# The African buffalo parasite *Theileria.* sp. (buffalo) can infect and immortalize cattle leukocytes and encodes divergent orthologues of *Theileria parva* antigen genes

R.P. Bishop [a, *, 1], J.D. Hemmink [b, 1, 2], W.I. Morrison [b], W. Weir [d], P.G. Toye [a], T. Sitt [a], P.R. Spooner [a], A.J. Musoke [a], R.A. Skilton [a], D.O. Odongo [a, c]

[a] *International Livestock Research Institute (ILRI), PO Box 30709, Nairobi, 00100, Kenya*
[b] *The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG Scotland, UK*
[c] *School of Biological Sciences, The University of Nairobi, PO Box 30197, Nairobi, 00100, Kenya*
[d] *College of Medical Veterinary and Life Sciences, University of Glasgow Glasgow, G61 1QH, UK*

## ABSTRACT

African Cape buffalo (*Syncerus caffer*) is the wildlife reservoir of multiple species within the apicomplexan protozoan genus *Theileria*, including *Theileria parva* which causes East coast fever in cattle. A parasite, which has not yet been formally named, known as *Theileria* sp. (buffalo) has been recognized as a potentially distinct species based on rDNA sequence, since 1993. We demonstrate using reverse line blot (RLB) and sequencing of 18S rDNA genes, that in an area where buffalo and cattle co-graze and there is a heavy tick challenge, *T.* sp. (buffalo) can frequently be isolated in culture from cattle leukocytes. We also show that *T.* sp. (buffalo), which is genetically very closely related to *T. parva*, according to 18s rDNA sequence, has a conserved orthologue of the polymorphic immunodominant molecule (PIM) that forms the basis of the diagnostic ELISA used for *T. parva* serological detection. Closely related orthologues of several CD8 T cell target antigen genes are also shared with *T. parva*. By contrast, orthologues of the *T. parva* p104 and the p67 sporozoite surface antigens could not be amplified by PCR from *T.* sp. (buffalo), using conserved primers designed from the corresponding *T. parva* sequences. Collectively the data re-emphasise doubts regarding the value of rDNA sequence data alone for defining apicomplexan species in the absence of additional data. 'Deep 454 pyrosequencing' of DNA from two *Theileria* sporozoite stabilates prepared from *Rhipicephalus appendiculatus* ticks fed on buffalo failed to detect *T.* sp. (buffalo). This strongly suggests that *R. appendiculatus* may not be a vector for *T.* sp. (buffalo*)*. Collectively, the data provides further evidence that *T.* sp. (buffalo). is a distinct species from *T. parva*.

## 1. Introduction

In certain regions of East Africa, such as those bordering the Maasai Mara game reserve, the Laikipia district in central Kenya and Lake Mburo National park in Uganda, cattle graze in areas that are also frequented by African Cape buffalo (*Syncerus caffer*). African buffalo are hosts of at least five species of *Theileria* (Allsopp et al., 1999; Norval et al., 1992; Bishop et al., 2004; Oura et al., 2011a). Some species can be transmitted to cattle by ixodid ticks in the genus *Rhipicephalus,* including the economically important pathogen *Theileria parva*, which is mainly transmitted by *Rhipicephalus appendiculatus*, whereas other *Theileria* species are transmitted by ticks of the genus *Amblyomma*. The heterogeneity among buffalo-derived *Theileria* parasites that infect leukocytes was first identified in a study using a panel of monoclonal antibodies to type isolates from naturally infected buffalo in Kenya (Conrad et al., 1987). This revealed two very distinct patterns of anti-schizont monoclonal antibody reactivity using immunofluorescence (IFAT) staining of *Theileria* schizont-infected cell lines. Further studies using multicopy DNA sequences as probes to analyse restriction fragment length polymorphism revealed similar diversity and identified several cloned cell lines from one buffalo (6834) that

gave very weak hybridisation with one of the probes employed (Conrad et al., 1989). A subsequent study revealed that the 18S ribosomal RNA gene sequence of the parasites in these cloned lines was distinct from *T. parva*, although it had the closest similarity to *T. parva* among the six *Theileria* species analyzed (Allsopp et al., 1993). This parasite was provisionally designated *T.* sp. (buffalo) (Allsopp et al., 1993). Subsequent studies have found this *Theileria* genotype to be well conserved and widespread in buffalo populations in both Uganda and South Africa, frequently in co-infections with *T. parva* (Chaisi et al., 2013; Mans et al., 2011a; Mans et al., 2015; Oura et al., 2011a,b; Pienaar et al., 2011b; Pienaar et al. 2014).

Serological detection of *T. parva* infection in cattle is based on an ELISA test that measures antibody responses to a schizont antigen that has been named the polymorphic immunodominant molecule, (PIM) (Toye et al., 1991; Katende et al., 1998). PIM is encoded by a single copy gene with relatively conserved N and C terminal ends and a hyper-polymorphic central domain that is rich in the amino acids glutamine and proline (Toye et al., 1995; Geysen et al., 2004). Most of the available murine anti-schizont monoclonal antibodies have been shown to be specific for this antigen (Toye et al., 1996).

We describe herein the genotype of parasites within *Theileria* schizont-infected leukocyte lines and in blood samples isolated from cattle at a ranch in the Rift Valley Province of Kenya, which is home to a resident herd of approximately 1000 buffalo that co-graze with cattle (Pelle et al., 2011). Analysis of the sequences of the p67 gene sequences from those leukocyte cultures containing *T. parva* (Obara et al., 2015) strongly suggest that they originate from ticks that had fed on buffalo (Nene et al., 1996). Several of the isolates were identified as *T.* sp. (buffalo) or mixed infections of this genotype together with *T. parva*. The genotyping was extended to orthologues of genes encoding antigens that are the target of antibody and cellular immune responses during *T. parva* infections in cattle. We further describe an analysis of two buffalo derived *Theileria* stabilates using high-throughput sequencing and discuss implications of the data with respect to the status of *Theileria* sp. (buffalo) as a species that is distinct from *T. parva*.

## 2. Materials and methods

### 2.1. Animals

Samples from a field study conducted at Marula farm near Naivasha in the Rift Valley province of central Kenya were used in the experiments. The study used *Bos taurus* (Friesian) cattle aged 6−8 months purchased from Marula farm where strict acaricidal control of ticks was practised. All cattle were initially shown to be free of antibodies to theilerial antigens using an indirect ELISA. Three groups of cattle were immunised against *T. parva* by the infection and treatment method (Radley et al., 1975) with the following stabilates: (1) 30 animals with T. *parva* Marikebuni stabilate 3014 (Morzaria et al., 1995); (2) 29 animals with *T. parva* Marikebuni stabilate 316 (Payne, 1999); (3) 27 animals with *T. parva* composite trivalent Muguga cocktail stabilate FAO 1 (Morzaria et al., 1999), containing the Muguga, Kiambu 5 and Serengeti stocks. An additional group of 27 animals − representing the non-immunised controls − were also included. Six weeks after immunisation, a total of 113 cattle, comprising 86 immunised and 27 control animals, were exposed to tick challenge in areas co-grazed by buffalo, other species of wild ruminants and cattle. Cattle were exposed during the day and returned to an enclosure at night. All cattle were monitored for temperature daily from day 17 after exposure. Any cattle showing enlarged lymph glands or with a fever of 39.5 °C or above were sampled by taking smears of aspirates from the affected lymph node. If schizonts were detected, then a contralateral lymph node was also sampled for schizont detection and blood smears were taken for detection of piroplasms. Lymph node aspirates were also collected in culture medium containing anticoagulant for establishment of cell cultures (see below). Blood samples for serology were taken weekly from the first day of immunisation and this was continued during the exposure period. These sera were screened for antibodies to *T. parva* using the indirect antibody detection ELISA based on the PIM antigen (Katende et al., 1998). All experimental procedures involving cattle were approved by the ILRI Institutional Animal Care and Use Committee (IACUC).

### 2.2. Sporozoite stabilates

Genomic DNA was prepared from sporozoite stabilates after removal of excess tick material by centrifugation for 10 min. Genomic DNA was extracted from the supernatant containing sporozoites using the DNeasy® TisKit (Qiagen, Dusseldorf Germany) according to the manufacturer's instructions. The sporozoite stabilates used were stabilate 3081 derived from buffalo 7014 captured in the Laikipia District (Morzaria et al., 1995) and stabilate 4110 from buffalo 7752. These were analysed by 18S rDNA 454 deep sequencing. Two additional DNA samples, one prepared from blood from a buffalo from the Kruger National park (SC03) and one from the Marula bovine cell line N79, both known to contain *T.* sp. (buffalo), were included as positive controls.

### 2.3. Theileria-infected cell lines

Samples for cell culture were transported to the laboratory within 24 h. Mononuclear cells were isolated from lymph node aspirates and/or blood samples by density gradient centrifugation, re-suspended in RPMI growth medium supplemented with 15% foetal calf serum and cultured at 37° C in 5% $CO_2$ in 24-well plates containing adherent cells from a foetal thymic cell line as feeder cells. The resultant established cell lines were cryopreserved using previously reported techniques (Brown, 1987). In some cases cloned parasitized cell lines derived from the cultures were used to increase the resolution of the 454 sequence analysis. . The cloned lines were derived from established parasitized lines originally isolated from cattle and buffalo from different regions of Kenya. They included cell lines from cattle in the Marula farm study (Table 1) and buffalo lines isolated previously from the Maasai Mara and Laikipia district (Table 2). Specifically, there were seven *T. parva* clones from the Marikebuni-cattle-derived stock and one from each of four other cattle stocks, eight clones from two buffalo-associated cattle *T. parva* stocks from Marula, nine *T. parva* cloned lines from four stocks of buffalo origin and nine clones from two *T.* sp. (buffalo) lines. Further details are provided in Table 2. The cell lines were cloned by limiting dilution in 96-well U-bottom plates in a mixture of 50% filtered supernatant from established buffalo-derived *T. parva* cell lines and 50% cell culture medium (RPMI 1640 medium enriched with glutamine, 2-mercaptoethanol, and 12.5% foetal calf serum) and $2 \times 10^5$ cells/per well γ-irradiated bovine PBMC as filler cells.

### 2.4. T. parva schizont characterisation with monoclonal antibodies

A panel of monoclonal antibodies was used to characterise theilerial isolates in an immunofluorescence test (IFAT) as previously described (Minami et al., 1983: Conrad et al., 1987).

### 2.5. Processing of DNA from blood samples and infected cell lines

Blood samples were processed as described by d'Oliveira et al.

**Table 1**
Theileria-infected cell lines from Marula farm and their reactivity with anti-PIM MAbs raised against T. parva Monoclonal Antibody number.

| Cell line | 1 | 2 | 3 | 4 | 7 | 10 | 12 | 15 | 20 | 21 | 22 | 23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N6 | + | − | − | + | + (<1%) | + | + | − | + | + | + | − |
| N13 | + | − | − | + | + | + | + | + (1%) | + | + | + (10%) | + (<1%) |
| N18 | + (<1%) | − | − | + (<5%) | + (<5%) | + | + | + (3%) | + (5%) | + | + | − |
| N20 | + | − | − | + | + | + | + | + | + | + | + | − |
| N33 | + | − | − | + | + | + | + | + (2%) | + | + | + | − |
| N36 | + (<1%) | − | − | + | + (20%) | + | + | + (10%) | + | + | + | − |
| N38 | + | − | − | + | + | + | + | + | + | + | + | − |
| N43 | + | − | − | + | + | + | + | + (2%) | + | + | + | − |
| N50 | + | − | − | + | + | + | + | + (5%) | + | + (40%) | + | − |
| N55 | + | − | − | + | + (5%) | + | + | + (1%) | + | + | + | − |
| N69 | + | − | − | + | + (10%) | + | + | + (5%) | + | + | + | − |
| N76 | + | − | − | + | + | + | + | + (<1%) | + | + | + | − |
| N77 | + | − | − | + | + | + | + | + (2%) | + | + (30%) | + | − |
| N79 | + | − | − | + | + (30%) | + | + | + (5%) | + | + | + | − |
| N86 | + (<1%) | − | − | + (<1%) | + (<1%) | + | + | + (<1%) | + (<1%) | + | + | − |
| N88 | + | − | − | + | + | + | + | + (1%) | + | + (10%) | + | − |
| N99 | + | − | − | + | + | + | + | + (2%) | + | + | + | − |
| N100 | + | − | − | + | + | + | + | + (4%) | + | + | + | − |
| N102 | + (<1%) | − | − | + (1%) | + (3%) | + | + | + (<1%) | + | + | + | − |
| N103 | + | − | − | + | + | + | + | + (1%) | + | + | + | − |
| N106 | + | − | − | + (70%) | + (70%) | + | + | + (5%) | + | + | + | − |
| N107 | − | − | − | − | − | + | + | + (2%) | + | + | + | − |

**Table 2**
Stocks and clones used for the phylogenetic analysis of Theileria sp (buffalo) and T. parva.

| Name/Animal | Clone | Location |
|---|---|---|
| **Cattle derived T. parva** | | |
| Muguga | Reference genome | Kenya |
| Marikebuni | A3, A7, B12, E43, F44, F53, I8, I38, J17 | Kenya |
| Mariakani | St 3231 clone 2/3 | Kenya |
| Boleni | St3230 clone 1:1 | Zimbabwe |
| Uganda | St 3645clone 1/2 | Uganda |
| **Cattle associated with African buffalo T. parva** | | |
| Marula N33 | Clone 2, 4, 5, 7 | Nakuru, Kenya |
| Marula N43 | Clone 1, 2, 5, 6 | Nakuru, Kenya |
| **Buffalo-derived T .parva** | | |
| Mara 3 | Clone 3 | Maasai Mara, Kenya |
| Mara 30 | Clone 5, 8, 11 | Maasai Mara, Kenya |
| Mara 42 | Clone 2,5 | Maasai Mara, Kenya |
| Buffalo 6998 | Clone 2,4,9 | Kenya |
| **Buffalo-derived T. sp (buffalo)** | | |
| Buffalo 6998 | Clone 3,8,10 | Kenya |
| Buffalo 6834 | Clone 1,5,7,10 | Laikipia, Kenya |

(1995) using 200 ul aliquots of blood. Prior to PCR, the samples were heated for 10 min at 100 °C to inactivate the proteinase K and centrifuged for 2 min. Genomic DNA was prepared from frozen pellets of $10^7$ cultured schizont-infected lymphocytes using the DNeasy® Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions.

### 2.6. Reverse line blot

Two sets of primers were separately used to amplify a 460–520 bp fragment of 18S and 16S SSU rRNA spanning the V4 variable region as originally described by Gubbels et al. (1999), but with slight modifications. All primers were manufactured by MWG (Germany). For the amplification of the 18S rRNA encoding gene of Theileria/Babesia, the forward primer used was (5′-GACA-CAGGGAGGTAGTGACAAG) and the reverse primer was (biotin-5′-CTAAGAATTTCACCTCTGACAGT). The reactions were performed in an automated DNA thermal cycler (MJ research), with an enabled hot lid.

The preparation, hybridization and subsequent removal of hybridised DNA from the membrane were performed as already described by Gubbels et al. (1999) as modified by Oura et al. (2004).

### 2.7. Nested 18s PCR assay

To confirm the data from the RLB, a semi-nested PCR assay was developed to differentiate between T. parva and T. sp (buffalo) using specific reverse primers in the second round (For T. parva (CTTATTTCGGACGGAGTTCG) and for T. sp. (buffalo) (CGCTTATTT-CAGACGGAGTTA). The first round of PCR employed Theileria/Babesia-specific primers (5′ GAGGTAGTGACAAGAAATAACAATA and 5′ CTAAGAATTTCACCTCTGACAGT) (Gubbels et al., 1999; Oura et al., 2004). 1 μl of PCR products was then used for a second round of PCR using the same forward primer and the species specific reverse primers. The presence of T. sp. (buffalo) was confirmed for some of the cell lines by sequencing of PCR products amplified using the species-specific primer, which were cloned using the pGEM-T Easy vector system (Promega Corporation, USA) according to the manufacturer's instructions and purified using the Promega PureYield Plasmid Miniprep System.

### 2.8. PCR amplification and sequencing of parasite antigen genes

**For p104, p67, and PIM loci**: PCR amplification was performed using Taq Expand™ high fidelity PCR reagents (Roche Diagnostics, Mannheim, Germany) in 100 μl reactions volumes containing 1X PCR buffer, 200 mM of each dNTP, 100 ng of each forward and reverse primer, 1.5 mM $MgCl_2$, 0.5 units of Expand High fidelity Taq DNA polymerase and 50 ng of DNA. PCR conditions and primers used in the p104 PCR were as previously described (Skilton et al., 2002). Primers ILO 6464 (5′CGC GGA TCC AAG ATC TTT CCC TTT TTA3′) and ILO 8413 (5′ CGG GGT ACC TTA ACA ACA ATC TTC GTT AAT GCG3′) were used to amplify approximately 1.5 kb of the PIM full length gene. Primers ILO 8222 (5′CGA CAC TGA ACG ATG CAA ATA3′) and ILO 8223 (5′GAG TTA TTG TTA GTG GAC GAT3′) were used to amplify approximately 2.2 kb encompassing the full length p67 gene (Nene et al., 1992, 1996). All PCR reactions were performed in a hot-lid MJ PCR thermo cycler (PTC-100, MJ research, CA). Amplicons were subsequently purified using the QIAquick PCR purification kit (QIAGEN, Germany), according to the manufacturer's instructions, and sequenced on an ABI 377 automated sequencer (Applied Biosystems Inc), using big dye terminator

chemistry. The twelve PIM sequences were submitted to GenBank with accession numbers KT258708; KT258709; KT258710; KT258711; KT258712; KT258713 KT258714; KT258715; KT258716; KT258717; KT258718; KT258719.

**Tp6, Tp7 and Tp8 loci:** PCR primers designed to amplify segments of three genes encoding *T. parva* antigens recognized by CD8 T cells (Tp6, Tp7 and Tp8) were used to analyse the sequences of these genes and the orthologues in *T.* sp. (buffalo). The primers and amplicon sizes were as follows: Tp6 - forward primer 5′ GTCCAA-TAATTTACGATGTGAG), reverse primer 5′CTTGTTTAGCCTCTACAGC − 376 bp amplicon; Tp7 - forward primer 5′TGAAGAAGGA CGACTCGCAC, reverse primer 5′TAAGCATTTCCCACTCACGC − amplicon 364 bp; Tp8 forward primer_5′ATCCACAACCA AGTGCCCAG, reverse primer 5′ACTGCGAAGGAGGTCAATCC − 348 bp amplicon. Each PCR reaction comprised 20 pmol of each primer, 0.5 units BIOTAQ polymerase (Bioline, UK), 2.0 μl 10 x PCR master mix and 50 ng genomic DNA in a total volume of 20 ul. A G-storm thermal cycler (Genetic Research instrumentation, Braintree, UK) with enabled hot lid was used for amplification. For the PCR reactions the annealing temperature was adjusted according to the primer pair used Tp6) −54 °C; Tp7), −58 °C and Tp8) −59 °C. The PCR products were purified for Sanger sequencing using the Promega Wizard Gel and PCR product purification System (Promega Corporation, USA). The Tp antigen sequences were submitted to GenBank and assigned accession numbers: Tp6 *(T. parva)* KT246308-KT246324; Tp6 orthologues *T.* sp. (buffalo) KT246325-KT246331; Tp7 *(T. parva)* KT246332-KT246348; Tp7 orthologues *T.* sp. (buffalo) KT246349-KT246352; Tp8 *(T. parva)* KT246353-KT246369; Tp8 orthologues *T.* sp. (buffalo) KT246370-KT24637. _ 2.9. *Sample preparation for Roche 454 sequencing of 18s rRNA amplicons.*

Primers used previously for amplification of a 375 bp product of the V4 variable region of the 18s rDNA gene (Gubbels et al., 1999; Oura et al., 2004).) were adapted for the preparation of samples for 454 sequencing by the addition of a sequencing adaptor and a multiplex identifier tag (MID) to form the so called fusion primer. The forward primer used was (5′ CGTATCGCCTCCCTCGCGCCATCAG-MID GAGGTAGTGACAAGAAATAACAATA) and the reverse primer was (5′ CTATGCGCCTTGCCAGCCCGCTCAG −MID-CTAAGAATTT CACCTCTGACAGT 3′). A unique combination of Multiplex Identifier (MID) tags was used for the amplification of each of the samples to assign reads to some of the samples. The sequences of the MID's were according the 454 (Roche) Technical Bulletin (TCB013-2009). The raw sequencing data was processed using a bioinformatics pipeline. Briefly, reads for individual PCR reactions were obtained using the sequences of the Multiplex identifiers (MIDs). Sequencing noise was reduced using algorithms available on the MOTHUR platform (Schloss et al., 2009) to maximise detection and rejection of chimeric sequences, three different algorithms were used: Uchime, Perseus, and Chimera slayer (Edgar et al., 2011; Haas et al., 2011; Quince et al., 2011). Only sequences detected at a minimum frequency of 0.5% of total reads were maintained in the final dataset.

## 2.9. Bioinformatics and phylogenetic analysis

Sequence alignments and phylogenetic analysis for the 18srDNA sequences, used the MEGA5 software package (Tamura et al., 2011). Sequences were aligned using the MUSCLE algorithm (Edgar, 2004). Maximum composite likelihood trees were constructed using 1000 bootstrap replicates as implemented in MEGA5; the optimal nucleotide substitution model was identified using data monkey. Some of the trees were rooted using orthologues of the gene of interest identified using BLAST. For the PIM sequences a Maximum Likelihood Tree constructed with RAxML (Stamatakis et al., 2014) using a GTR/G/I model with 100 bootstrap iterations, and visualised

using FigTree. (http://tree.bio.ed.ac.uk/software/figtree/). The tree was arbitrarily rooted between the *T. parva* and *T.* sp. (buffalo) clades.

## 3. Results

### 3.1. 1. Clinical reactions of cattle exposed to tick challenge at Marula farm

Fifty-three of the 113 tick-exposed cattle developed clinical disease and were treated with buparvaquone,: 40 were immunized animals and 13 were controls (Obara et al., 2015). Among the 22 animals from which lymph node isolates were made, all exhibited severe reactions, 14 were treated and either recovered or were euthanized. The other eight died overnight. Fourteen of these isolates were from vaccinated and eight from control animals. Most animals exhibited clinical and parasitological features typical of those induced by buffalo-derived *T. parva* (Norval et al., 1992), with low schizont parasitosis and low or, in some cases, no piroplasm parasitaemia. Piroplasm parasitaemia was detectable in only 26 of the 113 tick-exposed cattle. The clinical reactions of all 113 cattle are summarized in Supplementary Table S1. There were two cattle, N86 and N102, from which schizont-infected cell lines, were isolated that appeared to contain only *T.* sp. *buffalo*. Both were vaccinated with different Marikebuni stabilities, numbers 316 and 3014, respectively.

### 3.2. Parasite isolation and monoclonal antibody (MAb) typing

The results obtained from analyses of the established cell lines with the panel of 12 MAbs using an IFAT test, are presented in Table 1. The profiles were individually variable but the buffalo-derived *T. parva* isolates reacted (although sometimes giving only a % reaction) with all MAbs except number 23 (Table 1). The *T.* sp. (buffalo) parasites reacted strongly with only MAbs 10,12 and 20 as reported previously (Conrad et al., 1987), plus MAb number 21, which was not used in the earlier study. On the basis of observed partial reactions, a third category of isolates gave profiles suggesting a mixture of theilerial types in the cultures. The schizont-infected lymphocyte culture isolates that were pure *T.* sp. (buffalo), were maintained in culture for period of at least six months. Subsequent to this they were cryopreserved for further analysis.

### 3.3. Identification of T. sp. (buffalo) parasites in cattle by reverse line blot and analysis of 18s ribosomal subunit sequences

The 22 schizont-infected lymphocyte isolates were characterised by reverse line blot using PCR primers designed to detect all *Theileria* and *Babesia* species known to occur in cattle in East Africa, coupled with use of species-specific oligonucleotide probes. The data indicated that 17 lines hybridised only with a *T. parva* probe, two hybridised with both *T. parva* and *T.* sp. (buffalo) and three lines hybridised specifically with *T.* sp. (buffalo) (Fig. 1). The three lines that hybridised specifically to *T.* sp. (buffalo) were N107, N86 and N102 (Fig 1, lanes 20, 21 and 22), while N106 and N18 hybridised to oligonucleotides from both species. Three whole blood DNA samples from cattle N106, N69 and N86 were also tested by line blotting and all three hybridised only with the *T.* sp. (buffalo) probe (Fig. 1, lanes 23, 24 and 25).

The representation of *T. parva* and *T.* sp. (buffalo) in parasitized cell lines derived from 18 of these cattle was further investigated using a semi-nested PCR assay for the 18s rRNA subunit, employing primers designed to be specific for *T. parva* or *T.* sp. (buffalo) for the second round of amplification (see materials and methods). The specificity of the assay was confirmed by examining 3 cloned
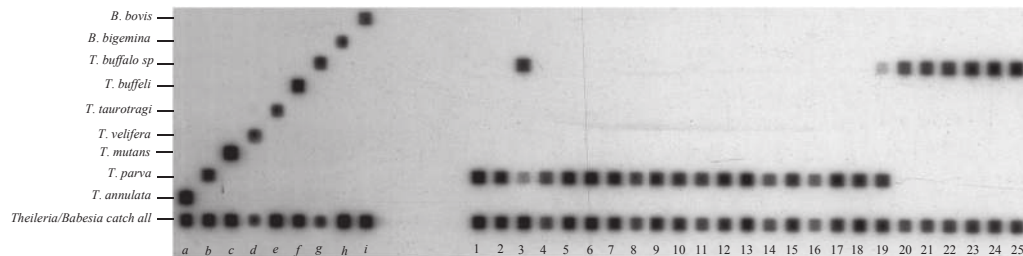
**Fig. 1.** Reverse line blot analysis of schizont cultures containing parasites isolated from Marula farm. The following species-specific oligonucleotide probes were used (a) *T. annulata*, (b) *T. parva*, (c) *T. mutans*, (d) *T. velifera*, (e) *T. taurotragi*, (f) *T. buffeli*, (g) *T. sp.* (buffalo). (h) *B. bigemina*, (i) *B. bovis*. The order of the experimental samples hybridized is DNA from cell culture isolates in lanes 1–22 was lane 1; (1) N6, (2) N13, (3) N18, (4) N20, (5) N33, (6) N36, (7) N38 (8) N43, (9) N50 (10) N55, (11) N69, (12) N76, (13) N77, (14) N79, (15) N88, (16) N99, (17) N100 (18) N103, (19) N106, (20) N107, (21) N86, (22) N102 and DNA extracted from whole cattle blood (23) N106 (24) N69 (25) N86.

*T. parva* infected lines and 3 cloned buffalo lines previously shown to be infected with *T.* sp. (buffalo) (Fig. 2). Fifteen of the 18 lines from cattle at Marula farm generated detectable products for *T. parva*. The 3 *T. parva*-negative lines (N18, N86 and N102) and a further 4 *T. parva* positive lines yielded PCR products with the *T.* sp. (buffalo) primers (N79, N88, N99, N107). Sequencing of the products from two of these samples confirmed that they originated from *T.* sp. (buffalo).

### 3.4. PCR amplification of genes encoding antigens that induce antibody responses in cattle

When DNA isolated from the 22 schizont-infected lymphocyte cell lines was amplified with the p104 primers that have previously been demonstrated to be specific for *T. parva* (Skilton et al., 2002) all except two generated a PCR product of the predicted size (Fig. 3, panel A). Consistent with RLB data, the two lines from which no product was generated were lines N86 and N102 (Fig. 3, panel A lanes 15 and 19), suggesting that these lines contained only *T.* sp. (buffalo). A faint band of the correct size was generated from N107 (Fig. 3, panel A, lane 22), suggesting the presence of a low level of *T. parva* schizont-infected cells that could not be detected by RLB.

When PCR was performed using primers designed to amplify the central variable region and part of the relatively conserved N and C terminal sections of PIM, a PCR product of approximately 1.5 kb was generated from all 22 schizont-infected lines (Fig. 3, panel B). A clearly visible PCR product was generated from lines N86 and N102 (Fig. 3, panel B, lanes 15 and 19), suggesting the presence of a PIM orthologue in *T.* sp. (buffalo).

The twenty two samples were also subjected to PCR with primers that amplified the near full length gene encoding p67 the major sporozoite surface antigen of *T. parva* (Nene et al., 1992, 1996). Nineteen samples produced a detectable PCR product of the predicted size (Fig. 3, panel C). The exceptions were lines N107 (Fig 3, panel c, lane 22) which appeared to contain a very low percentage of *T. parva*-infected cells that were only detectable using p104-PCR and lines N86 and N 102 (Fig 3, panel C, lanes 15 and 19, respectively). The absence of a PCR product from the latter two cell lines suggested that the specific PCR primers employed in this study do not amplify a p67 orthologue in *T.* sp. (buffalo). A similar failure to amplify p104 and p67 from T. sp. (buffalo) using primers designed from the *T. parva* genes encoding these antigens was reported by Pienaar et al. (2011b), using buffalo-derived blood samples from South Africa.

### 3.5. Cloning and sequencing of the PIM gene

. Twelve PIM nucleotide sequences were determined, two from N18 and one from each of ten other cell lines from different infected animals and predicted protein alignments, including three reference sequences were generated (data not shown). Sequences obtained from N86 and N102, in which only *T. sp. buffalo* was detected, exhibited a significant level of amino acid identity with *T. parva* in the relatively conserved N and C termini of the protein, indicating that the PCR product represented the orthologue of PIM. The N-terminal 103 amino acids in *T. parva* Muguga and *T. sp. buffalo* PIM
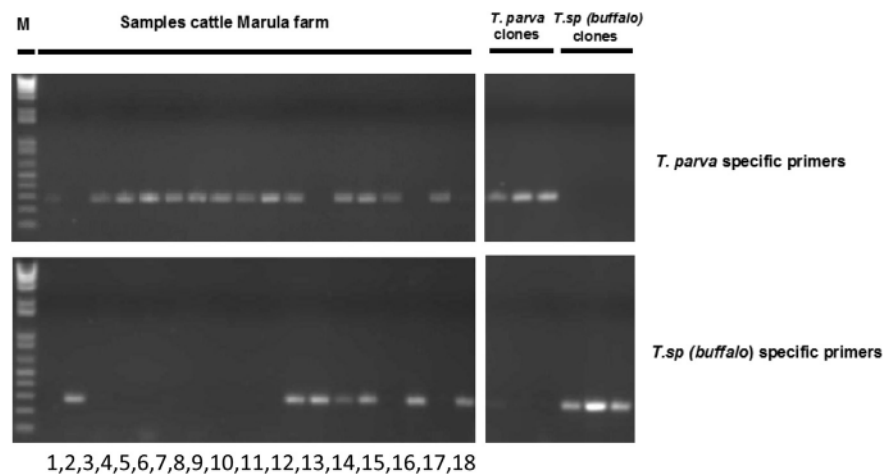


**Fig. 2.** Results of a semi-nested PCR assay used to amplify 18S ribosomal subunit DNA using primers specific for *T. parva* and *T.* sp. (buffalo). Samples are as follows: 1 )N13 2)N18 3) N20 4)N33 5)N36 6) N43 7)N50 8)N55 9) N69 10)N76 11) N79 12) N86 13) N88 14) N99 15)N100 16) N102 17) N103 18)N107 19–21) *T. parva* clones 22–24) *T.* sp. (buffalo) clones (documented in Table 2).
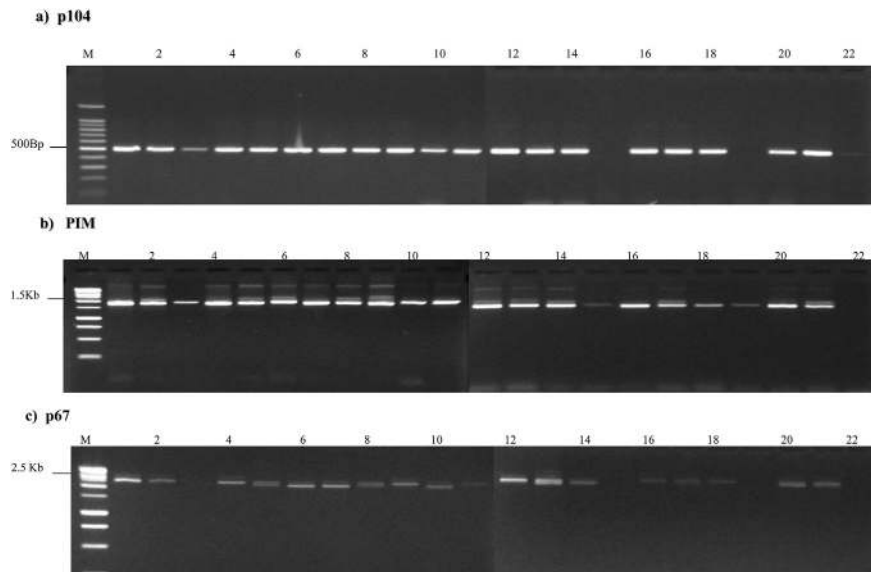
**Fig. 3.** PCR amplification of genes encoding *Theileria parva* antigens from Marula schizont-infected leukocyte cultures. Panel A, p104 primers; Panel B PIM, primers; Panel C p67 primers. The order of the schizont-infected lymphocyte samples is (1) N6; (2). N13; (3). N18; (4). N20; (5). N33; (6). N36; (7). N38; (8). N43; (9). N50; (10). N55; (11). N69; (12). N76; (13). N77, (14). N79; (15). N86, (16). N88; (17). N99; (18). N100; (19). N102; (20). N103; (21). N106; (22). N107.

exhibited 61% identity at the amino acid level, as compared to 94% between the equivalent section of the *T. parva* Muguga and *T. parva* Buffalo 7014 PIM. The *T. sp. buffalo* PIM orthologue sequences from N86 and N102 were identical at the amino acid level. The PIM sequences obtained from two isolates derived from animal N18, designated, (N18 and N18a) were much more closely related to N86 and N102 in the conserved sections of the protein than to the other eight sequences, all of which were derived from different animals, suggesting that they represented a *T. sp.* (buffalo) PIM orthologue fortuitously selected from a parasite population that appeared mixed according to RLB (Fig. 1). However, there were significant differences in the central variable region between the predicted N18 PIM protein as compared to the N86 and N 102 sequences. This suggests that the PIM orthologue of *T. sp. buffalo* can vary between isolates in a similar manner to that of T. *parva* PIM. The level of sequence identity of the other eight PIM gene sequences to three *T. parva* reference PIM sequences (derived from the *T. parva* stocks Muguga, Marikebuni and Kiambu V) indicated that they likely originated from *T. parva*. Phylogenetic analysis of the relatively conserved N-terminal PIM coding sequences using a maximum likelihood algorithm revealed a clear separation of the *T. sp.* (buffalo) and *T. parva* sequences into two distinct clades in a tree, with high bootstrap support (Fig. 4). The C-terminal and concatenated N and C-terminal regions of the molecule generated trees with similar topology (data not shown). The alignment of the full length PIM sequences is available in Supplementary Fig. 1. The PIM sequences have been deposited in GenBank (see materials and methods for accession numbers).

### 3.6. Potential immunological cross reactivity between T. sp. (buffalo) and T. parva in cattle

We have already described in section 3.2 characterisation of the Marula schizont-infected lymphocyte isolates with a panel of anti-schizont MAbs raised against *T. parva* (Table 1). The N86 and N106 *T. sp.* (buffalo) cell lines, in which the PIM orthologous was sequenced (see previous section), stained strongly with MAbs, 10, 12, 21 and 22, as did the *T. parva* isolates from Marula farm. Epitope 10 is located in the relatively conserved N-terminal region of PIM, while

epitope 12 is conformational and recognises non-contiguous residues within both the N-terminal and hyper variable glutamine/proline rich central variable regions. By contrast epitope 21 was mapped to the conserved C-terminal section of the protein, while the location of epitope 22 is unknown (Toye et al., 1996). Epitope 12 has been mapped to peptides 81–87 and 181–192 in the *T. parva* Muguga PIM protein sequence (Toye et al., 1996). Since both epitopes 10 and 12 of PIM are contained within the *T. parva* recombinant sequence (the N-terminus and part of the central variable region) that forms the basis of the indirect ELISA used for serological detection of exposure to *T. parva*, this suggests the potential for cross-reactivity between cattle exposed to *T. parva* and *T. sp.* (buffalo).

### 3.7. Phylogenetic relationship between T. parva and T. sp (buffalo) based on sequences of conserved genes encoding CD8 T cell antigens

In separate studies involving 454 sequencing of genes encoding *T. parva* antigens recognised by CD8 T cells (Graham et al., 2006), we identified 3 genes, Tp6, Tp7 and Tp8, that contained small numbers of synonymous point mutations but were almost completely conserved in their translated amino acid sequence. Amplicons of a segment of the orthologues of each of these 3 genes were generated from cloned buffalo cell lines confirmed as being infected with *T. sp.* (buffalo) and also from cloned cell lines infected with *T. parva*, originating from either cattle or buffalo (Table 2).

The sequences of the Tp6, Tp7 and Tp8 gene segments obtained from *T. parva* clones contained single nucleotide substitutions at 5, 9 and 4 polymorphic residues respectively. No single nucleotide substitutions were detected between *T. sp.* (buffalo) orthologues of Tp6, Tp7 and Tp8 in the limited number of sequences obtained. Comparison of the *T. parva* and *T. sp.* (buffalo) sequences revealed a greater degree of divergence with differences observed at 20, 31 and 14 residues in Tp6, Tp7 and Tp8 respectively. However, most of the nucleotide substitutions did not result in amino acid changes (0, 1 and 4 amino acid changes in the *T. sp.* (buffalo) sequences compared to Tp6, Tp7 and Tp8 respectively). Despite the limited sequence diversity, phylogenetic analysis using a maximum likelihood algorithm demonstrated clear clustering of *T. sp.* (buffalo)
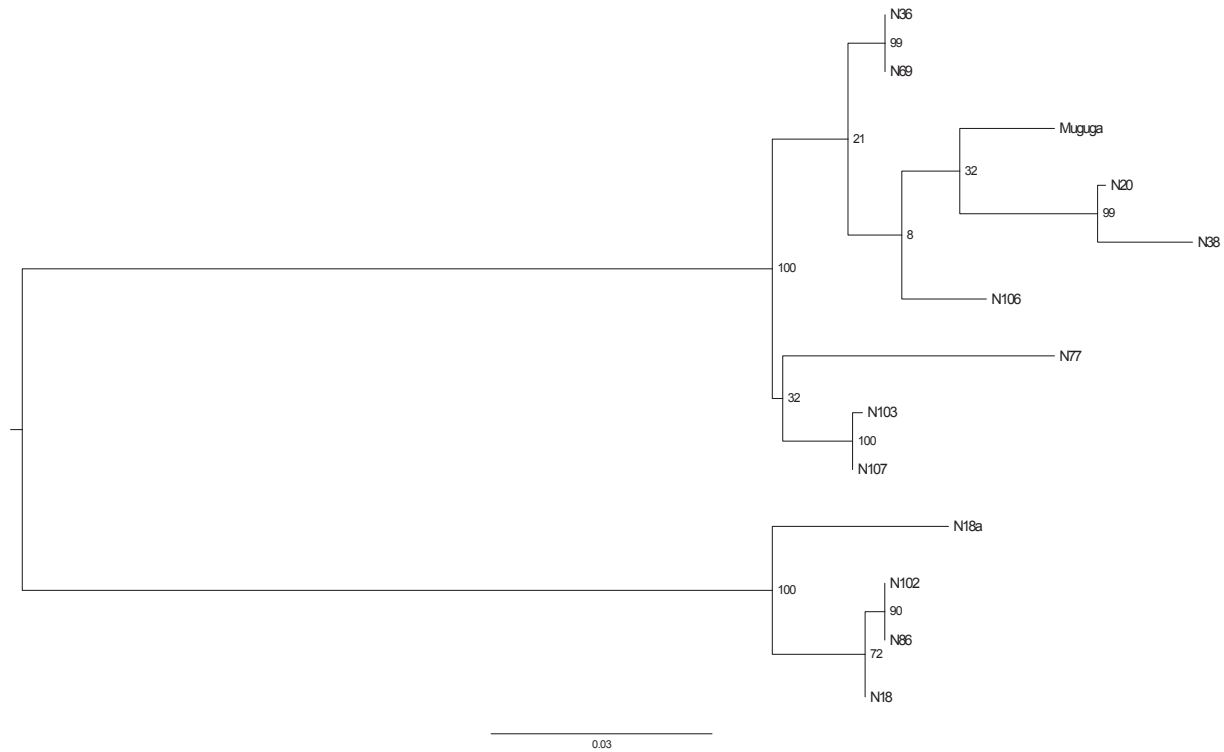
**Fig. 4.** Maximum Likelihood phylogenetic tree generated using N-terminal sequences of *T.* sp. (buffalo) and *T. parva* PIM antigen genes. Maximum composite likelihood trees were constructed using 1000 bootstrap replicates as implemented in MEGA5; the optimal nucleotide substitution model was identified using data monkey. The tree constructed with RAxML (Stamatakis et al., 2014) using a GTR/G/I model with 100 bootstrap iterations.

sequences separate from the *T. parka* sequences, with high levels of bootstrap support at the nodes separating the species (Fig. 5).

### 3.8. Screening of buffalo-derived stabilates of Theileria sporozoites for the presence of T. sp (buffalo)

In an attempt to determine whether available cryopreserved stabilates of *T. parva* prepared by feeding *R. appendiculatus* ticks on buffalo also contain *T.* sp. (buffalo), PCR amplicons of the 18s rRNA subunit from two buffalo-derived sporozoite stabilates were subjected to 454 sequencing (stabilates 3081 and 4110). Samples from stabilates 3081 and 4110 yielded 1479 and 607 sequence reads respectively, similar to or identical to the reference *T. parva* sequence; no *T.* sp. (buffalo) sequences were identified. The sequences obtained included a variant present in both stabilates, which differed by one nucleotide from the *T. parva* 18S rRNA gene reference sequence (111 and 58 reads from stabilates 3081 and 4110 respectively) and a further variant, also differing by a single nucleotide, in stabilate 4110 (45 reads). These sequences differed by two or three nucleotides from the *T.* sp (buffalo) reference sequence.

## 4. Discussion

*T.* sp. (buffalo), a parasite with a wide distribution in eastern and southern Africa has previously been detected at a high frequency in buffalo populations (Conrad et al., 1987; Allsopp et al., 1993; Pienaar et al., 2011a, b; Pienaar et al., 2014; Mans et al., 2015), and less frequently, also in cattle (Pienaar et al., 2011b; Githaka et al., 2014). However detection in cattle has previously been based only on PCR data and not parasite isolation. Herein we demonstrate for the first time that *T.* sp. (buffalo) schizont cultures can also be isolated from cattle in regions of Kenya where cattle and buffalo co-

graze. The presence of *T.* sp. (buffalo) in cell lines isolated from cattle demonstrates that, like *T. parva*, *T. taurotragi* and *Theileria annulata*, *T.* sp. (buffalo) is able to transform cattle cells.

Work on isolates from South African buffalo confirms that the species-specific section of the 18S rRNA gene is very similar in *T. parva* and *T.* sp. (buffalo), as originally shown for East African animals (Allsopp et al., 1993). A further variant *T. sp. (bougasvlei)* also appears to be present in South African buffalo (Zweygarth et al., 2009; Pienaar et al., 2011a; Mans et al., 2015), but has not yet been detected in East Africa. Additionally, use of *Theileria* cytochrome Oxidase III as a marker has also demonstrated that the two parasites frequently occur as co-infections in buffalo (Chaisi et al., 2013), although there is a high degree of inter-species variability in this sequence, making interpretation of data from some animals difficult. Interestingly, studies in South Africa have documented intermediate 18S sequences between *T. parva* and *T.* sp. (buffalo) (Mans et al., 2011a,b), suggesting that *Theileria* molecular epidemiology may differ regionally and that rDNA sequences alone are not definitive for speciation (Mans et al., 2015). The present study has identified polymorphism in genes encoding antigens that distinguish *Theileria* sp. (buffalo) from *T. parva*, including p104 and p67, as noted previously (Pienaar et al., 2011b), and also PIM. Given the exceptional hyper-variability of the central polymorphic domain of PIM between *T. parva* isolates (Toye et al., 1995; Geysen et al., 2004) it is interesting that T. sp. (buffalo) encodes an orthologue of the PIM gene, which could be amplified using primers derived from the conserved section of the *T. parva* PIM gene and is closer in sequence to that of *T. parva* than those previously described from other *Theileria* species. *Theileria* sp. (buffalo) exhibited 61% amino acid identity with the *T. parva* reference in the conserved N-terminal domain as compared to 40% with the *T. annulata* orthologue, (Knight et al., 1996). A high level of conservation of the genes encoding the Tp6, Tp7 and Tp8 antigens that
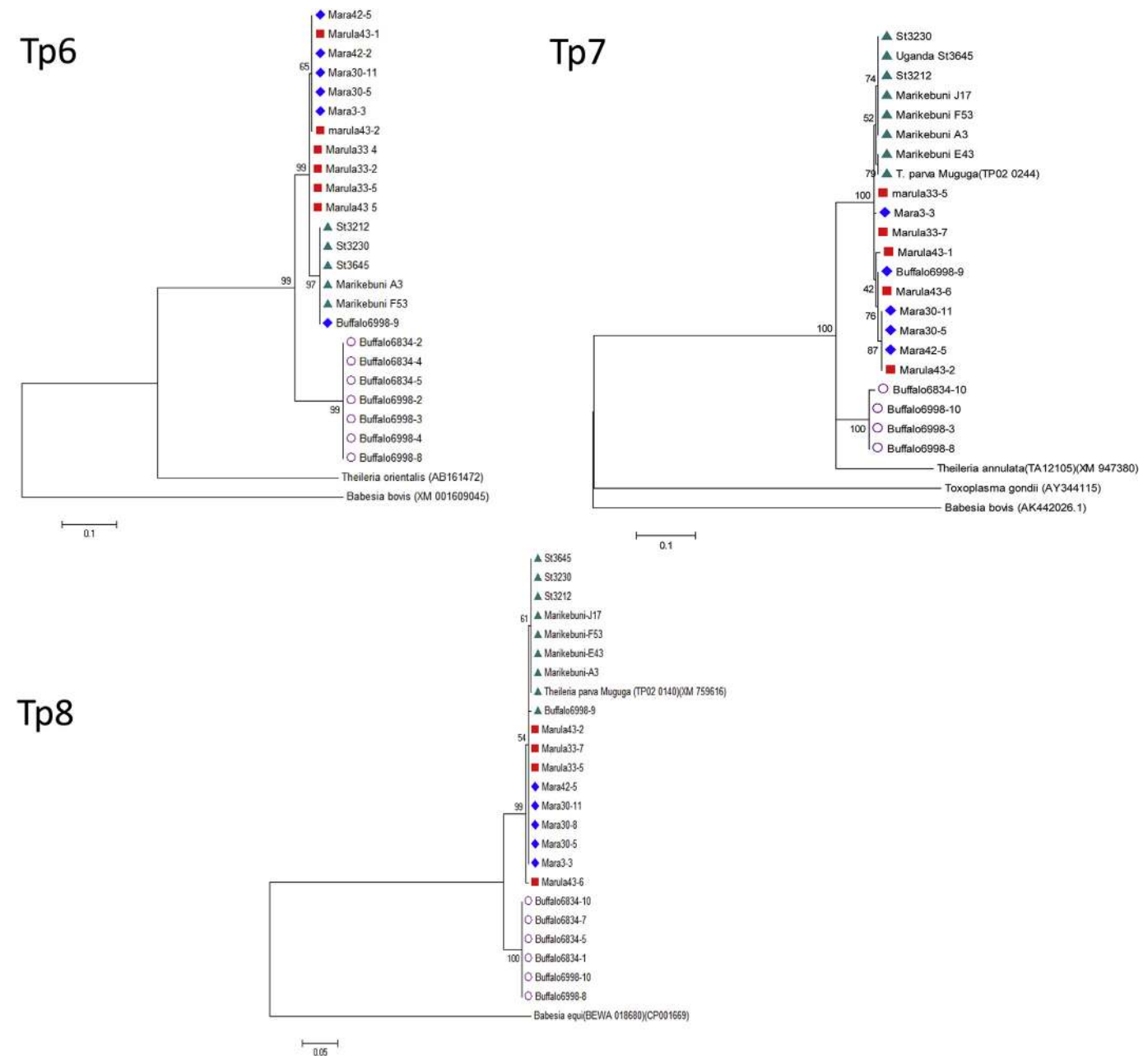
**Fig. 5.** Maximum Likelihood Phylogenetic trees illustrating the genetic relationships of T. *parva* CD8 T target antigen gene orthologues from *T.* sp. (buffalo). Panel (A) Tp6; Panel B Tp7: Panel C Tp8. Sequences were aligned and used to construct a maximum likelihood tree, at which the nodes were confirmed using 1000 bootstrap replications. The bootstrap values indicating the degree of support for each node are shown and also the GenBank accession numbers of the sequences. For Tp6, the tree was rooted using the prohibitin gene sequences present in *Babesia bovis* (XM001609045) and *Theileria orientalis* (AB161472). For Tp7, the tree was rooted using the putative Heat shock protein 90 gene sequences from *Toxoplasma gondii* (AY344115), *Babesia bovis* (AK442026) and *Theileria annulata* (XM_947380). For Tp8, the tree was rooted using an orthologue of Tp8 found in *Theileria equi* (CP001669).

are targets of bovine CD8 T cell responses in *T. parva* with the *T.* sp. (buffalo) orthologues also confirms the close relationship of these parasites. These genes are highly conserved within each species showing nucleotide substitutions in only a few residues. Nevertheless, phylogenetic analysis demonstrates a clear demarcation of the nucleotide sequences obtained for *T. parva* and *T.* sp. (buffalo), supporting the view that the two parasites indeed represent two separate species.

*T. parva* diagnostic primers derived from the p104 gene (Skilton et al., 2002) did not generate a product from the *T.* sp. (buffalo) schizont-infected lymphocyte lines N86 and N102, re-confirming their specificity and usefulness for *T. parva* surveillance. Primers

that amplify the p67 gene from all *T. parva* variants that have been tested, whether of cattle or buffalo origin, did not generate a detectable PCR product, from the *T.* sp. (buffalo) lines N86 or N102, although a product was amplified from other infected cell lines, containing either *T. parva* alone or a mixed infection with *T.* sp. (buffalo). This suggests that the p67 orthologue in (*T.* sp. *buffalo*) may be relatively divergent in sequence as a result of isolation and speciation. However, the differences may also be the result of minor differences in the primers derived from *T. parva* resulting in their failure to amplify the *T.* sp. (buffalo) orthologue. It is however very likely that there a positionally equivalent protein in *T.* sp. (buffalo) that may also be antigenic, since *T. annulata* has an

immunologically cross reactive orthologue known as SPAG1 (Knight et al., 1996) and there is a positional homologue present in the genome of the much more distantly related apicomplexan *Babesi bovis* that was identified by synteny analysis (Brayton et al., 2007). The divergence of the p67 and p104 antigen gene sequences relative to the similarity in the 18s gene, which is a widely used phylogenetic marker, suggests that complete genome sequencing would be informative in understanding the evolution of the Apicomplexa and would shed further light on the reliability of 18s rDNA as a phylogenetic marker in this group of protozoa. The genome sequence would also provide insight into the evolution of the more variable Tp CD8 T cell target antigens in two closely related, but apparently distinct populations that share the same buffalo host.

The extensive amino acid sequence identity between the N-terminal sections of *T. parva* PIM and the *T.* sp. (buffalo) homologue, coupled with the reactivity of some of the PIM-specific MAbs with *T.* sp. (buffalo), suggest that the presence of *T.* sp. (buffalo) could confound the results of serological analyses using the PIM-based ELISA in cattle in areas of heavy buffalo challenge. Furthermore, the schizonts of the two parasites are morphologically similar and thus parasitological analysis may also not be definitive. Therefore, in areas where buffalo are present, a real-time or conventional PCR assay (Odongo et al., 2010; Chaisi et al., 2013) may be required to confirm parasite identity. In South Africa a real time diagnostic test based on amplification of the 18s gene has been developed. The high degree of sequence similarity of the 18s rRNA gene of *T. parva* and *T.* sp (buffalo) can occasionally lead to mis-diagnosis of *T. parva* (Sibeko et al., 2008; Pienaar et al., 2011a). In the current study, the demonstration that PCR assays for p104 and p67 distinguish the two species and that the Tp6, Tp7 and Tp8 gene orthologues contain a number of nucleotide substitutions that were unique to *T.* sp. (buffalo) identify additional candidate genes that could be developed for differential diagnosis of these parasites. Given the high level of sequence similarity between the two species in the genes encoding the CD8 T cell target antigens, Tp6, Tp7 and Tp8 (Graham et al., 2006), it would be worthwhile to determine whether *T.* sp. (buffalo) induces immune responses in cattle that cross-react with *T. parva* and might convey a degree of immunity. It would also be interesting to investigate whether *T.* sp. (buffalo) contains orthologues of the more variable CD8 T cell target antigens (Graham et al., 2006; Pelle et al., 2011).

In common with certain other *Theileria* species in East Africa, for example *T. buffeli*, the tick vector involved in transmission of *T.* sp. (buffalo) still needs to be identified. High-throughput sequencing failed to detect *T.* sp. (buffalo) in two sporozoite stabilates, produced by feeding *R. appendiculatus* ticks on buffalo naturally infected with *Theileria* parasites. Unfortunately, samples were not available from the buffalo from which these stabilates were prepared to verify whether or not they were infected with *T.* sp. (buffalo). One of these buffalo had been captured from the Ol Pejeta ranch in the Laikipia district of Kenya; a recent study of samples from buffalo on the same site (now a Game Conservancy), demonstrated the presence of *T.* sp. (buffalo) as well as *T. parva* in all 8 animals examined (Hemmink H, unpublished data). Hence, the absence of the parasite in the two stabilates examined, although not conclusive, suggests that *R. appendiculatus* may well not be a suitable vector for the transmission of *T.* sp. (buffalo). The identification of the tick vector involved in transmission represents an area for future research. One approach would be to use PCR or RLB assays to survey dissected salivary glands from ticks collected from areas where *T.* sp. (buffalo) is known to occur. *T.* sp. (buffalo) has not been detected in cattle in regions where buffalo are absent, as indicated by RLB studies (Njiiri et al., 2015), suggesting that it is not transmissible between cattle. More targeted epidemiological studies to examine cattle

undergoing the acute phase of infection with *Theileria* parasites in regions where buffalo are present, will be required to determine the incidence of infection with this parasite in cattle under natural field conditions. However, the possible role of *T.* sp. (buffalo) in inducing pathogenesis in cattle will only be resolved definitively by obtaining *T.* sp. (buffalo) sporozoite isolates free of *T. parva*, to enable experimental testing in cattle. In addition to identification of the tick vector, it will be important to formally name this buffalo *Theileria* whose existence has been known for more than twenty years. The evidence that *T.* sp. (buffalo) is a species distinct from *T. parva*, as has been suggested previously (Pienaar et al., 2014; Mans et al., 2015), is strong. If these two *Theileria* populations were not reproductively isolated, their loci would rapidly be homogenized through sexual recombination.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2015.08.006.

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