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.

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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*

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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.

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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,

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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.

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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.

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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.

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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*.

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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.

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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).

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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.

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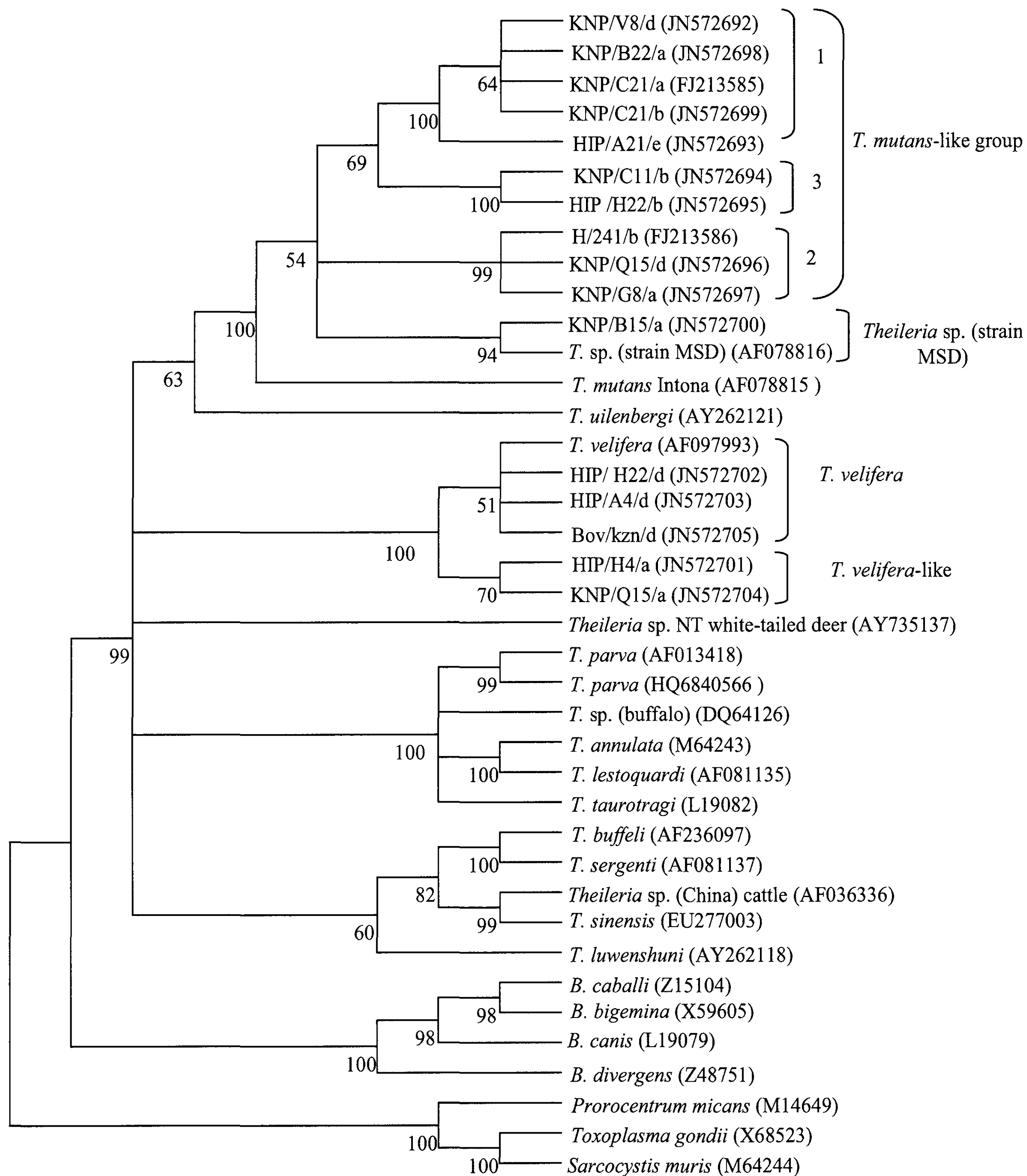

Figure 4.1: Phylogenetic tree showing the relationship of the *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera* 18S rRNA gene sequence variants identified in this study with other *Theileria* and *Babesia* species as indicated by maximum parsimony analysis. Bootstrap values indicate the degree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

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                         510       520       530       540       550       560       570       580       590       600
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

T. mutans                                                                           CTTGCGTCTCCGAATGTT
AF078815          AGTTGAATTTCTGCCGCATCGCGGCGGCCCTCCCGGGCCCAGCGGTTGCGGCTTATTTCGGACTCGCTTGCGTCTCCGAATGTTTACTTTGAGAAAATTA
Theileria sp. (strain MSD)
AF078816          .................CC...C.......--........G...-.C..............CGA.-..C...C.G.........................
KNP/B15/a         .................CC...C.......--........GA..-.C..............CGA.-..C...C.G.........................
T. mutans-like 1                                                                    CTTGCGATGCCGAATGTT
KNP/C21/a         ..............T.......CA......-.A...........-...........................ATG.........................
KNP/C21/b         ..............T.......CA......-.A...........-...........................ATG.........................
KNP/B22/a         ..............T.......CA......-.A...........-...........................ATG.........................
KNP/V8/d          ..............T.......CA......-.A...........-...........................ATG.........................
HIP/A21/e         ..............T.......CA......-.A...........-...........................ATG.........................
T. mutans-like 2/3                                                              TTGCGTGCATCTCCGAATTGTT
H/241/b    ┐      .................CC..AC.A.A...-.A.......GA..-C..................T..G...A............................
KNP/Q15/d  │ 2    .................CC..AC.A.A...-.A.......GA..-C..................T..G...A............................
KNP/G8/a   ┘      .................CC..AC.A.A...-.A.......GA..-C..................T..G...A............................
KNP/C11/b  ┐ 3    ..............A.......CA......-.A......AC...-...................T..G...A............................
HIP/H22/b  ┘      ..............A.......CA......-.A......AC...-...................T..G...A............................
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T. velifera                                CCTATTCTCCTTTACGAG
AF097993          TCGTAGTTGAATTTCTGCTACATTGCCTATTCTCCTTTACGAGTTTGGGTCTTTTGTGGCTTATCTGGG-TTCGCTTGC-TTCCCGGTGTTTTACTTTGA
HIP/A4/d          .....................................................................-.........G....................
Bov/KZN/d         .....................................................................-.........G....................
HIP/H22/d         .....................................................................-.........G....................
HIP/H4/a          ...........................CT........................................-.........G....................
KNP/Q15/a         ...........................CT........................................-.........G....................
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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.

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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.

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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

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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).

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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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