

Molecular detection of selected tick-borne agents and genetic diversity of *Theileria parva* in African buffaloes from Mozambique

Detecção molecular de agentes selecionados transmitidos por carrapatos e diversidade genética de *Theileria parva* em búfalos africanos de Moçambique

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Abstract

The African buffalo (*Syncerus caffer*) is an important reservoir of tick-borne pathogens and may contribute to pathogen transmission at wildlife–livestock interfaces. This study investigated the occurrence of *Ehrlichia* spp., *Coxiella burnetii*, *Babesia* spp., *Theileria parva* and the *Theileria orientalis* complex in 87 African buffaloes from the Marromeu Reserve, Mozambique, using molecular assays. In addition, the genetic diversity of *T. parva* was assessed based on distinct molecular markers. *Theileria parva* DNA was detected in 35/87 (40.2%) samples. Novel *p104* and *p67* haplotypes not previously reported in African buffaloes or cattle were detected among the sampled animals. The detected *p67* sequences were distributed among groups 1, 2, and 3, encompassing different allele types. No sample was positive for *Ehrlichia* spp., *E. ruminantium*, *T. orientalis* complex, *Babesia bigemina*, *Babesia bovis*, or *C. burnetii*. These findings demonstrate a high molecular occurrence and substantial genetic diversity of *T. parva*, reinforcing the role of African buffaloes as key reservoirs of the causative agent of Corridor Disease.

Keywords: African buffaloes, *Theileria parva*, *Ehrlichia ruminantium*, *Babesia* spp., *Coxiella burnetii*, *Theileria orientalis* complex.

Resumo

O búfalo-africano (*Syncerus caffer*) é um importante reservatório de patógenos transmitidos por carrapatos e pode contribuir para a transmissão de patógenos nas interfaces entre animais selvagens e rebanhos domésticos. Este estudo investigou a ocorrência de *Ehrlichia* spp., *Coxiella burnetii*, *Babesia* spp., *Theileria parva* e do complexo *Theileria orientalis* em 87 búfalos-africanos da Reserva de Marromeu, Moçambique, utilizando ensaios moleculares. Além disso, avaliou-se a diversidade genética de *T. parva*. DNA de *T. parva* foi detectado em 35 das 87 amostras (40,2%). Entre os animais amostrados foram detectados novos haplótipos dos genes *p104* e *p67*, não relatados anteriormente em búfalos selvagens africanos ou bovinos. As sequências *p67* detectadas distribuíram-se pelos grupos 1, 2 e 3, abrangendo diferentes tipos alélicos. Nenhuma amostra mostrou-se positiva para *Ehrlichia* spp., *E. ruminantium*, complexo *T. orientalis*, *Babesia bigemina*, *Babesia bovis* ou *C. burnetii*. Esses achados demonstram uma alta ocorrência molecular e uma diversidade genética substancial de *T. parva*, reforçando o papel dos búfalos-africanos como reservatórios-chave do agente causador da Doença do Corredor.

Palavras-chave: Búfalos africano selvagens, *Theileria parva*, *Ehrlichia ruminantium*, *Babesia* spp., *Coxiella burnetii*, complexo *Theileria orientalis*.

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Introduction

The African buffalo (*Syncerus caffer*) is one of the most emblematic species of the southern African megafauna (Woodburn et al., 2021). These animals inhabit a wide range of habitats, from dry savannas to tropical montane forests (Cornélis et al., 2014; 2023). While estimates indicate the African buffalo population exceeds 500,000 individuals, its geographic distribution has contracted significantly since the 19th century (Cornélis et al., 2014). This range decline is driven by compounding anthropogenic pressures, including habitat conversion to pasturelands, poaching, disease outbreaks, and political instability, alongside severe climatic events such as droughts (Cornélis et al., 2014). Currently, approximately 70% of the global African buffalo population is restricted to protected areas (Cornélis et al., 2014, 2023). This species plays a role as a reservoir or carrier of a large number of tick-borne pathogens, including *Theileria* spp., *Babesia* spp. and *Anaplasma* spp. (Andrew & Norval, 1989; Allsopp et al., 1999; Eygelaar et al., 2015; Machado et al., 2016). As African buffaloes and cattle now coexist across many interfaces, the mutual transmission of pathogens may increase (Kock et al., 2014).

The genus *Theileria* (Piroplasmida: Theileriidae) comprises tick-borne hemoprotozoa that infect mammals worldwide, causing major losses to livestock production in tropical regions due to high mortality, reduced meat and milk yield, and treatment costs (Bishop et al., 2004). *Theileria parva*, *Theileria annulata*, *Theileria taurotragi*, *Theileria mutans*, *Theileria velifera*, and *Theileria orientalis/Theileria buffeli/Theileria sergenti* can infect bovids, but with different levels of pathogenicity (Morzaria, 1989; Gebrekidan et al., 2020). *Theileria annulata*, whose main hosts are represented by the Asian water buffalo (*Bubalus bubalis*), infects cattle and causes a lymphoproliferative disease known as tropical theileriosis in subtropical and tropical regions, where tick vectors of the genus *Hyalomma* are present (Ullah et al., 2021). Endemic areas include North Africa, southern Europe (Mediterranean countries), the Middle East, and parts of Central and South Asia, such as Pakistan, India, Iran, and Turkey, reflecting the broad range of *Hyalomma* ticks that transmit this apicomplexan protozoan (Ravindran et al., 2023). This geographic pattern closely aligns with reports of infection prevalence in cattle across these regions (Bishop et al., 2004; Gharbi et al., 2020; Liu et al., 2022; Valente et al., 2022).

African buffaloes are reservoirs of *T. parva*, the etiological agent of Corridor Disease in cattle, a disease prevalent in South Africa in areas where African buffaloes and cattle share grazing lands with the presence of tick vectors (Chaisi et al., 2013). The destruction of lymphocytes during the replication of *T. parva* may facilitate the infection by other hemoparasites (McKeever, 2009), given that *T. parva* actively suppresses the inflammatory response (Okagawa et al., 2012; Henrichs et al., 2016).

Molecular occurrence of 83.5% for *T. parva*, 55.8% for *Theileria* sp. (buffalo), 1.8% for *Theileria taurotragi*, 5.8% for *Theileria buffeli*, and 2.2% for *Theileria mutans* were reported among cattle and African buffaloes from South Africa and Mozambique (Greater Limpopo Transfrontier Game Park - GLTP) (Chaisi et al., 2013). In Uganda, *T. parva* (43.4%), *T. mutans* (97.6%), *T. buffeli* (25.3%), *T. velifera* (94%), and *Theileria* sp. (buffalo) (48.2%) were detected in African buffaloes (Oura et al., 2011). Molecular occurrence of 81% for *T. mutans*, 61% for *Theileria* sp. (sable), 40% for *T. parva*, 13% for *Theileria* sp. (buffalo), and 11% for *T. velifera* was reported among African buffaloes from Kruger National Park, South Africa (Debeila, 2012). Among African buffaloes from Hluhluwe-iMfolozi Park, South Africa, the occurrence detected was 55% for *T. mutans*, 54% for *T. velifera*, 53% for *T. parva*, 53% for *Theileria* sp. (sable), 49% for *Theileria* sp. (buffalo), and 49% for *T. buffeli* (Debeila, 2012). Among African buffaloes from northern Botswana, the occurrence detected was 60% for *T. parva* and 37% for *T. mutans* (Eygelaar et al., 2015). *Babesia occultans* (Piroplasmida: Babesiidae) was detected in 40.6% out of 120 African buffaloes sampled in northern Botswana (Eygelaar et al., 2015).

The genus *Ehrlichia* spp. (Rickettsiales: Anaplasmataceae) comprises tick-borne obligate intracellular Gram-negative bacteria that can cause disease in animals and humans (Diarra et al., 2023). *Ehrlichia ruminantium*, which causes high mortality and extensive economic losses in Africa (Kelly et al., 2011), has been molecularly detected in 6% of African buffaloes from northern Botswana (Eygelaar et al., 2015). Even though *Ehrlichia minasensis* has been detected in cattle from Ethiopia (Hailemariam et al., 2017), Kenya (Peter et al., 2020), and Egypt (AL-Hosary et al., 2020), this agent has never been reported in African buffaloes. Although our research group has detected *Anaplasma marginale*, and genotypes closely related to *Anaplasma platys* and *Anaplasma phagocytophilum* in 72.2%, 10.3%, and 1%, respectively, of African buffaloes sampled in the Marromeu Reserve in Mozambique (Machado et al., 2016), studies on the occurrence of *Ehrlichia* spp. in African buffaloes in this region are nonexistent.

Coxiella burnetii (Legionellales: Coxiellaceae), the causative agent of Q fever, is an obligate intracellular Gram-negative Gammaproteobacterium that infects various cell types (mainly phagocytes) in humans and animals (van Schaik et al., 2013; Abdel-Moein & Hamza, 2017; Eldin et al., 2017). This agent can undergo pseudo-sporulation,

which provides high resistance and persistence in the environment (Bielawska-Drózd et al., 2013). Although inhalation of aerosols contaminated with this agent is considered the main route of transmission, vector-borne transmission by hematophagous ectoparasites is also possible (Eldin et al., 2017). This pathogen is shed into the environment through milk, feces, urine, and especially through birthing products (mainly placenta and parturient fluidw) of animal reservoirs (cattle, sheep, and goats) (Rodolakis, 2009; Angelakis & Raoult, 2010; Guatteo et al., 2011; Eldin et al., 2017). Studies on the detection of *C. burnetii* in African buffaloes are nonexistent.

Given the importance of livestock production in Mozambique, the knowledge on the occurrence and genetic diversity of tick-borne agents in wild ruminants, which may act as reservoirs for these pathogens, is highly relevant for guiding control and prophylactic measures aimed at reducing the economic losses caused by these pathogens in livestock in developing countries. The present study aimed to investigate, using molecular methods, the occurrence of tick-borne bacterial agents (*Ehrlichia* spp. and *C. burnetii*) and protozoa (*Babesia* spp. and *Theileria* spp.) in African buffaloes from Mozambique. In addition, the genetic diversity of *T. parva* was assessed based on distinct molecular markers.

Material and Methods

Ethical aspects

The present study was approved by the Animal Use Ethics Committee of São Paulo State University “Júlio de Mesquita Filho” (UNESP), Jaboticabal Campus (protocols numbers 01952/18 and 003891/23) and was approved by the Animal Use Ethics Committee of University of São Paulo (USP), with the protocol registered under No. 009 on page 03 of book 03 for the use of animals in experimentation.

Sampled species and study area

Blood samples from African buffaloes were collected, by convenience, when Carlos L. Pereira was the Director of Conservation at Gorongosa National Park, Mozambique. Animal handling and welfare during fieldwork with Cape buffaloes were conducted in accordance with the national legislation on the use of animals for research, implemented by the National Administration of Conservation Areas (ANAC) of Mozambique. In 2011, blood samples were collected from 87 African buffaloes (*S. caffer*) in the Marromeu Reserve, Mozambique (18°45'33.1"S 35°57'32.1"E). This reserve is a special buffalo protection area located in the Marromeu district (Sofala Province). The sampled animals were young, male and female, and apparently healthy. Approximately 10 mL of blood were collected from each animal before they were transferred from the Marromeu Reserve (an area where contact with cattle occurred) to Gorongosa National Park (an area without cattle contact). The EDTA blood was mixed (v/v) with ethanol, transported to the laboratory, and subsequently stored at -20 °C until an aliquot (~1 mL) was shipped to Brazil aiming at performing the molecular assays (Machado et al., 2016).

DNA extraction

DNA extraction from the African buffalo blood samples was performed by mixing the samples (v/v) with ethanol and incubating them in lysis buffer (1% SDS, 100 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0, and 350 mg/mL proteinase K) at 37 °C for 18 h, followed by centrifugation at 14,000 g for 5 min (Machado et al., 2016). DNA was purified using the Wizard Purification Systems kit (Promega). The extracted DNA samples had their concentration and 260/280 and 260/230 ratios measured using a spectrophotometer (Nanodrop, Thermo Scientific) and were stored at -20 °C until PCR assays were performed (Machado et al., 2016).

Endogenous PCR control

To rule out the occurrence false-negative results due to the presence of inhibitors, DNA blood samples from African buffaloes were subjected to a conventional PCR (cPCR) targeting the endogenous mammalian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene, following the protocol described by Birkenheuer et al. (2003) (Supplementary File 1). Only samples positive in this PCR assays were further subjected to downstream PCR assays for the selected tick-borne pathogens.

Detection and molecular characterization of *Theileria parva*

DNA blood samples from African buffaloes were subjected to a quantitative real-time PCR (qPCR) targeting the variable V4 region of the *T. parva* 18S rRNA gene (147 bp) (Papli et al., 2011) (Supplementary File 1). The number of DNA copies/ μ L was quantified using a G-block (Integrated DNA Technologies, Coralville, Iowa, USA) containing the *T. parva* target sequence. Serial dilutions were prepared to obtain standards with different concentrations of the target DNA sequence (2.0×10^7 to 2.0×10^0 copies/ μ L). The number of G-block copies was determined using the equation $(X \text{ g}/\mu\text{L DNA} / [\text{G-block size (bp)} \times 660]) \times 6.022 \times 10^{23} \times \text{copies of G-block}/\mu\text{L}$. All DNA samples were initially tested in duplicates. Any duplicates with a Cq difference higher than 0.5 were retested in triplicates. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the formula $(E = 10^{-1/\text{slope}})$ (Bustin et al., 2009). Ultrapure water (Promega) was used as a negative control.

All samples positive in the *T. parva*-qPCR assay were subjected to four cPCR assays for molecular characterization, targeting the *p104* gene (630 bp), a fragment of the conserved region of the transmembrane C-terminal region of the *Tpr1* gene (393 bp), the region encoding the N-terminal end of the *p67* gene (600 bp), and the region encoding the C-terminal end of the *p67* gene (300 bp) (Pienaar et al., 2011) (Supplementary File 1).

Conventional PCR assays for the *Theileria orientalis* complex

DNA blood samples from *S. caffer* were subjected to a cPCR for the detection of *Theileria orientalis* targeting the *mosp* gene (776 bp) (Ota et al., 2009) (Supplementary Material 1). gBlocks (Integrated DNA Technologies, Coralville, IA, USA) containing an insert of the target fragment of the *mosp* gene from *T. orientalis* were used as positive controls. Sterile ultrapure water (Invitrogen, Carlsbad, CA, USA) was used as a negative control.

Nested PCR assays for *Babesia bigemina* and *Babesia bovis*

Samples were subjected to nested (n)PCR assays for the detection of *B. bigemina*, targeting the roptry-associated protein-1 (*rap-1a*) gene (412 bp) (Terkawi et al., 2011), and *B. bovis*, targeting the spherical body protein 2 (*sbp-2*) gene (580 bp) (AbouLaila et al., 2010) (Supplementary File 1). DNA samples from the Jaboticabal strains of *B. bigemina* and *B. bovis* were used as positive controls. Ultrapure water (Promega) was used as a negative control.

PCR assays for *Ehrlichia* spp.

Samples were subjected to a PCR for the detection of *Ehrlichia* spp. targeting the *dsb* gene (378 bp) (Doyle et al., 2005) (Supplementary Material 1). DNA from *E. canis* (Jaboticabal strain) and ultrapure water (Invitrogen) were used as positive and negative controls, respectively.

Specific molecular screening for *E. ruminantium* was performed using a semi-nested PCR (snPCR) targeting the *pCS20* gene (280 bp) (Faburay et al., 2007) (Supplementary Material 1). DNA samples from cattle naturally infected with *E. ruminantium* (Matos et al., 2019) were used as positive controls, whereas ultrapure water (Invitrogen®) was used as a negative control.

qPCR assay for *Coxiella burnetii*

DNA blood samples from *S. caffer* were subjected to a qPCR targeting the repetitive insertion element *IS1111* of *C. burnetii* (295 bp) (Klee et al., 2006). The number of DNA copies/ μ L was quantified using a G-block (Integrated DNA Technologies, Coralville, Iowa, USA) containing the target sequence for *C. burnetii* DNA amplification. A standard curve was included on each plate, following the same criteria described for the *T. parva* qPCR. Ultrapure water (Promega) was used as the negative control for all reactions. *Coxiella burnetii* DNA kindly provided by Prof. Marcelo Labruna (FMVZ-USP) was used as the positive control.

Agarose gel electrophoresis

The products obtained from the cPCR, snPCR, and nPCR assays were subjected to horizontal electrophoresis on a 1.0% agarose gel stained with ethidium bromide (0.5 μ L/mL) in TBE running buffer, pH 8.0 (44.58 mM Tris-base; 0.44 mM boric acid; 12.49 mM EDTA). Electrophoresis was performed at 90 V / 150 mA for 60 minutes. A 100 bp molecular weight marker (Kasvi) was used to determine the size of the amplified products. The results were

visualized and analyzed using a UV transilluminator coupled to an image analysis software system (ChemiDoc MP Imaging System, BIO-RAD).

Purification and sequencing of amplicons

Amplicons were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent kit (Thermo Scientific, San Jose, CA, USA), following the manufacturer's recommendations. Sequencing of the amplified products was performed using an automated technique based on the chain-termination method with dideoxynucleotides (Sanger et al., 1977). The same primers used in the PCR assays were employed for sequencing. Reactions were conducted on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) at the Center for Biological Resources and Genomic Biology (CREBIO), Faculty of Agricultural and Veterinary Sciences – FCAV/UNESP, Jaboticabal.

Phylogenetic, genetic diversity and haplotype network analyses

Analysis of the electropherograms generated by sequencing and the construction of consensus sequences were performed using the Phred-Phrap software version 23 (Ewing & Green, 1998; Ewing et al., 1998), applying a minimum Phred quality score of 20 for base calling. The BLASTn program (Altschul et al., 1990) was used to compare the obtained sequences with those previously deposited in GenBank. Sequences were saved in FASTA format and aligned with other sequences of the same gene retrieved from the GenBank database using the MAFFT software (Kato et al., 2002). FASTA alignments were converted to Nexus format using the Alignment Transformation Environment software (Glez-Peña et al., 2010). Maximum likelihood analyses were performed using the IQ-TREE cluster (Nguyen et al., 2015). The best-fitting evolutionary model was selected using the jModelTest 2 software (Guindon & Gascuel, 2003; Darriba et al., 2012). Clade support for maximum likelihood analyses was assessed through bootstrap analyses (Felsenstein, 1985) with 1,000 replicates. Editing and rooting (via outgroup) of the phylogenetic trees were performed using Treegraph 2.0.56-381 beta (Stöver & Müller, 2010).

The DnaSP software version 5.10.1 (Librado & Rozas, 2009) was used to calculate the number of *T. parva* haplotypes for the *p104* and N-terminal region of the *p67* gene, as well as nucleotide diversity (π), the level of polymorphism (haplotype diversity – [dh]), the number of haplotypes (h), and the mean number of nucleotide differences (K) among the sequences obtained.

Sequences obtained for the *p104* and *p67* (N-terminal region) genes of *T. parva*, along with those previously deposited in GenBank, were analyzed using PopART ("Population Analysis with Reticulate Trees") (Leigh & Bryant, 2015). Sequences were grouped according to their locality in DnaSP version 5.10.1 (Librado & Rozas, 2009) and saved in NEXUS format. The NEXUS file was then imported into PopART, and genotype networks were inferred using the Median-Joining method described by Bandelt et al. (1999).

Results

All African buffalo blood DNA samples tested positive in the cPCR assay targeting the mammalian *gapdh* gene.

Molecular detection and genetic diversity of *Theileria parva*

A total of 35/87 (40.2%) African buffalo blood samples tested positive in the qPCR assay targeting the variable V4 region of the *T. parva* 18S rRNA gene. Absolute quantification of *T. parva* 18S rRNA DNA ranged from 4.4×10^{-1} to 9.91×10^3 copies/ μ L. Amplification efficiencies ranged from 91.6% to 92.5%; R^2 values ranged from 0.992 to 0.999; slope varied between –3.516 and –3.541; and y-intercept ranged from 36.401 to 37.919.

In the PCR assays for *T. parva* molecular characterization, among the 35 positive samples from the initial screening, 23 (65.7%) were positive for the *p104* gene; 31 (88.6%) were positive in the PCR targeting the conserved region of the C-terminal transmembrane domain of the *Tpr1* gene; 22 (62.8%) were positive in the PCR based on the C-terminal region of the *p67* gene; and 18 (51.4%) samples were positive in the PCR targeting the N-terminal region of the *p67* gene. The BLASTn analysis results for the *Tpr1*, *p104*, *p67* C-terminal region, and *p67* N-terminal region sequences of *T. parva* obtained from the African buffaloes are presented in Supplementary File 2.

The phylogenetic analysis performed with seven *T. parva* *p104* sequences obtained in the present study, along with additional sequences retrieved from GenBank, showed that the identified sequences were positioned in four groups (Figure 1). One sequence (buffalo 84 – PV994212) obtained in this study clustered in a sister clade to *T. parva*

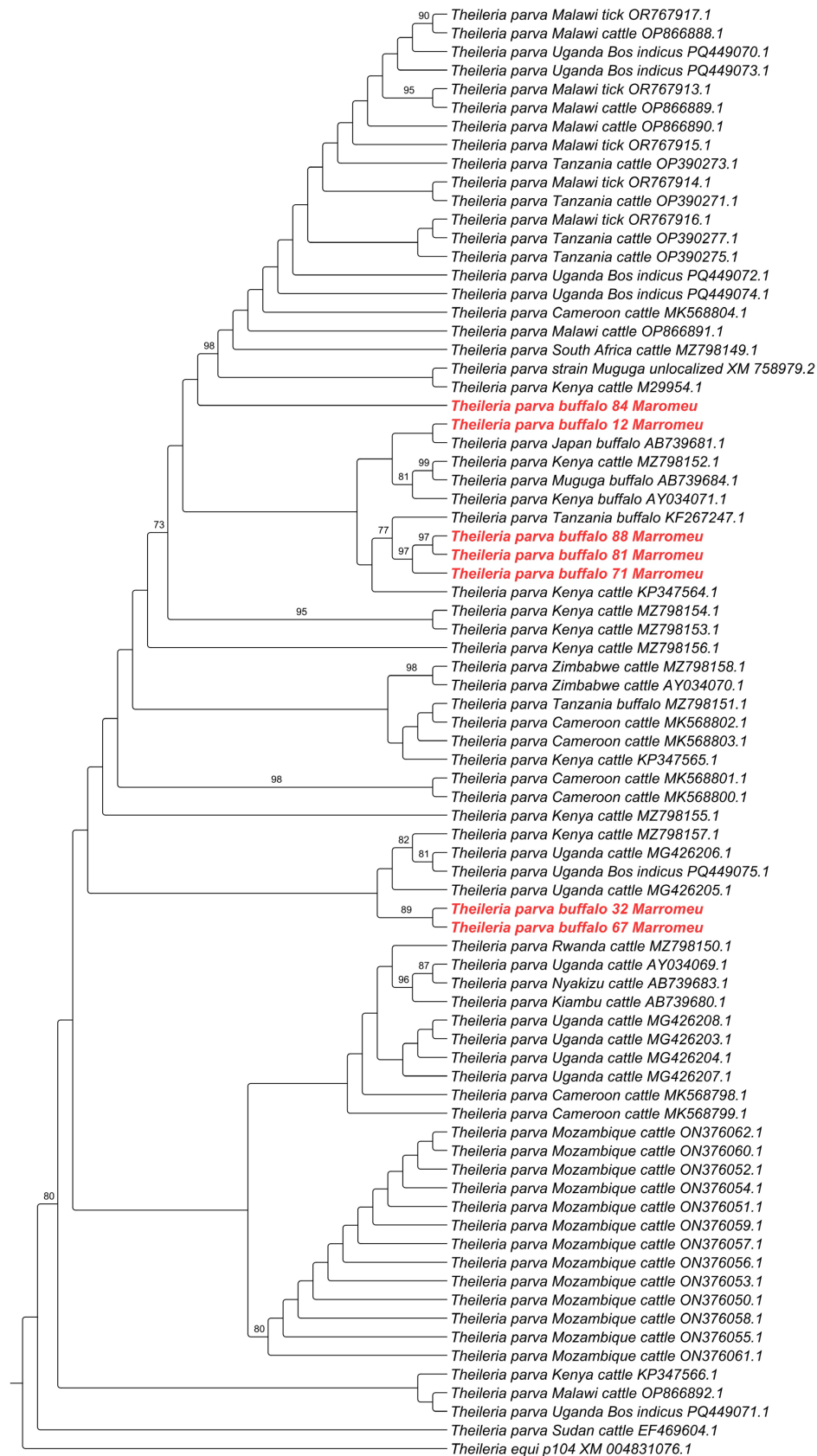


Figure 1. Phylogenetic tree based on an alignment of *T. parva* sequences of the *p104* gene (1,050 characters after alignment, due to the presence of gaps). The tree was inferred using the Maximum Likelihood (ML) method and the HKY+F+G4 evolutionary model. Sequences detected in the present study are highlighted in red. Only bootstrap values >70 were shown on the tree. A *Theileria equi* (XM004831076.1) sequence was used as an outgroup.

sequences from cattle and ticks from different African countries. Another sequence (buffalo 12 – PV994207) grouped within the same clade as *T. parva* previously detected in African buffalo in Kenya. Three sequences (buffaloes 88, 81, and 71 – PV994210, PV994211 and PV994213) clustered together in a clade that was sister to a sequence obtained from a African buffalo from Tanzania. The remaining two sequences (buffaloes 32 and 67 – PV994208 and PV994209) clustered in the same clade, which was sister to *T. parva* detected in cattle from Kenya and Uganda.

The phylogenetic analysis performed with eight *T. parva* *p67* gene sequences obtained in the present study together with additional sequences retrieved from GenBank showed that the sequences identified here were positioned in different groups (Figure 2). Two sequences from this study (buffaloes 50 and 82), positioned into allele type 1, group 2, clustered together in a single clade, which was recovered as a sister clade to *T. parva* sequences detected in cattle and African buffaloes from South Africa and Mozambique, respectively. Another sequence from this study (buffalo 88), positioned into allele type 1, group 1, clustered alone in a distinct clade, which showed to be a sister clade to *T. parva* sequences detected in cattle and African buffaloes from Africa. Two sequences (buffaloes 25 and 64), positioned into allele type 2, group 2, also clustered together in a single clade. This clade formed a sister group to *T. parva* sequences detected in African buffaloes from Mozambique and South Africa, and showed to be a sister to two other sequences obtained in the present study (buffaloes 9 and 48a). Another sequence obtained in this study (buffalo 96), positioned into allele type 1, group 3, clustered alone in a single clade, which showed to be a sister clade to several clades comprising *T. parva* sequences detected in cattle and African buffaloes from Africa.

The haplotype analysis of the *p104* gene performed with 69 sequences, including seven sequences detected in the present study, identified 33 different haplotypes. Haplotypes #1, #4, #5, #7, #8, #9, #13, #24, #26, and #32 were composed of more than one sequence. The sequences obtained in the present study were represented by haplotypes #13, #14, #15, #16, and #17 (Figure 3). Furthermore, some of the detected haplotypes were composed of sequences identified in more than one location, namely haplotypes #4, #5, #7, #9, #24, and #26 (Figure 3). The haplotypes detected in the present study clustered either with other haplotypes also detected in the present study or appeared alone, indicating that the haplotypes identified have not been previously detected in cattle or African buffaloes from other regions.

The haplotype analysis of the *p67* gene performed with 84 sequences, including seven sequences detected in the present study, identified 36 different haplotypes. Haplotypes #1, #5, #12, #15, #19, #21, #28, #31, and #36 were composed of more than one sequence. The sequences obtained in the present study comprised the following

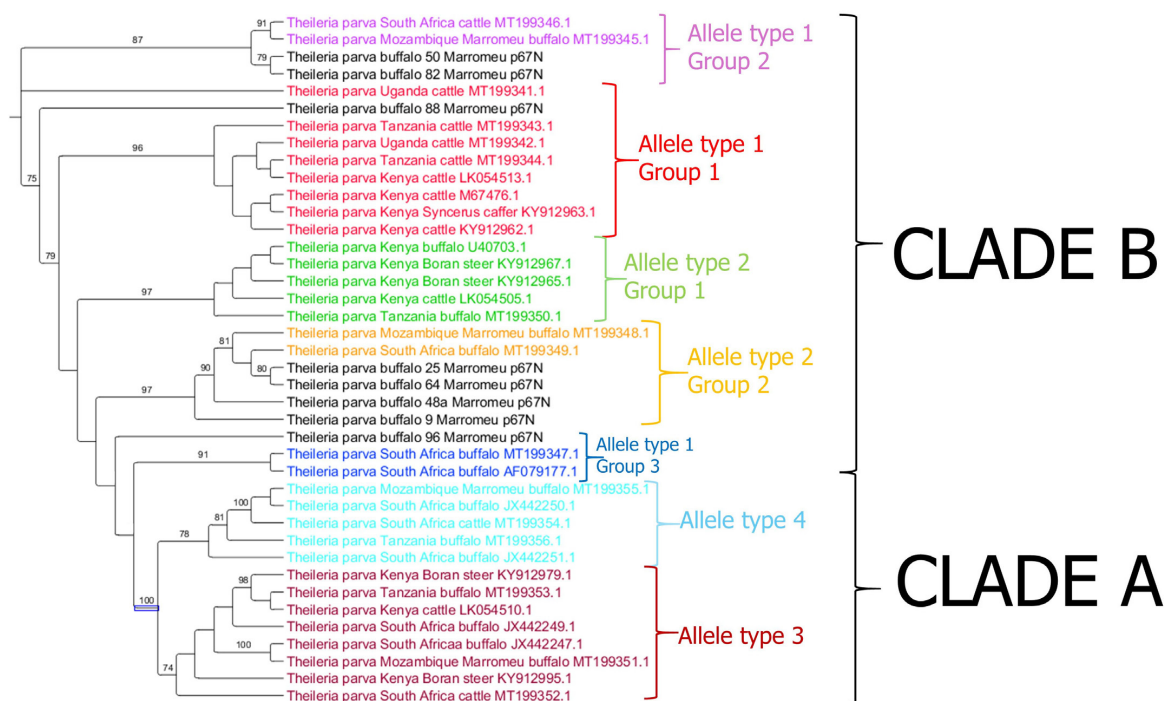


Figure 2. Phylogenetic tree based on an alignment of *T. parva* sequences using the *p67* gene (757 bp after alignment). The tree was inferred using the Maximum Likelihood (ML) method and the HKY+F+G4 evolutionary model. The sequences detected in the present study are highlighted in black, underlined, and in bold. Only bootstrap values >70 were shown on the tree.

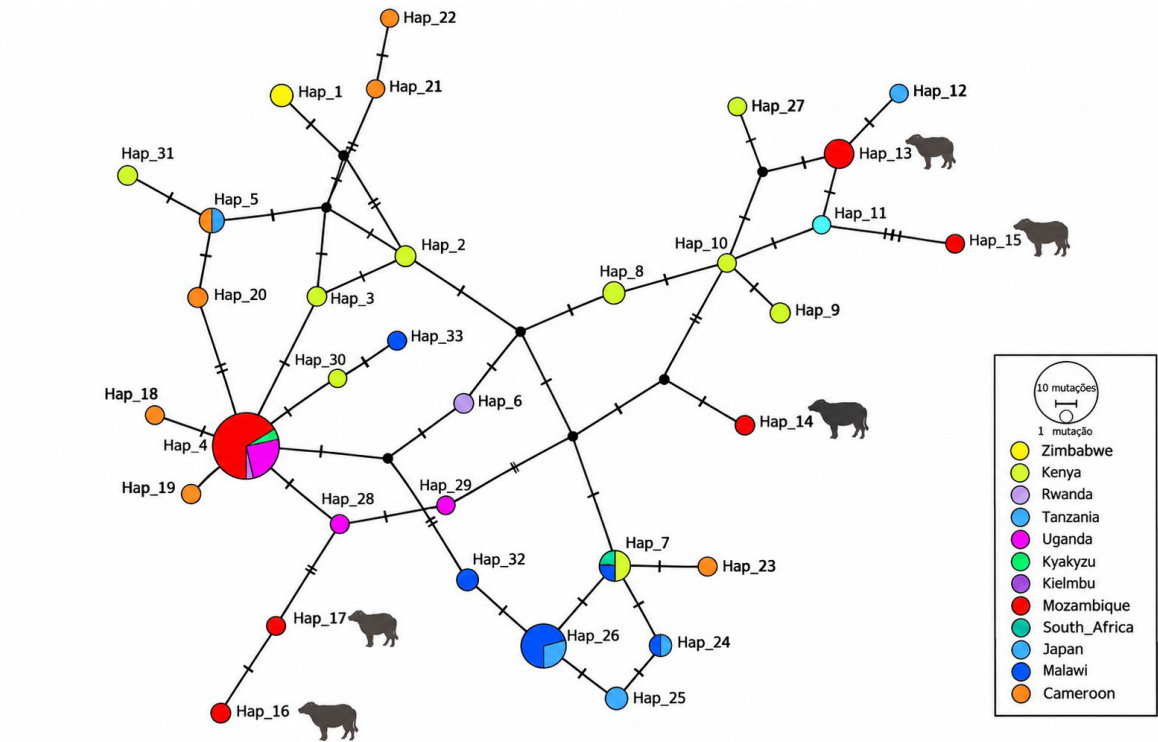


Figure 3. Haplotype diversity of the *p104* gene of *Theileria parva* detected in the present study compared with those previously deposited in GenBank. The analysis was performed using the DnaSP6 software. Haplotype inference and graphical representation were generated using the TCS method implemented in PopART. The haplotypes identified in the present study are highlighted with a African buffalo icon.

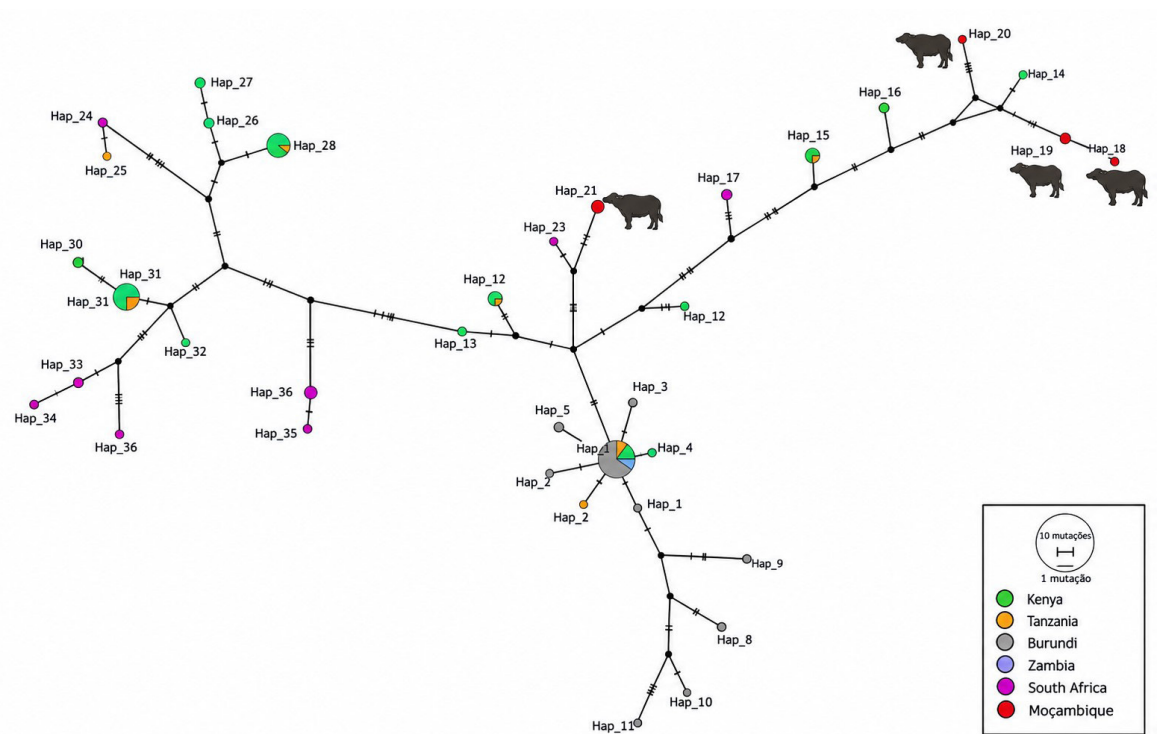


Figure 4. Haplotype diversity among *Theileria parva* *p67* gene sequences detected in the present study compared with those previously deposited in GenBank. The analysis was performed using DnaSP6. Haplotype inference and graphical representation were carried out using the TCS network method implemented in PopART. The haplotypes identified in the present study are highlighted with a buffalo icon.

haplotypes: #18, #19, #20, and #21 (Figure 4). In addition, some haplotypes were formed by sequences detected in more than one locality, namely haplotypes #1, #12, #15, #28, and #31 (Figure 4). The haplotypes detected in the present study were grouped with other haplotypes also detected in the present study or appeared alone,

Table 1. Genetic diversity and polymorphisms of the *Theileria parva* p104 and p67 gene sequences detected in African buffaloes from Mozambique.

Gene	pb	N	VS	GC%	g	Gd (average ± DP)	π (average ± SD)	K
p104	255	69	24	43,8	33	0,904±0.029	0,01921±0.00119	4,82182
p67	253	84	89	39,6	36	0,910±0.022	0,09370±0.00488	17,70884

N = number of sequences analyzed; VS = number of variable sites; GC% = G+C content; g = number of haplotypes; Gd = haplotype diversity; DP = standard deviation; π = nucleotide diversity (per site); K = average number of nucleotide differences.

indicating that the haplotypes identified here have not been previously detected in cattle or African buffaloes from other regions.

The results of the genetic diversity analyses conducted with the p104 and p67 gene sequences of *T. parva* can be found in Table 1.

Molecular detection of *Ehrlichia* spp., *Ehrlichia ruminantium*, *Theileria orientalis*, *Babesia bigemina*, *Babesia bovis* and *Coxiella burnetii*

All African buffalo blood DNA samples tested negative in the molecular assays for the detection of *Ehrlichia* spp. (*dsb*), *E. ruminantium* (*pCS20*), *T. orientalis* (*mpsp*), *B. bigemina* (*rap-1a*), *B. bovis* (*sbp-2*), and *C. burnetii* (*IS1111*).

Discussion

This study found a molecular occurrence of 40.2% for *T. parva* among African buffaloes sampled in the Marromeu Reserve, Mozambique, corroborating the results of previous studies conducted in Uganda (43.4%) (Oura et al., 2011) and in the Kruger National Park, South Africa (45.9%) (Chaisi et al., 2013). On the other hand, a molecular occurrence of 83.5% was found among African buffaloes sampled across several parks in South Africa and in the Greater Limpopo Transfrontier Game Park (GLTP) in Mozambique (Chaisi et al., 2013). In addition, 60% and 53% African buffaloes from Botswana (Eygelaar et al., 2015) and Hluhluwe-iMfolozi Park, South Africa (Debeila, 2012), were positive for *T. parva*.

In African wildlife–livestock interface regions where only cattle were treated with acaricides, *T. parva* continued to pose a problem for cattle (Walker et al., 2003). Significantly higher levels of antibodies against *T. parva* were found in cattle in unfenced areas compared to animals kept in locations with physical separation between wildlife and cattle in Zimbabwe (Caron et al., 2013). According to Jori et al. (2013), *T. parva* is less likely to be transmitted when African buffaloes are physically separated from cattle. Therefore, future surveillance efforts should be directed primarily toward cattle located in areas without physical separation from African buffaloes (Jori et al., 2013).

The different positivity rates observed among the four PCR assays used for additional molecular characterization (88.6% for *Tpr1*, 65.7% for *p104*, 62.8% for *p67-C*, and 51.4% for *p67-N*) are therefore not unexpected, as amplification success depends on the specific target region and the analytical sensitivity of each molecular assay. In particular, the screening qPCR targets a relatively short fragment (147 bp) of the variable V4 region of the *T. parva* 18S rRNA gene, whereas the PCR assays aiming at additional molecular characterization assays require amplification of substantially larger fragments (300–630 bp). Samples containing relatively low amounts of parasite DNA, or DNA with some degree of degradation, might therefore remain detectable by the short qPCR due to the high sensibility, while failing to produce a detectable product for one or more of the larger conventional PCR targets.

Five *T. parva* haplotypes (obtained from seven sequences) for the p104 gene and four haplotypes (obtained from eight sequences) for the p67 gene were identified in the present study. When analyzed together with sequences previously deposited in GenBank, Hd values of 0.904 and 0.910 were observed, demonstrating high genetic diversity for *T. parva* based in these two molecular markers in Africa. The p104 and p67 haplotypes of *T. parva* detected in

the African buffaloes from Marromeu have not been previously detected in African buffaloes and cattle from other locations. The *p104* haplotypes detected in African buffaloes sampled in Marromeu were not shared with those previously described in cattle from Mozambique.

The present study corroborates previous studies that demonstrated genotypic differences between *T. parva* isolates derived from African buffaloes and those from cattle (Nene et al., 1996), showing that *T. parva* isolates from the former exhibit higher genetic diversity than those isolated from the latter (Bishop et al., 1994; Oura et al., 2011). These findings can be explained by the fact the *S. caffer* represent the natural and ancestral reservoir of this apicomplexa protozoa. This long-term host–parasite association has allowed the accumulation and maintenance of multiple *T. parva* haplotypes, resulting in high genetic heterogeneity in African buffalo-derived isolates, which are often associated with subclinical infections (Morrison et al., 2020). In contrast, *T. parva* isolates infecting cattle represent a restricted subset of this diversity, likely arising from spillover events from the sylvatic cycle, followed by genetic bottlenecks and host-driven selection. This reduced diversity in cattle reflects a more recent host adaptation and has been frequently associated with increased pathogenicity, as cattle have not coevolved with African buffalo-derived genotypes (Morrison et al., 2020). The extensive genetic diversity maintained in African buffalo populations also has important implications for disease control, as it may compromise vaccine efficacy and facilitate the emergence of novel variants capable of infecting cattle, particularly in areas where wildlife–livestock interfaces occur (Choopa et al., 2025). While *T. parva* isolates from cattle cause a disease known as East Coast Fever (ECF) in cattle, those derived from African buffaloes cause a disease known as Corridor Disease (CD). These diseases differ in severity and lethality: whereas CD is characterized as an acute infection in cattle, leading to death in a short period of time, ECF is a milder, chronic disease and not as lethal as CD (Cook et al., 2021).

In the present study, the sequences detected in African buffaloes clustered within group 1, allele type 1; group 2, allele types 1 and 2; and group 3, allele type 1. Previous studies based on sequence polymorphisms in the central variable region of the *p67* gene from *T. parva* isolates derived from cattle and African buffaloes detected the presence of four related *p67* allele groups, designated as groups 1, 2, 3, and 4. The different allele types are distinguishable based on two indels. The *p67* gene of *T. parva* isolates from African buffaloes is characterized by the presence of four alleles, whereas isolates from cattle possess only one allele, known as allele 1. These findings demonstrate higher genetic diversity in *T. parva* isolates from African buffaloes compared with those from cattle (Mukolwe et al., 2020).

Although the *mpsp* gene is the most commonly molecular marker used in the molecular screening for *T. orientalis* complex, we did not detect DNA of these protozoa in the present study. Nevertheless, previous studies have documented substantial genetic diversity within the *T. orientalis/T. buffeli* complex infecting water buffaloes and African buffaloes across different geographic regions. In southeastern Asia, the N-1 genotype has been reported in water buffaloes (Sansamur et al., 2025), whereas the N-2 genotype has been identified in water buffaloes from southern Asia (Vinodkumar et al., 2016). In addition, genetically diverse *T. orientalis/T. buffeli*-related taxa have been detected in African buffaloes in southern Africa (Chaisi et al., 2013). In northern Brazil, the N-1, N-2, and N-4 genotypes have also been reported in water buffaloes (Alves et al., 2026).

The absence of *T. orientalis* complex DNA in African buffalo blood samples the present study may be related to a genuinely low molecular occurrence, limited geographical distribution, low parasitemia or absence of competent tick vectors in the studied area. In addition, the conventional PCR assay targeting a single molecular marker might have limited capacity to detect low abundant, divergent, mixed or previously undescribed genotypes (Chaisi et al., 2013). The use of next-generation sequencing of PCR amplicons by Illumina platform should be used in the future to unravel the genetic diversity, epidemiology, and population biology of the *T. orientalis* complex among African buffaloes.

Babesia bigemina and *B. bovis* were not molecularly detected in the African buffaloes sampled in the present study, corroborating the results of previous works conducted in Botswana (Penzhorn, 2006) and Uganda (Oura et al., 2011). On the other hand, *B. occultans* and *B. bovis* were detected using the Reverse Line Blot technique in 40.6% and 3.6% of African buffaloes sampled in Botswana (Eygelaar et al., 2015). When three African buffaloes were inoculated with a pathogenic cattle-derived isolate of *B. bigemina*, none showed clinical signs of the disease, although the parasite was detected in blood smears for four days, indicating its multiplication. The carrier state for *B. bigemina* was demonstrated in two African buffaloes for 28 and 147 days, respectively, through sub-inoculation into splenectomized calves; however, no parasites were found after 28 days post-inoculation in the third African buffalo (Karbe et al., 1979). Future studies should be conducted to investigate the innate resistance of this wild ruminant species to infestations by tick vectors and infections by *B. bovis* and *B. bigemina* (Oura et al., 2011), as has been observed in Asian water buffaloes (*B. bubalis*) (Benitez et al., 2018). Although African buffaloes exhibit innate

resistance to infestations by *Rhipicephalus microplus*, in some cases these animals can still be found infested by this tick species (Iseki et al., 2007). In Mozambique, *R. appendiculatus*, *R. decoloratus*, and *R. zambeziensis* have been previously found parasitizing African buffaloes (Moyo et al., 2018).

In the present study, all African buffalo blood DNA samples tested negative in the PCR assay for *Ehrlichia* spp. based on the *dsb* gene, which has been used for the detection of *E. minasensis* (André et al., 2020; Osman et al., 2025). In Africa, this agent has been detected in cattle from southwestern Ethiopia (Hailemariam et al., 2017), Kenya (Peter et al., 2020), Egypt (AL-Hosary et al., 2020), in ticks collected from cattle, sheep, and goats in Ghana (Addo et al., 2023), and more recently in camels, goats, and sheep from Somalia (Osman et al., 2025). The lack of detection of *E. minasensis* in African buffaloes sampled in the present study might be associated with the innate resistance of these animals to infestation by *R. microplus*, the tick incriminated as the vector for this agent, as recently suggested for Asian water buffalo sampled in southeastern Brazil (Secato et al., 2025).

None of the sampled African buffaloes tested positive in the snPCR assay for *E. ruminantium*, corroborating the results obtained by by Oura et al. (2011) in Uganda. On the other hand, Eygelaar et al. (2015) found a 6% occurrence of *E. ruminantium* in African buffaloes in northern Botswana. Unlike cattle, African buffaloes appear not to maintain *E. ruminantium* infections (Henrichs et al., 2016). These animals have developed immunological resistance and coadaptation over millennia of coexisting with the bacterium (Peter et al., 2002; Allsopp, 2010). The African buffalo's immune system eliminates the severe form of the disease and restricts the pathogen to the vascular endothelium of internal organs (Peter et al., 2002; Pfitzer et al., 2004). As a consequence, the bacterium rarely circulates in peripheral blood and maintains an extremely low bacterial load (subclinical infection) (Peter et al., 2002; Allsopp, 2010). This biological behaviour hampers conventional PCR and blood smears from detecting the presence of the agent, yielding false-negative results in animals that serve merely as asymptomatic carriers (Allsopp, 2010; Kasari et al., 2010).

The absence of *C. burnetii* positivity in the present study could be explained by the use of blood samples instead of feces, urine, saliva, vaginal secretions, placenta, amniotic fluid, and milk, biological materials in which infected animals shed the infectious agent (Abiri et al., 2019). Indeed, reproductive secretions and milk are considered better biological samples for the detection of *C. burnetii*, playing an important role in its transmission and dissemination (Berri et al., 2000). Additionally, the transmission of *C. burnetii* is highly influenced by landscape, climate, animal movement, and high population density (Nusinovici et al., 2015). Future studies should be conducted aiming to investigate the occurrence of *C. burnetii* in rectal and vaginal swabs from African buffaloes.

Finally, some limitations of the present study should be acknowledged. Unfortunately, ectoparasites were not collected from African buffaloes at the time of sampling, which would have contributed to a better understanding of the epidemiology of tick-borne pathogens studied herein. Noteworthy, archived whole blood samples are often collected opportunistically or during specific research or management activities (the translocation of African buffaloes in this study in particular) rather than through systematic surveillance, which may limit the representativeness of the study population. Archived samples reflect the epidemiological situation at the time of collection and may not accurately represent the current occurrence or distribution of tick-borne agents. Nonetheless, the availability of archived blood samples from *S. caffer* constitutes a valuable resource for epidemiological investigations of tick-borne agents, as molecular screening can be performed without the need for additional field sampling, which is often limited by the technical complexity, high costs, logistical constraints, and risks associated with capturing and immobilizing these wild animals.

Conclusion

The high molecular occurrence and genetic diversity of *T. parva* in African buffaloes sampled in the Marromeu Reserve, Mozambique, reflects the important role these animals play as reservoirs for this agent. The *p104* and *p67* haplotypes of *T. parva* detected in African buffaloes from Marromeu were not shared with those previously reported in African buffaloes and cattle from other locations.

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Data availability

All data generated in this study are included in this published article. The sequences of *Theileria parva* have been deposited in Genbank under Accession numbers PV994207; PV994208; PV994209; PV994210; PV994211; PV994212; PV994213.

Ethics declaration

This study was approved by the Ethics Committee on Animal Use of the São Paulo State University “Júlio de Mesquita Filho” (UNESP) – Jaboticabal campus (protocols numbers 01952/18 and 003891/23) and was approved by the Animal Use Ethics Committee of University of São Paulo (USP), with the protocol registered under No. 009 on page 03 of book 03 for the use of animals in experimentation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

Caroline Tostes Secato: writing – review & editing, writing – original draft, visualization, validation, software, methodology, investigation, formal analysis, data curation, conceptualization. Marta Maria Geraldine Teixeira: writing – review & editing, methodology, investigation, formal analysis, data curation, conceptualization. Ana Cláudia Calchi: writing – review & editing, software, methodology. Abraão Isaque da Silva: writing – review & editing, writing – original draft, visualization, validation, methodology, investigation, data curation, conceptualization. Carlos Lopes Pereira: writing – review & editing, validation, methodology. Rosângela Zacarias Machado: writing – review & editing, supervision, resources, project administration, investigation. Marcos Rogério André: writing – review & editing, writing – original draft, validation, supervision, resources, project administration, investigation, funding acquisition, data curation, conceptualization.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary File 1. Description of the molecular assays (target gene, identification and sequence of primers/probes, thermal conditions, amplicon size, and reference) used for the endogenous gene, screening and characterization of tick-borne agents investigated in the present study.

Supplementary File 2. BLASTn results of the sequences of the Tpr1 and p104 genes, and the C-terminal and N-terminal regions of the p67 gene of *Theileria parva* detected in buffaloes from Marromeu, Mozambique.

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