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PROTOZOOLOGY - ORIGINAL PAPER

Genetic diversity of *Babesia bovis* in beef cattle in a large wetland in Brazil

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Abstract

Babesia bovis is the etiological agent of bovine babesiosis, a disease transmitted by *Rhipicephalus microplus*, which affects cattle herds in tropical and subtropical regions of the world, causing significant economic losses due to decreasing meat and milk yield. This study used molecular techniques to determine the occurrence and genetic diversity of *B. bovis*, based on the genes encoding the spherical body protein (*sbp-2*) and the merozoite surface antigens (MSAs) genes, in a herd of 400 Nellore (*Bos indicus*) sampled from beef cattle farms in the Pantanal region, state of Mato Grosso do Sul, Midwestern Brazil. The results of the nested PCR assays based on the *sbp-2* gene indicated that 18 (4.5%) calves were positive for *B. bovis*; out of them, while 77.7% (14/18) were positive for the *B. bovis msa-2b* fragment, 66.6% (12/18) were positive for the *msa-2c* fragment. The phylogenetic analysis based on the maximum likelihood method using 14 sequences from *msa-2b* clones and 13 sequences from *msa-2c* clones indicated that the sequences detected in this study are clearly distributed in different cladograms. These findings corroborated the diversity analysis of the same sequences, which revealed the presence of 14 and 11 haplotypes of the *msa-2b* and *msa-2c* genes, respectively. Furthermore, the entropy analyses of amino acid sequences revealed 78 and 44 high entropy peaks with values ranging from 0.25 to 1.53 and from 0.27 to 1.09 for MSA-2B and MSA-2C, respectively. Therefore, the results indicate a low molecular occurrence of *B. bovis* in beef cattle sampled in the Brazilian Pantanal. Despite this, a high degree of genetic diversity was found in the analyzed *B. bovis* population, with possibly different haplotypes coexisting in the same animal and/or in the same studied herd.

Keywords Bovine babesiosis · *Babesia bovis* · Genetic diversity · MSA · Pantanal

Introduction

Bovine babesiosis, transmitted by *Rhipicephalus microplus* Canestrini 1888 (Acari: Ixodidae), is a hemoparasitosis caused by the protozoa *Babesia bovis* and *Babesia bigemina*. This disease affects cattle herds in tropical and subtropical regions of the world, causing significant economic losses that result from decreasing meat and milk yields. In Brazil, it is considered an endemic disease, with seroprevalence ranging from 56.4 to 97.9% and reaching annual economic losses of about 3.5 million dollars, especially in areas of enzootic instability (Trindade et al. 2010; Grisi et al. 2014). In addition to production drop, the expenses for treating clinically affected animals and for controlling the disease vector add to the economic losses caused by the disease, making it one of the limiting factors for improving cattle productivity in tropical and subtropical areas (Rodrigues et al. 2005; Taylor et al. 2010).

The Brazilian Pantanal covers an area of 140,000 km^2 shared between the States of Mato Grosso and Mato Grosso do Sul. The Pantanal region is considered one of the largest wetland systems in the world, being internationally recognized for its exuberance and richness of biodiversity

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(Alvarenga 1980; Junk et al. 2006). Some species of ticks in the region share wild and domestic animals as hosts (Bechara et al. 2000; Ramos et al. 2016). The main economic activity of the region is the extensive production of beef cattle, with a herd estimated at four million animals (Abreu et al. 2008).

The *B. bovis* merozoites have, on their surface, at least five glycoproteins belonging to the family of variable merozoite surface antigens (VMSA), involved in the hemoparasite invasion of the erythrocytes. The family of the variable merozoite surface antigen of the *B. bovis* includes the *msa-1* gene and the *msa-2* loci. While the *msa-1* is a single genome copy gene, the *msa-2* comprises four tandem genes, defined as *msa-2a1*, *msa-2a2*, *msa-2b*, and *msa-2c* (Florin-Christensen et al. 2002). These antigens are highly immunogenic and contain neutralizing-sensitive epitopes, and for this reason, these have been considered as candidates for the development of *B. bovis* vaccines. However, the high genetic diversity of such surface antigens among the different *B. bovis* isolates (Hines et al. 1992) is considered the main difficulty for developing methodologies for the immune control against *B. bovis* infection (Tattiyapong et al. 2016). In this context, the *msa* genes may represent genetic markers for studying the genetic diversity of *B. bovis* (Timms et al. 1990; Genis et al. 2009; Altangerel et al. 2012).

Previous studies using distinct geographical isolates of *B. bovis* as well as isolates from outbreaks and vaccine samples have shown that polymorphism of essential antigenic components is responsible for vaccine failure (Berens et al. 2005; Bock et al. 1992). Although several diversity studies using the *msa* gene family have been conducted worldwide (Genis et al. 2009; Lau et al. 2010; Altangerel et al. 2012; Simking et al. 2013; Sivakumar et al. 2013; Tattiyapong et al. 2014; Molad et al. 2014; Nagano et al. 2013; Matos et al. 2017), little is known on the genetic variability of *B. bovis* isolates in Brazil. To date, only three studies have evaluated the genetic diversity of *B. bovis* in Brazil. While one of these studies indicated low genetic diversity among *B. bovis* isolates from the states of Bahia, São Paulo, Rio Grande do Sul, Mato Grosso do Sul, and Rondônia, other studies showed high genetic diversity among samples from the states of Bahia and Sao Paulo, especially between the sequences of the *msa-2b* and *msa-2c* genes (Ramos et al. 2012; Nagano et al. 2013; Matos et al. 2017).

Considering the scarcity of studies investigating the genetic diversity of *B. bovis* in Brazil, coupled with the fact that Brazil is one of the largest exporters of animal products to the world, the present study aimed to investigate the occurrence and genetic diversity of *B. bovis* in a population of beef cattle sampled in farms in the Brazilian Pantanal. The results are expected to improve the knowledge on the genetic structure of the *B. bovis* population, as well as the selection efforts of MSAs suitable for the development of vaccines and the understanding of the parasite escape mechanisms to the immune response.

Material and methods

Research authorization

This research followed the Ethical Principles on Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Estadual Paulista "Júlio de Mesquita Filho" (Protocol 12375/15).

Study site and blood sampling

Blood samples were collected from Nellore herds of *Bos indicus* from five beef cattle farms in the central Pantanal Region, Nhecolândia sub-region (18° 59′ 15″ S, 56° 37′ 03″) in MS, Brazil (Fig. 1). As there is no data available on the *B. bovis* prevalence in this state, an expected prevalence of 50% was assumed, according to Stevenson (2005). The sample size was determined by the systematic random sampling method, where the minimum number of animals had 5% absolute precision and 95% confidence interval, as indicated by the formula below:

$$n = z^2(1\text{–Py}) \times \text{Py}/d^2$$
$$n = 3,84\text{–}192,08.\ 50/25n = 384;\ \text{approximately 400 animals}$$

where

z	coefficient of confidence ($z = 1.96$)
n	sample size
Py	expected prevalence (50%)
d^2	desired absolute precision (5%)

Blood samples were collected from 400 apparently healthy animals in two categories as follows: cow (> 6 years) and calves (7–12 months). All farms presented animals intensely or moderately infested with *Rhipicephalus microplus* ticks (data not shown), which were identified using previously described taxonomic keys (Barros-Battesti et al. 2006; Nava et al. 2014). Unfortunately, systematic counts of ticks were not performed. Macrocyclic lactones (avermectins) were widely used for the control of ticks. Although the animals were apparently healthy, accurate physical examinations were not performed. Microhematocrit analysis (Douglas and Wardrop 2010) showed that all sampled animals showed HT values higher than 24%, except for one cow that showed to be negative in the nested PCR for *B. bovis*.

Blood samples from 400 animals (200 cows and 200 calves) were collected directly from the jugular or caudal veins in two different months: August 2016 (261 sampled animals) and April 2017 (139 sampled animals). The selected

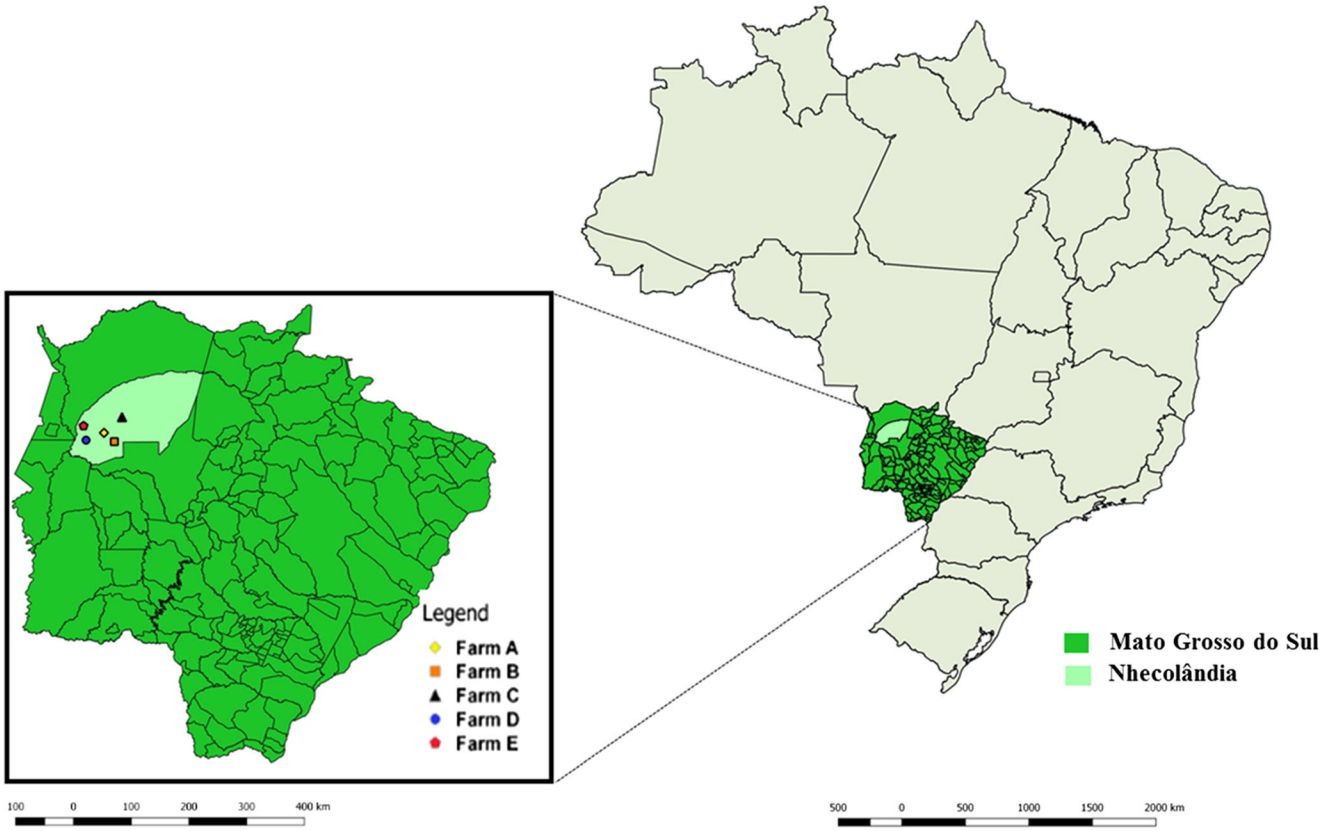

Fig. 1 Map showing the Nhecolândia sub-region in the central Pantanal region in Mato Grosso do Sul in Brazil, and the farms where the blood samples were collected from beef cattle

animals were not sampled twice. Ten milliliters of blood samples was collected from each animal. The samples used in the molecular tests for DNA extraction were collected in EDTA-containing tubes and stored at − 70 °C until the DNA extraction.

Molecular assays

DNA extraction and endogenous reaction control

DNA was extracted from 250 μL of whole blood in EDTA following the genomic DNA isolation protocol previously described by Kuramae-Izioka (1997). To check for DNA purity and concentration, the 260/280 and 260/230 ratios, the DNA samples were evaluated in a spectrophotometer (Nanodrop-Thermo Scientific) and were stored at − 20 °C for subsequent PCR assays. To confirm the presence of amplifiable DNA in each sample and discard the occurrence of false negative results due to problems in the extraction of DNA, each one was used as template in a PCR internal control assay based on the mammals *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene, as described by Birkenheuer et al. (2003). All of the primers pairs used are listed in Table 1.

PCR screening for *Babesia bovis* based on the *sbp-2* (spherical body protein) gene

The positive samples in the abovementioned conventional PCR on the *gapdh* gene were submitted to the nested PCR for *B. bovis* based on the *sbp-2* spherical body protein gene, as described by Aboulaila et al. (2010). The fragment amplified in the second reaction is approximately 600 bp in size. The DNA sample of *B. bovis* obtained from an experimentally infected calf blood (Machado et al. 1997) and ultra-sterile water were used as positive and negative controls, respectively.

Babesia bovis amplification reactions directed to the *msa-2b* and *msa-2c* (merozoite surface antigen) genes

The molecular characterization and genetic diversity study of *B. bovis* was performed in the *sbp-2*-PCR-positive DNA samples using conventional PCR assays targeting the *msa-2b* and *msa-2c* genes as previously described (Tattiyapong et al. 2014). The DNA sample of *B. bovis* obtained from an experimentally infected calf blood (Machado et al. 1997) and ultrapure sterilized water were used as positive and negative controls, respectively.

Table 1 Sequence of the primers used in the PCR assays, the size of the respective products, and references

Gene	Sequence	Product size	Reference
gapdh (glyceraldehyde-3-phosphate dehydrogenase)	Forward (5′-CCTTCATTGACCTCAACTACAT-3′) Reverse (5′-CCAAAGTTGTCATGGATGACC-3′)	Approximately 450 bp	(Birkenheuer et al. 2003).
sbp-2 spherical body protein	F (5′-CCGAATTCCTGGAAGTGGATCTCATGCAACC-3′) R (5′-ATCTCGAGTCACGAGCACTCTACGGCTTTGCA G-3′) nested F (5′-CGAATCTAGGCATATAAGGCAT-3′) nested R (5′-ATCCCCTCCTAAGGTTGGCTAC-3′)	Approximately 600 bp	(Aboulaila et al. 2010).
msa-2b merozoite surface antigen	F 5′-ATGATCGGGAAAATCTTCTTGTTAA-3′ R 5′-TTAAAATGCAGAGAGAACGAAGTAGC-3′	780–843 bp	(Altangerel et al. 2012; Sivakumar et al. 2013)
msa-2c merozoite surface antigen	F 5′-ATGGTGTCTTTTAACATAATAACC-3′ R 5′-TTAAAATGCAGAGAGAACGAAGTAGC-3′	792–798 bp	

All PCR products were submitted to horizontal electrophoresis in 1.0% agarose gel stained with ethidium bromide (0.625 μL/mL) in TEB run buffer pH 8.0 (44.58 M Tris-base; 44 M boric acid, 12.49 mM EDTA) at 90 V/150 mA for 50 min. To determine the amplified products, a 100 base pair molecular weight marker was used (Thermo Scientific, San Jose, CA, USA). The results were visualized and analyzed in an ultraviolet light transilluminator coupled to an image analysis program (Chemi-Doc, Bio-Rad®).

Cloning and sequencing reactions

Five amplicons obtained in the *msa-2b* and *msa-2c* gene-based PCR assays were subjected to pGEM-T Easy vector cloning (Promega® Madison, USA), following the manufacturer's recommendations. The ligation reaction with the plasmid was used to transform competent *E. coli* DH5α cells (10^9–10^{10} CFU/ng DNA), plated on Luria-Bertani agar (LB) (Invitrogen, Carlsbad, CA, USA) and cultured in LB broth (Invitrogen Carlsbad, CA, USA). Up to three clones of each positive sample were selected for sequencing. For extracting the plasmid DNA, the alkaline lysis method was used (Sambrook et al. 2001). Plasmid DNA extracted from the clones was subjected to a PCR assay using the primers M13 F (5′-CGCCAGGGTTTTCCCAGTCA CGAC-3′) and M13 R (5′-GTCATAGCTGTTTCCTGTGT GA-3′) (Lau et al. 2010) that flank the multiple cloning site of the plasmid pGEM T-easy and, therefore, included the inserts of the *msa-2b* and *msa-2c* genes.

The amplified products of the reactions were purified with the "Silica Bead DNA Gel Extraction" Kit (Fermentas, São Paulo, SP), following the manufacturer's recommendations. Sequencing of the amplified products was performed in both directions using an automated technique based on the dideoxynucleotide chain termination method (Sanger et al. 1977). The oligonucleotides used in the sequencing were the same as those used in the PCR of plasmid DNA, namely M13 F and M13 R, which flank the multiple cloning site of the pGEM T-easy vector and amplify a fragment up to 1100 bp. Sequencing was conducted on the ABI PRISM 3700 DNA Analyzer sequencer (Applied Biosystems).

Sequence analysis

Phylogenetic inferences

The alignment of sequencing data and the consensus sequence were obtained using the PhredPhrap software (Ewing et al. 1998). Subsequently, the BLASTn analysis tool was used to compare the sequences obtained in this work with others of the same gene in the "GenBank" database (Benson et al. 2002). The consensus sequences saved as FASTA were aligned with other homologous sequences from the same gene retrieved from the GenBank database using the MAFFT software (Katoh et al. 2002). Phylogenetic inference on the *msa-2b* and *msa-2c* gene sequences was obtained from maximum likelihood analyses using the RaxML BlackBox cluster 7.6.3 (Stamatakis et al. 2008). The analyses were carried out via the CIPRES portal (Miller et al. 2010). The bootstrap was accessed with 1000 replicates and the best evolutionary model was selected based on the Akaike Information Criterion (AIC) in the jModeltest 2.1.3 software (Darriba et al. 2012). The phylogenetic trees were edited and rooted (via external group) in the Treegraph 2.0.56-381 beta software (Stover and Muller 2010).

Genetic diversity

Identity analysis between sequences

The identity percentage between the nucleotide sequences of the *msa-2b* and *msa-2c* clones obtained in this study was found using the Sequence identity matrix tool in the Bioedit v. 7.0.5.3 (Hall 1999).

Entropy of amino acids

Entropy analysis was performed to verify the variability between the amino acid sequences. To that end, the nucleotide

sequences of the *msa-2b* and *msa-2c* clones were converted into amino acids in the ExPASy software (Gasteiger et al. 2003), aligned and then submitted to said analysis using the "Entropy (H (x)) plot" via Bioedit v. 7.0.5.3 (Hall 1999).

Haplotype diversity

The obtained alignment of the nucleotide sequences of the *msa-2b* and *msa-2c* genes was used to calculate the nucleotide diversity (π), polymorphism level (diversity of haplotypes—[Dh]), number of haplotypes, and the average number of nucleotide differences (K), using the DnaSP v5 software (Librado and Rozas 2009). In addition, the nucleotide sequences were submitted to the TCS Network analysis (Clement et al. 2000), which was inferred from the Population Analysis with the Reticulate Trees (popART) program (Leigh and Bryant 2015).

Accession numbers

The sequences of *Babesia bovis* generated in this study were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MH475362 to MH475365, MK361205 to MK361214 for *msa-2b* and MH751587 to MH751591, MK305090 to MK305092, MK353800 to MK353804 for *msa-2c*.

Results

Quality of extracted DNA samples and PCR of the mammalian *gapdh* gene

The mean DNA concentration and the absorbance (260/280 and 260/230 nm) ratios of the DNA samples were 25.14 ng/μL (SD ± 10.14), 1.8 nm and 1.33 nm (SD ± 0.12 and ± 0.56), respectively. The 400 DNA samples extracted from the bovine blood were positive for the endogenous gene (*gapdh*) as shown by the PCR results.

Occurrence of *Babesia bovis* in sampled cattle

Of the 400 bovine blood samples submitted to screening, 4.5% (18/400) were positive as shown by the nPCR results targeting the *sbp-2* gene. All positive blood samples were from calves.

Amplification reactions for *Babesia bovis* based on the *msa-2b* and *msa-2c* genes

Of the 18 positive blood samples from calves, 77.7% (14/18) were positive to the *B. bovis msa-2b* fragment, where as 66.6% (12/18) were positive to the *msa-2c* fragment.

Analysis of the sequence identity by BLASTn

Among the *B. bovis* positive samples submitted to gene cloning, clones of the *msa-2b* (14 clones) and *msa-2c* (13 clones) genes were sequenced and compared to other sequences previously deposited in the GenBank, using the Blastn analysis tool. All analyzed sequences (*msa-2b* and *msa-2c*) shared 90–99% identity with other sequences of said *B. bovis* genes detected in cattle.

Phylogenetic analysis

Phylogenetic analysis of *msa-2b* gene sequences

Phylogenetic analysis using the maximum likelihood method and the GTR + G evolution model was performed based on 14 sequences of the *msa-2b* gene obtained in this study and 22 sequences of the *msa-2b* gene from previous studies conducted in Brazil and other parts of the world. Three sequences of the *msa-2c* gene from *B. bovis* were used as an external group. Sequences employed in the phylogeny were obtained from samples #4 (3 clones), #168 (3 clones), #189 (3 clones), #190 (3 clones), and #191 (2 clones). The cladogram revealed six clades, supported by bootstrap values ranging from 40 to 100%. The *B. bovis* sequences detected in the present study were positioned in 4 clades (1, 2, 3, and 4) (Fig. 2). The sequences obtained from sample #189 (189-clone 1, 189-clone 2, and 189-clone 3) clustered in clade 1, demonstrating phylogenetic proximity. However, while clones 1 and 2 appear as a sister group, clone 3 positioned farthest from these and closer to the CA 109.2 isolate sequence detected in Vietnam (LC004318). Also, this same clade clustered clones belonging to samples #168 and #190 (168-clone 1 and 190-clone 1), along with sequences detected in Vietnam and the Philippines, under LC004328 and AB819788 accession numbers, respectively. The second clones from samples #168 and #190 (168-clone 2 and 190-clone 2) appeared in the second clade, clustered with a sequence from Vietnam, and together with the Pantanal sequences obtained from sample #4 (4-clone 1, 4-clone 2, and 4-clone 3). The sequence #190-clone 3 obtained in the present study was positioned in the third clade, as a sister group of the Tabasco isolate, detected in Mexico. This third clade also grouped sequences detected in Thailand, Mexico, and one previously detected in Taiaçu, in the state of São Paulo. The 191-clone 1, 191-clone 2, and 168-clone 3 sequences from Pantanal were positioned in clade 4, clustered with sequences previously detected in the states of Rio de Janeiro and São Paulo (KX160815, KU522560), respectively; this clade also had sequences from Sri Lanka and Thailand. With only two sequences each, clade 5 had AB745712 (Thailand) and AB786821 (Vietnam) while clade 6 had DQ173953 and DQ173958 detected in Australia.

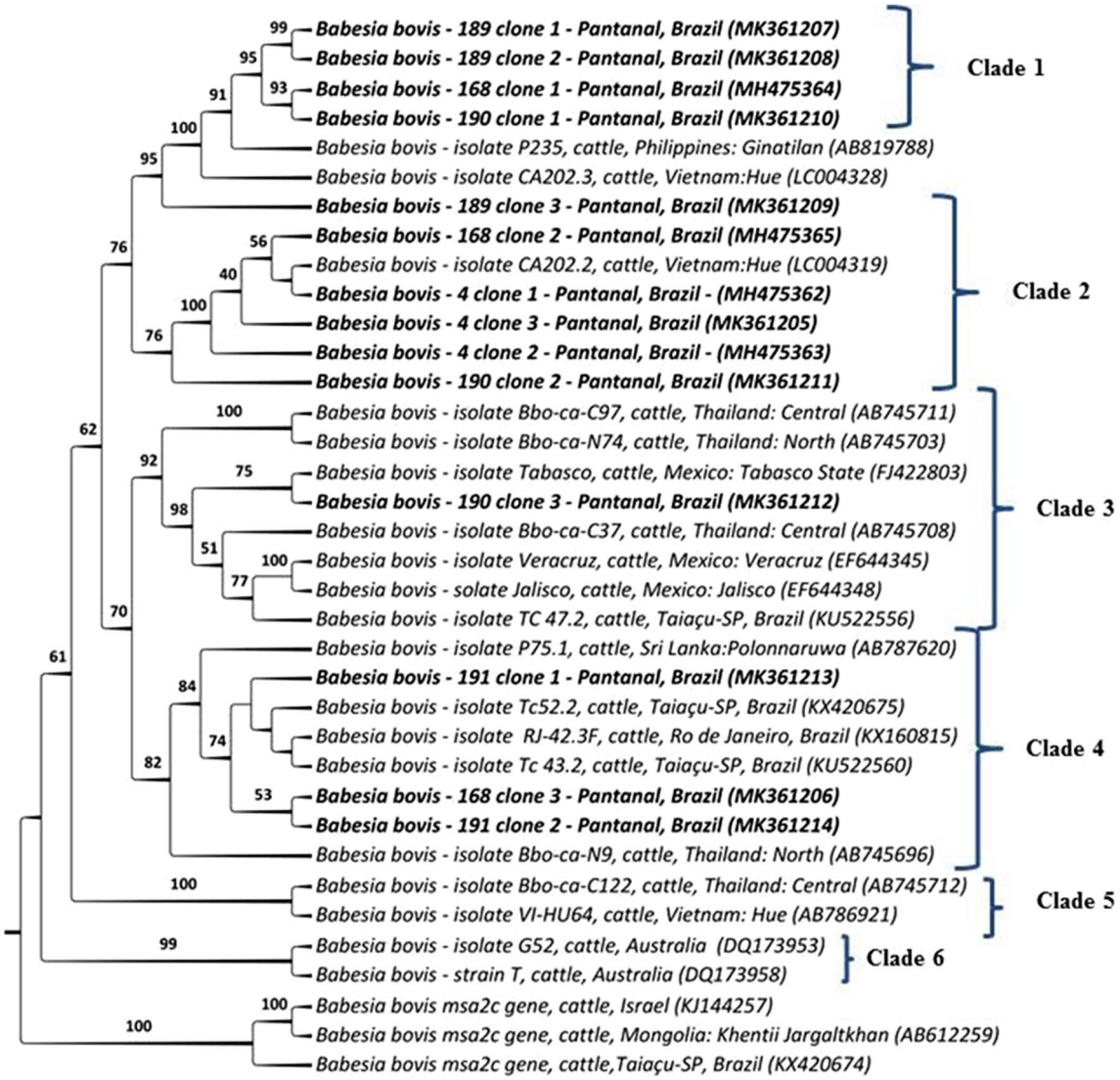

Fig. 2 Phylogenetic analysis of *B. bovis msa-2b* gene sequences. The phylogenetic tree was inferred using the maximum likelihood method and the GTR + G evolution model. The sequences amplified in this study are highlighted in bold. The numbers on each node correspond to the bootstrap values accessed with 1000 replicas. Three sequences of the *B. bovis msa-2c* gene were used as an external group

Phylogenetic analysis of *msa-2c* gene sequences

Phylogenetic analysis of maximum likelihood and evolutionary model TIM3 + G were based on 13 sequences obtained in this study and 25 sequences from previous studies conducted in Brazil and other parts of the world. Three sequences of the *msa-2b* gene from *B. bovis* were used as an external group. Sequences of the present study were obtained from samples #10 (3 clones), #138 (3 clones), #139 (3 clones), #168 (2 clones), and #179 (2 clones). The cladogram revealed 9 clades, supported by bootstrap values ranging from 43 to 100% (Fig. 3). The *B. bovis* sequences of beef cattle sampled in the Brazilian Pantanal were positioned in 6 clades (1, 2, 3, 6, 7, and 9). Two clones from sample #10 (10-clone 1 and 10-clone 2) clustered into the first clade, along with sequences previously detected in the Philippines, Vietnam, and Israel. The 3 clones of the #139 sample (139-clone 1, 139-clone 2, and 139-clone 3) clustered together with a sequence from sample #10 (10-clone 3), all four clustered with the sequences detected in the Philippines, Sri Lanka, and Mexico. The 179-clone 1 and 179-clone 2 sequences obtained from samples #179 formed alone clades 9 and 3, respectively. The remaining (138-clone 1, 138-clone 2, 138-clone 3, and 168-clone 2) sequences from samples #138 and #168 were clustered together in clade 7 along with sequences detected in Turkey and Mexico (GU004533, KC515390, KC515389, and EF640958).

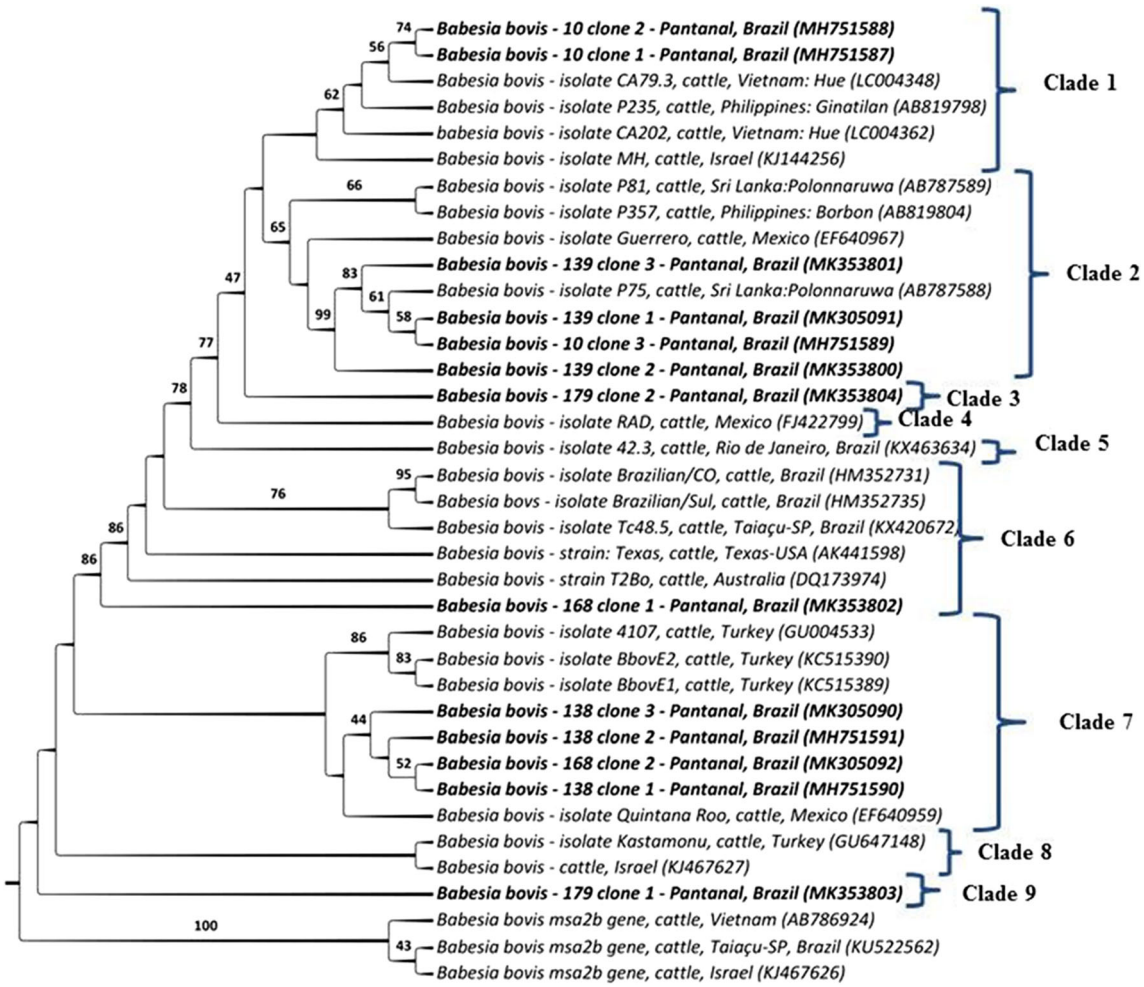

Fig. 3 Phylogenetic analysis of *B. bovis msa-2c* gene sequences. The phylogenetic tree was inferred from the maximum likelihood method and the TIM3 + G evolution model. The sequences amplified in this study are highlighted in bold. The numbers on each node correspond to the bootstrap values accessed with 1000 replicas. Three sequences of the *B. bovis msa-2b* gene were used as an external group

Identity analysis of the sequences

Identity of the *Babesia bovis msa-2b* nucleotide sequences

The identity ranged between 58 and 98.2% for the *B. bovis msa-2b* sequences obtained from blood samples from beef cattle from the Brazilian Pantanal (Table 2). The lowest identity percentage (58%) corresponded to the comparison of clone-1 and clone-3 sequences of sample #190 with clone-3 of sample # 4. On the other hand, the highest identity percentage (98.2%) refers to two clones of the same sample (clones 1 and 2 of sample #189). The analysis of *B. bovis* sequences obtained from the same animal, i.e., clones from the same sample, shows that the highest divergence percentages between clones were detected in the sequences obtained from the *msa-2b* gene. The divergence between the sequences ranged from 4.1 to 22.8%, 27.3 to 33.9%, 1.8 to 5%, 19.6 to 32.7%, and 7.6 to 30.5% in clones obtained from samples #4, #168, #189, #190, and #191, respectively. The highest divergence between *msa-2b* clones was 33.9% observed for the sequences of clones 1 and 2 of sample #168, whereas the lowest divergence was 1.8%, observed between clones 1 and 2 of sample #189.

Identity of *Babesia bovismsa-2c* nucleotide sequences

The identity of the msa-2c sequences obtained in the Brazilian Pantanal varied between 75.1 and 99.8% (Table 3). The lowest identity percentage (75.1%) was observed for the sequences 179-clone 2 and 138-clone 3, whereas the highest (99.8%) referred to sequences 139-clone 3 and 10-clone 3. The divergence variation between the sequences obtained from clones of the same sample was 3 to 5.5%, 0.6 to 2.3%, 2 to 8.7%, 14.3% and 12.3% in the clones obtained from samples #10, #138, #139, #168, and #179, respectively. The greatest divergence between *msa-2c* clones was 14.3% between clones 1 and 2 of sample #168 while the lowest was 0.6% between clones 2 and 3 of sample #138.

Table 2 Identity and divergence matrix of the *Babesia bovis msa-2b* nucleotide sequences detected in beef cattle sampled in the Brazilian Pantanal

Identity

Divergence	Clade 1					Clade 2					Clade 3	Clade 4		
Sample; clone	189; 1	189; 2	189; 3	168; 1	190; 1	168; 2	4; 1	4; 3	4; 2	190; 2	190; 3	191; 1	168; 3	191; 2
189; 1	ID	98.2%	96%	93.7%	90.9%	69.7%	64.8%	61.9%	63.2%	74.7%	69.2%	70.6%	70.2%	64.6%
189; 2	1.8%	ID	95%	94.8%	92%	69.3%	65.5%	61.6%	63.9%	75.6%	68.3%	70.4%	68.8%	65.3%
189; 3	4%	5%	ID	91.6%	88.7%	70.9%	66.5%	62.5%	65%	76.8%	68.5%	70.1%	69.4%	64.2%
168; 1	6.3%	5.2%	8.4%	ID	96.3%	66.1%	67%	58.5%	68.3%	80%	72.2%	73.7%	72.7%	69.4%
190; 1	9.1%	8%	11.3%	3.7%	ID	65.3%	69.1%	58%	70.8%	80.4%	69.5%	71%	70.4%	71.9%
168; 2	30.3%	30.7%	29.1%	33.9%	34.7%	ID	83.5%	80.7%	81.1%	83.2%	64.9%	70%	68.9%	66.5%
4; 1	35.2%	34.5%	33.5%	33%	30.9%	16.5%	ID	79.1%	95.9%	77.6%	65.9%	69.9%	68.4%	71.8%
4; 3	38.1%	38.4%	37.5%	41.5%	42%	19.3%	20.9%	ID	77.2%	68.1%	58%	62.3%	61.5%	59.2%
4; 2	36.8%	36.1%	35%	31.7%	29.2%	18.9%	4.1%	22.8%	ID	79%	66%	70.5%	71.4%	74.8%
190; 2	25.3%	24.4%	23.2%	20%	19.6%	16.8%	22.9%	31.9%	21%	ID	67.3%	71.4%	70.6%	70.6%
190; 3	30.8%	31.7%	31.5%	27.8%	30.5%	35.1%	34.1%	42%	34%	32.7%	ID	79.4%	78.4%	73%
191; 1	29.4%	29.6%	29.9%	26.3%	29%	30%	30.1%	37.7%	29.5%	28.6%	20.6%	ID	97.1%	92.4%
168; 3	29.8%	31.2%	30.6%	27.3%	29.6%	31.1%	31.6%	38.5%	28.6%	29.4%	21.6%	2.9%	ID	93.6%
191; 2	35.4%	34.7%	35.8%	30.6%	28.1%	33.5%	28.2%	40.8%	25.2%	29.7%	27%	7.6%	6.4%	ID

Table 3 Identity and divergence matrix of the *Babesia bovis msa-2c* nucleotide sequences detected in beef cattle sampled in the Brazilian Pantanal

Identity

Divergence	Clade 1		Clade 2				Clade 3	Clade 6	Clade 7			Clade 9
Sample; clone	10; 1	10; 2	139; 3	139; 1	10; 3	139; 2	179; 2	168; 1	138; 3	138; 2	168; 2	138; 1	179; 1
10; 1	ID	97%	94.6%	95.7%	94.5%	91.4%	81.3%	91.2%	91.2%	91.4%	90.7%	91.8%	81.2%
10; 2	3%	ID	97.3%	97.9%	97.2%	91.1%	78.9%	91.5%	93.9%	94.1%	90.5%	92.5%	81.2%
139; 3	5.4%	2.7%	ID	98%	99.8%	91.3%	77.5%	92.2%	94.9%	94.4%	89.2%	93.6%	79.8%
139; 1	4.3%	2.1%	2%	ID	97.9%	92.1%	78.4%	90.6%	93.2%	93.5%	90.2%	92%	80.7%
10; 3	5.5%	2.8%	0.2%	2.1%	ID	91.2%	77.4%	92.1%	94.8%	94.2%	89.1%	93.5%	79.1%
139; 2	8.6%	8.9%	8.7%	7.9%	8.8%	ID	84.8%	84%	86.5%	86.2%	93.6%	85.4%	87.4%
179; 2	18.7%	21.1%	22.5%	21.6%	22.6%	15.2%	ID	80.1%	75.1%	75.4%	81%	75.7%	87.7%
168; 1	8.8%	8.5%	7.8%	9.4%	7.9%	16%	19.9%	ID	92.7%	92.2%	85.7%	93%	78%
138; 3	8.8%	6.1%	5.1%	6.8%	5.2%	13.5%	24.9%	7.3%	ID	99.4%	92.1%	97.9%	84.1%
138; 2	8.6%	5.9%	5.6%	6.5%	5.8%	13.2%	24.6%	7.8%	0.6%	ID	92.4%	97.7%	84.5%
168; 2	9.3%	9.5%	10.8%	9.8%	10.9%	6.4%	19%	14.3%	7.9%	7.6%	ID	90.7%	89%
138; 1	8.2%	7.5%	6.4%	8%	6.5%	14.6%	24.3%	7%	2.1%	2.3%	9.3%	ID	82.3%
179; 1	18.8%	18.8%	20.2%	19.6%	20.9%	12.6%	12.3%	22%	15.9%	15.5%	11%	17.7%	ID

Entropy analysis of MSA-2B and MSA-2C amino acids

Amino acid entropy analysis, performed from the sequence alignment of *msa-2*b and *msa-2c* clones, is shown in Fig. 4a, b. The charts show 78 and 44 high entropy peaks distributed along the amino acid sequence for the MSA-2B and MSA-2C, respectively. In the present study, the entropy value ranged from 0.25 to 1.53 for MSA-2B and from 0.27 to 1.09 for MSA-2C.

Analysis of *Babesia bovis* haplotypes detected in the Brazilian Pantanal

A total of 14 haplotypes with $\pi = 0.1735$, Dh = 1000, and $K = 100.11318$ were identified among the 14 sequences of the analyzed *msa-2b* gene. On the other hand, of the 13 *msa-2c* gene sequences analyzed, 11 haplotypes with $\pi = 0.03220$, Dh = 0.9794, and $K = 20.0256$ (Table 4) were identified. Furthermore, haplotypes #5 and #7 were formed by two sequences only, #5 by clones 2 and 3 of sample #139, and #7 by 2 and 3 of sample #138.

Analysis of haplotypes based on the *msa-2b* and *msa-2c* genes of *Babesia bovis* sequences detected in Brazil and worldwide

The *B. bovis* sequences obtained in the Brazilian Pantanal were highly diverse compared to those previously detected in Brazil and worldwide. The haplotype networks for both genes obtained from the TCS Network tool are shown in Figs. 5 and 6.

The haplotypes were analyzed based on 33 sequences of *B. bovis msa-2b* detected in Brazil, including the 14 sequences obtained in this study; the results indicated 25 different haplotypes with $\pi = 0.18027$, Dh = 0.9773 and $K = 83.46$ (Table 4). Only haplotypes #1, #8, #11, #14, and #20 consisted of more than one sequence. Haplotype #1 consisted of sequences AB819788 and LC004328, detected in the

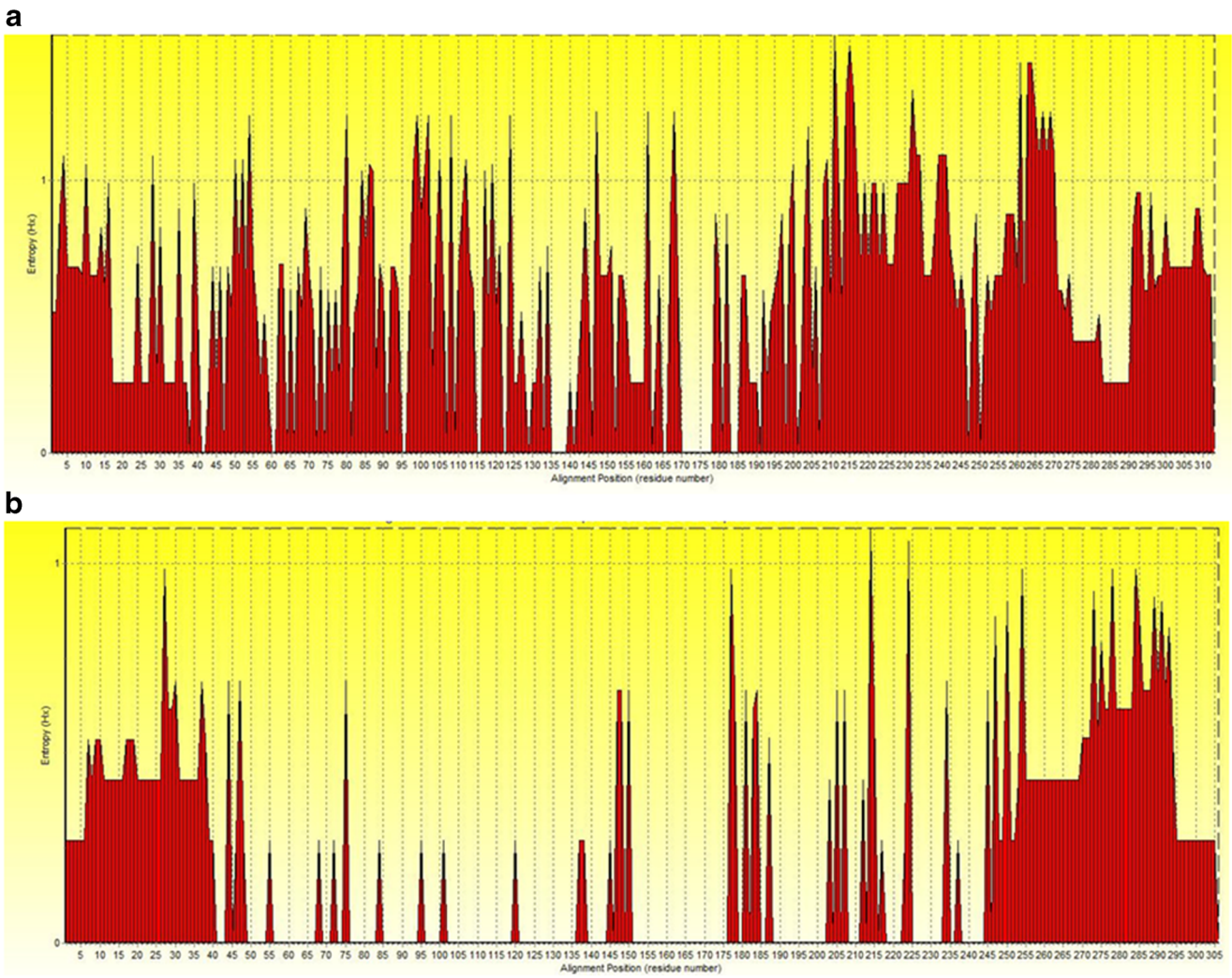

Fig. 4 **a** Amino acid entropy plot obtained from the *msa-2b* gene sequences. **b** Amino acid entropy plot obtained from the *msa-2c* gene sequences

Table 4 Polymorphism and genetic diversity of the *B. bovis msa-2b* and *msa-2c* sequences detected in beef cattle in the Brazilian Pantanal and worldwide

Gene	(pb)	*N*	VS	GC%	h	Dh (mean ± SD)	π (mean ± SD)	*K*
Sequences within the Brazilian Pantanal								
msa-2b	938	14	255	37.2	14	1000 ± 0.027	0.1735 ± 0.0154	100.1318
msa-2c	916	11	51	38.7	11	0.9794 ± 0.039	0.03220 ± 0.0028	20.0256
Sequences worldwide								
msa-2b	979	33	244	36.9	25	0.9773 ± 0.015	0.18027 ± 0.0062	83.46
msa-2c	1005	37	117	38.9	31	0.9880 ± 0.010	0.03294 ± 0.00377	19.76

N, number of analyzed sequences; VS, number of variable sites; GC, G + C content; h, number of haplotypes; Dh, diversity of haplotypes; SD, standard deviation; π, nucleotide diversity (per site); *K*, nucleotide difference number

Philippines and Vietnam while haplotype #8 had 3 sequences detected in the Pantanal (clone 1 of sample #4, clone 2 of sample #4, and clone 2 of sample #168), whereas haplotype #11 consisted of sequences DQ173958 and DQ173953 from Australia and haplotype #14 consisted of the clone 3 sequences from samples #190 and AB745708 from Pantanal and Thailand respectively. Finally, haplotype #20 consisted of the sequences KX160815, KU522560, KX420675, and clone 3 of sample 168, originated from Taiaçu-SP and Pantanal-MS.

The analysis of haplotypes based on 37 sequences of *B. bovis msa-2c* detected in the world, including the 13 sequences obtained in this study, indicated the presence of 31 different haplotypes with $\pi = 0.03294$, Dh = 0.9880, and $K = 19.76$. Four haplotypes consisted of more than one sequence. Haplotype #1 comprised the sequences LC004362 and LC004348, detected in Vietnam. Haplotype # 9 comprised the sequences AK441598, KX420672, and HM352731, which were detected in Texas (USA), Taiaçu in SP (Brazil) and Mato Grosso do Sul (Brazil), respectively. Haplotype #16 consisted of the AB787588 sequences from Sri

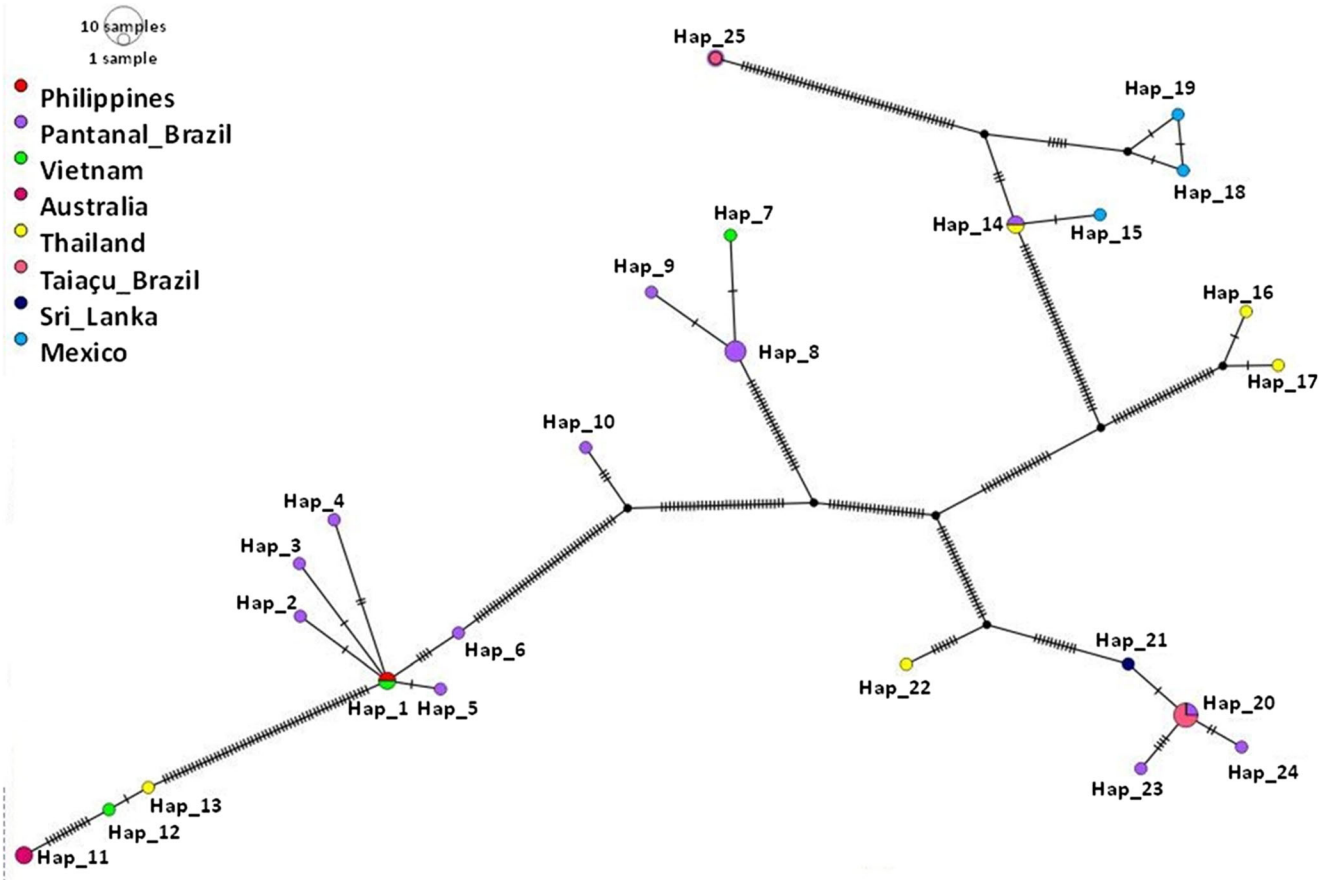

Fig. 5 TCS network of haplotypes based on the *Babesia bovis msa-2b* gene sequences detected in Brazil and worldwide. (The black spots are median-vectors; small traits between one haplotype and another represent mutational event)

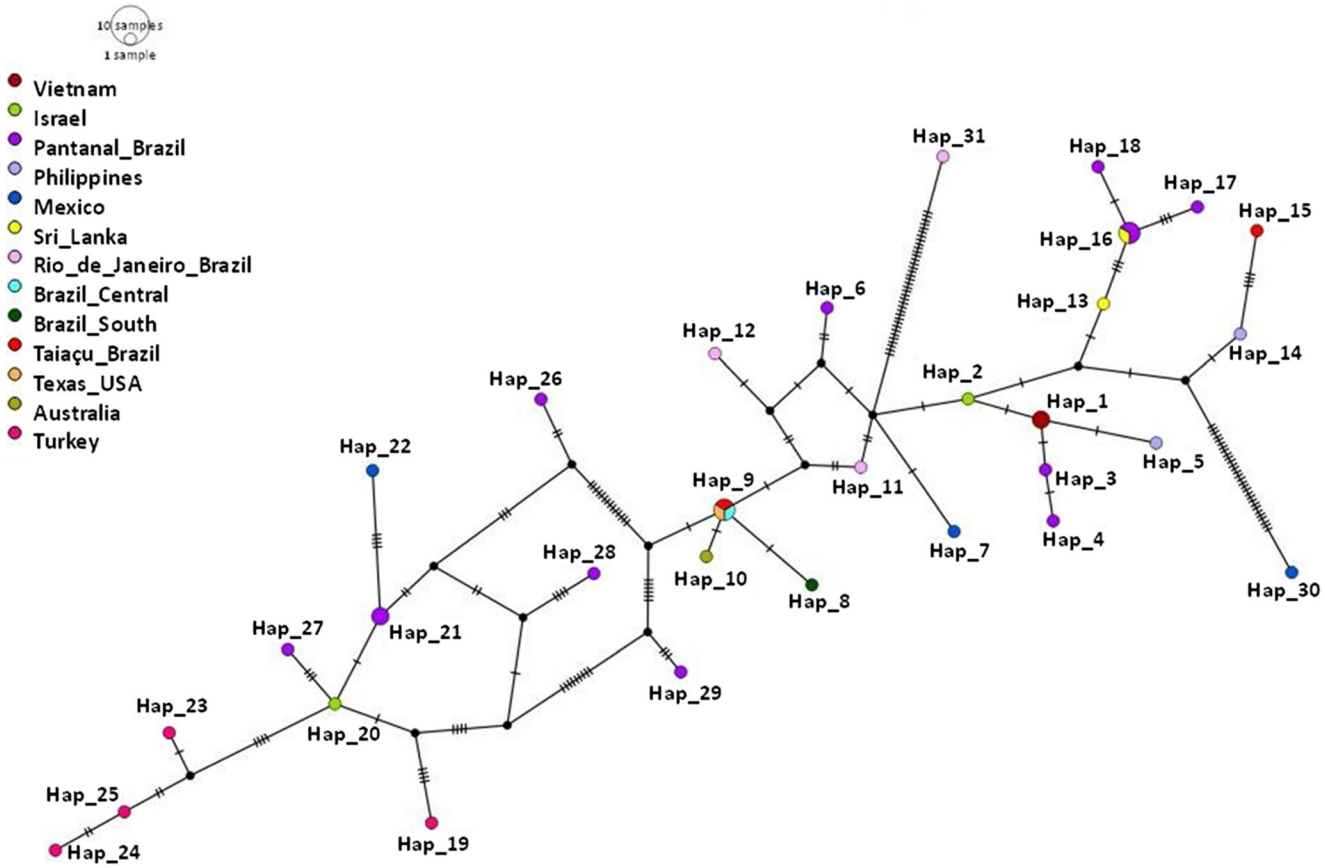

Fig. 6 TCS network of haplotypes based on the *Babesia bovis msa-2c* gene sequences detected worldwide. (The black spots are median-vectors; small traits between one haplotype and another represent mutational event)

Lanka and two sequences detected in the Pantanal (clone 2 of sample #139 and clone 3 of sample #139), and haplotype #21 indicated two sequences detected in the Pantanal, clones 2 and 3 of sample #138.

Discussion

The present study showed a low molecular occurrence of *B. bovis* in cattle sampled in the Brazilian Pantanal. The molecular assay results showed that of the animals sampled, 4.5% (18/400) were positive for *B. bovis* based on the *sbp-2* gene. The percentage of positive animals in this study was similar to the 3.6% (11/308) reported in a herd of water buffaloes in the Amazon region (Silveira et al. 2016) and lower than the 33.2% (67/202) reported in milk cattle sampled in Parnaiba, Piauí, both based on the 18S rRNA gene. Although the nPCR method used in this study to detect *B. bovis* is considered highly sensitive (Mosqueda et al. 2012), the low molecular occurrence found could be attributed to a parasitemia level below the limit of detection of the technique used. According to Aboulaila et al. (2010), the limit of detection of the nPCR targeting the *sbp-2* gene was 2.7×10^{-2} infected bovine red blood cells (as low as 1 fg (1×10^{-9}) per test of the *B. bovis* genomic DNA). Therefore, considering the high sensitivity of the used nPCR assay, we could hypothetize that the low number of animals presenting *B. bovis* DNA in blood samples might have been resulted from the innate resistance of Nelore cattle to *R. microplus* attachment to their skin (Spickett et al. 1989; Martinez et al. 2006; Gasparin et al. 2007; Veríssimo et al. 2015). It is known that the final stage of *B. bovis* sporogony in *R. microplus* ticks only occurs after the tick larvae starting feeding (Jalovecka et al. 2018), which could resulted, as a consequence, in low numbers of inoculated infecting sporozoites. Although the susceptibility of particular breeds to *B. bovis* infection is not known, previous studies have suggested that, following the same pattern of resistance to the *R. microplus* tick, zebu animals are more resistant than taurine to infection by this piroplasmid (Bock et al. 1997; Bock et al. 1999; Bilhassi et al. 2014).

This study analyzed gene fragments encoding the *msa-2b* and *msa-2c* merozoite surface proteins in a bovine population sampled in the Brazilian Pantanal to determine the genetic diversity of *B. bovis* in this region. Our results show that the *B. bovis* population is highly diverse in the Pantanal region, with the presence of probably more than one distinct haplotype in the same animal. Previous studies report that the MSA gene family are appropriate markers of genetic diversity for *B. bovis* (Berens et al. 2005; LeRoith et al. 2005; Borgonio et al. 2008;

Genis et al. 2008; Genis et al. 2009; Simking et al. 2013). The results obtained in the screening PCR assay (*sbp-2* gene) and in PCR protocols based on the *msa-2b* and *msa-2c* genes were in disagreement for ten samples, of which four and six positive samples in the screening turned out negative in the *msa-2b* and *msa-2c* PCR assays, respectively. The screening PCR assay used to detect *B. bovis* DNA in the samples was based on the *sbp-2* (spherical body protein 2) gene (AbouLaila et al. 2010), which shows 12 truncated copies corresponding to the 5′ end and a complete copy in the *B. bovis* genome (Brayton et al. 2007; AbouLaila et al. 2010). Therefore, the amplification is more sensitivity to this gene compared to those based on the genes encoding merozoite surface proteins, which are single copy in the *B. bovis* genome. In addition, the nested PCR technique used for the screening assay, which is based on two successive amplifications, has higher sensitivity and specificity than PCR for MSA's genes performed with only one reaction.

In general, in both phylogenetic analyses, all *msa* nucleotide sequences detected in beef cattle from the Pantanal were distributed in several different clades, as recently demonstrated by Matos et al. (2017), who used *msa-2b* as a *B. bovis* genetic marker in blood samples from dairy cattle in the state of São Paulo, southeastern Brazil. The sequences detected in the present study were clustered with sequences from other countries, suggesting that none of the haplotypes found in this study is unique to Brazil and that the *msa-2b* and *msa-2c* sequences of *B. bovis* circulating in the Pantanal region share genetic traits with all sequences previously detected worldwide. The heterogeneous phylogenetic positioning of the sequences detected in the Pantanal is supported by the identity analysis between the sequences and corroborated by the high value of nucleotide diversity per site, especially for the *msa-2b* gene.

The *msa-2b* phylogeny positioned most of the sequences detected in this study in clades #1 and #2, suggesting a probable predominant haplotype. In contrast, the *msa-2c* phylogenetic analysis allocated single sequences detected in the Pantanal in three clades, which were positioned alone in clades #3 and #9; while in clade #6, a sequence detected in the Pantanal was clustered with sequences previously detected in Brazil, Texas (USA), and Australia. These findings indicate phylogenetic proximity of the *B. bovis* haplotypes circulating in different countries and in Brazilian Pantanal. Although previous studies performed in Brazil considered that the *msa-2c* is a conserved gene region compared to other *msa* loci (Ramos et al. 2012; Matos et al. 2017), a high genetic diversity has been reported for this genic locus in *B. bovis* strains detected in cattle from Australia (Berens et al. 2005), the Philippines (Tattiyapong et al. 2014), and Israel (Molad et al. 2014). These findings show that the genetic diversity among *B. bovis* populations varies according to the geographical region.

On the other hand, the Australian *msa-2b* sequences, which included the *B. bovis* T vaccine isolate, were allocated in clade #6, separately from all sequences detected in the Pantanal. This result suggests that the *msa-2b* haplotypes previously detected in Australia have a more distant phylogenetic relationship compared to the genotypes detected in this study, suggesting a potential obstacle for producing a single vaccine to be used worldwide. Comparing all phylogenetic analyses and haplotype networks consisting of the same sequences, it is concluded that the spatial haplotype network supports the data presented in the phylogeny since it shows the proximity of the phylogenetically close groups and indicates the dimension of the mutational events separating them. The Pantanal sequence group distributed throughout the network was distant from haplotypes #11, #12, and #13, which were positioned in clades that lacked the Pantanal sequences. In addition, it is still possible to verify that haplotypes #2, #3, #4, #5, and #6, formed by sequences of the present study, possibly originated directly from haplotype #1, displaying few mutational events among themselves. All other haplotypes constituted by the Pantanal sequences originated from the "median vector" (shown in the haplotype network as black spots), which represents intermediate haplotypes in the evolutionary route to the present ones, but were not contemplated in the sampling.

Similar to *msa-2b* gene, the spatial haplotype network of the *msa-2c* gene corroborated the phylogenetic data since the phylogenetically related groups were close, allowing to infer the size of the mutational events that separate them. However, unlike the *msa-2b* network, the occurrence of mutational events separating our sequences was much lower than those previously detected in *msa-2c* in Brazil and worldwide, since such events were almost exclusively restricted to haplotypes #30 and #31. Corroborating the phylogenetic positioning, the *msa-2c* haplotypes from the Pantanal were well distributed throughout the network. However, in addition to a smaller number of mutational events among the *msa-2c* sequences of the Pantanal, a greater number of haplotypes originated directly from other samples, without a "median-vector," as observed in the *msa-2b* haplotype network. Thus, it is inferred that haplotypes #3 and #4 may have originated from haplotype #1, consisting of *B. bovis* sequences from Vietnam. Haplotype #27 appears to have originated from haplotype #20, consisting of a sequence from Israel. Haplotype #16 with sequences from the Pantanal and Sri Lanka appears to have split to give rise to, in one direction, haplotypes #17 and #18 from the Pantanal, and in the other direction, another haplotype from Sri Lanka. These high spatial distribution data are further supported by the polymorphism and diversity analyses of the Pantanal sequences.

Additionally, the analyses of the amino acid sequences from the original nucleotide sequences ratifies the high polymorphism with 78 and 44 entropy peaks reaching up to 1.53 in *msa-2b* and 1.09 in *msa-2c*, respectively. The high genetic diversity of the *msa* genes observed in the different haplotypes circulating in Brazil and worldwide (Genis et al. 2009; Lau et al. 2010; Altangerel et al. 2012; Simking et al. 2013; Sivakumar et al. 2013; Tattiyapong et al. 2014; Molad et al. 2014; Nagano et al.

2013; and, Matos et al. 2017) may be related to the parasites' escape from infected host's immune response (Altangerel et al. 2012). The verified genetic heterogeneity may result in antigenic variations among *B. bovis* populations, limiting the use of such antigens as recombinant vaccines or even related to the protection failure of vaccine isolates, as already pointed out by Berens et al. (2005) and LeRoith et al. (2005).

Conclusion

The present study indicated a low molecular occurrence of *B. bovis* in beef cattle sampled in the Brazilian Pantanal. Despite this, a high degree of genetic diversity was found in the analyzed *B. bovis* population, with the possible presence of different haplotypes coexisting in the same animal and in the studied herd.

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Compliance with ethical standards

This research followed the Ethical Principles on Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Estadual Paulista "Júlio de Mesquita Filho" (Protocol 12375/15).

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