CHAPTER 5

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

5.1 Abstract

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

5.2 Introduction

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

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

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

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

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

5.3 Materials and Methods

5.3.1 DNA samples

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

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

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

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

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

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

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

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

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

5.3.4 Sequence and phylogenetic analyses

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

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

5.3.5 GenBank Accession numbers

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

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

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

* Partial sequences were not included in the phylogenetic trees.

5.4 Results

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

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

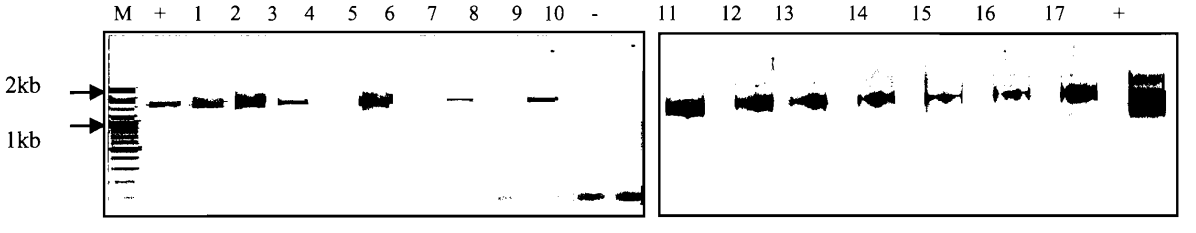

Figure 5.1: Agarose gel eletrophoresis analyis of amplicons of the full-length 18S rRNA gene (1-10) and ITS region (11-17) of *Theileria* spp. of buffalo from field samples. M = 100 bp plus marker, + = *T. parva* positive control, - = water negative control.

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

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

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

5.4.2 Sequence and phylogenetic analyses of the 18S rRNA genes

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

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

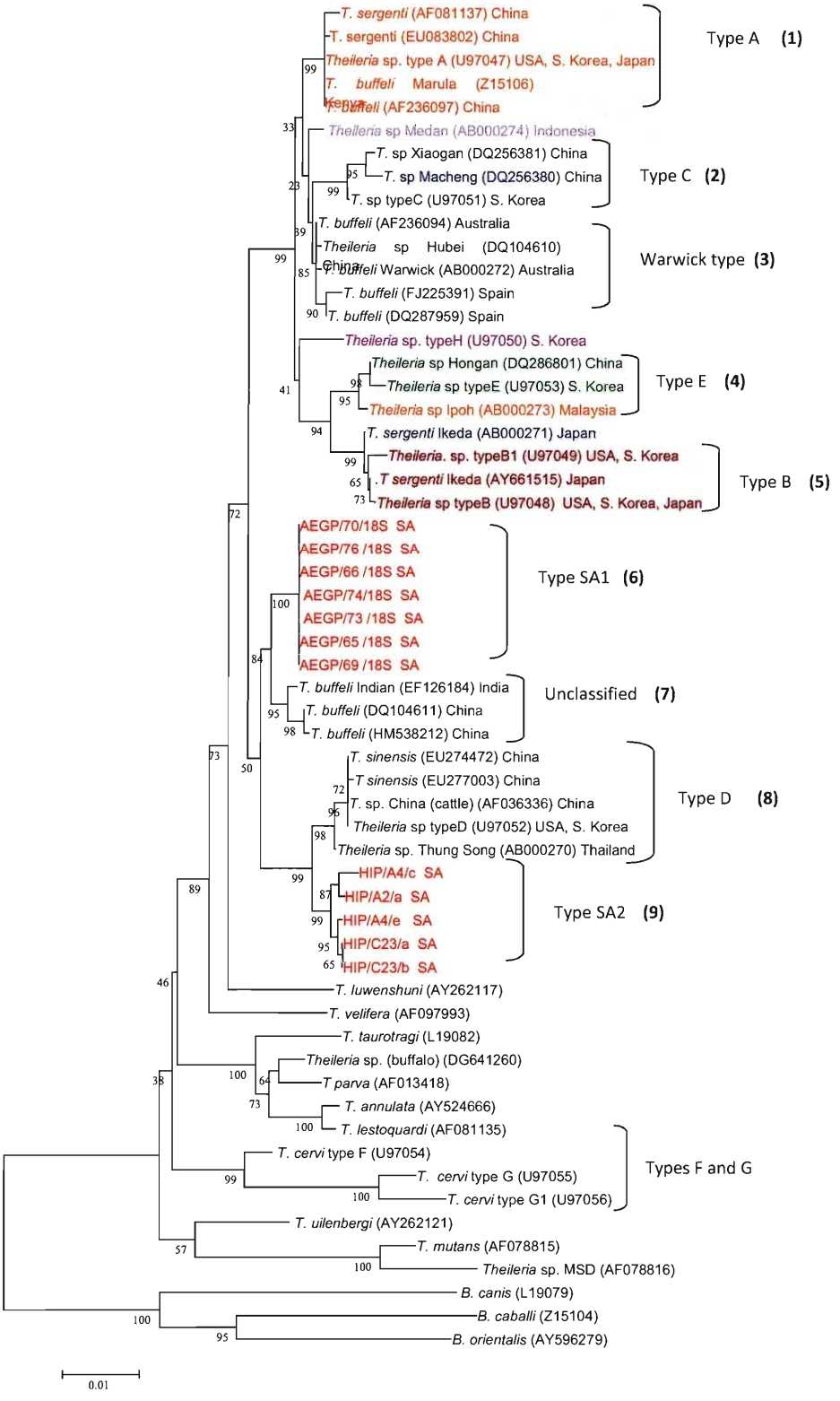

Figure 5.2: Phylogenetic relationships of novel *T. buffeli*-like and *T. sinensis*-like 18S rRNA gene sequences from South Africa (red) with known *T. buffeli* sequences as inferred by the Neighbor-joining method. GenBank accession numbers are indicated in parenthesis. Numbers in brackets are designated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

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

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

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

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

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

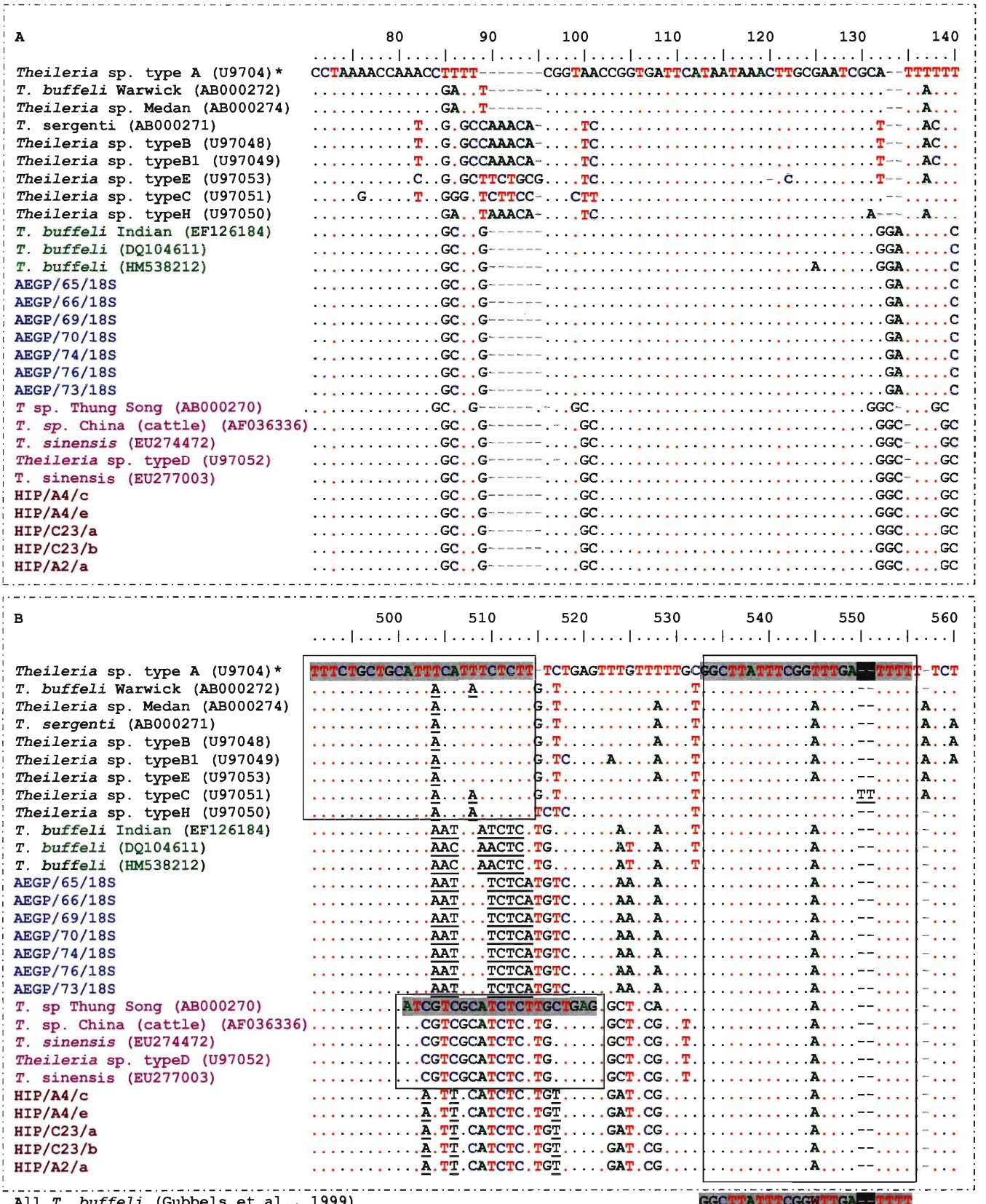

Figure 5.3: Alignment of novel *T. buffeli*-like (blue) and *T. sinensis* (brown) 18S rRNA gene sequences with known *T. buffeli* sequences (black, green and pink) showing V1 variable (A) and V4 hypervariable (B) regions of the gene. RLB probes were designed from the V4 hypervariable region (B). Blocks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

5.4.4 Sequence and phylogenetic analyses of the novel ITS sequences

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

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

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

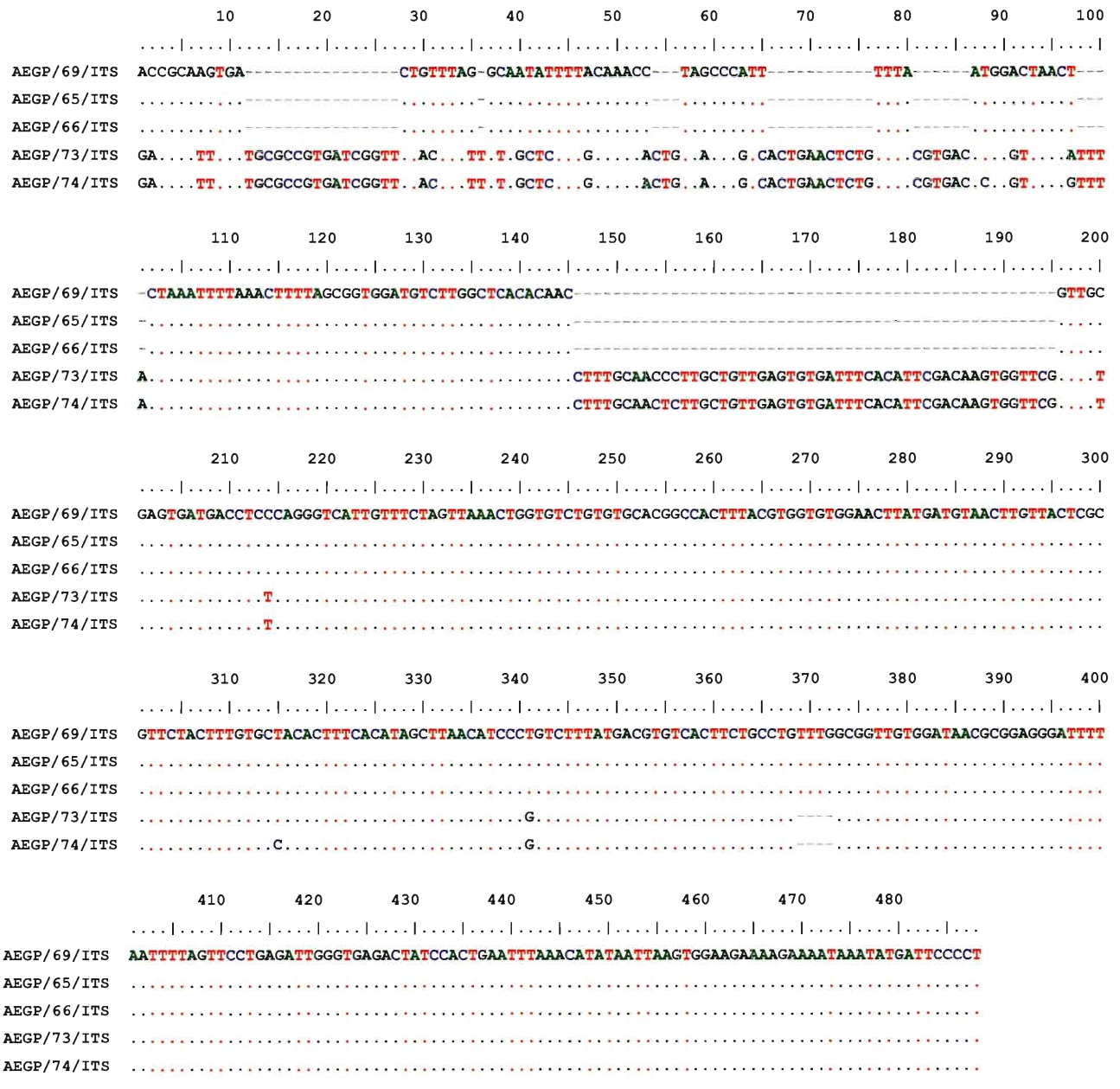

Figure 5.4: Sequence alignment of the complete ITS2 region of novel *Theileria* spp. from the Addo Elephant Game Park. The dots indicate conserved nucleotides; gaps (-) indicate missing nucleotides and were introduced to maintain homology.

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

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

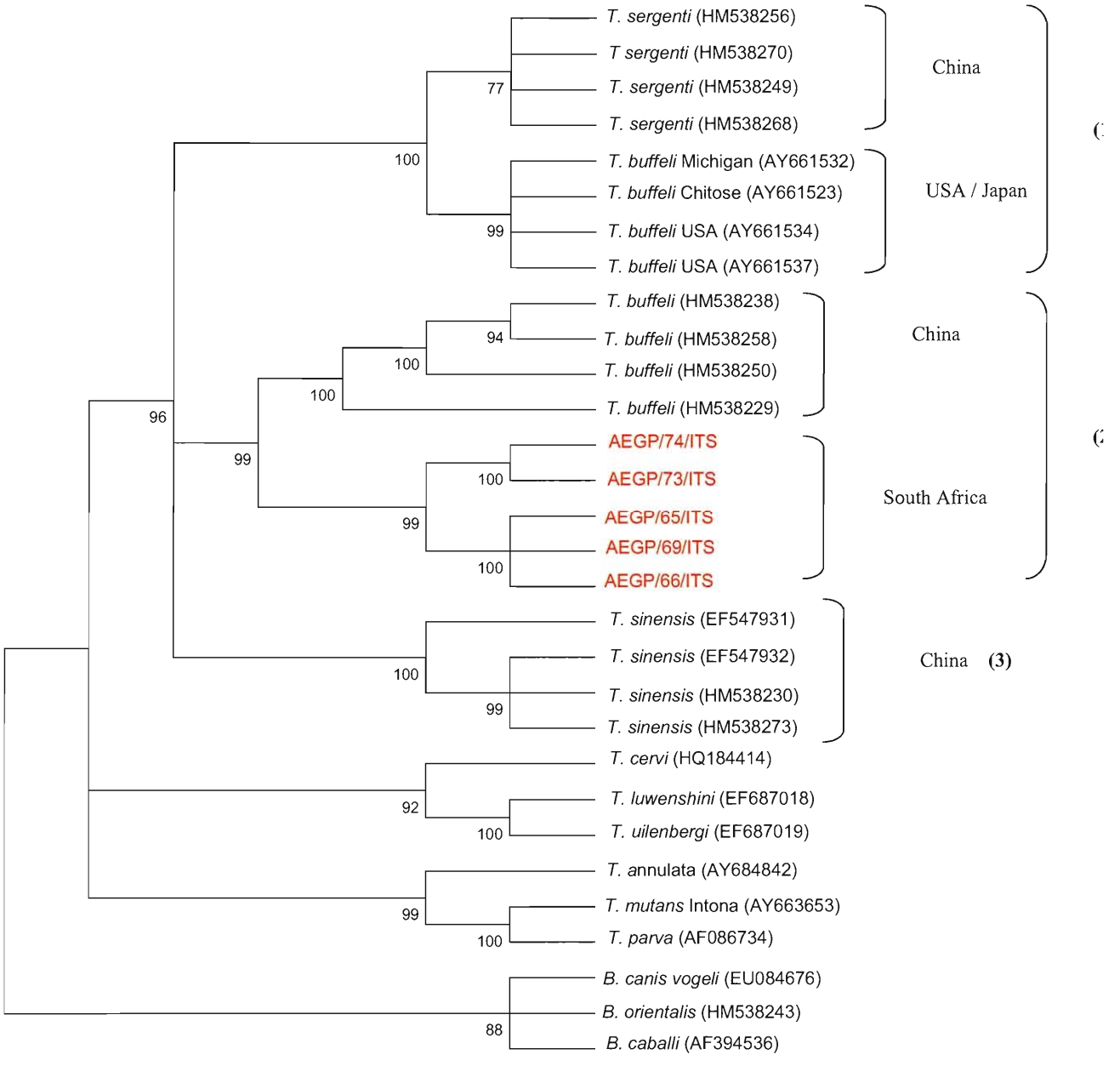

Figure 5.5: Phylogenetic relationships of novel *T. buffeli*-like ITS sequences from South Africa (red) with known *T. buffeli*-like ITS sequences from Genbank (accession numbers in parenthesis) as determined by Bayesian inference. The numbers in brackets are designated cluster numbers. Posterior probabilities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

5.5 Discussion

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

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

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

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

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

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

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

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

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

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

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

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

5.5.3 *Theileri buffeli*-like ITS genotypes

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

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

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

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

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

5.5.4 Classification of the novel *Theileria* spp. genotypes

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

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

5.6 Conclusion

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

5.7 References

AKTAS, M., BENDELE, K.G., ALTAY, K., DUMANLI, N., TSUJI M., HOLMAN, P.J., 2007. Sequence polymorphism in the ribosomal DNA internal transcribed spacers differs among *Theileria* species. Veterinary Parasitology 147, 221-230.

ALLSOPP, M.T., CAVALIER-SMITH, T., DE WAAL D.T., ALLSOPP, B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108, 147-152.

ALTAY, K., AYDIN, M.F., DUMANLI, N., AKTAS, M., 2008. Molecular detection of *Theileria* and *Babesia* infections in cattle. Veterinary Parasitology 158, 295-301.

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W., LIPMAN, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

BAI, Q., LIU, G., HEN, G., 1995. An unidentified *Theileria* species infective for cattle discovered in China. Chinese Journal of Veterinary Science 1995. 15, 15-21.

BAI, Q., LIU, G.Y., YIN, H., ZHAO, Q.Z., LIU, D.K., REN, J.X., LI, X., 2002a. *Theileria sinensis* sp. nov.: study on classical taxonomy. Acta Animal Husbandry and Veterinary Science 33, 73–77.

BAI, Q., G,Y., YIN, H., ZHAO, Q.Z., LIU, D.K., REN, J.X., LI, X., 2002b. *Theileria sinensis* nov: study on molecular taxonomy. Acta Animal Husbandry and Veterinary Science 33, 185–190.

BENDELE, K.G., 2005. Molecular characterization of *Theileria* spp.using ribosomal RNA. MSc. Thesis: Texas A & M University, USA.

BONFIELD, J.K., SMITH, K.F., STADEN, R., 1995. A new DNA sequence assembly program. Nucleic Acids Research 23, 4992-4999.

BOSMAN, A.M., OOSTHUIZEN, M.C., PEIRCE, M.A., VENTER, E.H., PENZHORN, B.L., 2010. *Babesia lengau* sp. nov., a novel *Babesia* species in cheetah (*Acinonyx jubatus*, Schreber, 1775) populations in South Africa. Journal of Clinical Microbiology 48, 2703-2708.

BRIGIDO, C., PEREIRA DA FONSECA, I., PARREIRA, R., FAZENDEIRO, I., DO ROSARIO, V.E., CENTENO-LIMA, S., 2004. Molecular and phylogenetic characterization of *Theileria* spp. parasites in autochthonous bovines (Mirandesa breed) in Portugal. Veterinary Parasitology 123, 17-23.

CHAE, J., LEE, J., KWON, O., HOLMAN, P.J., WAGHELA, S.D., WAGNER, G.G., 1998. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene variable (V4) region among and within geographic isolates of *Theileria* from cattle, elk and white-tailed deer. Veterinary Parasitology 75, 41-52.

CHAE, J.S., ALLSOPP, B.A., WAGHELA, S.D., PARK, J.H., KAKUDA, T., SUGIMOTO, C., ALLSOPP, M.T., WAGNER, G.G., HOLMAN, P.J., 1999a. A study of the systematics of *Theileria* spp. based upon small-subunit ribosomal RNA gene sequences. Parasitology Research 85, 877-883.

CHAE, J., LEVY, M., HUNT, J.,JR, SCHLATER, J., SNIDER, G., WAGHELA, S.D., HOLMAN, P.J., WAGNER, G.G., 1999b. *Theileria* sp. Infections associated with bovine fatalities in the United States confirmed by small-subunit rRNA gene analyses of blood and tick samples. Journal of Clinical Microbiology 37, 3037-3040.

CHAE, J.S., WAGHELA, S.D., CRAIG, T.M., KOCAN, A.A., WAGNER, G.G., HOLMAN, P.J., 1999c. Two *Theileria cervi* SSU RRNA gene sequence types found in isolates from white-tailed deer and elk in North America. Journal of Wildlife Diseases 35, 458-465.

CHAISI, M.E., SIBEKO, K.P., COLLINS, N.E., POTGIETER, F.T., OOSTHUIZEN, M.C., 2011. Identification of *Theileria parva* and *Theileria* sp. (buffalo) 18S rRNA gene sequence variants in the African Buffalo (*Syncerus caffer*) in southern Africa. Veterinary Parasitology 182, 150-162.

CHANSIRI, K., KAWAZU, S.I., KAMIO, T., TERADA, Y., FUJISAKI, K., PHILIPPE, H., SARATAPHAN. N., 1999. Molecular phylogenetic studies on *Theileria* parasites based on small subunit ribosomal RNA gene sequences. Veterinary Parasitology 83, 99-105.

COLLINS, N.E., ALLSOPP, B.A., 1999. *Theileria parva* ribosomal internal transcribed spacer sequences exhibit extensive polymorphism and mosaic evolution: application to the characterization of parasites from cattle and buffalo. Parasitology 118, 541-551.

COSSIO-BAYUGAR, R., PILLARS, R., SCHLATER, J., HOLMAN, P.J., 2002. *Theileria buffeli* infection of a Michigan cow confirmed by small subunit ribosomal RNA gene analysis. Veterinary Parasitology 105, 105-110.

CRIADO, A., MARINEZ, J., BULING, A., BARBA, J.C., MERINO, S., JEFFERIES, R., IRWIN, P.J., 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome b genes. Veterinary Parasitology 142, 238-247.

DE ROJAS, M., UBEDA, J.M., CUTILLAS, C., MORA, M.D., ARIZA, C., GUEVARA, D., 2007. Utility of ITS1-5.8S-ITS2 identification and phylogenetic inference within the *Rhinonyssus coniventries* species complex (Acari: Rhinonyssidae). Parasitology Research 100, 1041-1046.

FUJISAKI, K., KAWAZU, S., KAMIO, T., 1994. The taxonomy of the bovine *Theileria* spp. Parasitology Today 10, 31-33.

FUUJISAKI, K., 1992. A review of the taxonomy of *Theileria sergenti/buffeli/orientalis* group parasites in cattle. Journal of Protozoology Research 2, 87-96.

GIMENEZ, C., CASADO, N., CRIADO-FORNELIO, A., ALVAREZ DE MIGUEL, F., DOMINGUEZ-PENAFIEL, G., 2009. A molecular survey of Piroplasmida and *Hepatozoon* isolated from domestic and wild animals in Burgos (northern Spain). Veterinary Parasitology 162, 147-150.

GUBBELS, J.M., VOS, A.P., WEIDE, M., VISERAS, J., SCHOULS, L.M., VRIES, E., JONGEJAN F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

GUBBELS, M.J., HONG, Y., WEIDE, M., QI, B., NIJMAN, I.J., GUANGYUAN, L., JONGEJAN F., 2000. Molecular characterisation of the *Theileria buffeli/orientalis* group. International Journal for Parasitology 30, 943-952.

GUBBELS M.J., YIN H., BAI Q., LIU G., NIJMAN I.J., JONGEJAN F., 2002. The phylogenetic position of the *Theileria buffeli* group in relation to other *Theileria* species. Parasitology Research 88, S28-32.

HALL, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series. 41, 95-98.

HILLIS, D.M., DIXON, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Quarterly Review of Biology 66, 411-453.

HILPERTSHAUSER, H., DEPLAZES, P., MELI, M.L., HOFMANN-LEHMANN, R., LUTZ, H., MATHIS, A., 2007. Genotyping of *Babesia bigemina* from cattle from a non-endemic area (Switzerland). Veterinary Parasitology 145, 59-64.

HOLMAN, P.J., BENDELE, K.G., SCHOELKOPF, L., JONES-WITTHUHN, J., JONES, S.O., 2003. Ribosomal RNA analysis of *Babesia odocoilei* isolates from farmed reindeer (*Rangifer tarandus tarandus*) and elk (*Cervus elaphus canadensis*) in Winsconsin. Parasitology Research 91, 378-383.

HORAK, I.G., POTGIETER, F.T., WALKER, F.T., JANE, B., DE VOS, V., BOOMKER, J., 1983. The ixodid tick burdens of various large ruminant species in South African nature reserves. The Onderstepoort Journal of Veterinary Research 50, 221-228.

HORAK, I.G., BOOMKER, J., SPICKETT, A.M., DE VOS, V., 1992. Parasites of Domestic and Wild Animals In South Africa. XXX. Ectoparasites of Kudus in the Eastern Transvaal Lowveld and the Eastern Cape Province. Onderstepoort Journal of Veterinary Research 59, 259-273.

HORAK, I.G., BOOMKER, J., FLAMAND, J.R.B., 1995. Parasites of domestic and wild animals in South Africa XXXIV. Arthropod parasites of nyalas in north-eastern KwaZulu-Natal. Onderstepoort Journal of Veterinary Research 62, 171-179.

KAMAU, J., SALIM, B., YOKOYAMA, N., KINYANJUI, P., CHIHIROSUGIMOTO, C., 2011. Rapid discrimination and quantification of *Theileria orientalis* types using ribosomal DNA internal transcribed spacers. Infection, Genetics and Evolution 11, 407-414.

KATOH, K., KUMA, K., TOH, H., MIYATA, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33, 511-518.

KAWAZU, S., KAMIO, T., KAKUDA, Y.T., SUGIMOTO, C., FUJISAKI, K., 1999. Phylogenetic relationships of the benign *Theileria* species in cattla and Asian buffalo based on the major piroplasm surface protein (p33/34) gene sequences. International Journal for Parasitology 29, 33-50.

LEW, A.E., ANDERSON, G.R., MINCHIN, C.M., JESTON, P.J., JORGENSEN, W.K., 2003. Inter- and intra-strain variation and PCR detection of the internal transcribed spacer 1 (ITS-1) sequences of Australian isolates of *Eimeria* species from chickens. Veterinary Parasitology 112, 33-50.

LIU, Q., ZHOU, Y.Q., HE, G.S., OOSTHUIZEN, M.C., ZHOU, D.N., ZHAO, J.L., 2010a. Molecular phylogenetic studies on *Theileria* spp. isolates (China) based on small subunit ribosomal RNA gene sequences. Tropical Animal Health and Production 42, 109-114.

LIU, A., GUAN, G., LIU, Z., LIU, J., LEBLANC, N., LI, Y., GAO J., MA, M., NIU, Q., REN, Q., BAI, Q., YIN, H., LUO, J., 2010b. Detecting and differentiating *Theileria sergenti* and *Theileria sinensis* in cattle and yaks by PCR based on major piroplasm surface protein (MPSP). Experimental Parasitology 126, 476-481.

MASHISHI, M.S.K., (Ed.) 2002. Diseases shared by wildlife and livestock. Department of Agriculture, South Africa, 3pp.

M'GHIRBI, Y., HURTADO, A., BARANDIKA, J.F., KHLIF, K., KETATA, Z., BOUATTOUR, A., 2008. A molecular survey of *Theileria* and *Babesia* parasites in cattle, with a note on the distribution of ticks in Tunisia. Parasitology Research 103, 435-442.

MORZARIA, S.P., YOUNG, A.S., KIMBER, C.D., 1977. The serological relationship of a British *Theileria* with other *Theileria* species using the indirect fluorescent antibody test. Research in Veterinary Science 22, 330-333.

NIU, Q., LUO, J., GUAN, G., LIU, Z., MA, M., LIU, A., GAO, J., REN, Q., LI, Y., QIU, J., YIN, H., 2009. Differentiation of two ovine *Babesia* based on the ribosomal DNA internal transcribed spacer (ITS) sequences. Experimental Parasitology 121, 64-68.

RONQUIST, F., HUELSENBECK, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572-1574.

SAITO-ITO, A., TAKADA, N., ISHIGURO, F., FUJITA, H., YANO, Y., MA, X., CHEN, E., 2008. Detection of Kobe-type *Babesia microti* associated with Japanese human babesiosis in field rodents in central Taiwan and southeastern mainland China. Parasitology 135, 691.

SAITOU, N., NEI, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406-425.

SARATAPHAN, N., KAKUDA, T., CHANSIRI, K., ONUMA, M. 2003. Survey of benign *Theileria* parasites of cattle and buffaloes in Thailand using allele-specific polymerae chain reaction of Major Piroplasm Surface Protein Gene. Journal of Veterinary Medical Science 65, 133-135.

STADEN, R., BEAL, K.F., BONFIELD, J.K., 2000. The Staden package, 1998. Methods in Molecular Biology 132, 115-130.

STEWARD, N.P., UILENBERG, G., DE VOS. A.J., 1996. Review of Australian species of *Theileria*, with special reference to *Theileria buffeli* of cattle. Tropical Animal Health and Production 28, 81-90.

STOCKHAM, S.L., KJEMTRUP, A.M., CONRAD, SCHMIDT, D.A., SCOTT, M.A., ROBINSON, T.W., TYLER, J.W., JOHNSON, G.C., CARSON, C.A., CUDDIHEE, P., 2000. Theileriosis in a Missouri beef herd caused by *Theileria buffeli*: case report, herd investigation, ultrastructure, phylogenetic analysis, and experimental transmission. Veterinary Pathology 37, 11-21.

SUN, C., LIU, Z., GAO, J., GUAN, G., MA, M., LUO, J., YIN, H., 2008. Investigations into the natural infection rate of *Haemaphysalis qunghaiensis* with Piroplasma using neste PCR. Experimental and Applied Acarology 4, 107-114.

SWOFFORD, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

TAMURA, K., DUDLEY, J., NEI, M., KUMAR, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24, 1596-1599.

THOMPSON, J.R., MARCELINO, L.A., POLZ, M.F., 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Research 30, 2083-2088.

UILENBERG, G., PERIE, N.M., SPANJER, A.A., FRANSSEN F.F., 1985. *Theileria orientalis*, a cosmopolitan blood parasite of cattle: demonstration of the schizont stage. Research in Veterinary Science38, 352-360.

UILENBERG, G., 1995. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. Veterinary Parasitology 57, 19-41.

UILENBERG, G., 2011. *Theileria sergenti*. Veterinary Parasitology 175, 386.

YIN, H., GUAN, G., MA, M., LUO, J., LU, B., YUAN, G., BAI, Q., LU, C., YUAN, Z., PRESTON, P., 2002. *Haemaphysalis qinghaiensis* ticks transmit at least two different *Theileria* species: one is infective to yaks, one is infective to sheep. Veterinary Parasitology 107, 29-35.

YIN, H., LUO, J., SCHNITTGER, L., LU B., BEYER, D., MA, M., GUAN, G., BAI, Q., LU, C., AHMED, J., 2004. Phylogenetic analysis of *Theileria* species transmitted by *Haemaphysalis qinghaiensis*. Parasitology Research 92, 36-42.

WANG, L.X., HE, L., FANG, R., SONG, Q.Q., TU, P., JENKINS, A., ZHOU, Y.Q., ZHAO, J.L., 2010. Loop-mediated isothermal amplification (LAMP) assay for detection of *Theileria sergenti* infection targeting the p33 gene. Veterinary Parasitology 171, 159-162.

ZAHLER, M., SCHEIN, E., RINDER, R.G., 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of different vector specificity and pathogenicity to dogs. Parasitology Research 84, 544-548.