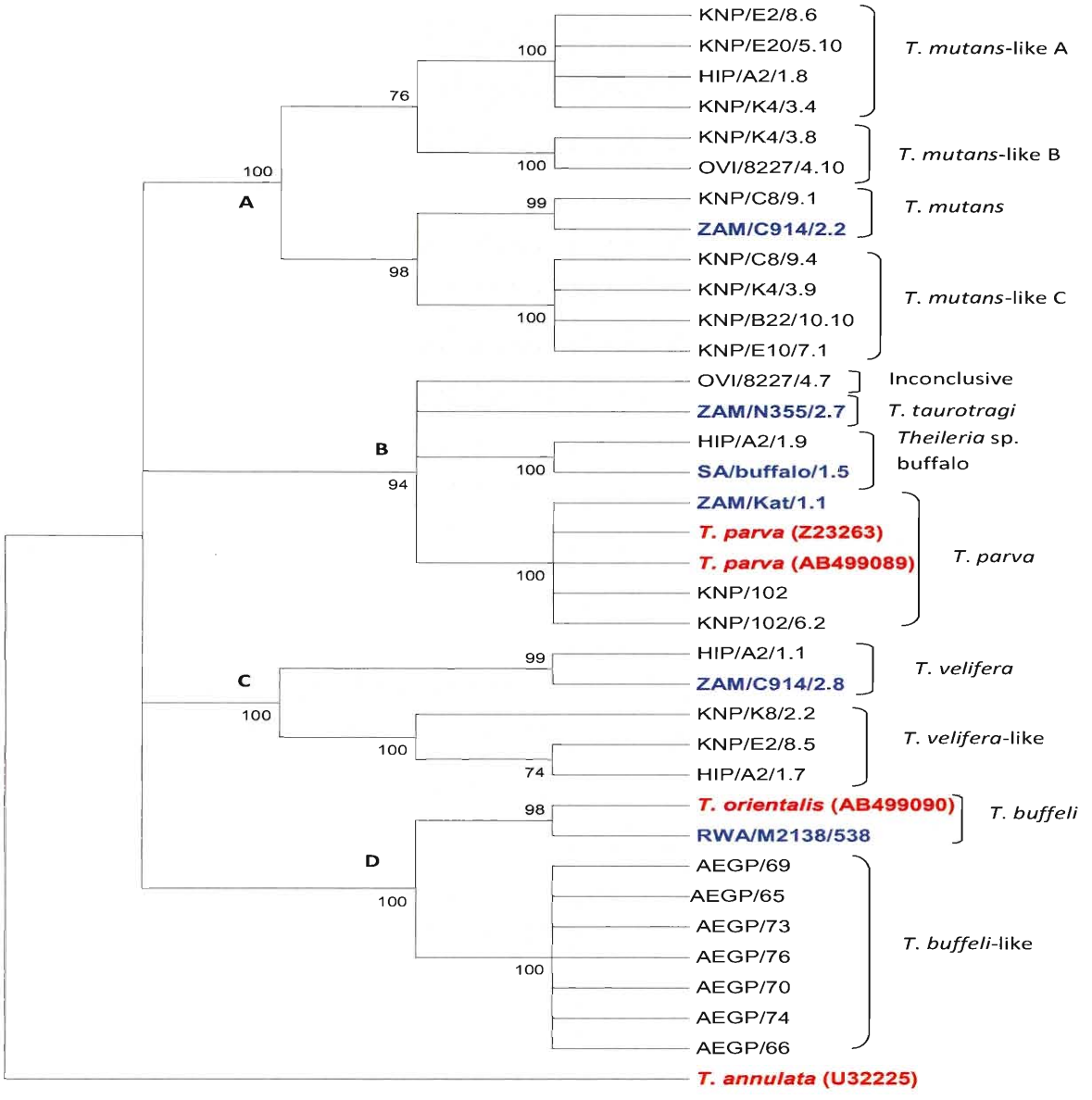
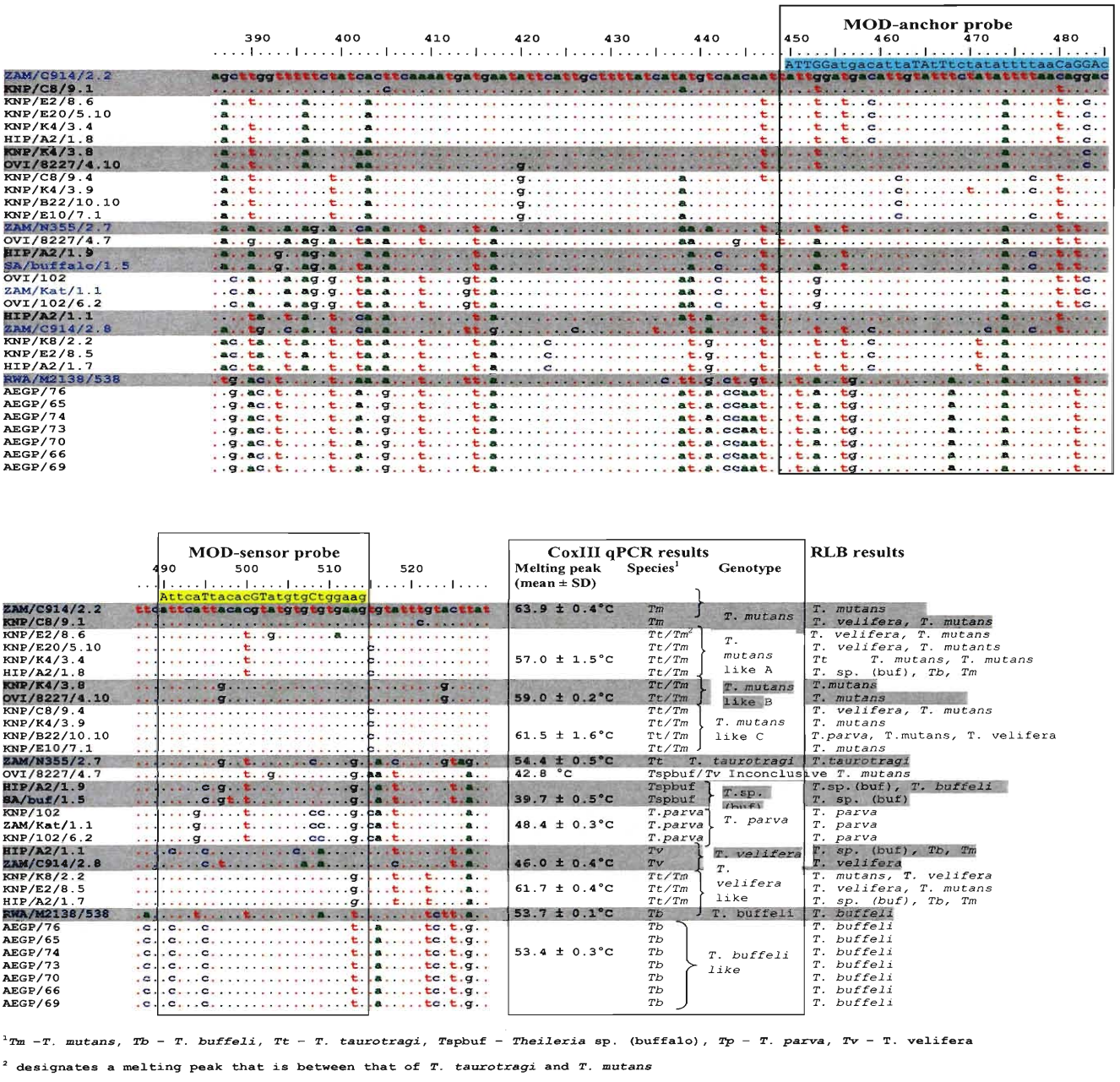


Figure 7.3: Phylogenetic relationships of the cox III gene sequence variants of *Theileria* spp. of buffalo and cattle in South Africa identified in this study (black) with *Theileria* control sequences (blue) and published *Theileria* spp. (red) as indicated by neighbor joining analysis. Bootstrap values indicate the degree of support for each cluster. The tree was outgroup rooted using the cox III gene sequence of *T. annulata.*



[1]*Tm* – *T. mutans*, *Tb* – *T. buffeli*, *Tt* – *T. taurotragi*, *Tspbuf* – *Theileria* sp. (buffalo), *Tp* – *T. parva*, *Tv* – T. velifera

[2] designates a melting peak that is between that of *T. taurotragi* and *T. mutans*

Figure 7.4: Sequence alignment of the cox III gene of *Theileria* spp. indicating the sequences of the modified anchor (light blue) and sensor (yellow) probes. Cox III sequences were obtained from clones from control samples and clones from selected African buffalo samples. Differences are based on the cox III sequence of *T. mutans* (ZAM/C9142.2). The cox III qPCR result obtained for each clone is shown along with the cox III genotype identified by phylogenetic analysis. The RLB results obtained from the sample from which the cox III clones were derived are also shown.

7.5 Discussion and Conclusion

FRET technology has previously been used for the development of diagnostic assays to simultaneously identify co-infecting piroplasmids (*Theileria* and *Babesia* spp.) in different hosts. Criado-Fornelio et al. (2009) developed a qPCR assay, based on the 18S rRNA gene, for the simultaneous identification of *Babesia bovis*, *Babesia divergens*, *Babesis major*, *Babesia bigemina*, *Theileria annae* and an unidentified *Theileria* sp. in bovines. The qPCR by Wang et al. (2010), also based on the 18S rRNA gene, differentiates *B*abesia *gibsoni*, *Babesia canis canis*, *Babesia canis vogeli* and *Babesia canis rossi* in canines. In addition to the identification of *T. parva* in infected animals, the 18S qPCR by Sibeko et al. (2008) can simultaneously detect *T. taurotragi* and *T. annulata* when the *Theileria*-genus primer set is used. The cox III qPCR assay of Janssens (2009) was developed for the simultaneous detection and differentiation of *T. parva* and five co-infecting *Theileria* spp., namely *T. annulata*, *T. velifera*, *T. taurotragi*, *T. mutans* and *T. buffeli* in infected cattle based on differences in their melting temperatures (Janssens, 2009). This assay was later modified, by the development of new primer and probe sets, to include the detection of *Theileria* sp. (buffalo) in buffalo (unpublished results). In this study the modified cox III qPCR assay was compared with the 18S qPCR assay for the specific detection of *T. parva*, and with the RLB assay for the 'universal' detection and discrimination of *Theileria* spp. in cattle and buffalo samples.

The analytical sensitivities of the qPCR assays for the detection of *T. parva* were determined as 100% at parasitemia of 8.79×10^{-4}% for the 18S qPCR assay (Sibeko et al., 2008), and between 4.1×10^{-5} and 4.1×10^{-4}% for the cox III qPCR assay (Janssens, 2009), indicating that the latter assay might be slightly more sensitive than the former. More *T. parva* infections were identified by the cox III qPCR than the 18S rRNA assay. Both qPCR assays have previously been reported as being more sensitive than the 18S- and cox III RLFP-PCR assays in detecting *T. parva* (Sibeko et al., 2008; Janssens, 2009).

The cox III qPCR assay was also more sensitive than the RLB assay in the detection of *T. parva* and *Theileria* sp. (buffalo) infections. Real-time PCR has previously been reported to be more sensitive than the RLB assay in detecting *Theileria* and *Babesia* spp. (Sibeko et al., 2008; Bhoora et al., 2009). Additionally, the cox III qPCR is less laborious, less time-consuming and more cost-effective than the RLB assay as it requires the use of a single hybridization probe pair to detect all the *Theileria* spp., whereas the RLB requires a probe for each species or genotype that is identified.

The cox III qPCR assay has the potential of replacing or complementing the RLB assay for the simultaneous detection of *Theileria* spp. of cattle and buffalo in the future. However, the cox III qPCR assay used in this study was shown to be less specific than the RLB in the detection of other *Theileria* spp. that infect the African buffalo.

Sequence and phylogenetic analyses of the cox III gene in our study indicated the presence of a single *T. parva* genotype in cattle and buffalo, and therefore the cox III qPCR assay can specifically detect *T. parva* infections in these hosts where the parasitemia is above the detection limit of the assay. However, sequence polymorphism in the cox III genes of the other *Theileria* species decreases the specificity of the assay for these species; hence their apparent low prevalences as indicated by the cox III qPCR results, and the discrepancies between cox III qPCR and RLB assay results.

The identification of *T. taurotragi* by the cox III qPCR assay in buffalo was unexpected. To our knowledge this species has never been isolated from the African buffalo. In addition, the RLB assay did not detect *T. taurotragi* in any of these samples. The RLB result was confirmed by sequence analysis: none of the cox III sequences derived from buffalo samples grouped together with the *T. taurotragi* control sequence. Sequencing and phylogenetic analyses of clones that had non-specific cox III melting peaks between the *T. taurotragi* and *T. mutans* peaks were identified as *T. mutans*-like genotypes. This limitation of the cox III assay may be overcome by designing probes from a region within the cox III gene that is more variable between the two species. Although the cox III qPCR assay can distinguish between the different *Theileria* spp., failure of the assay to accurately distinguish between the different species variants is another limitation of the test.

As with the 18S rRNA gene (Chapters 4, 6), the greatest heterogeneity in the cox III gene was observed within the *T. mutans* group. Although a direct comparison between the two genes could not be made as different samples (with the exception of sample KNP/B22) were analysed and pure parasite stocks are not available, we also identified a *T. mutans* cox III genotype (identical to cox III *T. mutans* from cattle) and three *T. mutans*-like cox III genotypes. However, unlike the 18S rRNA gene where all the genotypes were exclusively obtained from buffalo samples, we identified a *T. mutans*-like B cox III genotype from a cattle sample (OVI/8227/4.10).

Further studies are needed to determine if sequence OVI/8227/4.7 represents a unique sequence in cattle as the clone had a unique melting peak that was between those of *Theileria* sp. (buffalo) and *T. velifera*. Although we identified a single *T. parva* genotype in both cattle (Muguga strain) and

buffalo, analysis of more clones might reveal the presence of more *T. parva* cox III genotypes as Mans et al. (2011) identified 8 cattle and 3 buffalo *T. parva* 18S rRNA genotypes.

The identification of a *T. velifera* genotype in cattle and buffalo, and a *T. velifera*-like genotype in buffalo is concurrent with our previous results on the 18S rRNA gene (Chapter 4). Low parasitemia might explain why the cox III assay failed to identify *T. velifera* from sample HIP/A2 by RLB and cox III qPCR assays. Similarly, the very faint *T. velifera* signal obtained from sample KNP/K8 might be due to low parasitemia. Sequence variations between the *T. velifera* control sequence and the *T. velifera*-like sequences explains the failure of the cox III assay to identify *T. velifera* in the field samples. All three *T. velifera*-like sequences had melting peaks between those of *T. buffeli* and *T. mutans* by the cox III qPCR. This result was obtained with the *T. mutans*-like C sequences and might be due to the fact that there is only one nucleotide difference in the sensor probe area of two genotypes.

The design of species-specific primers and probes has been restricted by the lack of cox III sequence data. When this study commenced, the only *Theileria* spp. for which cox III sequences were available in GenBank were *T. parva*, *T. annulata*, *T. equi* and *T. orientalis*. Sequence data obtained from this study will therefore allow for the design of new primers and probes for effective differentiation between the different species and their variants.

In conclusion, there is extensive sequence variation within the cox III gene of *Theileria* spp. of the African buffalo. Although the gene is a good marker in phylogenetic studies of closely related species, it might not be a suitable gene for use in a diagnostic assay, particularly in *Theileria* spp. of buffalo where there is a lot of variation. The observed microheterogeneity within genotypes is possibly due to *Taq* polymerase error (Zahler et al., 1998; Aktas et al., 2007). It is possible that analysis of more samples could reveal more variation as the cox III gene is a fast evolving gene. Whether the observed variation represents new species or just variants of known species is uncertain and cannot be determined based on the results of a single gene. Our results further indicate the importance of the identification and characterization of all local genotypes of a gene before the development of diagnostic assays as suggested by Bhoora et al. (2009). The sensitivity and specificity of the cox III qPCR assay for the identification of benign and non-pathogenic *Theileria* spp. in the African buffalo could be improved by the development of primers from a conserved area of the gene and probes from variable areas of the gene.

7.6 References

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CHAPTER 8

General Discussion, Conclusions and Recommendations

8.1 Identification and molecular characterization of pathogenic, mildly pathogenic and benign *Theileria* spp. of the South African buffalo

The 18S rRNA gene is part of the ribosomal functional core and is exposed to similar selective forces in all living organisms (Moore and Steitz, 2002). Generally rRNA gene sequences are easy to access and their repetitive arrangement within the genome provides excess amounts of template DNA for PCR (Meyer et al., 2010). It is therefore one of the most frequently used molecular markers for PCR amplification, molecular epidemiology and characterization, and phylogenetic studies of living organisms (Meyer et al., 2010).

The results of our molecular epidemiology study on the 18S rRNA genes of *Theileria* parasites of the South African buffalo using the RLB hybridization assay have confirmed the findings of previous studies that buffalo are commonly co-infected with different *Theileria* spp., namely, *T. parva*, *Theileria* sp. (buffalo), *T. mutans*, *T. velifera* and *T. buffeli* (Allsopp et al., 1993; Gubbels et al., 1999; Oura et al., 2004). Molecular characterization of partial (V4 hypervariable) (Chapter 6) and full-length (Chaisi et al., 2011; Chapters 3, 4, 5) 18S rRNA gene sequences of *Theileria* spp. of the African buffalo indicated extensive sequence variation within the 18S rRNA genes of these species. Co-infections with several genotypes of a single species were also common.

Based on the results of the partial and full-length 18S rRNA sequences, we described the occurrence of at least 13 distinct 18S rRNA *Theileria* genotypes of the African buffalo (Chapters 3, 4, 5, 6). Seven of these genotypes differed from known or previously reported sequences (Chapter 6) and were recently reported as novel (unique) by Mans et al. (2011). Our study was limited to the analysis of a smaller number of samples from buffalo, while Mans et al. (2011) analysed many more samples from both buffalo and cattle. The phylogenetic positions of some of these genotypes, and the fact that they show distinct host, and geographical differences, indicates that they may represent new species. However, additional biological and morphological data are needed in order to confirm this statement.

Although we don't know the actual mechanisms that bring about the observed diversity in the 18S rRNA gene, in their review, Deitsch et al. (1997) indicated that gene sequence diversity may be due to random mutation of nucleotides during sexual reproduction or through inter- and intra-genetic recombination and the random assortment of alleles during sexual reproduction. Studies on the ITS region of *Theileria* spp. of cattle indicated higher levels of genetic variation in the ITS region in the pathogenic *Theileria* species (*T. annulata* and *T. parva*) than in the mildly pathogenic and non-pathogenic species (Collins and Allsopp, 1999; Aktas et al., 2007). This has been attributed to higher levels of parasitemia during acute disease, leading to the ingestion of greater numbers of organisms by the tick and therefore a more diverse pool during gametogenesis in the tick vector which then results in a greater opportunity for recombination to occur during sexual reproduction in the tick vector (Collins and Allsopp, 1999). Aktas et al. (2007) also indicated that it is possible that a higher rate of parasite proliferation in the pathogenic species increases the likelihood of random mutation.

The effect of the 18S rRNA gene heterogeneity on the epidemiology of theileriosis in South Africa is unknown and should be investigated in order for the development of more efficient control strategies for this disease. Although *T. mutans* is generally regarded as a benign species, some strains of this species have been associated with severe disease in cattle (Young et al., 1978; Paling et al., 1981; Saidu, 1981). It would therefore be interesting to see whether there in an association between *T. mutans* genotypes and disease.

8.1.1 Implications of the sequence variation of the V4 hypervariable region of the 18S rRNA gene on the diagnosis of pathogenic and non-pathogenic *Theileria* species of the African buffalo

The RLB hybridization assay by Gubbels et al. (1999) is a practical tool for the simultaneous detection and differentiation of *Babesia* and *Theileria* spp. in infected animals. We designed two new RLB probes, designated *T. mutans*-like 1 and *T. mutans*-like 2/3, for the specific detection of these novel genotypes in order to determine their prevalence in the South African cattle and buffalo populations (Chapter 4). However, the new probes cross-reacted with the *T. mutans* target DNA despite the 3 nucleotide differences between the new probes and the original *T. mutans* sequence.

In order to discriminate between the different *T. mutans* genotypes, we recommend that new RLB probes should be designed from a more variable area of the 18S rRNA gene which is located upstream of the current probe area (Chapter 4). Similarly, new RLB probes could be designed for

all the novel genotypes identified in this study, and for *Theileria* sp. strain (MSD) in order to determine their prevalence in the South African buffalo and cattle populations. Alternatively, a real-time PCR assay could be developed for genotyping as quantitative real-time PCR (qPCR) assays are known to be more sensitive than the RLB assay in the identification of *Theileria* spp. from various hosts (Sibeko et al., 2008, Bhoora et al., 2009, Chaisi et al., 2011).

Although the RLB hybridization assay has previously been used for the identification of new *Theileria* spp. and species variants, cross-reactions occur between some of the currently used probes with certain genotypes (Mans et al., 2011; personal observation), resulting in false positives. It is also not possible to use the RLB assay to identify novel species or species variants in mixed infections, which commonly occur in cattle and buffalo, as a positive signal of a known species does not indicate the possibility of the presence of a novel genotype (Chapter 4; Mans et al., 2011). The latter problem can be resolved by random sequencing of the 18S rRNA gene (Mans et al., 2011).

Our study and others (Mans et al., 2011; Pienaar et al., 2011a) have indicated that despite the extensive sequence variation within the 18S rRNA gene of *Theileria* spp., only *Theileria* sp. (buffalo) and *Theileria* sp. (bougasvlei) can compromise the sensitivity of the 18S rRNA hybridization assay of Sibeko et al. (2008) for the detection of *T. parva* in mixed infections. This problem has been partially overcome by the recent development of the Hybrid II assay (Pienaar et al., 2011b). Although the primers used in the Hybrid II assay amplify both *Theileria* sp. (buffalo) and *T. parva*, two distinct melting peaks are obtained for these species and *Theileria* sp. (bougasvlei) is not amplified by the new primer set. The Hybrid II assay is therefore more specific than the hybridization assay in the diagnosis of *T. parva* infections in cattle and buffalo. A different target gene that sufficiently differentiates between these species should be used to develop a more sensitive *T. parva* assay (Chapter 3; Chaisi et al., 2011).

8.2 Molecular characterization and phylogeny of *T. buffeli*-like and *T. sinensis*-like genotypes of the African buffalo (*Syncerus caffer*) based on their 18S rRNA gene and internal transcribed spacer (ITS) sequences

Classification of the *Theileria buffeli/T. sergenti/T. orientalis* group of benign parasites of cattle and buffalo is complicated and confusing. These species have a cosmopolitan distribution, and although they have previously been identified from cattle and buffalo in South Africa, very little is known about these organisms in this country. Chapter 5 therefore provides useful genetic information

towards the proper phylogeny and classification of this group based on their 18S rRNA and ITS gene sequences. Although the phylogenetic groupings of the *T. buffeli*-like ITS sequences were consistent with those of the 18S rRNA gene sequences for clones obtained from samples originating from the Hluhluwe iMfolozi Park, a direct comparison of 18S rRNA gene and ITS sequences amongst clones was not possible due to mixed infections. An integrated phylogenetic study of sequence data from different genetic markers might provide additional phylogenetic information for defining species, subspecies and/or strains of this complex group of benign bovine *Theileria* spp. (Aktas et al., 2007).

8.3 Evaluation of the cox III qPCR assay for the simultaneous identification and differentiation of *Theileria* spp. in buffalo

In an effort to develop a more sensitive diagnostic assay for *T. parva* infections in cattle, Janssens (2009) developed two qPCR assays based on the cox III gene. The first assay simultaneously identifies and differentiates, by melting curve analysis, between all known *Theileria* spp. of cattle. This assay was later modified to include the identification of *Theileria* sp. (buffalo) in buffalo samples. Both buffalo and cattle are of socio-economic importance in South Africa, they share the same *Theileria* spp. and therefore diagnostic tools should target the identification of these species in both animals. Our results (Chapter 7) indicate that the modified cox III assay is sensitive and specific in detecting *T. parva* infection from cattle and buffalo samples as indicated by Janssens (2009), and an added advantage over existing diagnostic assays for *T. parva* is that it can simultaneously identify and differentiate this pathogen from the other co-infecting *Theileria* spp. However, extensive sequence variation observed within the cox III gene of the other *Theileria* spp. of buffalo renders the assay unsuitable for the identification of these parasites in buffalo in South Africa. The novel cox III gene sequences could be used to improve the specificity of the assay for the identification and differentiation of all known *Theileria* spp. and their variants in buffalo. It will be important to characterize the cox III gene of *Theileria* spp. of cattle in South Africa before a more specific assay is developed.

This study highlights the complexities involved in the diagnosis of *T.parva* in cattle and buffalo in South Africa, and provides invaluable information towards the classification of the complex *T. buffeli*/*T. sergenti*/*T. orientalis* group of benign species. The sequence data generated from this project will allow for the development of a more specific and sensitive diagnostic assay for *T. parva* which will assist the veterinary regulatory authorities in the control of Corridor disease in South Africa

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APPENDICES

Appendix A: A comparison of the results obtained from the identification of *Theileria* spp. of buffalo and cattle by the RLB, 18S qPCR, and modified cox III qPCR assays. Highlighted areas indicate areas where data is not available.

Samples	RLB Results		18S RT-PCR	FRET RT-PCR	
	T. parva	Others	*T. parva*	*T. parva*	Others
KNP/102	+		+	+	
OVI/9433	+		+	+	
OVI/8227		**T/B catchall probe**	+	+	*T. mutans*
OVI/9424	+		+	+	
OVI/8389	-		-	+	*T. mutans*
KZN/bov	-	*T.* sp (sable), *T. velifera, T. mutans, T. taurotragi*			
AEGP/59	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/63	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/64	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/65	-	T. buffeli	-	-	*T. buffeli*
AEGP/66	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/67	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/69	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/70	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/72	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/73	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/74	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/75	-	*T. buffeli*	-	-	*T. buffeli*
AEGP/76	-	*T. buffeli*	-	-	*T. buffeli*

Sample	RLB assay		18S qPCR	cox III qPCR	
	T. parva	Others		*T. parva*	Others
KNP/B1	+				
KNP/B5	+	*T. mutans*		+	
KNP/B11	+			+	
KNP/B15	+		+	+	
KNP/B17	+	*T. mutans, T. velifera*	+	+	
KNP/B20	-	**T/B catchall probe**	-	+	
KNP/B22	+	*T.* sp (buffalo), *T. mutans, T. velifera*	+	+	*T*. sp (buffalo)
KNP/C2	+	*T.* sp (buffalo), *T. mutans, T. velifera*	+	+	*T*. sp (buffalo)
KNP/C5	+	*T. mutans*	+	+	*T*. sp (buffalo)
KNP/C8	-	*T. velifera, T. mutans*	+	+	*T. mutans*, Non-specific
KNP/C11	+		+	+	
KNP/C14	+	*T. mutans, T. velifera*			
KNP/C16	+	*T. mutans, T. velifera*	+	+	
KNP/C18	+	*T. mutans, T. velifera*	+	+	
KNP/C21	-	**T/B catchall probe**			
KNP/C24	+	*T. mutans*	+	+	
KNP/C26	-	*T. mutans, T. velifera*	+	+	Non-specific
KNP/E2	-	*T. mutans, T. velifera*	-	-	Non-specific
KNP/E3	-	**T/B catchall probe**		+	*T. taurotragi*
KNP/E5	-	*T. mutans, T. velifera*	+	+	Non-specific
KNP/E7	-	*T. mutans*	+	+	Non-specific
KNP/E10	-	*T. mutans*	-	-	Non-specific
KNP/E13	-	*T. mutans, T. velifera*	-	-	Non-specific
KNP/E18	-	*T. mutans*	+	+	*T.* sp (buffalo)
KNP/E20	-	*T. mutans, T. velifera*	+	+	Non-specific
KNP/E23	-	*T. velifera*	+	+	Non-specific
KNP/G1	+	*T. mutans, T. velifera*	+	+	
KNP/G2	+	*T. mutans*	+	+	
KNP/G5	-	*T.mutans, T. velifera*	+	+	Non-specific
KNP/G6	+	*T.mutans*	+	+	
KNP/G8	+	*T.* sp (buffalo), *T.mutans, T.velifera*	+	-	*T*. sp (buffalo); Non-specific
KNP/G10	-	*T.mutans, T. velifera*	+	+	Non-specific
KNP/G11	+	*T. mutans, T. velifera*	+	+	
KNP/G12	+	*T. mutans, T. velifera*	+	+	

Sample	RLB assay		18S qPCR	cox III qPCR	
	T. parva	Others		*T. parva*	Others
KNP/G14	-	*T. mutans, T. velifera*	+	+	
KNP/J1	-	*T. mutans, T. velifera*	+		Non-specific
KNP/J4	+	*T. mutans, T. velifera*	+	+	
KNP/J7	-	**T/B catchall probe**		+	*T. taurotragi*
KNP/J10	-	*T. mutans*; *T. velifera*		+	*T. taurotragi*
KNP/K1	+	*T. mutans, T. velifera*	+	+	Non-specific
KNP/K4	-	*T. mutans*	-	+	*T. taurotragi*
KNP/K8	-	*T. mutans*		+	*T. taurotragi*
KNP/K10	-	*T. mutans, T. velifera*	+	+	Non-specific
KNP/Q4	+	*T.* sp (buffalo),*T. mutans*	+	+	
KNP/Q7	-	*T. mutans*	+	+	*T*. sp (buffalo)
KNP/Q13		**Negative**	-	+	*T*. sp (buffalo)
KNP/Q15	+	*T.* sp (buffalo)	+		
KNP/Q18	+	*T.* sp (buffalo), *T.mutans*	+	+	*T. taurotragi*, Non-specific
KNP/Q22	+	*T.* sp (buffalo), *T. mutans*	+	+	*T*. sp (buffalo)
KNP/V1	+	*T.* sp (buffalo), *T. mutans*	+	+	*T*. sp (buffalo)
KNP/V2	+	*T.* sp (buffalo)	+	+	*T*. sp (buffalo)
KNP/V3	+	*T.* sp (buffalo), *T. mutans*	+	-	*T*. sp (buffalo)
KNP/V4	+	*T. mutans*	+	+	*T*. sp (buffalo)
KNP/V6	-	*T.* sp (buffalo)	-	-	*T*. sp (buffalo)
KNP/V7	-	*T.* sp (buffalo)	-	+	*T*. sp (buffalo)
KNP/V8	+	*T.* sp (buffalo), *T. mutans*	+		
KNP/V9	-	*T. mutans*	-	-	

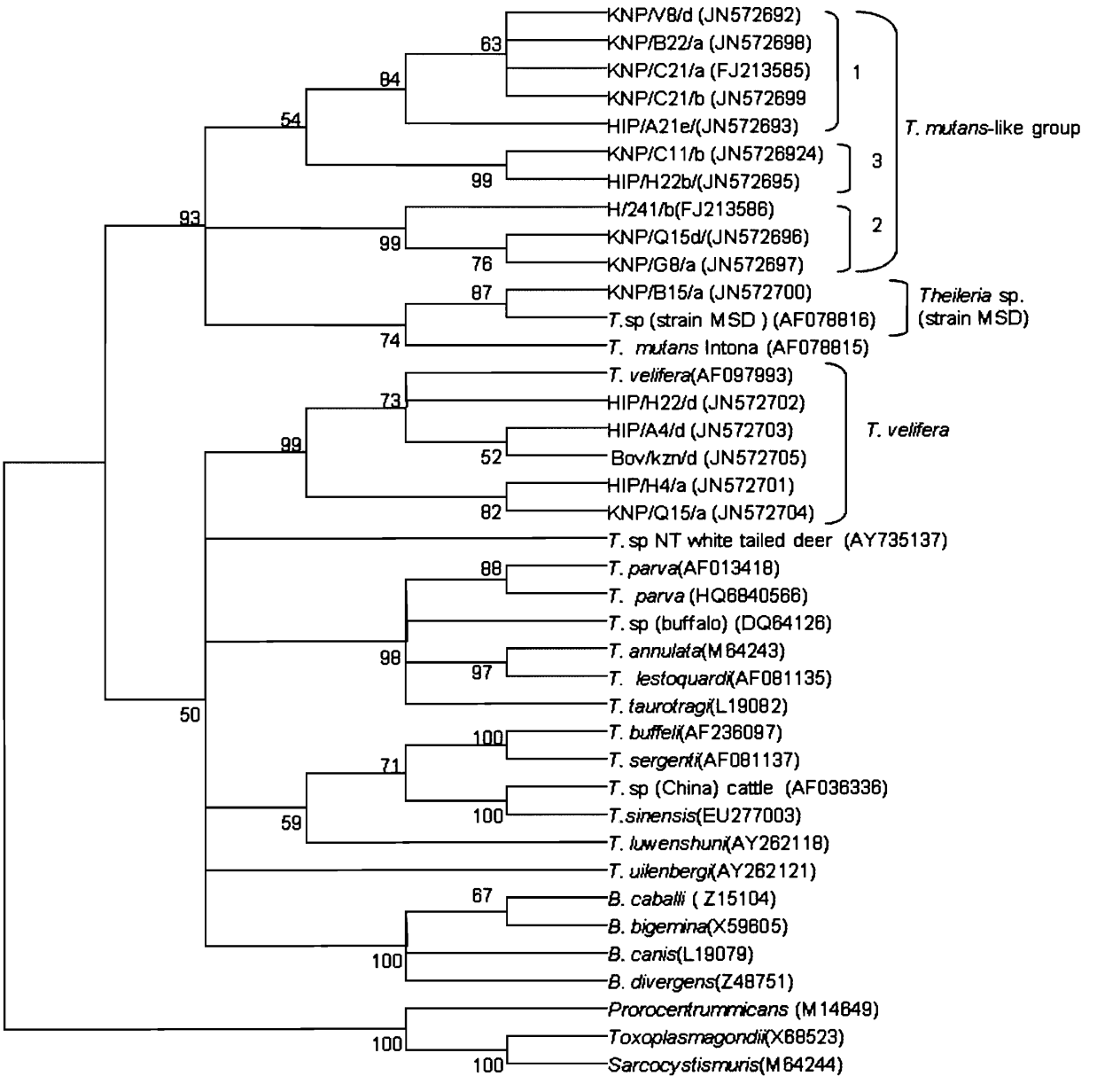
Samples	RLB Results		18S RT-PCR	FRET RT-PCR	
	T. parva	Others	*T. parva*	*T. parva*	Others
HIP/A2	-	*T.* sp (buffalo), *T. buffeli*	-	+	*T.* sp (buffalo)
HIP/A4	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A5	+	*T.* sp (buffalo), *T. buffeli, T. velifera, T. mutans*	+	+	*T.* sp (buffalo)
HIP/A6	+	*T.* sp (buffalo), *T. buffeli, T. mutans*	+	+	*T.* sp (buffalo)
HIP/A11	+	*T.* sp (buffalo), *T. buffeli, T. mutans, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A12	+	*T.* sp (buffalo), *T. buffeli, T. mutans*	+	+	*T.* sp (buffalo)
HIP/A13	-	*T.* sp (buffalo), *T. buffeli, T. mutans*	-	+	*T.* sp (buffalo)
HIP/A14	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A17	-	*T.* sp (buffalo), *T. buffeli*	-		*T.* sp (buffalo)
HIP/A19	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A21	-	*T.* sp (buffalo) (faint signal)	-		
HIP/A22	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A25	-	*T.* sp (buffalo), *T. mutans*	+	+	*T.* sp (buffalo)
HIP/A27	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A28	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A29	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A30	-	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A33	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A34	-	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/A36	-	*T. buffeli*	+	+	*T.* sp (buffalo)
HIP/A38	-	*T.* sp (buffalo), *T. buffeli*	+	+	*T.* sp (buffalo)
HIP/A41	+	*T.* sp (buffalo), *T. buffeli*	+	+	*T.* sp (buffalo)
HIP/A45	-	T/B catchall probe only	+	+	*T.* sp (buffalo)
HIP/B7		**Negative**	-	+	
HIP/B11	-	**T/B catchall probe only**	+	+	*T.* sp (buffalo)
HIP/B20		**Negative**	-	+	
HIP/B31	-	T/B catchall probe only	+	+	
HIP/B32	+	*T. sp (buffalo), T. buffeli, T. mutans, T. velifera*	+	+	*T.* sp (buffalo)
HIP/B44	-	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/B46	-	-	-	+	
HIP/B48	+	*T. buffeli, T. velifera*	+	+	*T.* sp (buffalo)
HIP/B49	+	*T. buffeli*	+	+	*T.* sp (buffalo)
HIP/B52	-	*T.* sp (buffalo), *T. buffeli*	+	+	*T.* sp (buffalo)
HIP/B56	-	*T.* sp (buffalo), *T. buffeli, T. mutans*	+	+	*T.* sp (buffalo)
HIP/B58	+	*T.* sp (buffalo), *T. buffeli, T.mut, T. vel*	+	+	*T.* sp (buffalo)
HIP/B60	-	*T.* sp (buffalo), *T. buffeli*	+	+	*T.* sp (buffalo)
HIP/B62	-	*T. buffeli*	+	+	*T.* sp (buffalo)
HIP/B64	+	*T.* sp (buffalo), *T. mutans, T. velifera*	+	+	*T.* sp (buffalo)
HIP/B65	+	*T.* sp (buffalo), *T. buffeli, T. mutans*	+	+	*T.* sp (buffalo)
HIP/B66	+		+	+	*T.* sp (buffalo)

Samples	RLB Results		18S RT-PCR	FRET RT-PCR	
	T. parva	Others	*T. parva*	*T. parva*	Others
HIP/B68	+		+	+	*T*. sp (buffalo)
HIP/B70		**Negative**	-	+	*T*. sp (buffalo)
HIP/B75		**Negative**	-	+	
HIP/B77		**Negative**	+	+	
HIP/B78	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T*. sp (buffalo)
HIP/B79	+	*T. buffeli, T. mutans, T. velifera*	+	+	*T*. sp (buffalo)
HIP/C2	-	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T*. sp (buffalo)
HIP/C4	-	**T/B catchall probe only**	+	+	*T*. sp (buffalo)
HIP/C5	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T*. sp (buffalo)
HIP/C8	+	*T.* sp (buffalo), *T. buffeli, T. velifera*	+	+	*T*. sp (buffalo)
HIP/C9	+	*T. sp (buffalo), T. buffeli, T. mutans, T. vel*	+	+	*T*. sp (buffalo)
HIP/C11	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/C13	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/C15	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/C19	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/C23	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H24		**Negative**	+	+	
HIP/C27	-	*T. buffeli*	+	+	
HIP/H6		**T/B catchall probe only**	+	+	*T. taurotragi*
HIP/H16		**Negative**		+	
HIP/H18	+	*T.* sp (buffalo), *T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H19	-	*T.* sp (buffalo), *T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H21		**Negative**	-	+	*T*. sp (buffalo)
HIP/H22	+	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H25	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H27		**Negative**	+	+	
HIP/H30		**Negative**	+	+	*T*. sp (buffalo)
HIP/H32	+	*T.* sp (buffalo), *T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H33	-	*T. buffeli*	+	+	*T*. sp (buffalo)
HIP/H35		**Negative**	-	+	*T*. sp (buffalo)
HIP/H36		**Negative**	-	+	*T*. sp (buffalo)
HIP/H41	-	*T. buffeli*	+	+	*T*. sp (buffalo)

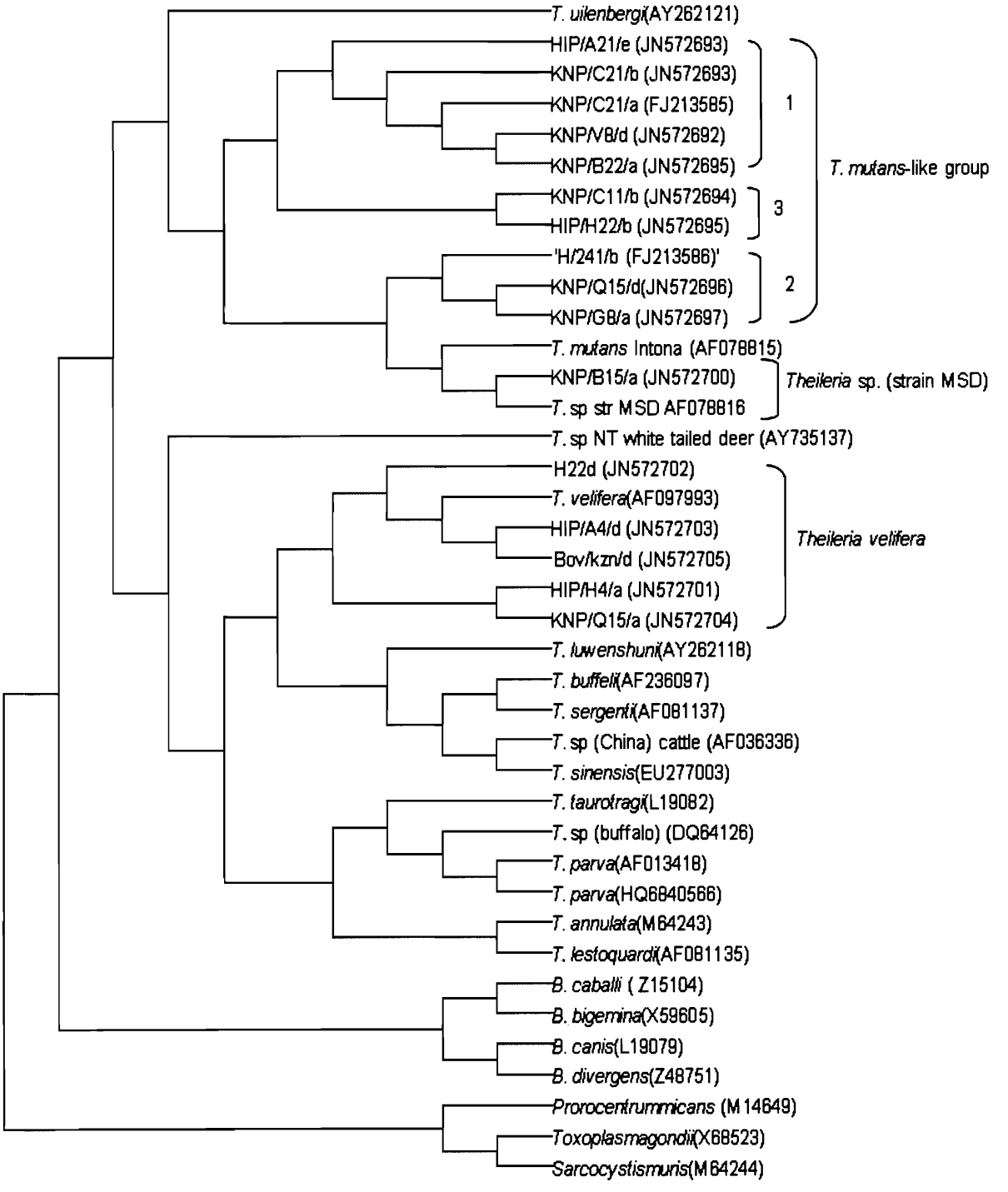
Sample	RLB Results		18S RT-PCR	FRET RT-PCR	
	T. parva	Others	*T. parva*	*T. parva*	Others
GLTP/ 2	+	*Theileria* sp. (buffalo), *T. velifera, T. mutans*	+	+	
GLTP/ 3	+	*T. velifera, T. mutans*	+	+	
GLTP/ 4	+	*T. mutans*	+	+	
GLTP/ 5	+	*T. velifera, T. mutans*	+	+	*Theileria* sp. (buffalo)
GLTP/ 6	+		+	+	
GLTP/ 7	+	*T. velifera, T. mutans*		+	
GLTP/ 8	-	*T. velifera, T. mutans*	-	-	Non-specific
GLTP/ 9	+	*Theileria* sp. (buffalo), *T. mutans*	+	+	
GLTP/ 10	+	*T. mutans*	+	+	
GLTP/ 11	-	*T. mutans*	+	-	*Theileria* sp. (buffalo)
GLTP/ 12	-	*T. mutans*	-	+	Non-specific
GLTP/ 13	+		+	+	
GLTP/ 14	+	*T. velifera*	+	+	
GLTP/ 15	+		+	+	
GLTP/ 16	+		+	+	
GLTP/ 17	+			+	Non-specific
GLTP/ 19	+		+	+	
GLTP/ 20	+	*Theileria* sp. (buffalo)	+	+	
GLTP/ 23	-	*Theileria* sp. (buffalo)	+	+	
GLTP/ 25	-	*Theileria* sp. (buffalo)	-	+	Non-specific
GLTP/ 26	-	*Theileria* sp. (buffalo)	-	+	Non-specific
GLTP/ 28		**Negative**	-	+	Non-specific
GLTP/ 29	+	*Theileria* sp. (buffalo)	+	+	
GLTP/ 34	+		+	+	
GLTP/ 36	+	*Theileria* sp. (buffalo)	+	+	
GLTP/ 38	+		+	-	*Theileria* sp. (buffalo)
GLTP/ 39	+		+	+	
GLTP/ 40	+		+	+	
GLTP/ 42	+	*T. mutans*	+	+	
GLTP/ 44	+		+	+	
GLTP/ 46	+		+	+	
GLTP/ 47	+		+	+	
GLTP/ 49	+	*T. mutans*	+	+	

Appendix B: Phylogenetic trees showing the relationship of the *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera* 18S rRNA gene sequence variants identified in this study with other *Theileria* and *Babesia* species as indicated by (a) neighor-joining, (b) maximum likelihood and (c) bayesian analyses. Bootstrap values indicate the degree of support for each cluster. The trees were outgroup rooted using *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii*.

(a) Neighbor joining tree



(b) Maximum likelihood tree



(c) Bayesian tree