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

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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.

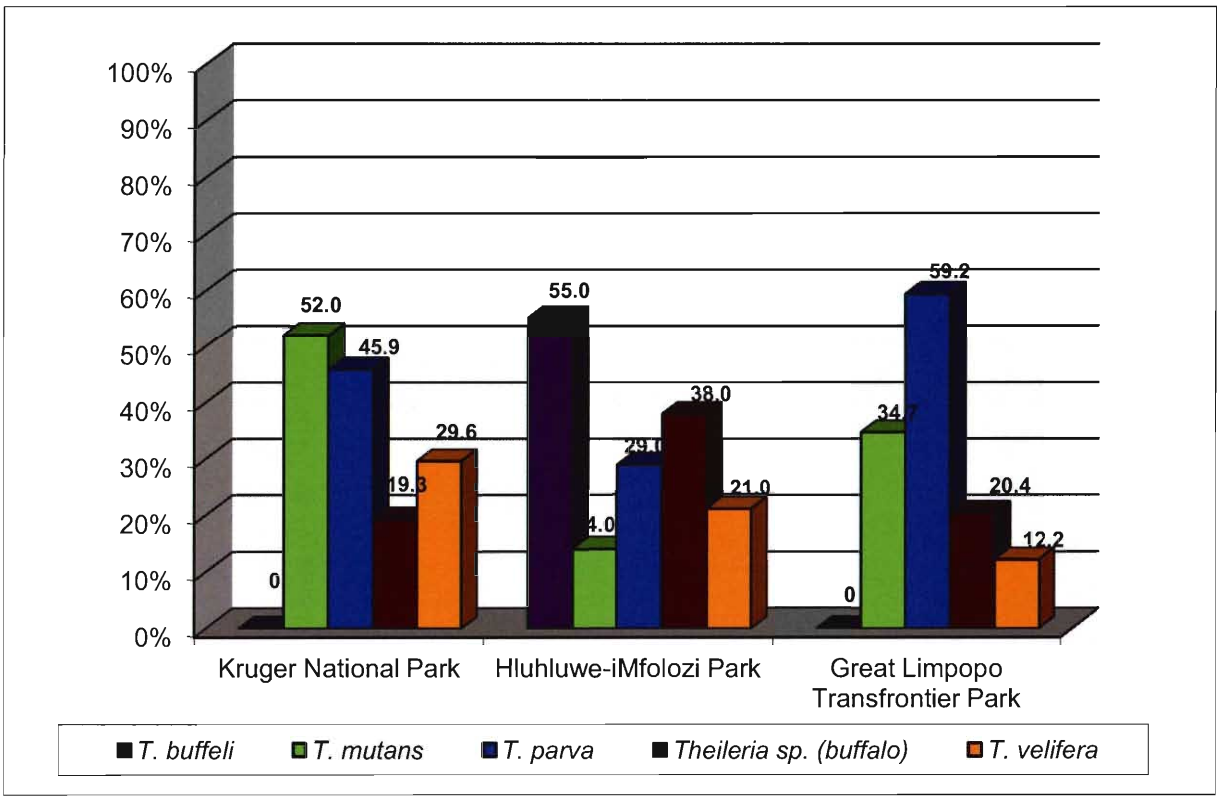


Figure 3.1: Occurence of *Theileria* spp. infections in buffalo blood samples from the Kruger National Park (KNP), Hluhluwe-iMfolozi Game Park (HIP) and the Greater Limpopo Transfronteir Park (GLTP), South Africa, as determined by the Reverse Line Blot hybridization assay.

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%)

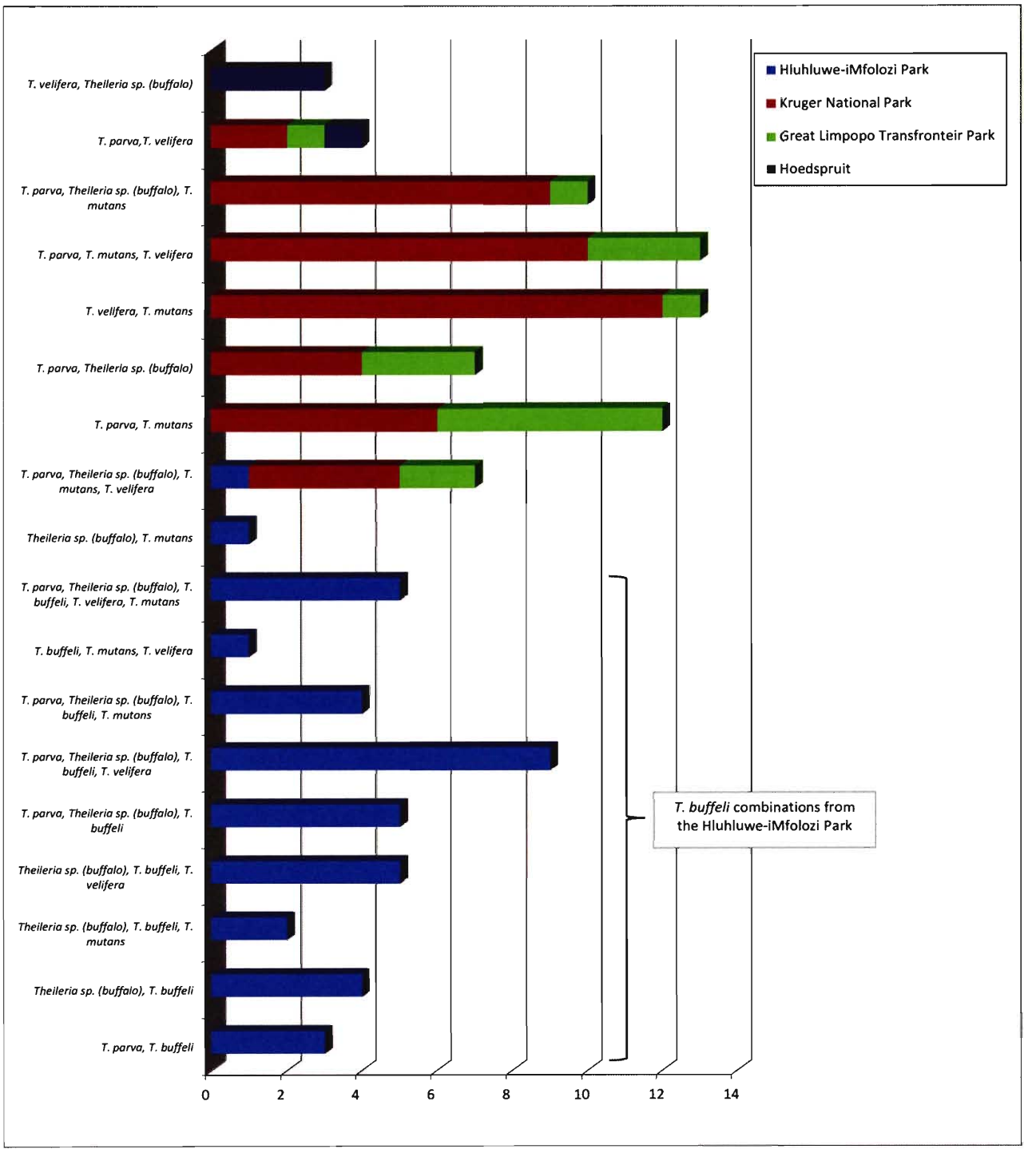


Figure 3.2: Composition of mixed *Theileria* spp. infections, as determined using the RLB hybridization assay, in buffalo blood samples from the Kruger National Park, Hluhluwe-iMfolozi Park, Greater Limpopo Transfrontier Park and a private game farm in Hoedspruit.

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.

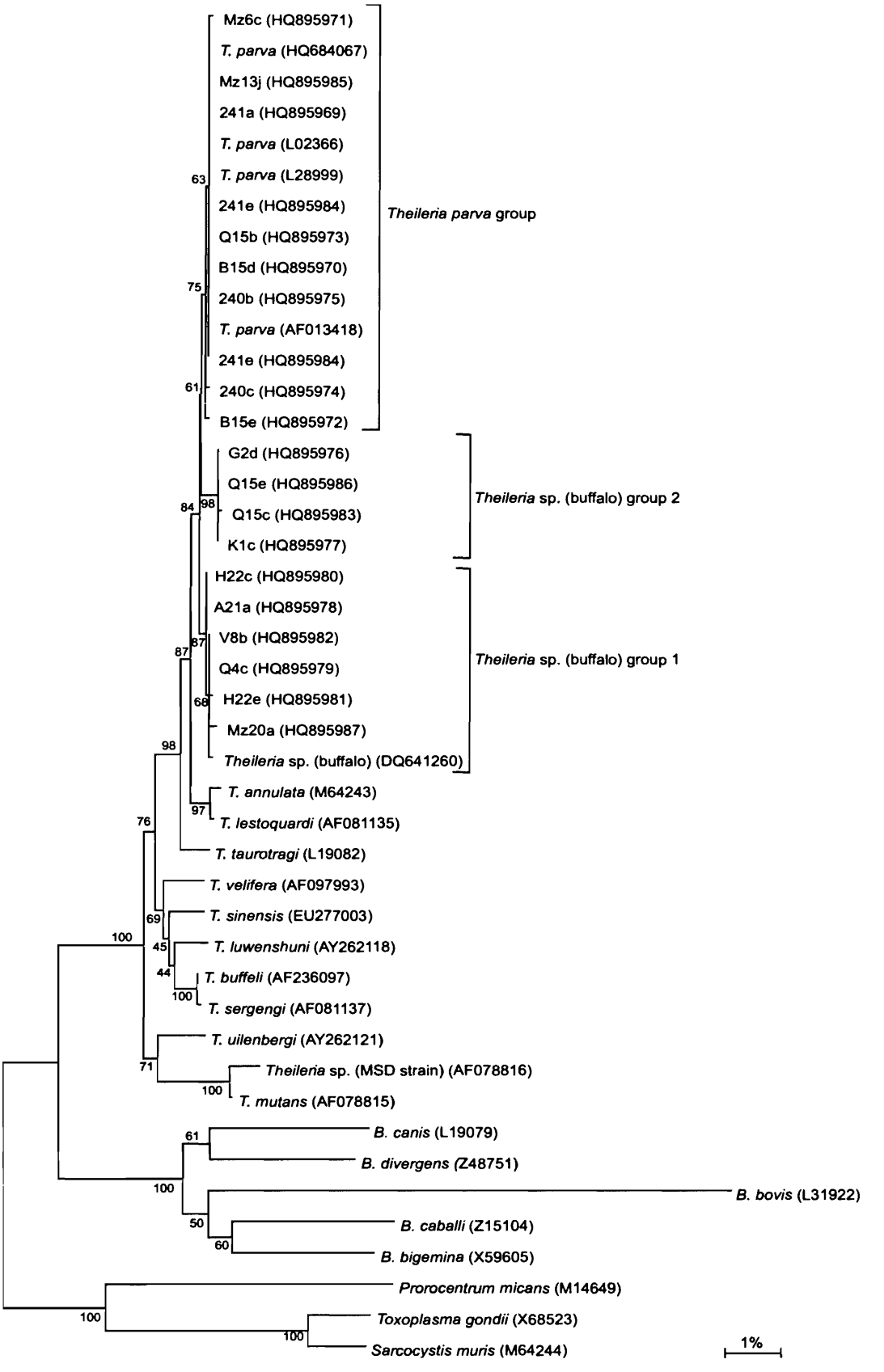
The previously published 18S rRNA gene sequences of *T. parva* and *Theileria* sp. (buffalo) differ by 11 bp (Table 3.4). Estimated evolutionary divergences between the *T. parva* and *Theileria* sp. (buffalo) sequences obtained in this study were compared by determining the number of base differences 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).

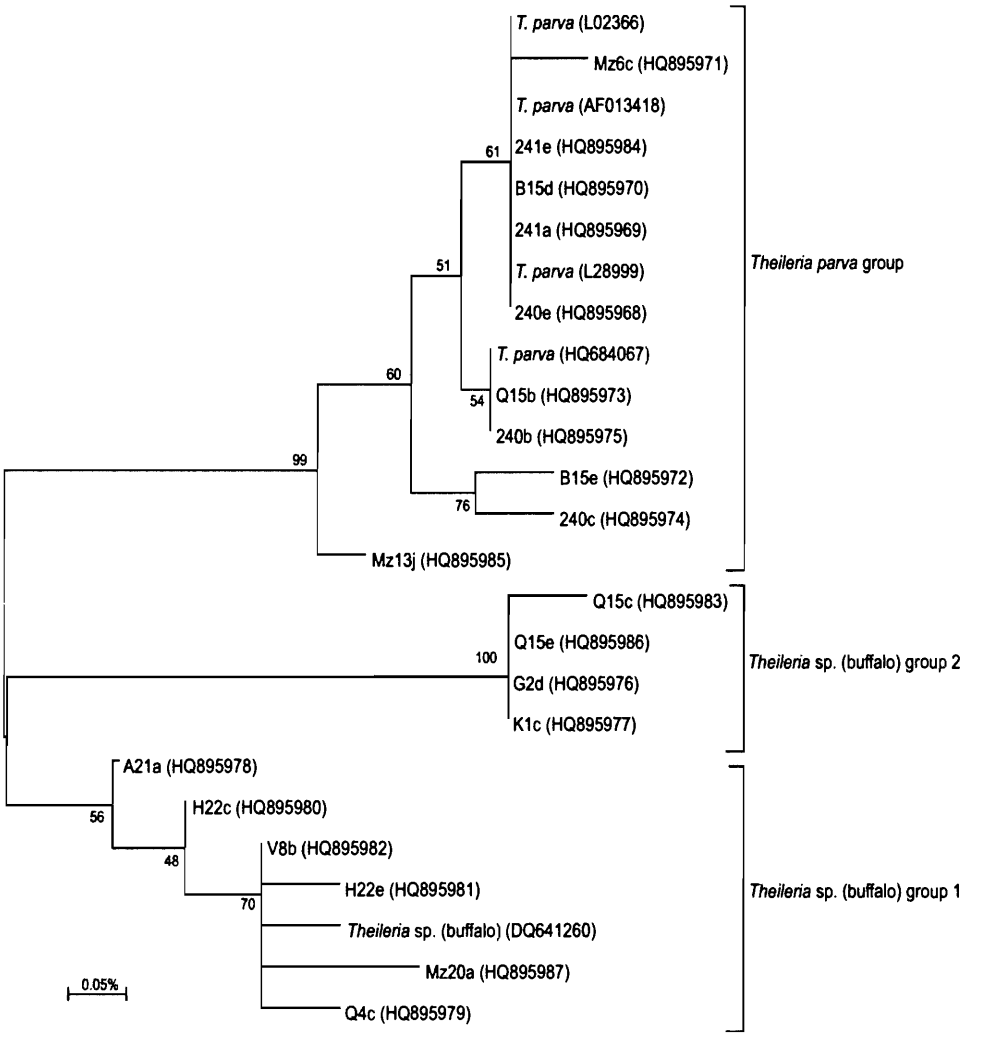
Table 3.4: Estimates of evolutionary divergence between the *T. parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequences. The number of base differences per sequence from analysis between sequences is shown. All results are based on the pairwise analysis of 25 sequences. Analyses were conducted in 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.

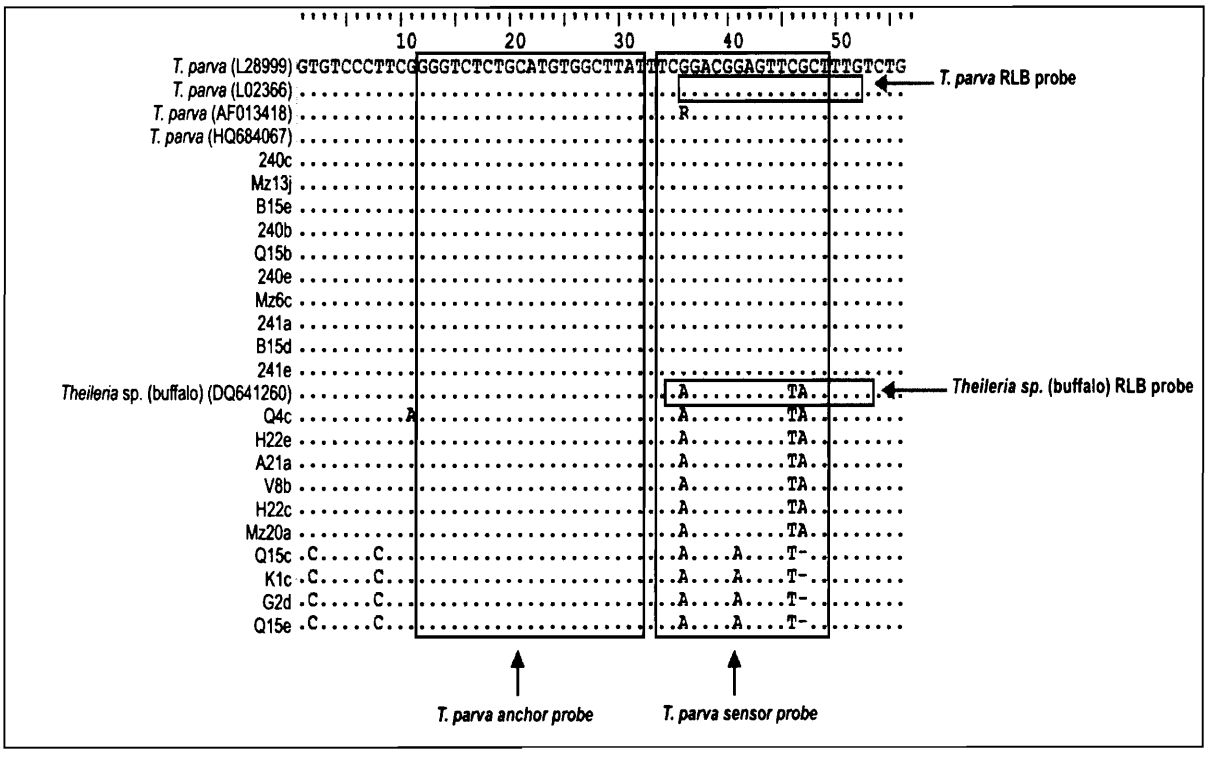
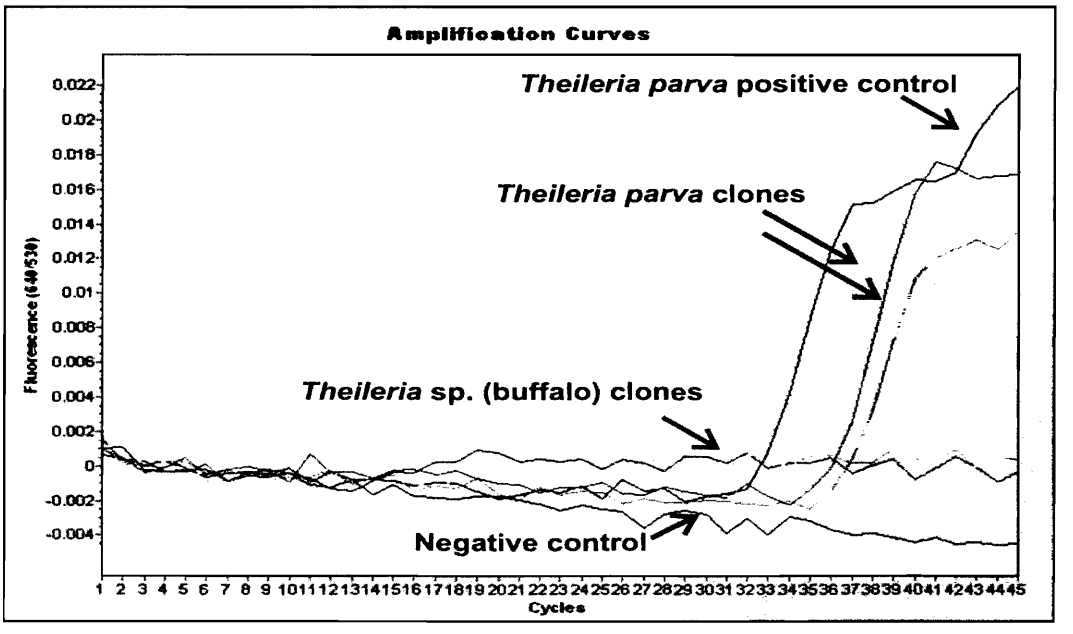


Figure 3.4: Nucleotide alignment of a 56 bp region of the V4 hypervariable region of the published 18S rRNA gene of *T. parva* and *Theileria* sp. (buffalo), as well as the variants identified in the study. Regions where the *T. parva* real-time PCR and RLB probes were designed are indicated with boxes.

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)

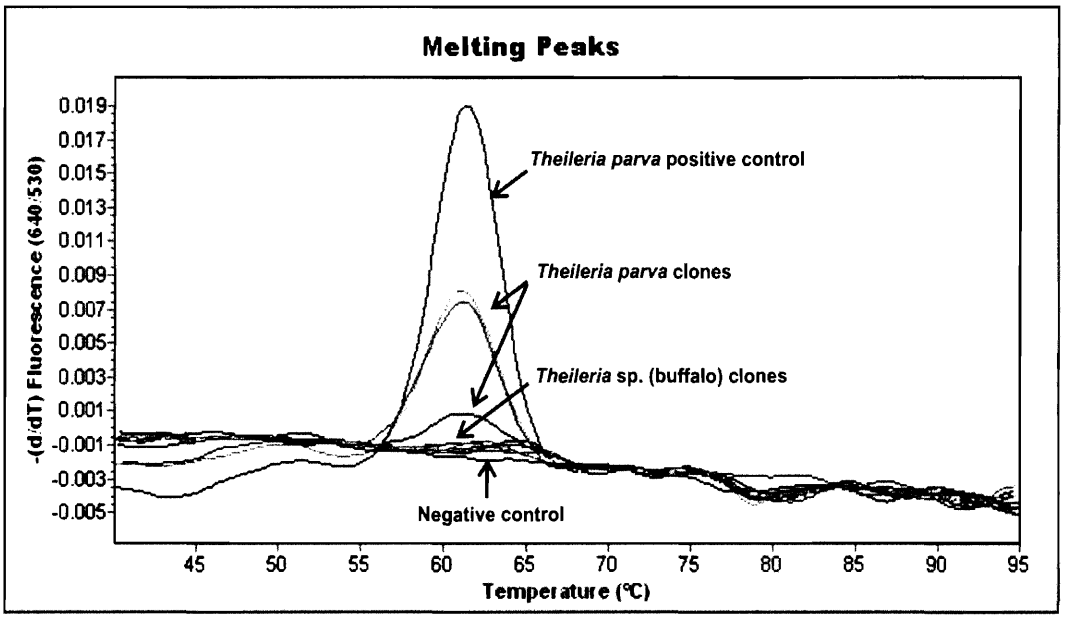


Figure 3.5: *Theileria parva*-specific real-time PCR results obtained with the cloned *T. parva* and *Theileria* sp. (buffalo) variants obtained in this study. (a) Amplification curves showing increase in fluorescence at 640 nm for both *T. parva* and *Theileria* sp. (buffalo) clones. (b) Melting curve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

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