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Genetic diversity of piroplasmids species in equids from island of São Luís, northeastern Brazil

Diversidade genética de espécies de piroplasmídeos em equídeos na ilha de São Luís, Nordeste do Brasil

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Abstract

Equine piroplasmosisis, a tick-borne disease caused by the intra-erythrocytic protozoans *Babesia caballi* and *Theileria equi,* has economic importance due to the international trade and the increased movement of horses all over the world. The goal of this study was to evaluate the occurrence of phylogenetic diversity of *T. equi* and *B. caballi* genotypes among infected equids from São Luís Island, state of Maranhão, northeastern Brazil. Between December of 2011 and June of 2012, EDTA-blood and serum samples were collected from 139 equids (90 donkeys, 39 horses and 10 mules). From 139 serum samples submitted to ELISA assay, IgG antibodies to *T. equi* and *B. caballi* were detected in 19.4% (27/139) and 25.2% (35/139), respectively. Among sampled animals, 21.6% (30/139) and 55.4% (77/139) were positive for cPCR assays for *T. equi* and *B. caballi*, based on *ema-1* and *rap-1* genes, respectively. Overall, the *T. equi* sequences (n=7) submitted to Maximum Likelihood analysis (based on a 18S rRNA fragment of 1700 bp after alignment) grouped into three main groups, which were subdivided in eight clusters. The present work showed that different genotypes of *T. equi* and *B. caballi* circulate among equids in Brazil.

Keywords: *Babesia caballi*, *Theileria equi*, phylogeny, 18S rRNA.

Resumo

A piroplasmose equina, uma doença transmitida por carrapatos e causada pelos protozoários intra-eritrocíticos *Babesia caballi* e *Theileria equi*, tem importância econômica devido ao comércio internacional e ao aumento do movimento de cavalos em todo o mundo. O objetivo do presente estudo foi mostrar a diversidade filogenética de *T. equi* e *B. caballi* infectando cavalos, burros e jumentos na Ilha de São Luís, Estado do Maranhão, Nordeste do Brasil. Entre dezembro de 2011 e junho de 2012, amostras de sangue com EDTA e soro de foram coletadas de 139 equídeos (90 jumentos, 39 cavalos e 10 burros). Dentre as 139 amostras de soro submetidas ao ensaio de ELISA, foram detectados anticorpos IgG contra *T. equi* e *B. caballi* em 19,4% (27/139) e 25,2% (35/139), respectivamente. Entre os animais amostrados, 21,6% (30/139) e 55,4% (77/139) foram positivos por meio dos ensaios de cPCR para *T. equi* e *B. caballi*, com base nos genes *ema-1* e *rap-1*, respectivamente. No geral, as sequências *T. equi* (n = 7) submetidas à análise de Máxima Verossimilhança (baseada em um fragmento do 18S rRNA de 1700 pb, após o alinhamento) foram agrupadas em três grupos principais, os quais foram subdivididos em oito grupos. O presente trabalho mostrou que diferentes genótipos de *T. equi* e *B. caballi* circulam entre equídeos no Brasil.

Palavras-chave: *Babesia caballi*, *Theileria equi*, filogenia, 18S rRNA.

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Introduction

Equine piroplasmosisis is a tick-borne disease caused by intra-erythrocytic protozoans, namely *Babesia caballi* and *Theileria equi*, which is associated with an illness characterized by fever, anemia, dyspnea, icterus, hepato-and splenomegaly, edema, intravascular hemolysis, petecheal hemorrhages in mucosal surfaces, hemoglobinuria, and even death (SCHEIN, 1988; UILENBERG, 2006).

This disease has economic importance due to the international trade and the increased movement of horses all over the world (BASHIRUDDIN et al., 1999; NICOLAIEWSKY et al., 2001). Nowadays, some disease-free countries usually require serological test in horses in order to prevent seropositive animals from entering their territories (BÖSE et al., 1995; BRÜNING, 1996). Serological techniques, such as indirect fluorescent antibody (IFA) test and enzyme-linked-immunosorbent assay (ELISA), have been considered as preferable tests to check the exposure of horses to piroplasmids for international trade by the World Organization trade for Animal Health (OIE, 2011).

In Brazil, *T. equi* and *B. caballi* are mainly transmitted by *Rhipicephalus* (*Boophilus*) *microplus* and *Anocentor nitens*, respectively (GUIMARÃES et al., 1998; BATTSETSEG et al., 2002). In this country, the prevalence among horses ranges from 22 to 100% for *T. equi* (TENTER & FRIEDHOFF, 1986; PFEIFER BARBOSA et al., 1995; RIBEIRO et al., 1999; HEUCHERT et al., 1999; HEIM et al., 2007; GOLYNSKI et al., 2008; KERBER et al., 2009; BALDANI et al., 2010; SALVAGNI et al., 2010; MACHADO et al., 2012), and from 70 to 90% for *B. caballi* (PFEIFER BARBOSA et al., 1995; HEIM et al., 2007; KERBER et al., 2009; MACHADO et al., 2012), indicating that both parasites are endemic among horses.

Horses that recover from an acute phase of infection become reservoirs for both parasites (DE WAAL, 1992). On the other hand, donkeys usually show an asymptomatic form of piroplasmosis, showing a lower parasitaemia when compared to horses (KUMAR et al., 2009; MACHADO et al., 2012).

In the last years, molecular techniques, such as reverse line blot (RLB), hybridization (NAGORE et al., 2004) and quantitative real time PCR (BHOORA et al., 2010) have been developed for the diagnosis of equine piroplasmosis. The antigenic polymorphisms of merozoite surface antigens have been commonly observed between different isolates of hemoprotozoan parasites (CARCY et al., 2006). Most of the studies of molecular occurrence of equid piroplasmids have targeted *ema-1* gene for *T. equi* and *rap-1*gene for *B. caballi.* Additionally, 18S rRNA gene has been extensively used for broad phylogenetic classification (BATTSETSEG et al., 2002; UETI et al., 2003).

Despite the endemicity of both parasites among horses in Brazil, the epidemiology of piroplasmosis remains poorly studied among donkeys and mules. The goal of this study was to evaluate the occurrence of phylogenetic diversity of *T. equi* and *B. caballi* genotypes among infected equids from São Luís Island, state of Maranhão, northeastern Brazil.

Materials and Methods

Animals and area of study

Between December of 2011 and June of 2012, a molecular and serological study was performed in São Luís Island (latitude -2° 31′ 48″ S, longitude - 44° 18′ 10″ W), state of Maranhão, northeastern Brazil (Figure 1). For this purpose, EDTA-blood and serum samples were collected from 139 equids (90 donkeys, 39 horses and 10 mules), sampled by convenience. Among sampled equids, 23 animals were abandoned donkeys (17 females and 6 males) caught by the technical staff from the prefecture of São Luís city and kept in Universidade Estadual do Maranhão (UEMA). Additionally, 116 equids, including 67 donkeys (37 females and 20 males), 39 horses (11 females and 28 males), and 10 mules (5 females and 5 males) were working animals belonging to coachman. All equids sampled were adult animals, whose age ranged from five to eight years old.

DNA extraction

DNA was extracted from 200µL of each whole blood sample using the QIAamp DNA Blood Mini kit (QIAGEN, Valencia, California, USA), according to the manufacturer's instructions.

Molecular detection of equid piroplasmids

Each sample of extracted DNA was used as template in nested PCR assays, using previously described specific protocols based on *ema-1* gene for *T. equi* (NICOLAIEWSKY et al., 2001) and *rap-1* gene for *B. caballi* (IKADAI et al., 1999; BATTSETSEG et al., 2001). Briefly, five microliters of DNA was used as a template in 25 µL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0.6 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U of *Taq* DNA polymerase (Life Technologies) and 0.5 µM each primer (Integrated DNA Technologies) (Table 1). PCR amplifications were performed at 94°C for 4 min followed by 40 repetitive cycles of 94°C for 1 min, 56°C for 1 min for *B. caballi* and 60°C for 1 min for *T. equi*, and 72°C for 1 min, followed by a final extension at 72°C for 5 min.

Molecular characterization of equid piroplasmids

In order to perform the molecular characterization of the equid piroplasmids, eleven positive samples (seven for *T. equi* and four for *B. caballi*) were randomly selected and submitted to previously described conventional PCR protocols for *T. equi* and *B. caballi* based on 18S rRNA (~1600 bp) (BHOORA et al., 2009) (Table 1). The primers namely NBabesia1F: 5'-AAGCCATGCATGTCTAAGTATAAGCTTTT-3' and 18SRev-TB: 5'- GAATAATTCACCGGATCACTCG-3' were initially used for this purpose. The reactions were performed using five microliters of DNA as a template in 25 µL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.0 U Taq DNA

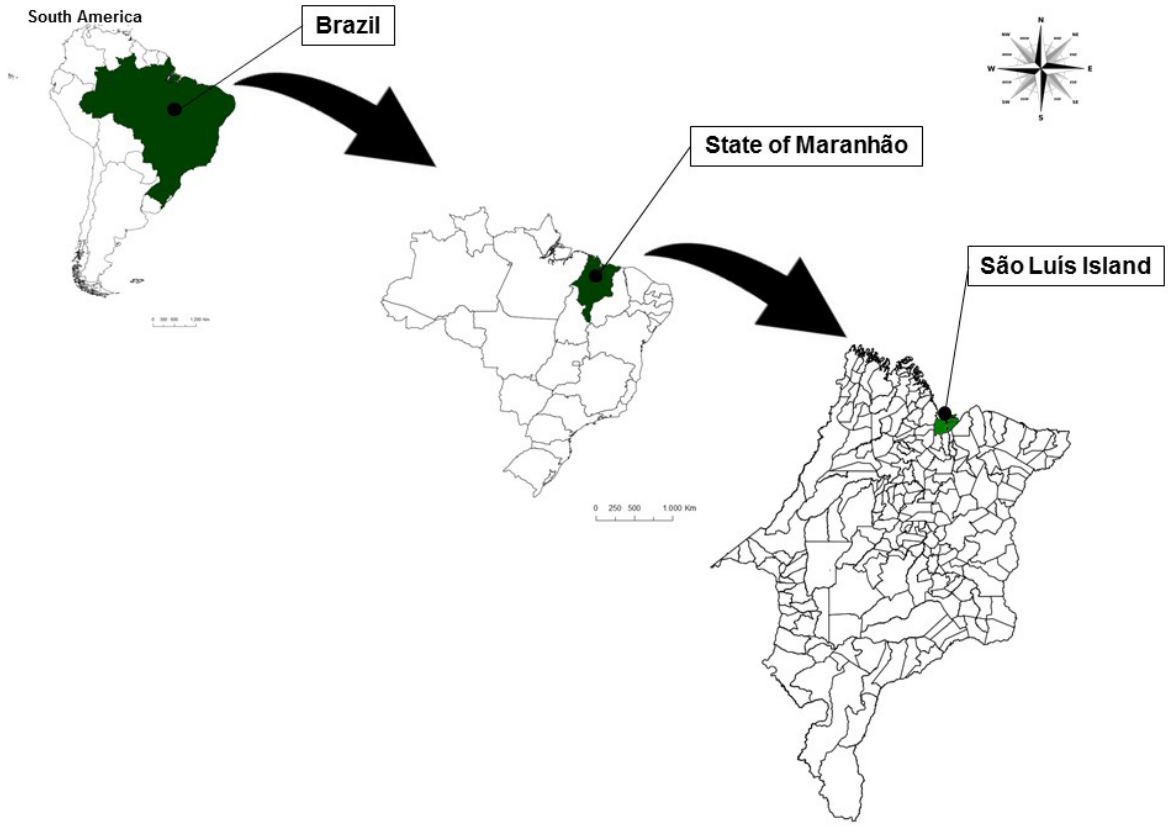

Figure 1. Map of Brazil and Maranhão state showing the São Luís Island where the equids were caught.

Polymerase (Life Technologies, Carlsbad, CA, USA) and 0.5 μM of each primer (Integrated DNA Technologies). PCR amplifications were performed by an initial cycle of 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min, and an extension final by 72 °C for 7 min. Additionally, DNA samples were submitted to another three nested PCR assays in order to achieve the whole 18S rRNA gene fragment of *T. equi* and *B. caballi*. The amplifications conditions were the same previously described, except the primers and annealing temperature (Table 1). *T. equi* and *B. caballi* DNA samples obtained from naturally infected donkeys (MACHADO et al., 2012) and ultra-pure sterile water were used as positive and negative controls, respectively, in all PCR assays described above. In order to avoid the PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated rooms. Ethidium-bromide stained gels were imaged under ultraviolet light using the Image Lab Software version 4.1 (Bio-Rad®).

Sequencing and phylogenetic analyzes of equine piroplasm

18S rRNA-amplicons obtained were purified using Silica Bead DNA Gel Extraction Kit (Fermentas, São Paulo, SP, Brazil). The purified PCR products were subsequently sequenced in both directions using the same PCR primers (forward and reverse). Sanger sequencing at CREBIO (Centro de Recursos Biológicos e Biologia Genômica – FCAV/UNESP) was performed using the ABI Prism 310 Genetic Analyser–Applied Byosystem/Perkin Elmer. Consensus sequences were obtained through the analysis of the sense and antisense sequences using the CAP3 program (HUANG & MADAN, 1999). Comparisons with sequences deposited in GenBank were done using the basic local alignment search tool (BLASTn – using default parameters) (ALTSCHUL et al., 1990). The 18S rRNA sequences were aligned with sequences published in GenBank using Clustal/W (THOMPSON et al., 1994) and adjusted in Bioedit v.7.0.5.3 (HALL, 1999). Phylogenetic inference based on Maximum Likelihood criterion (ML) was inferred with RAxML-HPC BlackBox 7.6.3 (STAMATAKIS et al., 2008) through the CIPRES Science Gateway (MILLER et al., 2011). Akaike information criterion (AIC) was used in Mega 5.05 to identify the most appropriate model of nucleotide substitution. GTRGAMMA + I model was chosen as the most appropriate for the Maximum Likelihood analysis of the 18S rRNA alignment. The sequences amplified in the present study were deposited in GenBank database under accession numbers: KY952226-KY952236.

Enzyme-linked immunosorbent assay (ELISA)

In order to detect IgG antibodies to *T. equi* and *B. caballi*, the serum samples were submitted to ELISA assays using commercial kits (Imunodot diagnóstico, IMUNOTEST®), in according to the manufacturer's instructions. Briefly, 100μL of crude *T. equi* and *B. caballi* recombinant antigen (concentration of 10 μg/mL

Table 1. Primers and annealing temperatures used for amplification of equine piroplasmids *rap-1, ema*-1 and 18S rRNA genes.

Primers	Primer sequences (5'-3')	Target gene	Anneling temperature (°C)	Amplicon size (bp)	References
BC48F1[a]	ACGAATTCCCACAACAGCCGTGTT	*rap-1*	56	530	Ikadai et al. (1999)
BC48R3[a]	ACGAATTCGTAAAGCGTGGCCATG				
BC48F11[a]	GGGCGACGTGACTAAGACCTTATT			430	Battsetseg et al. (2001)
BC48R31[a]	GTTCTCAATGTCAGTAGCATCCGC				
EMAE-F[b]	CCGCCCTTCACCTCGTTCTCAA	*ema-1*	60	396	Nicolaiewsky et al. (2001)
EMAE-R[b]	TCTCGGCGGCATCCTTGACCTC				
EMAI-F[b]	CCGTCTCCGTTGACTTGGCCG			102	
EMAI-R[b]	GGACGCGCTTGCCTGGAGCCT				
NBabesia1F[c]	AAGCCATGCATGTCTAAGTATAAGCTTTT	18S rRNA	58	800	Oosthuizen et al. (2008)
BT18S3R[c]	GAATAATTCACCGGATCACTCG				Bhoora et al. (2009)
BT18S2F[c]	GGGTTCGATTCCGGAGAGGG		55		Oosthuizen et al. (2008)
BT18S2R[c]	CCCGTGTTGAGTCAAATTAAGCCG				Matjila et al. (2008)
BT18S3F[c]	GGGCATTCGTATTTAACTGTCAGAGG		55		Oosthuizen et al. (2008)
18SRev-TB[c]	CCTCTGACAGTTAAATACGAATGCCC				Matjila et al. (2008)

[a]Primers used for specific detection of *B. caballi*; [b]Primers used for specific detection of *T. equi*; [c]Primers used for molecular characterization of equids piroplasmids.

and 2.5 µg/mL, respectively), were diluted in a 0.05M sodium bicarbonate-carbonated buffer (pH 9.6 and added to each well of a micro-ELISA plate (Nunclon™ Surface; Nunc, Denmark). After incubation at 4°C during 18 hours, the excess of antigen was removed by washing with PBS Tween-80 at 0.05%. Blocking of the uncoated sites in the well was achieved by the addition of 200 µL of PBS Tween-80 containing 6% skim milk for 2 h at 37°C. The blocking agent was removed, the plate was washed with PBS Tween-80 at 0.05%, and individual equids sera diluted (1:100) in PBS Tween-80 with 5% skim milk (PBS-TWSM) were added to each well and then incubated for 90 min at 37°C. After washing the plates, 100 µL of alkaline phosphatase conjugated anti-horse IgG (Sigma–Aldrich, St. Louis, Missouri, USA) diluted 1:30.000 in PBS-TW-SM were added to each well and then incubated for 90 min at 37°C. The plates were washed and the appropriate substrate (p-Nitrophenyl phosphate) (Sigma–Aldrich, St. Louis, Missouri, USA) was added. Serum samples of previously positive and negative donkeys were used as controls at ELISA assays (MACHADO et al., 2012). Absorbance at 405nm and were read after 45 minutes incubation at room temperature using an ELISA reader (Dynex Technologies). The cut-off value (0.343 and 0.346, for *T. equi* and *B. caballi*, respectively) were determined as being 2.5 times the mean absorbance value of negative controls, where readings above the cutoff value were considered positive, according to Machado et al. (1997).

Results

Molecular and serological occurrence for equids piroplasmids

Among all equids' blood samples analyzed, 21.6% (30/139) and 55.4% (77/139) were positive for cPCR assays for *T. equi* and *B. caballi*, respectively (Table 2). Additionally, co-positivity in PCR assays for both piroplasmid species was observed in 13.6% (19/139) of animals sampled (Table 3). From 139 serum samples submitted to ELISA assay, IgG antibodies to *T. equi* and *B. caballi* were detected in 19.4% (27/139) and 25.2% (35/139), respectively (Table 2). Only two animals (1.4%) showed antibodies for both *T. equi* and *B. cabalii* (Table 3). Fourteen (10%) animals were positive for both *T. equi*-ELISA and cPCR assays. Lastly, 17 (12.2%) animals were positive for both *B. caballi*-ELISA and cPCR assays. The occurrence of DNA or antibodies anti-piroplasms among the different equids species is shown in the Table 2.

Phylogenetic analysis

Out of the 30 positive samples for *T. equi*, seven (23.3%) 18S rRNA amplicons were randomly selected and submitted to sequencing and ML analyses. These sequences, when analyzed by BLASTn (using default parameters), shared identity ranging from 99% to 100% with *T. equi* sequences previously detected in Spain (AY150062), USA (CP001669 – whole genome) and Brazil (KU240071). Among the 77 positive samples for *B. caballi*, four (5.2%) 18S rRNA sequences were sequenced. The BLASTn analyses reveled that all sequences shared 99% identity with *B. caballi* (AY534883) amplified from an equine blood sample from Spain. All sequences amplified in the present study showed query coverage ranging from 99% to 100%.

Overall, the *T. equi* sequences (n=7) submitted to ML analysis (based on a fragment of 1700 bp after alignment) grouped in three main groups (#1, #2 and #3), which were subdivided in eight clusters (A-H) (Figure 2). Among the seven sequences amplified, two of them detected in donkeys (#139 and #148) were positioned near to sequences previously detected in horses from Rio de Janeiro, southeastern Brazil, and to other two sequences detected in horses from South Africa, constituting the group #1 and cluster E. Five sequences (#40, #44, #85 and #91, detected in horses, and #119, detected in a mule), belonging to group #3 and cluster H, were

Table 2. Number and percentage of equids positive to *T. equi* and *B. caballi.*

Animals	cPCR+		Elisa+	
	T. equi	***B. caballi***	***T. equi***	***B. caballi***
Mules	2/10 (20%)	6/10 (60%)	1/10 (10%)	4/10 (40%)
Horses	15/39 (38.5%)	23/39 (60%)	17/39 (43.5%)	3/39 (7.7%)
Donkeys	13/90 (14.4%)	48/90 (53.3%)	9/90 (10%)	28/90 (31.1%)
Total	30/139 (21.6%)	77/139 (55.4%)	27/139 (19.4%)	35/139 (25.2%)

Table 3. Number and percentage of equids co-positive to *T. equi* and *B. caballi.*

Animals	cPCR+	Elisa+
	T. equi* + *B. caballi	***T. equi* + *B. caballi***
Mules	1/10 (10%)	0/10 (0%)
Horses	11/39 (28.2%)	0/39 (0%)
Donkeys	7/90 (7.7%)	2/90 (2.2%)
Total	19/139 (13.6%)	2/139 (1.4%)

positioned near to sequences previously identified in equids from Brazil (south and southeastern regions), USA, Spain and South Africa (Figure 2). *Theileria equi* genotypes belonging to group #2 were not represented by any of the sequences from our study. Additionally, four *B. caballi* sequences (#142, #143, #151 and #157), all detected in donkeys, submitted to ML analysis (based on a fragment of 1720 bp after alignment), were positioned in the same group (group #1) and nearest to *B. caballi* sequences from Brazil, USA, Mongolia and Spain (Figure 3).

Discussion

In the present study, the frequency of piroplasm infection in donkeys, horses and mules from São Luís Island, Maranhão state, northeastern Brazil, was investigated by molecular and serological techniques. Both *T. equi* and *B. caballi* circulate in equids sampled in the studied area.

The low percentage of positive donkeys for piroplasmids found in the present study corroborates with a previous work carried out in the state of São Paulo, southeastern Brazil (MACHADO et al., 2012), suggesting low levels of transmission in this animal species in both studied areas.

Herein, we found an overall low seroprevalence for *T. equi* (19.4%) and *B. caballi* (25.2%) among sampled equids. Horses showed a higher seroprevalence to *T. equi* when compared to that found among donkeys and mules. On the other hand, donkeys and mules showed a higher seroprevalence to *B. caballi* when compared to that found among sampled horses. These seroprevalence rates were lower than those found among donkeys sampled in São Paulo state for both *T. equi* and *B. caballi* (MACHADO et al., 2012; PIANTEDOSI et al., 2014; LAUS et al., 2015) and in horses from southeastern (BALDANI et al., 2010; SALVAGNI et al., 2010; PFEIFER BARBOSA et al., 1995; HEUCHERT et al., 1999; HEIM et al., 2007; KERBER et al., 2009) and southern regions (VIEIRA et al., 2013) of Brazil.

Previously, the occurrence of antibodies for *B. caballi* was more prevalent than that one found for *T. equi* in horses parasitized by *Dermacentor nitens, Amblyomma cajennense* and *Rhipicephalus (Boophilus) microplus* ticks (KERBER et al., 2009). Additionally, while the parasitism by *D. nitens* was statistically associated with *B. caballi* positivity, the presence of *Amblyomma cajennense* in horses was associated with infection and/or exposure to *T. equi* (KERBER et al., 2009). In fact, the intrastadial transmission of *T. equi* by *A. cajennense* male ticks has been confirmed between uninfected and infected horses in the USA (SCOLES & UETI, 2013). Unfortunately, an accurate assessment of tick infestation on sampled equids was not performed, precluding any inferences about tick species found in animals and positivity for *T. equi* and *B. caballi*. Future studies aiming at assessing the vectors involved in piroplasmids transmission among equids in northeastern Brazil are much needed.

The occurrence of *T. equi* DNA in donkey blood samples in the present study was lower than that found among horses and mules. On the other hand, the occurrence of *B. caballi* DNA in donkey blood samples were higher than that found among horses and mules. While the number of seropositive animals for *T. equi* and *B. caballi* was lower than the number of PCR positive animals in the present study, the opposite was reported by Machado et al. (2012) among equids sampled in the state of São Paulo.

The 18S rRNA gene is widely used for phylogenetic analysis of equine piroplasms (BHOORA et al., 2009; HALL et al., 2013; MUNKHJARGAL et al., 2013). Herein, the genetic composition and the phylogenetic positioning of *T. equi* and *B. caballi* sequences amplified among equids from São Luís, Maranhão state, Brazil was assessed.

In the present study, three distinct main groups were identified by phylogenetic analysis of *T. equi* and *B. caballi* 18S rRNA gene sequences. *T. equi* and *B. caballi* 18S rRNA sequences detected in equids from Brazil clustered within two and one groups, respectively. Although fewer *B. caballi* sequences were analyzed, less genetic variation within *B. caballi* sequences seemed to occur when compared to *T. equi* sequences. These findings are in agreement with previous reports (CRIADO-FORNELIO et al., 2004; BHOORA et al., 2009).

Although Heim et al. (2007), when analyzing the *T. equi ema-1* gene sequences (800bp) and *B. caballi* BC-48 gene sequences (700bp), have reported no differences within each species or

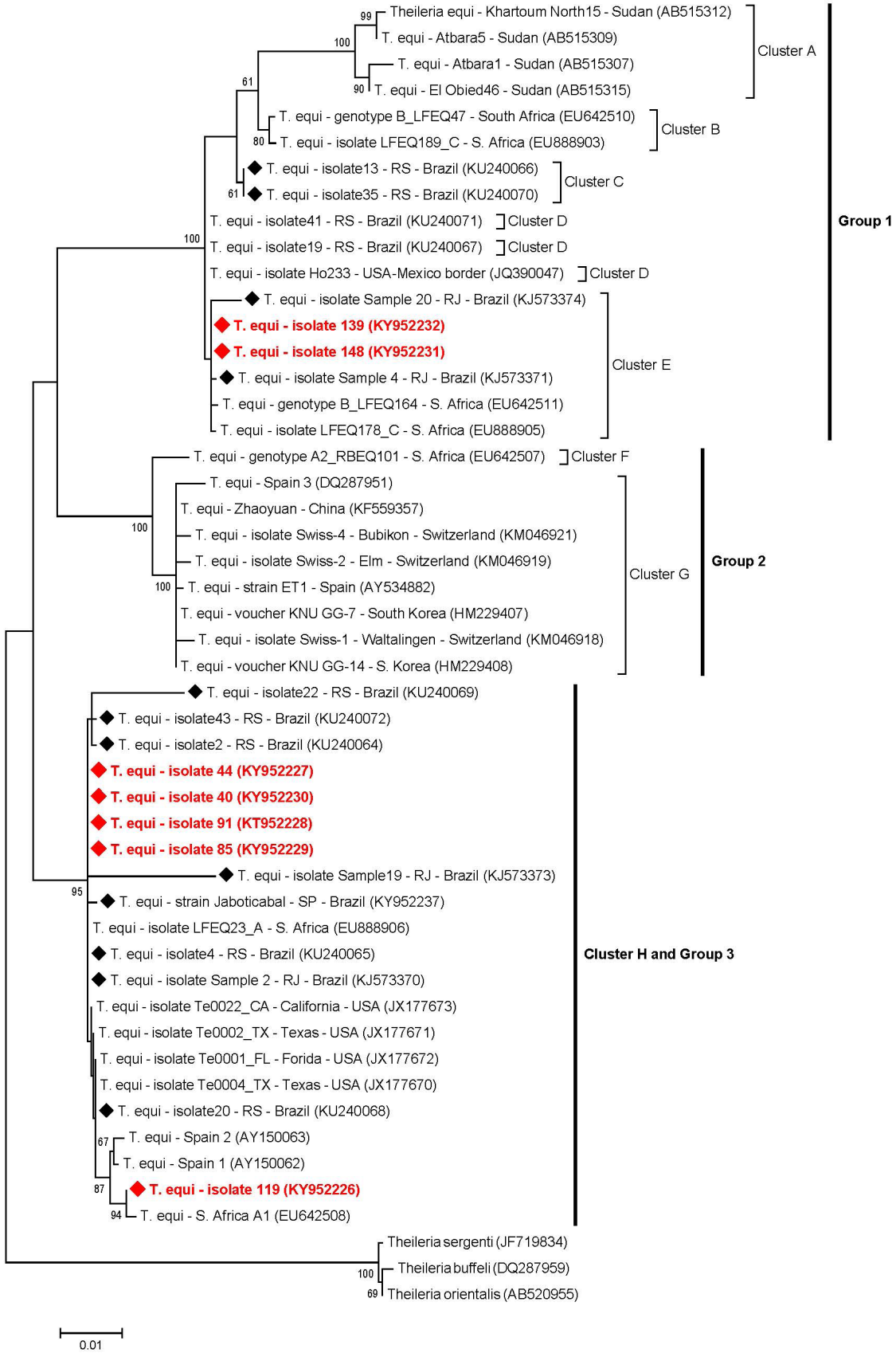

Figure 2. Phylogenetic relationships within the *T. equi* sequences based on a 1700 bp fragment of the 18S rRNA gene. The phylogenetic tree was inferred by using the maximum likelihood method. The sequences detected in the present study are highlighted in red. The numbers at the nodes correspond to boostrap values higher than 60% accessed with 1.000 replicates. *Theileria* species was used as outgroup.

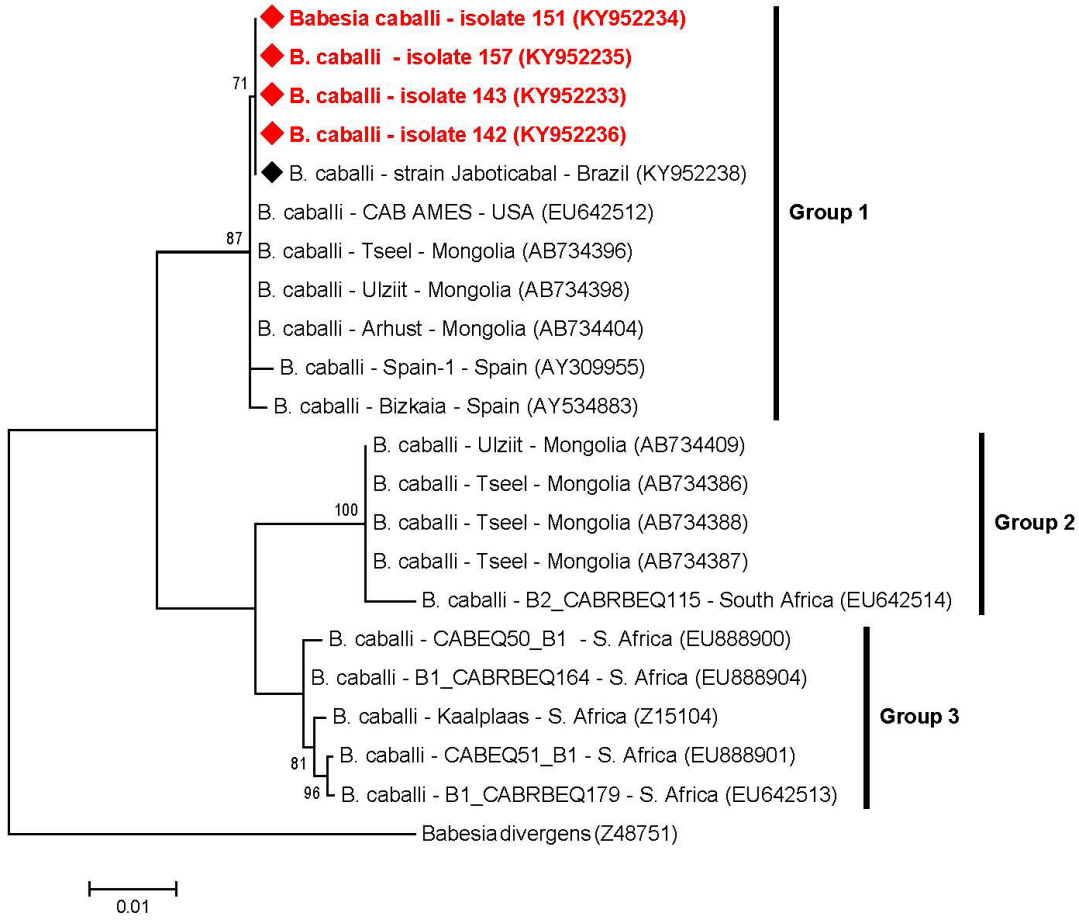

Figure 3. Phylogenetic relationships within the *B. caballi* sequences based on a 1720 bp fragment of the 18S rRNA gene. The phylogenetic tree was inferred by using the maximum likelihood method. The sequences detected in the present study are highlighted in red. The numbers at the nodes correspond to boostrap values higher than 60% accessed with 1.000 replicates. *Babesia divergens* was used as outgroup.

among known sequences available and, therefore, no diversity was observed among the isolates, the results of the phylogenetic analysis performed in the present study showed a high genetic diversity among the *T. equi*-18S rRNA sequences amplified in equids from different Brazilian regions. Additionally, the findings also revealed that the genetic diversity of *T. equi* sequences seems to be not geographically delimited in the Brazil, as suggested by the positioning of some sequences amplified from different regions (Maranhão, Rio Grande do Sul, Rio de Janeiro and São Paulo states), which clustered all together.

The heterogeneity observed among the *T. equi*-18S rRNA sequences amplified in equids from Brazil is in agreement with previous studies performed in Spain (NAGORE et al., 2004), South Africa (BHOORA et al., 2009), Greece (KOUAM et al., 2010), Mongolia (MUNKHJARGAL et al., 2013), Tunisia (ROS-GARCÍA et al., 2013) and Israel (KETTER-RATZON et al., 2017). On the other hand, Hall et al. (2013) showed that *T. equi*-18S rRNA sequences from different USA regions clustered in a single cluster, exhibiting very little diversity within this group, suggesting a limited introduction of a small number of *T. equi* genotypes into the USA (HALL et al., 2013).

Even though it is not possible to use the 18S rRNA gene sequences diversity to classify *T. equi* as different species (CRIADO-FORNELIO et al., 2003; NAGORE et al., 2004), it is extremely necessary that future studies evaluate the pathogenic potential of these different genotypes infecting equids around the world.

Conclusion

The present work showed that different genotypes of *T. equi* and *B. caballi* circulate among equids in Brazil. Future studies aiming at elucidating the differences in pathogenic potential of different piroplasmids genotypes are much needed. Additionally, the phylogenetic positioning of these genotypes according to mitochondrial genes will contribute to the elucidation of the taxonomy of Piroplasmida.

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