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

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Abstract

The African buffalo (*Syncerus caffer*) is a natural reservoir host for both pathogenic and non-pathogenic *Theileria* species. These often occur naturally as mixed infections in buffalo. Although the benign and mildly pathogenic forms do not have any significant economic importance, their presence could complicate the interpretation of diagnostic test results aimed at the specific diagnosis of the pathogenic *T. parva* in cattle and buffalo in South Africa. The 18S rRNA gene has been used as the target in a quantitative real-time PCR (qPCR) assay for the detection of *T. parva* infections. However, the extent of sequence variation within this gene in the non-pathogenic *Theileria* spp. of the Africa buffalo is not well known. The aim of this study was, therefore, to characterize the full-length 18S rRNA genes of *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera* and to determine the possible influence of any

sequence variation on the specific detection of *T. parva* using the 18S rRNA qPCR. The reverse line blot (RLB) hybridization assay was used to select samples which either tested positive for several different *Theileria* spp., or which hybridized only with the *Babesia*/*Theileria* genus-specific probe and not with any of the *Babesia* or *Theileria* species-specific probes. The full-length 18S rRNA genes from 14 samples, originating from 13 buffalo and one bovine from different localities in South Africa, were amplified, cloned and the resulting recombinants sequenced. Variations in the 18S rRNA gene sequences were identified in *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera*, with the greatest diversity observed amongst the *T. mutans* variants. This variation possibly explained why the RLB hybridization assay failed to detect *T. mutans* and *T. velifera* in some of the analysed samples.

Key words: African buffalo, *Theileria mutans*, *Theileria velifera*, *Theileria* sp. (strain MSD), reverse line blot hybridization assay, 18S rRNA gene, phylogenetic analysis

1. Introduction

Theileriosis is a widespread disease of wild and domestic ruminants caused by tick-transmitted apicomplexan parasites of the genus *Theileria* (Mehlhorn and Schein, 1984). It is recognized as a major threat to the livestock industry as some members of the genus may cause severe disease and mortality, and others mild or subclinical infections in their respective hosts (Uilenberg, 1999). *T. parva*, which appears to have evolved in the African buffalo (*Syncerus caffer*), is by far the most pathogenic and is of significant economic importance in eastern, central and southern Africa, where it causes East Coast fever (ECF), January disease and Corridor disease in cattle (Uilenberg et al., 1982). *T. parva* is transmitted

by *Rhipicephalus appendiculatus*, *Rhipicephalus zambesiensis* and *Rhipicephalus duttoni* (Lawrence et al., 1994).

In South Africa, Corridor disease occurs when *T. parva* is transmitted from the African buffalo to cattle by tick vectors. It is an acute, usually fatal disease in cattle and is a controlled disease in South Africa. African buffalo are also thought to be the original hosts of other *Theileria* spp., namely, *Theileria mutans*, *Theileria velifera*, *Theileria buffeli,* and *Theileria* sp. (buffalo) (Norval et al., 1992). *T. mutans* (Theiler, 1906) is a parasite of buffalo, it is infective to cattle and has been shown to cause latent infections in sheep (Young et al., 1978). It occurs in parts of sub-Saharan Africa and is transmitted by different species of *Amblyomma* ticks (Uilenberg et al., 1982). Historically, *T. mutans* was implicated in all benign bovine *Theileria* infections worldwide. However, transmission, serology and phylogenetic studies have indicated that this parasite is an African species and is different from benign *Theileria* species isolated from cattle in other parts of the world (Chae et al., 1999). Although generally considered a benign species in buffalo, some strains of *T. mutans* have been associated with disease in cattle (Saidu, 1981). Invasion of the brain capillaries by *T. mutans* can result in a form of benign bovine theileriosis known as turning sickness (Seifert, 1996).

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

A quantitative real-time PCR (qPCR) test that is based on the 18S rRNA gene has been developed (Sibeko et al., 2008) and is currently used, together with other tests, for the detection of *T. parva* in cattle and buffalo in South Africa. Recently, 18S rRNA gene sequence variants of *T. parva* and *Theileria* sp. (buffalo) were identified by Mans et al. (2011). These authors also reported sequence variations in the 18S V4 hypervariable region of *T. buffeli, T. velifera* and *T. mutans* of cattle and buffalo in South Africa. Although these sequence variants do not compromise the specificity of the qPCR test, the specificity could be compromised if there are other as yet unidentified strains of *Theileria* spp. with 18S rRNA sequences similar to *T. parva* in the qPCR probe region. The aim of the current study was to determine the sequence variation in the 18S rRNA of *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera*.

2. Materials and Methods

2.1 Blood samples

Thirteen buffalo blood samples originating from the Kruger National Park (KNP), Hluhluwe-iMfolozi Game Park (HIP), and a private game ranch in the Hoedspruit area (H) in Mpumalanga (Table 1) were analysed. The samples were selected from a previous study (Chaisi et al., 2011) and had either tested positive for several different *Theileria* spp. using the reverse line blot (RLB) hybridization assay or only hybridized with the *Theileria* and/or *Babesia* genus-specific probe and not with any of the *Theileria* and/or *Babesia* species-specific probes (Table 1). Additionally, a bovine (KZN/bov) blood sample from a farm in KwaZulu-Natal, which tested positive for *Theileria* sp. (sable), *T. mutans*, *T. taurotragi* and *T. velifera* using the RLB hybridization assay, was included in the study. Genomic DNA was extracted from the blood samples using the High Pure Template Preparation kit (Roche

Table 1: RLB hybridization assay, cloning and sequencing results of buffalo blood samples selected for 18S rRNA gene sequence analysis. The samples originated from the Kruger National Park (KNP), Hluhluwe-iMfolozi game park (HIP), a private game ranch near Hoedspruit (H), and a farm in the KwaZulu-Natal province (KZN).

Sample	**RLB results (Chaisi et al., 2011)**	**Clone (size in bp)**	**Phylogenetic classification**
KNP/B15	*T. parva*	a (1580)	*Theileria* sp. (strain MSD)-like
KNP/B22	*T. parva*, *Theileria* sp. (buffalo), *T. velifera*, *T. mutans*	a (1579)	*T. mutans*-like 1
KNP/C11	*T. parva*	b (1579)	*T. mutans*-like 3
KNP/C21	*Theileria*/*Babesia* genus-specific probe only	a (1638)	*T. mutans*-like 1
		b (1576)	*T. mutans*-like 1
KNP/G8	*T. parva*, *Theileria* sp. (buffalo), *T. velifera*, *T. mutans*	a (1582)	*T. mutans*-like 2
KNP/Q15	*T. parva*, *Theileria* sp. (buffalo)	a (1590)	*T. velifera*-like
		d (1582)	*T. mutans*-like 2
KNP/V8	*T. parva*, *Theileria* sp. (buffalo)	d (1558)	*T. mutans*-like 1
HIP/A4	*T. parva*, *Theileria* sp. (buffalo), *T. buffeli*, *T. velifera*	d (1593)	*T. velifera*
HIP/A21	*Theileria* sp. (buffalo) (faint signal)	e (1579)	*T. mutans*-like 1
HIP/H4	*T. parva*, *T. buffeli*	a (1587)	*T. velifera*-like
HIP/H22	*T. parva*, *T. buffeli*	b (1579)	*T. mutans*-like 3
		d (1593)	*T. velifera*
H/241	*Theileria* sp. (buffalo), *T. velifera*	b (1582)	*T. mutans*-like 2
KZN/bov	*Theileria* sp. (sable), *T. mutans*, *T. taurotragi*, *T. velifera*	d (1588)	*T. velifera*

Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. DNA was eluted in 100 µl elution buffer and stored at -20°C.

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

A fragment of approximately 1700 bp of the 18S rRNA gene was amplified using the forward primer Nbab-1F and reverse primer TB 18S-Rev, specific for *Theileria* and *Babesia* spp. (Oosthuizen et al., 2008). The PCR conditions were as previously reported (Chaisi et al., 2011). Purified amplicons were ligated into the pGEM-T Easy Vector and transformed into JM109 High Efficiency cells (Promega, Madison, WI). At least five white colonies per sample were selected and screened by colony PCR using primers RLB-F2 and RLB-R2 (Nijhof et al., 2003). The colony PCR mixture and cycling conditions were as previously described for the RLB hybridization assay (Chaisi et al., 2011), except that colony DNA was used as template. Recombinant plasmid DNA was extracted from overnight bacterial cultures and the full-length 18S rRNA gene of selected clones was sequenced using vector primers (SP6 and T7) (Chaisi et al., 2011) and internal primers (RLB-F2, RLB-R2, Nbab_1F, 18SRev-TB, BT18S_2F, BT18S_3F, BT18S_4F, BT18S_4R) (Oosthuizen et al., 2008). Purified products were analyzed on an ABI3100 genetic analyzer at the ARC-OVI (South Africa) sequencing facility.

2.3 Sequence and phylogenetic analyses

The sequences were assembled and edited using the GAP4 program of the Staden package (version 1.6.0 for Windows) (Staden et al., 2000). Full-length 18S rRNA gene consensus sequences were obtained from 16 clones and were submitted to GenBank with accession numbers FJ213585, FJ213586 and JN572692 – JN572705. A search for homologous sequences was performed using BLASTn (www.ncbi.nlm.gov/BLAST/). Novel sequences were aligned with published sequences from GenBank using the MAFFT (version 5) multiple sequence alignment programme employing the FFT-NS-i algorithm (Katoh et al., 2005). The alignment was manually edited using BioEdit (version 7) (Hall, 1999). The genetic distances

between the sequences were estimated by determining the number of base differences between sequences using MEGA4 (Tamura et al., 2007).

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

3. Results

3.1 18S rRNA gene sequence analysis

Sixteen 18S rRNA gene sequences were obtained from the thirteen buffalo samples and the bovine sample selected for the current study. The BLASTn sequence homology search indicated that 11 of these sequences were closely similar to the 18S rRNA sequences of *T. mutans* Intona (AF078815) *Theileria* sp. (strain MSD) (AF078815) and *Theileria uilenbergi* (AY262121), and five sequences were identical or closely similar to the 18S rRNA of *T. velifera* (AF0978993) (Table 1).

Comparisons of the novel sequences to *T. mutans* and *Theileria* sp. (strain MSD) over a region of 1562 bp indicated that the *Theileria* sp. (strain MSD)-like sequence (KNP/B15/a) differed from those of *Theileria* sp. (strain MSD) and *T. mutans* by 7 and 17 nucleotides, respectively. The ten *T. mutans*-like sequences differed from the *T. mutans* and *Theileria* sp.

(strain MSD) sequences by 18-23 and 25-30 base positions, respectively. There were up to 29 nucleotide differences within the *T. mutans*-like sequences. Micro-heterogeniety of 1–7 bp mismatches was observed between the *T. velifera* sequences.

3.2 Phylogenetic analysis

Neighbor-joining, maximum likelihood, maximum parsimony and Bayesian phylogenetic analyses were used to reveal the relationships between the *T. mutans*, *Theileria* sp. (strain MSD) and *T. velifera* 18S rRNA gene variants and related *Theileria* and *Babesia* species. There were no differences in the groupings of the *T. mutans* variants but the branching of the variants differed in the different trees. A representative tree generated by maximum parsimony is shown in Figure 1. Sequence KNP/B15/a grouped closely with *Theileria* sp. (strain MSD). Although the ten *T. mutans*-like sequences grouped with *T. mutans* (Intona) and *Theileria* sp. (strain MSD), they formed three distinct clades, designated *T. mutans*-like 1, 2 and 3 (Figure 1). These groupings were also evident in the sequence alignment of the V4 hypervariable region of the 18S rRNA gene (Figure 2).

In comparison to the *T. mutans* group, there seems to be less sequence variation in the 18S rRNA gene sequences within *T. velifera*, although this may be merely due to the smaller number of sequences analysed. The *T. velifera* sequences formed two clades. Clade 1 consisted of sequences originating from parasites from both bovine (*T. velifera* AF097993, KZN/bov/d) and buffalo (HIP/H22/d, HIP/A4/d), while clade 2 consisted of only buffalo-derived (HIP/H4/a and KNP/Q15/a) sequences (Figure 1). The two nucleotide differences in the RLB probe area between the *T. velifera* and *T. velifera*-like sequence possibly prevented annealing of the RLB probe to the PCR amplicon, resulting in failure of the RLB assay to identify *T. velifera* in the original samples. This grouping was further confirmed by the

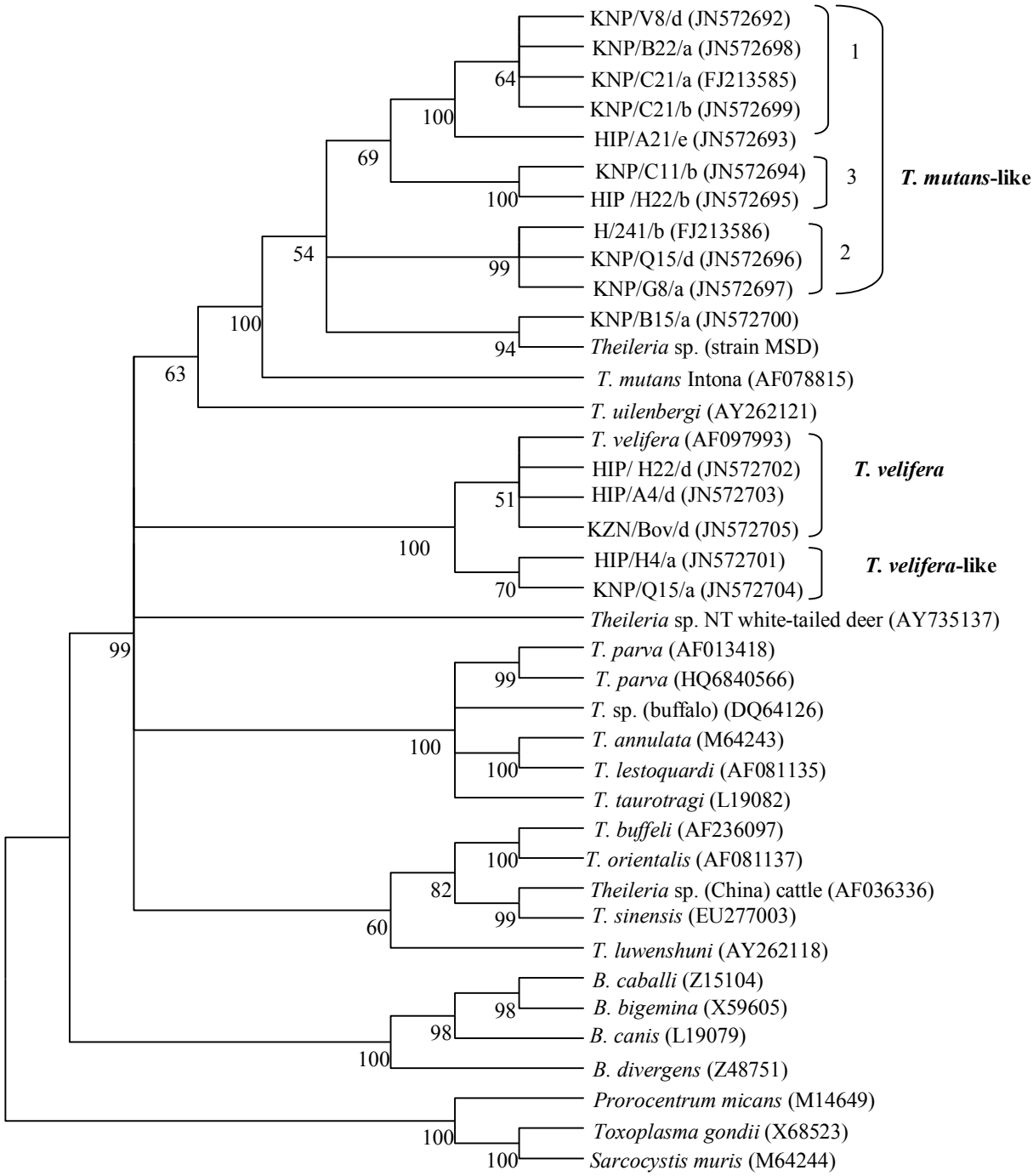


Figure 1: : Phylogenetic tree showing the relationship between the *T. mutans*, *Theileria* (strain MSD) and *T. velifera* 18S rRNA sequence variants identified in this study with other *Theileria* and *Babesia* species as indicated by maximum parsimony analysis. Bootstrap values indicate the degree of support for each cluster. The tree was rooted using the 18S rRNA gene sequences of *Prorocentrum micans*, *Sarcocystis muris* and *Toxoplasma gondii*.

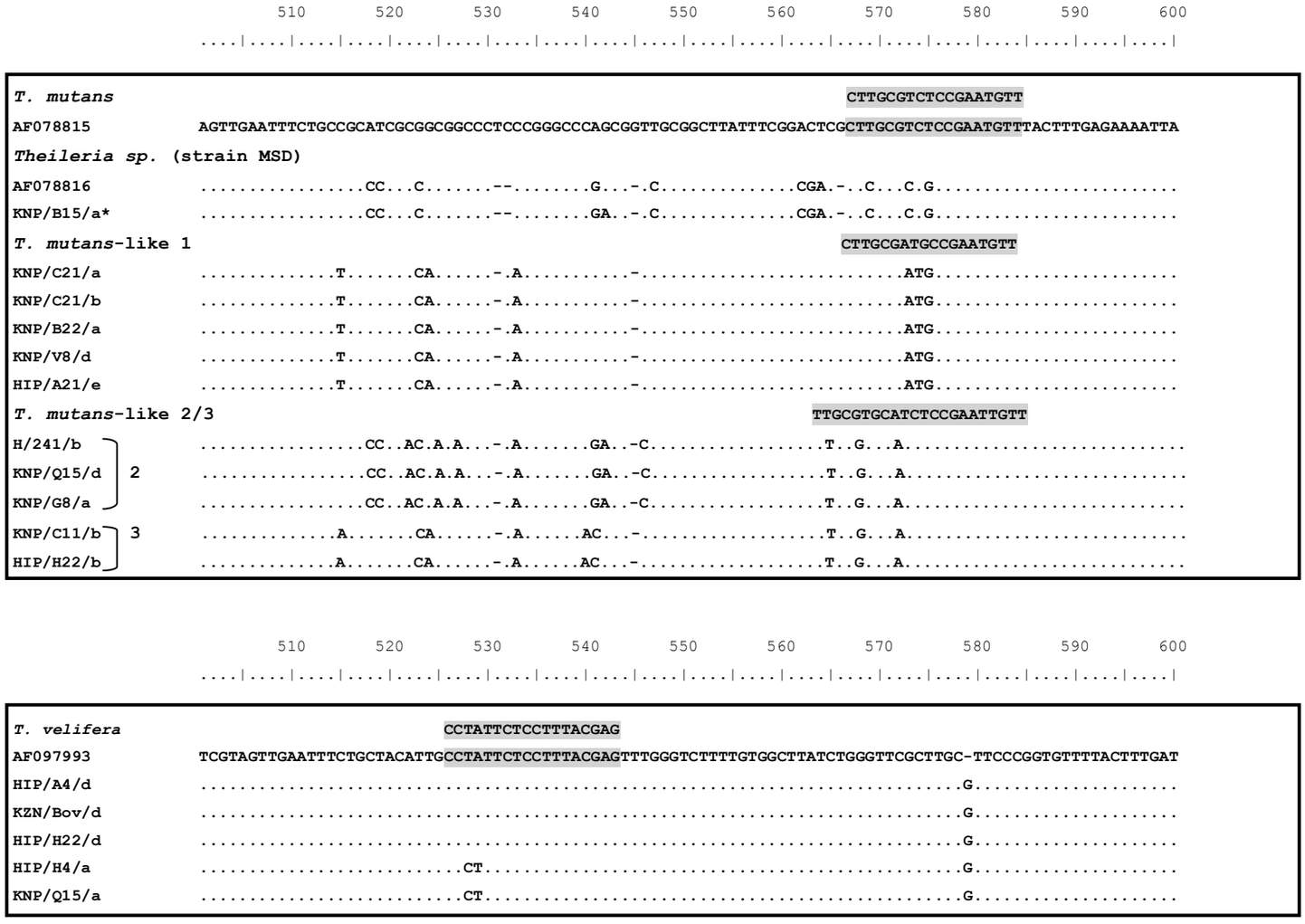
sequence alignment; in the RLB probe region, clade 1 sequences were identical to the *T. velifera* RLB probe sequence while clade 2 sequences differed from the *T. velifera* probe sequence at 2 nucleotide positions (Figure 2).

4. Discussion

Extensive heterogeneity in the 18S rRNA gene of *T. mutans* was observed in the South African buffalo population. The three *T. mutans*-like variants seem to have a wide distribution in southern Africa as they have been identified not only from buffalo populations in the Limpopo, Mpumalanga and KwaZulu-Natal provinces (this study), but also in the Free State province and Zimbabwe (Mans et al., 2011).

Although no attempts have been made to clarify the identity of *Theileria* sp. (strain MSD) after its first description by Chae et al. (1999), the identification of similar sequences in buffalo and cattle indicates that this genotype is circulating in some buffalo and cattle populations in southern Africa. The availability of a specific RLB probe in future studies would enable the detection of this genotype in cattle and buffalo.

The presence of the *Theileria* and *Babesia* genus specific probe in the RLB assay allowed for the identification of *T. mutans*-like sequences in sample KNP/C21. However, novel *T. mutans*-like and *T. velifera*-like sequences were also identified from clones of samples which tested negative for these species by RLB hybridization assay, but were positive for other *Theileria* spp. This illustrates that new species or variants of species cannot be detected by the RLB assay when dealing with mixed infections containing both known species and novel variants. The RLB probes currently used to detect *T. mutans* and *T. velifera* do not detect all



The numbers correspond to the actual position of the nucleotides within the 18S rRNA gene

* Denotes the geographical area of the sample/sample number/clone
KNP – Kruger National Park, HIP – Hluhluwe-iMfolozi Game Park, H – Private Game Farm in the Hoedspruit area,
KZN – KwaZulu-Natal

Figure 2: Nucleotide alignment of a 100 bp region of the V4 hypervariable region of published 18S rRNA gene sequences of *T. mutans* and *T. velifera*, as well as their variants identified in this study. The sequences of the RLB probes are highlighted.

of the sequence variants that we have identified. New RLB probes should therefore be designed for use in future RLB experiments to detect all of the sequence variants identified.

The 18S rRNA gene sequence of *T. parva* under the qPCR primer and probes differs from the sequences of the novel *T. mutans* and *T. velifera* 18S variants identified in this study, suggesting that these parasites would not be detected by the *T. parva*-specific qPCR assay. Samples containing novel *T. mutans* and *T. velifera* variants were tested using the *T. parva*-specific qPCR as part of a previous study (Chaisi et al., 2011) and none of these were positive for *T. parva*. They would therefore not interfere with the diagnosis of *T. parva* infections in cattle and buffalo in South Africa.

Our study supports the recommendation that a thorough survey to determine heterogeneity in the target sequence should always be carried out prior to the development of molecular-based diagnostic tests. Although there is extensive variation within the 18S rRNA gene of the *T. mutans* group, the novel *T. mutans* and *T. velifera* 18S variants identified in this study do not compromise the specificity of the *T. parva*-specific qPCR assay used for diagnosis of *T. parva* in South Africa.

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