
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. (bougasvlei) 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 amplification (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 (Kato 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.

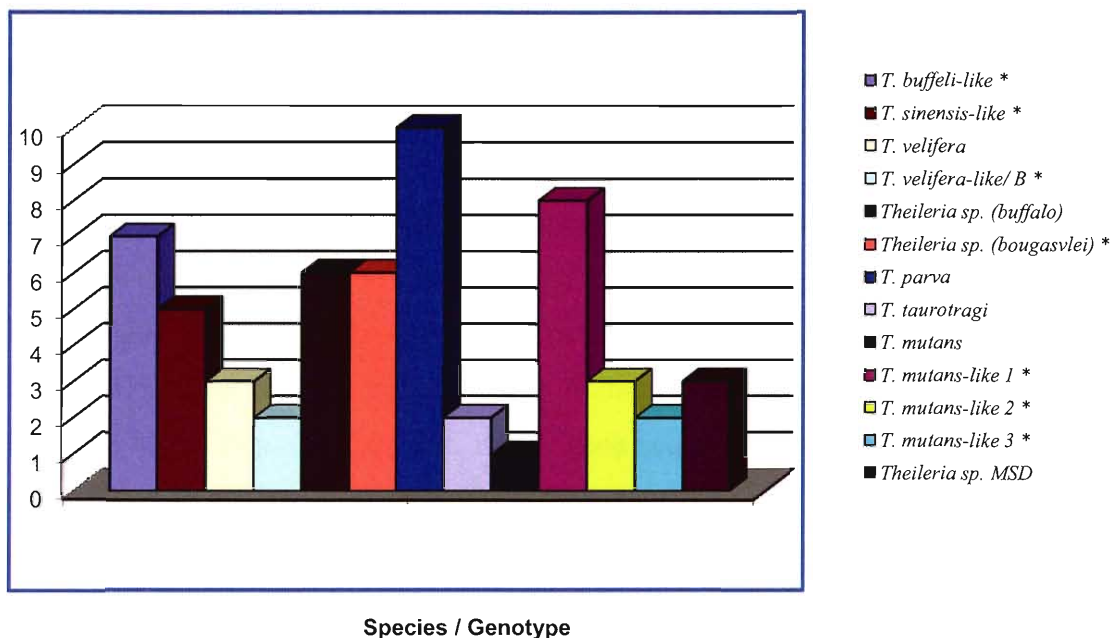


Figure 6.1: The frequency of known and novel (*) 18S rRNA gene sequences (n = 57) of *Theileria* spp. identified from buffalo and cattle samples (n = 31) from different geographical regions in South Africa, and from the GLTP in Mozambique.

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.

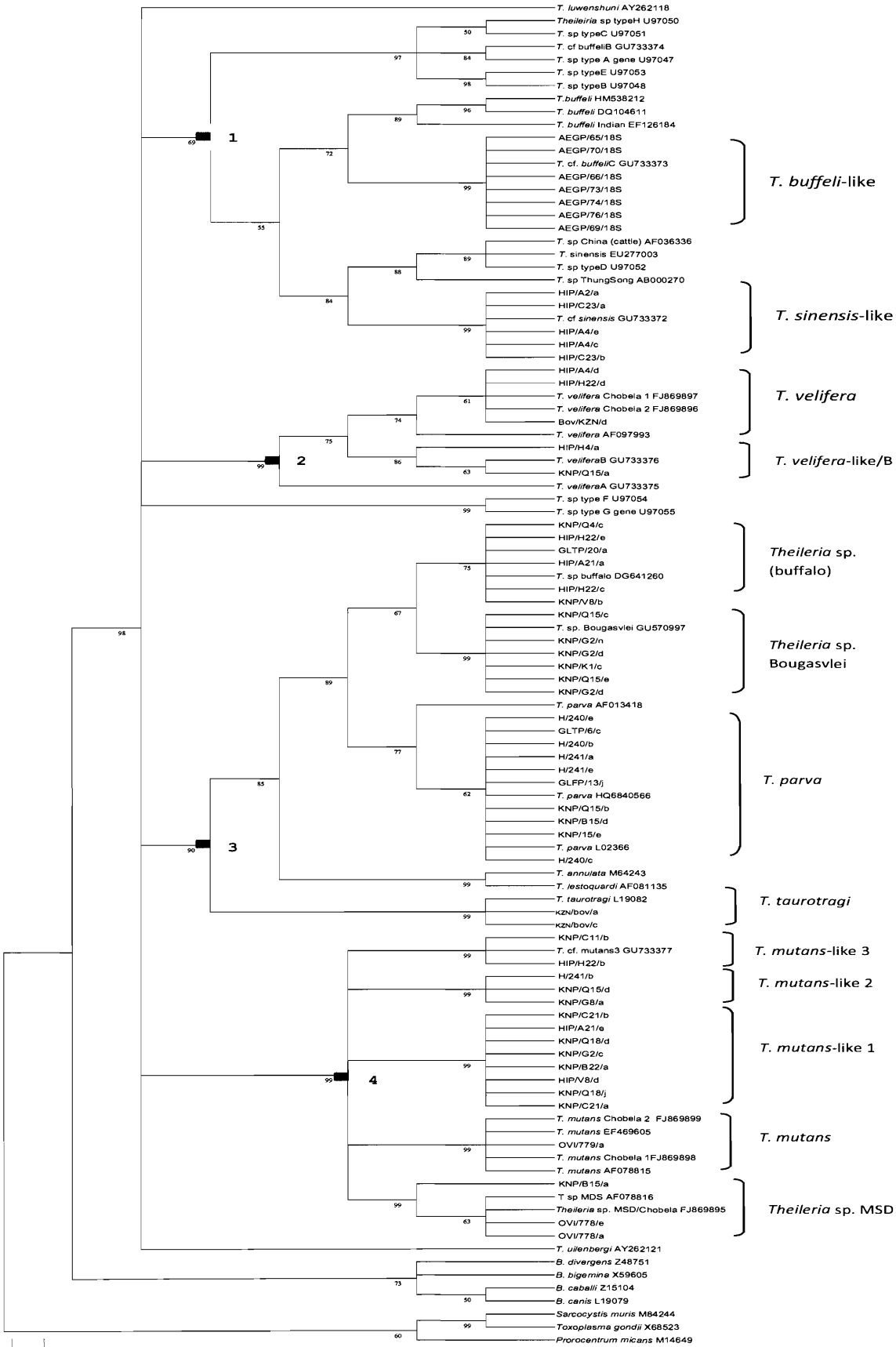


Figure 6.2: Phylogenetic tree showing the relationships between *Theileria* spp. variants identified in this study with known *Theileria* and *Babesia* species as indicated by Neighbour-joining analysis. Bootstrap values indicate the degree of support for each cluster. The tree was rooted using *Prorocentrum 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. <i>T. buffeli</i> -like													
2. <i>T. sinensis</i> -like	11												
3. <i>T. velifera</i>	17	24											
4. <i>T. velifera</i> -like/B	18	25	3										
5. <i>Theileria</i> sp. (buffalo)	21	22	25	24									
6. <i>Theileria</i> sp. (bougaslvei)	21	25	27	28	4								
7. <i>T. parva</i>	24	22	24	25	3	7							
8. <i>T. taurotragi</i>	24	22	27	27	10	9	11						
9. <i>T. mutans</i>	30	30	36	37	22	22	22	26					
10. <i>T. mutans</i> -like 1	30	29	36	38	25	22	25	28	7				
11. <i>T. mutans</i> -like 2	31	30	37	38	25	25	25	26	10	8			
12. <i>T. mutans</i> -like 3	30	27	36	37	22	22	22	28	6	15	11		
13. <i>Theileria</i> 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. (bougaslvei) 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.



	Forward primer		Anchor probe			Sensor probe	
	90	100	110	120	130	140	150
<i>T. parva</i> (HQ6840567)	TTTCTGCTGCATCGC	TGTCCTCC	TTGGGGTCTCTGC	ATGTGGCTTATT	TCGGACGGAGTT	TCGCTT	TTGTCGG
H/240/e (HQ895968)
H/241/a (HQ895969)
H/241/e (HQ895984)
GLTP/6/c (HQ895971)
KNP/Q15/b (HQ895973)
H/240/b (HQ895975)
KNP/B15/e (HQ895972)
H/240/c (HQ895974)
KNP/B15/d (HQ895969)
GLFP/13/j (HQ895985)
<i>Theileria sp. buffalo</i> (DG641260)
HIP/A21/a (HQ895978)
HIP/H22/c (HQ895980)
KNP/V8/b (HQ895982)
GLTP/20/a (HQ895987)
KNP/Q4/c (HQ895979)
HIP/H22/e (HQ895981)
<i>T. sp. bougasvlei</i> (GU570997)
KNP/G2/d (HQ895976)
KNP/K1/c (HQ895977)
KNP/Q15/c (HQ895983)
KNP/Q15/e (HQ895986)
KNP/G2/n
<i>T. taurotragi</i> (L19082)
KZN/bov/c
KZN/bov/a
<i>Theileria cf. buffeli</i> (GU733374)
<i>Theileria cf. buffeli</i> (GU733373)
AEGP/65/18S
AEGP/66/18S
AEGP/69/18S
AEGP/70/18S
AEGP/74/18S
AEGP/76/18S
AEGP/73/18S
<i>T. cf. sinensis</i> (GU733372)
HIP/A4/c
HIP/A2/a
HIP/A4/e
HIP/C23/a
HIP/C23/b
<i>T. velifera</i> (AF097993)
HIP/H22/d (JN572702)
KZN/bov/d (JN572705)
HIP/A4/d (JN572703)
<i>T. velifera</i> A (GU733375)
<i>T. velivera</i> B (GU733376)
HIP/H4/a (JN572701)
KNP/Q15/a (JN572704)
<i>T. mutans</i> (AF078815)
OVI/779/a
<i>T. mutans-like 1</i>
KNP/C21/a (FJ213585)
HIP/V8/d (JN572692)
KNP/B22/a (JN572698)
HIP/A21/e (JN572693)
KNP/C21/b (JN572699)
KNP/Q18/d
KNP/Q18/j
KNP/G2/c
<i>T. mutans-like 2</i>
H/241/b (FJ213586)
KNP/Q15/d (JN572696)
KNP/G8/a (JN572697)
<i>T. mutans-like 3</i> (GU733377)
KNP/C11/b (JN572694)
HIP/H22/b (JN5726925)
<i>Theileria sp. MDS</i> (AF078816)
KNP/B15/a (JN5726700)
OVI/778/a
OVI/778/e

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. (bougasvlei) 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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