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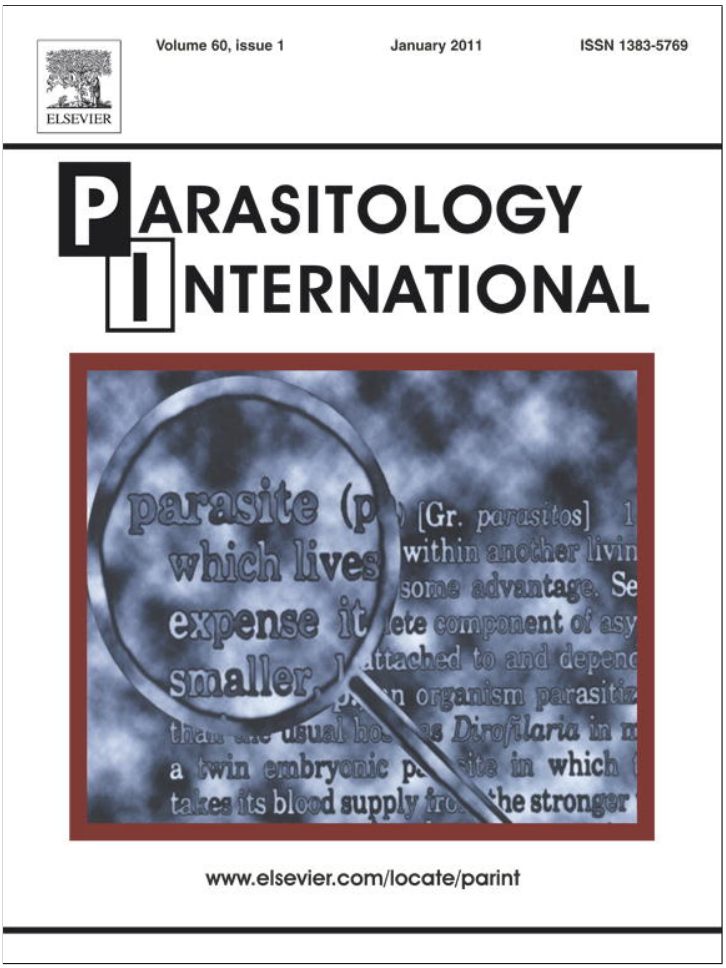

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Short communication

Genotypic variations in field isolates of *Theileria* species infecting giraffes (*Giraffa camelopardalis tippelskirchi* and *Giraffa camelopardalis reticulata*) in Kenya

Naftaly Githaka [a], Satoru Konnai [a], Robert Skilton [b], Edward Kariuki [c], Esther Kanduma [b,d], Shiro Murata [a], Kazuhiko Ohashi [a,*]

[a] Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan
[b] Biosciences Eastern and Central Africa-International Livestock Research Institute Hub (BecA-ILRI Hub), P.O. Box 30709-00100, Nairobi, Kenya
[c] Kenya Wildlife Service, P.O. Box 40241-00100, Nairobi, Kenya
[d] Department of Biochemistry, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya

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ABSTRACT

Recently, mortalities among giraffes, attributed to infection with unique species of piroplasms were reported in South Africa. Although haemoparasites are known to occur in giraffes of Kenya, the prevalence, genetic diversity and pathogenicity of these parasites have not been investigated.

In this study, blood samples from 13 giraffes in Kenya were investigated microscopically and genomic DNA extracted. PCR amplicons of the hyper-variable region 4 (V4) of *Theileria* spp. small subunit ribosomal RNA (18S rRNA) gene were hybridized to a panel of genus- and species-specific oligonucleotide probes by reverse line blot (RLB). Two newly designed oligonucleotide probes specific for previously identified *Theileria* spp. of giraffes found single infections in eight of the specimens and mixed infections in the remaining five samples. Partial 18S rRNA genes were successfully amplified from 9 samples and the PCR amplicons were cloned. A total of 28 plasmid clones representing the Kenyan isolates were analyzed in the present study and compared with those of closely-related organisms retrieved from GenBank. In agreement with RLB results, the nucleotide sequence alignment indicated the presence of mixed infections in the giraffes. In addition, sequence alignment with the obtained 18S rRNA gene sequences revealed extensive microheterogeneities within and between isolates, characterized by indels in the V4 regions and point mutations outside this region. Phylogeny with 18S rRNA gene sequences from the detected parasites and those of related organisms places *Theileria* of giraffes into two major groups, within which are numerous clades that include the isolates reported in South Africa. Collectively, these data suggest the existence of at least two distinct *Theileria* species among giraffes, and extensive genetic diversity within the two parasite groups.

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1. Introduction

Piroplasms in Kenyan giraffes were first reported by Brocklesby and Vidler [1] who identified *Theileria*-like piroplasms in both the Maasai (*Giraffa camelopardalis tippelskirchi*) and reticulated (*Giraffa camelopardalis reticulata*) giraffes, and one *Babesia* sp. in reticulated giraffes. Recently, Oosthuizen et al. [2] reported potentially pathogenic species of *Babesia* and *Theileria* suspected of causing fatalities in young giraffes in South Africa. In their report, each animal was found to be infected with a unique parasite, as deduced from 18S ribosomal RNA (18S rRNA) gene sequences, and suggested that more piroplasms may occur in giraffes [2]. The tick vectors, as well as, the epidemiology and pathology associated with these organisms in East African giraffes are not yet known.

Extensive genetic variation has been reported among field populations of different *Theileria* spp. [3–9]. Bhoora et al. [5] and Salim et al. [6] reported the occurrence of numerous genotypes of *Theileria equi*, the causative agent of equine piroplasmosis in horses, across geographic regions within a single country. Several unique genotypes and variants associated with disease outbreaks have also been reported recently in *Theileria orientalis*, the casual agent of bovine piroplasmosis in Asia and Australia [10]. This genetic heterogeneity within *Theileria* genus is believed to among other factors, arises from sexual recombination during gametogony in the vector-ticks [11]. In the present study, we report sequence heterogeneity in field isolates of *Theileria* spp. of the giraffes from Kenya.

Thirteen giraffe blood samples in EDTA-coated tubes were provided by the Veterinary Department, Kenya Wildlife Service (KWS), Nairobi, Kenya (Table 1). Genomic DNA was extracted from 500 μl of frozen blood or 300 μl of fresh blood with the Wizard Genomic DNA purification Kit (Promega, Madison, USA). Primers RLB F2 [5′-GAC ACA GGG

* Corresponding author. Tel.: +81 11 706 5215; fax: +81 11 706 5217.
E-mail address: okazu@vetmed.hokudai.ac.jp (K. Ohashi).

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Table 1
Summary of animal hosts from which giraffe blood samples were obtained, and GenBank accession numbers of corresponding *Theileria* isolates identified in this study. Samples that were positive in both touch-down PCR and reverse line blot (RLB) but failed to yield visible bands with 18S rRNA PCR were not cloned (ND).

Sample identification	Age (years)	Geographical origin	RLB screening	18S rRNA clone # (GenBank accession no.) [bp]
Giraffe #30	1	Garrisa, N.E.P.	Single infection	2(JQ928928)[1665], 5(JQ928927)[1361]
Giraffe #43	2	Garrisa, N.E.P.	Single infection	ND
Giraffe #44	5	Garrisa, N.E.P.	Mixed infection	1(JQ928926[a])[1433], 2(JQ928925)[1665], 4(JQ928924)[1672]
Giraffe #63	1.5	Garrisa, N.E.P.	Single infection	5(JQ928922[b])[1649], 6(JQ928921)[1665], 4(JQ928923)[1665]
Giraffe #64	2	Nairobi National Park	Single infection	ND
Giraffe #66	2	Nairobi National Park	Single infection	2(JQ928920)[1665], 3(JQ928919)[1653], 4(JQ928918[b])[1665], 6(JQ928917)[1665],
Giraffe #70	1.5	Nairobi National Park	Single infection	1(JQ928916)[1665], 6(JQ928914[b])[1644], 2(JQ928915)[1665]
Giraffe #81	Adult	Giraffe Centre, Nairobi	Mixed infection	1(JQ928913)[1665], 3(JQ928912)[1656], 6(JQ928911)[1665]
Giraffe #86	Adult	Giraffe Centre, Nairobi	Mixed infection	1(JQ928910)[1570], 3(JQ928909)[1665], 5(JQ928908)[1665], 6(JQ928907)[1614]
Giraffe #94	Juvenile	Giraffe Centre, Nairobi	Mixed infection	ND
Giraffe #104	Adult	Garrisa, N.E.P.	Single infection	ND
Giraffe #108	Adult	Garrisa, N.E.P.	Single infection	4(JQ928933[b])[1658], 1(JQ928934[b])[1664], 6(JQ928932)[1609]
Giraffe #115	Adult	Giraffe Centre, Nairobi	Mixed infection	6(JQ928929)[1665], 2(JQ928931[a])[1665], 3(JQ928930)[1184]

N.E.P.: North–Eastern Province. ND: No data.
[a] Isolates matching RLB probe *Theileria* sp. (giraffe) 2.
[b] Isolates matching RLB probe *Theileria* sp. (giraffe) 1.

AGG TAG TGA CAA G-3′] and biotin-labeled RLB R2 [5′-Biotin-CTA AGA ATT TCA CCT CTA ACA GT-3′] [12] were used to PCR amplify the V4 hypervariable region (V4) of parasite 18S rRNA gene from the giraffe DNA samples using a touchdown PCR protocol as described previously [13]. Genomic DNA from *Theileria parva*-infected cattle, Cape buffalo (*Syncerus caffer*) and eland (*Taurotragi orynx*) was used as positive controls at the PCR step for the subsequent RLB hybridization. The buffalo and eland positive controls were field samples from which *Theileria* sp. (buffalo) and *Theileria taurotragi*, respectively, had been previously detected while control genomic DNA for other *Theileria* spp. was not available. Sterile, deionized water was used as a negative control. Five μl of PCR products were electrophoresised through a 2% agarose gel, and stained with ethidium bromide prior to visualization under UV light.

To identify *Theileria* spp. occurring in giraffes, 18S rRNA gene sequences of *Theileria* spp. previously described in giraffes [2] were obtained from GenBank and used to design two species-specific probes, designated as *Theileria* sp. (giraffe) 1 and *Theileria* sp. (giraffe) 2. In addition to these two, probes specific for all *Theileria* and *Babesia* spp. (*Theileria*/*Babesia* catch-all), all *Theileria* spp. (*Theileria* catch-all), all *Babesia* spp. (*Babesia* 1 and 2) as well as several others that are specific for different *Theileria* spp. were included on the membrane for screening blood parasites (Table 2). Reverse line blot was conducted as detailed previously [19].

Table 2
List of oligonucleotide probes used in the RLB assay. GenBank accession numbers of *Theileria* spp. (giraffe) used to derive the newly designed probes are shown in parentheses.

Probe specificity	18S sequence (5′–3′)	Reference
Theileria/*Babesia* catch-all	TAATGGTTAATAGGARCRGTTG	Gubbels et al. 1999 [13]
Theileria catch all	ATTAGAGTGCTCAAAGCAGGC	Nagore et al. 2004 [14]
Babesia catch all 1	ATTAGAGTGTTTCAAGCAGAC	Bhoora et al. 2009 [4]
Babesia catch all 2	ACTAGAGTG TTT CAA ACAGGC	Brothers et al. 2011 [15]
Theileria parva	GGACGGAGTTCGCTTTG	Gubbels et al. 1999 [13]
Theileria taurotragi	TCTTGGCACGTGGCTTTT	Gubbels et al. 1999 [13]
Theileria sp. (buffalo)	CAGACGGAGTTTACTTTGT	Oura et al. 2004 [16]
Theileria annulata	CCTCTGGGGTCTGTGCA	Georges et al. 2001 [12]
Theileria buffeli	GGCTTATTTCGGWTTGATTTT	Gubbels et al. 1999 [13]
Theileria mutans	CTTGCGTCTCCGAATGTT	Gubbels et al. 1999 [13]
Theileria velifera	CCTATTCTCCTTTACGAGT	Gubbels et al. 1999 [13]
Theileria ovis	TTGCTTTTGCTCCTTTACGAG	Nagore et al. 2004 [14]
Theileria sp. (kudu)	CTCCATTGTTTCTTTCCTTTG	Nijhof et al. 2005 [17]
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Oosthuizen et al. 2008 [18]
Theileria sp. (giraffe) 1 (FJ213582 & FJ213584)	TTATTTCTCCTTGACGAGTT	This work
Theileria sp. (giraffe) 2 (FJ213583)	CTCTTTGATGGGCTTTTG	This work

Partial 18S rRNA genes from giraffe DNA samples were PCR-amplified, cloned, and sequences edited manually as described previously [19]. Basic local alignment search tool (BLAST) [20] was used to search for sequences similar to the 18S rRNA gene sequences determined in the present study. A nucleotide sequence alignment of the new sequences and a number of related organisms obtained from the GenBank were constructed using CLUSTAL W [21] in Geneious Pro 5.5 [22] and the alignment trimmed to remove ambiguous sequence ends. Similarity matrices were performed using the two-parameter method of Kimura [23] alongside the Jukes–Cantor correction model for multiple base changes [24]. Phylogenetic trees were constructed using the neighbor-joining method [25] implemented by Geneious Pro 5.5 in combination with the bootstrap method at 1000 replicates/tree. GenBank accession numbers for the new sequences and corresponding animal sources are listed in Table 1.

Blood smears revealed the presence of haemoparasites in the erythrocytes of specimen #115 whereas the rest of the blood specimens were too haemolyzed for microscopy. Genomic DNA was successfully extracted from all samples. A touchdown PCR with primers targeting the V4 region of the 18S rRNA gene yielded visible bands with 12/13 (92%) of the giraffe-derived DNA samples and for the three positive controls while no contamination was detected in the water negative control (Fig. 1).

A total of sixteen generic and species-specific probes were included in the RLB assay. Among the positive controls, strong signals corresponding to *T. parva*, *Theileria* sp. (buffalo) and *T. taurotragi* were visible on the membrane while additional bands were also observed in the case of the buffalo and eland genomic DNA. In the study samples, strong and distinct RLB signals were obtained with the *Theileria*/*Babesia* catch-all and *Theileria* catch-all probes. RLB with oligonucleotide probes designed to detect *Theileria* spp. of giraffes showed that *Theileria* DNA was present in all 13 samples. Between these two newly designed probes, *Theileria* sp. (giraffe) 1 hybridized with PCR amplicons from a total of 11 giraffe samples (#30, 43, 44, 63, 64, 66, 70, 81, 86, 94 and 115) whereas the second probe, *Theileria* sp. (giraffe) 2, hybridized with 7 samples (44, 81, 86, 94, 104, 108 and 115). In addition, out of the 13 samples, 5 (44, 81, 86, 94 and 115) yielded signals with both new probes. No signals were detected with the water negative control or with the other species-specific probes present on the membrane (Fig. 1).

Subsequently, partial 18S rRNA genes were successfully amplified and cloned from nine giraffe specimens. In total, 28 sequences (1184–1672 bp) representing parasite isolates from Kenyan giraffes were

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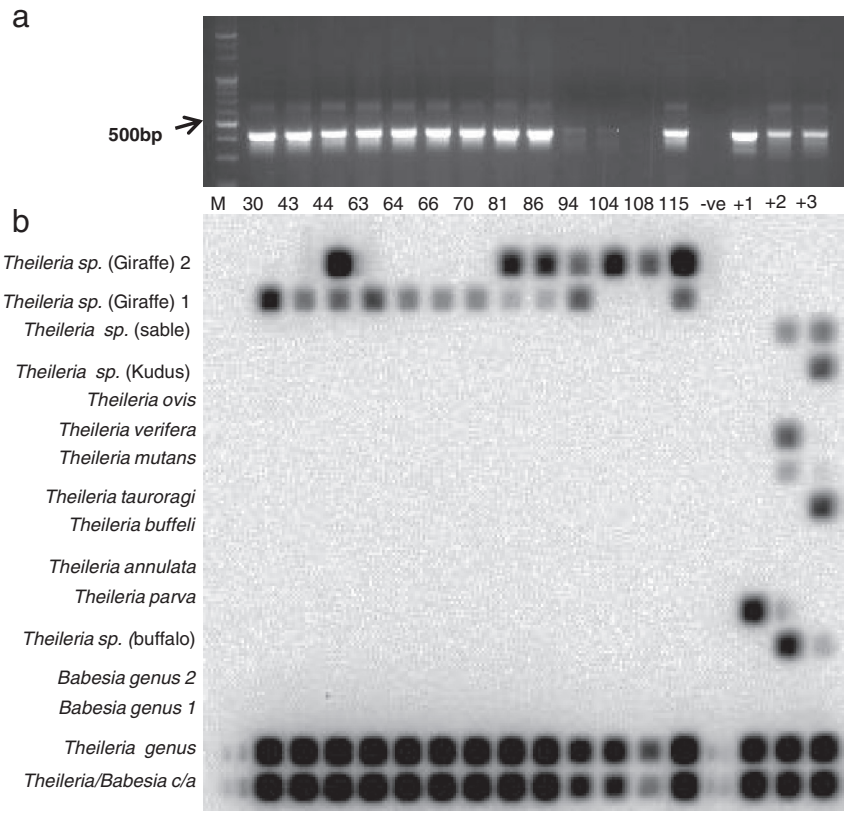

Fig. 1. Detection of haemoparasites in giraffes by reverse line blot (RLB) assay. a Touch-down PCR applied to giraffe-derived gDNA samples. +1, +2 and +3 are positive control samples from cattle, Cape buffalo and eland, respectively. M is a 100-bp DNA ladder. Lanes are labeled according to animal number. b RLB hybridization of PCR products derived from giraffe DNA. DNA probes were applied horizontally starting with the *Theileria*/*Babesia* catch-all and ending with *Theileria* sp. (giraffe 2).

assembled. Among the five samples diagnosed as dual infections with RLB, clones corresponding to the *Theileria* sp. (giraffe) 2 probe were overrepresented among the randomly sequenced clones, perhaps reflecting higher parasitemia that was supported by stronger signals on the RLB. BLAST searches with these sequences indicated that they were of *Theileria* origin; however, none of the 28 clones from this study yielded 100% identity with sequences in GenBank. Moreover, the BLAST searches showed the presence of multiple genetic variants of *Theileria* spp. in the giraffe.

Nucleotide sequence alignment of the 18S rRNA gene sequences obtained in the present study, and those reported in South Africa revealed extensive heterogeneity within and between isolates (92.0–99.9%), largely within the V4 region (Fig. 2). Genotypes identical to the ones reported in South African giraffes were found among isolates sequenced in the current study, consistent with the RLB data. Moreover, clones without sequences matching the two probes, were also found upon aligning 18S rRNA gene sequences obtained from the Kenyan giraffes and those of giraffe isolates reported previously.

Phylogenetic analyses were conducted with the aligned 18S rRNA gene sequences (Fig. 3). Furthermore, a phylogenetic tree comprising the 18S rRNA gene sequences from *Theileria* and *Babesia* spp. placed isolates from the giraffes within the *Theileria sensu stricto* clade (data not shown). From these analyses, *Theileria* spp. from the Kenyan giraffes were found to cluster into two major clades, which also carry *Theileria* isolates that were previously reported in giraffes in South Africa. Importantly, the South African genotypes (accession number FJ213582, FJ213584 and FJ213583) clustered with sequences derived from the Kenyan giraffes in a manner consistent with the RLB observation.

The finding of haemoparasites in a blood smear from one of the giraffes was consistent with a study by Brocklesby and Vidler [1] that reported the occurrence of *Theileria* and *Babesia* in Kenyan giraffes, and a recent study that reported pathogenic *Theileria* and *Babesia* in South African giraffes [2]. The giraffe blood samples analyzed in the present study yielded positive signals with the *Theileria* generic probe and also with one or both of the newly designed probes that are specific for *Theileria* spp. (giraffe) in the RLB assay, suggesting high prevalence of these parasites among giraffes. Oosthuizen et al. [2] reported novel *Theileria* from both healthy and clinically ill giraffes following translocation. Furthermore, in this study, RLB indicated that *Theileria* genotypes identical to one causing fatalities among South African giraffes are present in Kenya highlighting the need for surveillance against fatalities associated with these parasites. This is especially important for Kenya in the context of an ongoing restocking program that involves translocation of giraffes [Dr. Kariuki, unpublished data]. Although *Babesia* spp. have previously been reported among the giraffes [1,2], none of the specimens hybridized with the two *Babesia*-specific probes in the present study. This could indicate the absence of the specific tick vectors for these organisms at the localities where the study animals were resident although infestation of giraffes by multiple tick species is reportedly very common [26] and potentially leading to high rates of infection with blood-borne parasites.

The specificity and geographic coverage observed with the new probes further suggest that they could be used reliably to detect *Theileria* spp. occurring in giraffes. However, there is need to design more probes using *Theileria* isolates identified in the present study and those from South Africa. Moreover, studies with larger sample numbers are necessary since RLB has been found to discriminate between geographically diverse isolates with some blood-parasites, such as *T. equi* and *Babesia cabali* [4]. In addition, presently, only three positive controls (*T. parva*, *T.* sp. (buffalo) and *T. taurotragi*) were included in the RLB. Consequently, cross-hybridization between the new probes and other *Theileria* spp. cannot be ruled out entirely.

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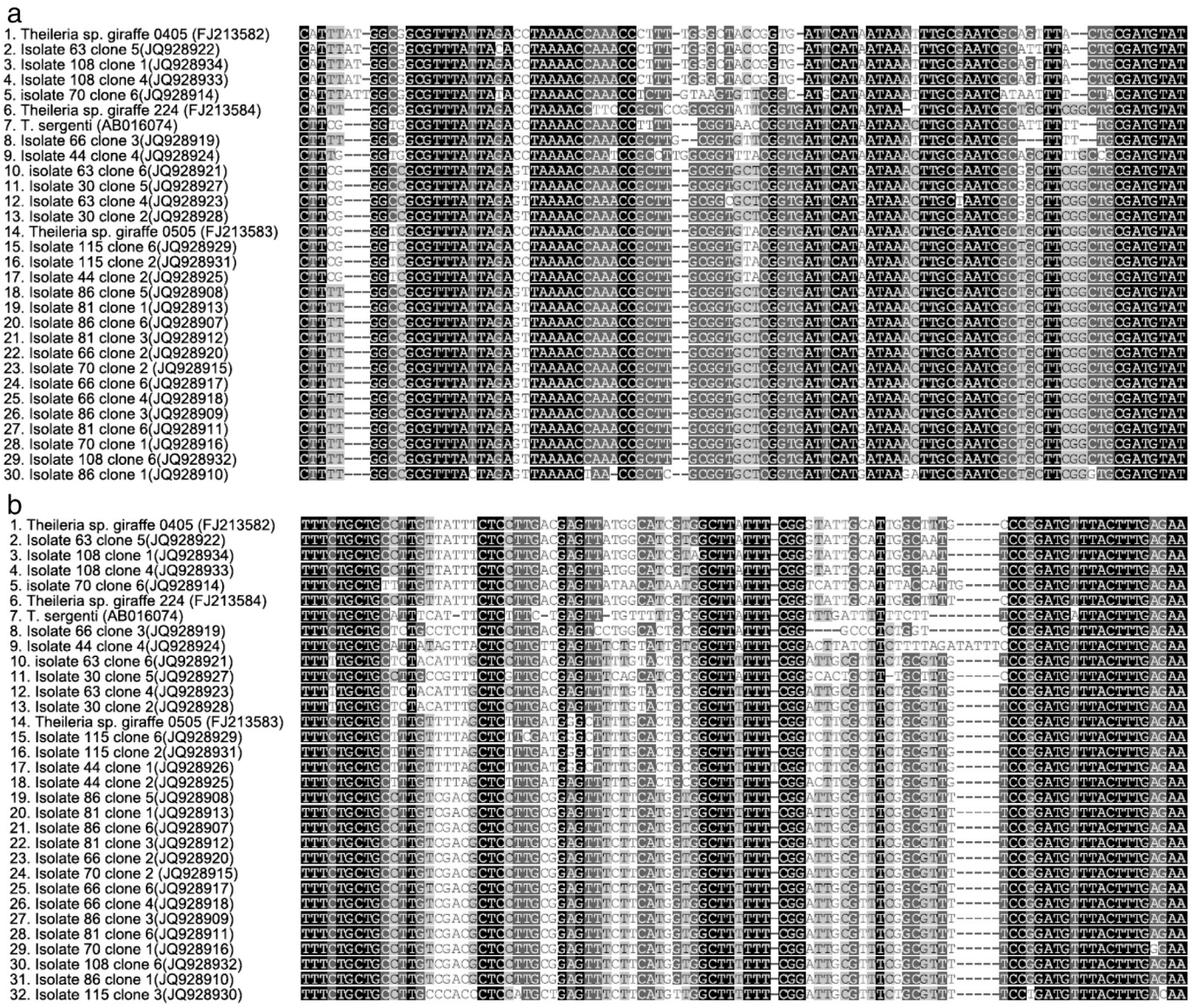

Fig. 2. Multiple sequence alignment extraction of the most variable regions of 18S rRNA genes obtained from *Theileria* sp. (giraffe) in the present study, and those previously identified in South Africa (GenBank accession numbers FJ213583, FJ213582 and FJ213584). a Nucleotide positions 180–279 and b, Nucleotide positions 620–719. Full-length 18S rRNA gene from *Theileria sergenti* (GenBank accession number AB016074) was used as reference for sequence length. The obtained length for sequences with accession numbers JQ928926 and JQ928930 fell short of the region represented in Fig. 2a.

Singular and mixed infections were evident from RLB, and were confirmed by sequencing of the 18S rRNA genes from parasite isolates in the current work. Moreover, more samples hybridized with the *Theileria* sp. (giraffe) 1 probe than *Theileria* sp. (giraffe) 2, whereas genotypes identical to both probes were also found in some isolates. The significance of this observation can only be postulated at the present; however, it is worth noting that the *Theileria* sp. (giraffe) 1 probe was derived from a South African isolate that was associated with acute disease onset and death in a young giraffe [2].

A significant number of sequence variants were found within and among isolates when the 18S rRNA gene sequences obtained from the Kenyan giraffes were aligned with isolates reported in South Africa. It is possible that these are mere variants rather than completely new species or sub-species since the observed heterogeneity did not impair the RLB assay. Mans et al. [27] has also shown that heterogeneity in the 18S rRNA gene does not seem to impair a real-time PCR detection assay specific for *T. parva*. Such genetic variation in haemoparasites is attributable to either parasite sexual recombination during gametogony [11], the presence of more than one transcription unit in the 18S rRNA gene [3], or point mutations [28].

The 18S rRNA gene sequences acquired in the present study were aligned with closely related parasites and subjected to phylogenetic analysis. The isolates from the present study cluster into two major clades that include the South African genotypes from which the new probes were designed. When analyzed alongside *Babesia* spp., *Theileria* isolates from giraffes fall well within the *Theileria sensu stricto* clade. Collectively, the RLB data and the phylogeny suggest that the South African parasites represent a sub-set of the genotypes present in Kenya, and it is likely that more isolates will be found if a larger number of giraffes from Southern Africa are sampled.

In summary, we report the occurrence of genetically diverse *Theileria* spp. in otherwise healthy giraffes in Kenya. Two RLB probes were designed and used to detect *Theileria* spp. in giraffes. Phylogeny of the giraffe *Theileria* suggests the existence of two distinct populations, and extensive heterogeneity within these two groups. Further studies are necessary to clarify the observed genetic diversity among the *Theileria*

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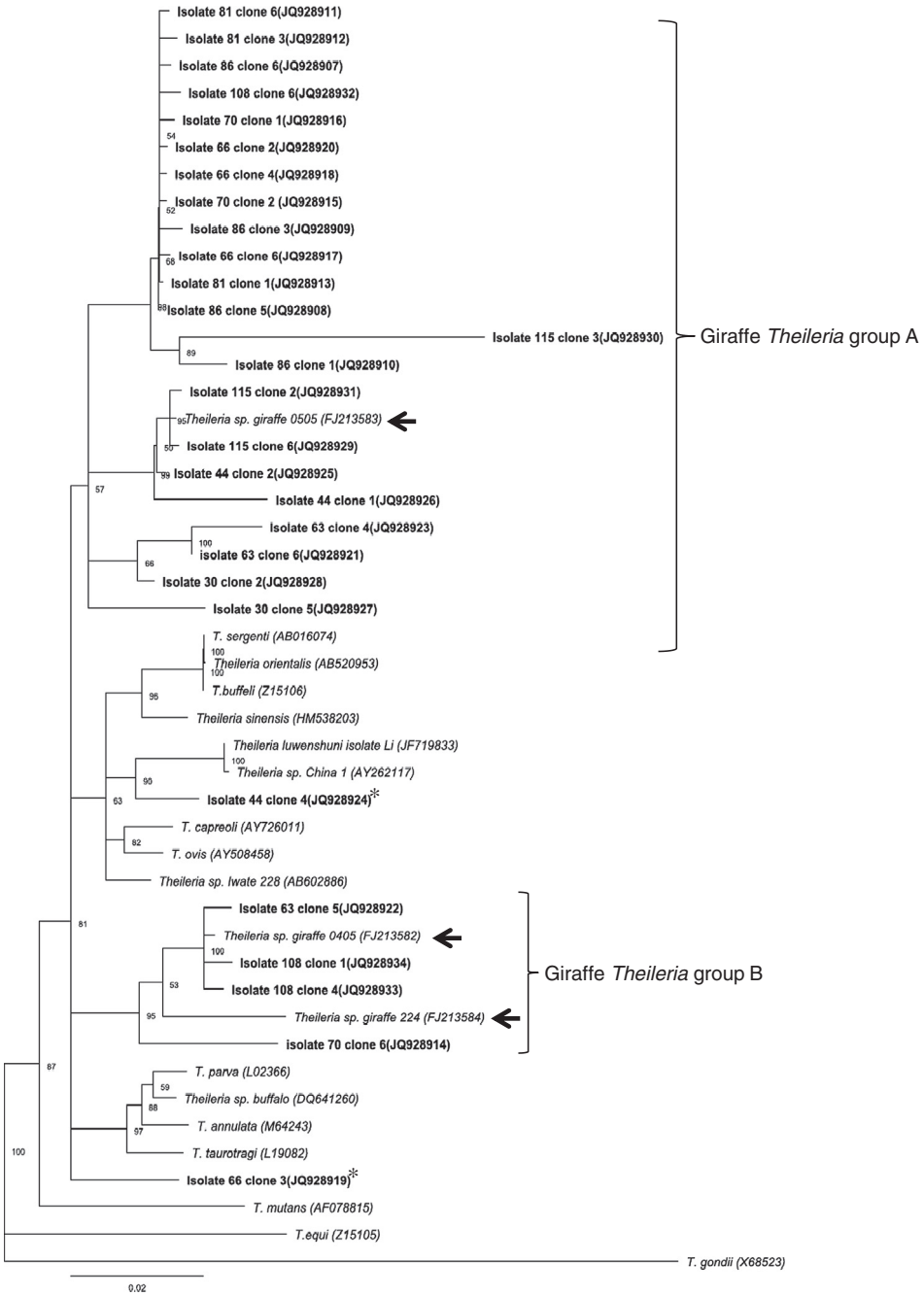

Fig. 3. Rooted neighbor-joining phylogenetic tree showing the relationship between genotypes of *Theileria* spp. (giraffe) identified in the present study and closely related *Theileria* species based on 18S rRNA gene sequences. The numbers represent the percentage of 1000 replications (bootstrap support) for which the same branching patterns were obtained. GenBank accession numbers for parasite sequences are in parentheses. Genotypes identified in this study are in bold, while the arrow indicates *Theileria* spp. (giraffe) that were previously reported in South Africa. The scale bar indicates substitutions per site. *These sequences are likely due to sequencing errors rather than unique isolates.

occurring in the giraffes, and to investigate the possible role of *Theileria* spp. in disease incidence among giraffes in East Africa.

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