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Occurrence and genetic diversity of hemoplasmas in beef cattle from the Brazilian Pantanal, an endemic area for bovine trypanosomiasis in South America

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ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are Gram-negative bacteria that parasitize the erythrocyte surface of a wide variety of mammals. The present study aimed at investigating the occurrence of hemoplasmas in beef cattle in the Brazilian Pantanal, an area endemic for bovine trypanosomiasis in South America. Additionally, the objective of this study was to characterize molecularly the genotypes of the found hemoplasmas. For this purpose, blood and serum samples of 400 beef cattle were collected from five properties in Corumbá, Nhecolândia sub-region, Mato Grosso do Sul, in Midwest Brazil. Blood samples underwent DNA extraction and standard 16S rRNA gene-based PCR assays for hemoplasmas. The sequences obtained were submitted to phylogenetic inferences, distance analysis, and genotype diversity. The Indirect Enzyme-Linked Immunoabsorbent Assay (iELISA) indicated the presence of anti-Trypanosoma vivax IgG antibodies in 89.75% of the animals sampled, confirming the endemicity of said agent in the studied region. Among the 400 bovine blood samples tested, 2.25% (9/400) were positive for hemoplasmas in cPCR. The phylogenetic analysis of the obtained sequences confirmed the presence of *'Candidatus* Mycoplasma haemobos' and *Mycoplasma* wenyonii DNA in 0.5% (2/400) and 1.75% (7/400) animals, respectively. Five genotypes of *M. wenyonii* and one of '*Candidatus* M. haemobos' were detected among the sequenced amplicons. The present study showed low molecular occurrence of hemoplasmas in beef cattle sampled in the Brazilian Pantanal, an area endemic for bovine trypanosomiasis. Despite of the conservation of the 16S rRNA gene, there was considerable diversity of hemoplasma genotypes infecting the sampled beef cattle.

1. Introduction

The ever-increasing proximity between domestic and wild animals with humans has allowed the exchange of vectors and pathogens between different species. This increase of vector traffic increases the spreading of transmitted pathogens as well, causing health problems and mortality to a wide range of animals, including humans [1]. In this scenario, hemotropic mycoplasmas, also known as hemoplasmas, are emerging as important pathogens in human and veterinary medicine [2–4].

Hemoplasmas are bacteria of the genus *Mycoplasma* characterized by the absence of cell wall and epi-erythrocytic location [5], which facilitates transmission via hematophagous arthropod vectors, such as ticks, flies, and mosquitoes. Although the Brazilian climate favors the development of these vectors, the arthropods involved in the transmission of these agents remain unknown [6].

Abbreviations: '*C*Mh', '*Candidatus* Mycoplasma haemobos'; *Mw*, *Mycoplasma wenyonii*; ML, Maximum likelihood; BI, Bayesian inference

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Cattle farming is one of the main economic sectors of Brazil, representing 6% of the Gross Domestic Product (GDP) while handling annually approximately 400 billion reais [7]. The cattle herd in Brazil has approximately 218.2 million animals. Additionally, in 2017, Brazil produced 9.8 million tons of meat and ranked second in meat production worldwide, after the United States, which produced about 11.38 million tons [8]. Among the hemotropic mycoplasma species described so far, *Mycoplasma wenyonii* and *'Candidatus* Mycoplasma haemobos' have been detected in cattle from England [9], Japan [10], China [11], Switzerland [12] and Brazil [13,14]. Both species may cause hemolytic anemia in this group of ruminants [15].

The Brazilian Pantanal is the largest floodplain in the world, with a seasonal climate divided into a rainy season from October to April, and a dry period from May to September [16], and considered endemic for *Trypanosoma vivax* [17,18]. *Trypanosoma vivax* infection is correlated with immunosuppression in hosts and may increase its susceptibility to secondary infections [19]. Beef cattle ranching is the main economic activity in the Pantanal region [20], with predominantly Nellore herds.

Although hemoplasmas have been detected in domestic and wild ruminants around the world, few studies have been conducted regarding the epidemiology, genetic diversity and ecology of such agents. Since *T. vivax* has been associated with immunosuppression and predisposes to secondary infections, we hypothetized that a high occurrence of hemoplasmas would be found in cattle in Pantanal wetland. Therefore, this study aimed at investigating the occurrence and genetic diversity of hemoplasmas in beef cattle sampled in the Brazilian Pantanal.

2. Material and methods

2.1. Study site and sampling

Blood samples were collected from beef cattle (*Bos taurus indicus*) reared under extensive management from five properties selected conveniently in the central region of Pantanal Sul Matogrossense, in the Nhecolândia subregion (18º59'15" S; 56º37'03") (Fig. 1). Since there is no data on the prevalence of hemoplasmas in cattle in this state, an expected prevalence of 50% was assumed according to Stevenson [21]. The sample size was determined using the systematic random sampling method, where the minimum number of animals had an absolute accuracy of 5% and 95% confidence interval as indicated by the formula below:

$$n = z^2 (1 - Py) \times Py / d^2$$

where:z = confidence coefficient (z = 1,96);n = sample size;Py = expected prevalence (50%);d = desired absolute precision (5%) [21].

Therefore, according to the formula, the number of animals to be sampled would be $n = 1{,}96^2$ (1-0,5) x 0,5 / 0,052 : n = 384.

For this purpose, blood samples collected from 400 animals were split into two categories, cow (≥ 6 years) and calves (7–12 months). The categories were determined according to the availability of animals in front of the extensive breeding and rearing system for fattening, where females are kept in the herd as matrices and male calves are quickly sold for fattening on another farm.

In August 2016 and April 2017, the bovine blood samples were collected directly from the jugular or caudal vein in tubes containing EDTA (ethylenediaminetetraacetic acid) anticoagulant for DNA extraction, which was then submitted to molecular assays for hemoplasmas. Blood samples for separating the serum to perform the serological test for *Trypanosoma vivax* were also collected in tubes without anticoagulant. All samples were stored at −70 °C. The project was developed according to the norms of the National Council for Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee on the Use of Animals (CEUA) under protocol nº 12375/15.

2.2. Indirect Immunoenzymatic Assay (iELISA) for detecting the anti-Trypanosoma vivax IgG antibodies

The indirect ELISA technique (iELISA) was used to determine the

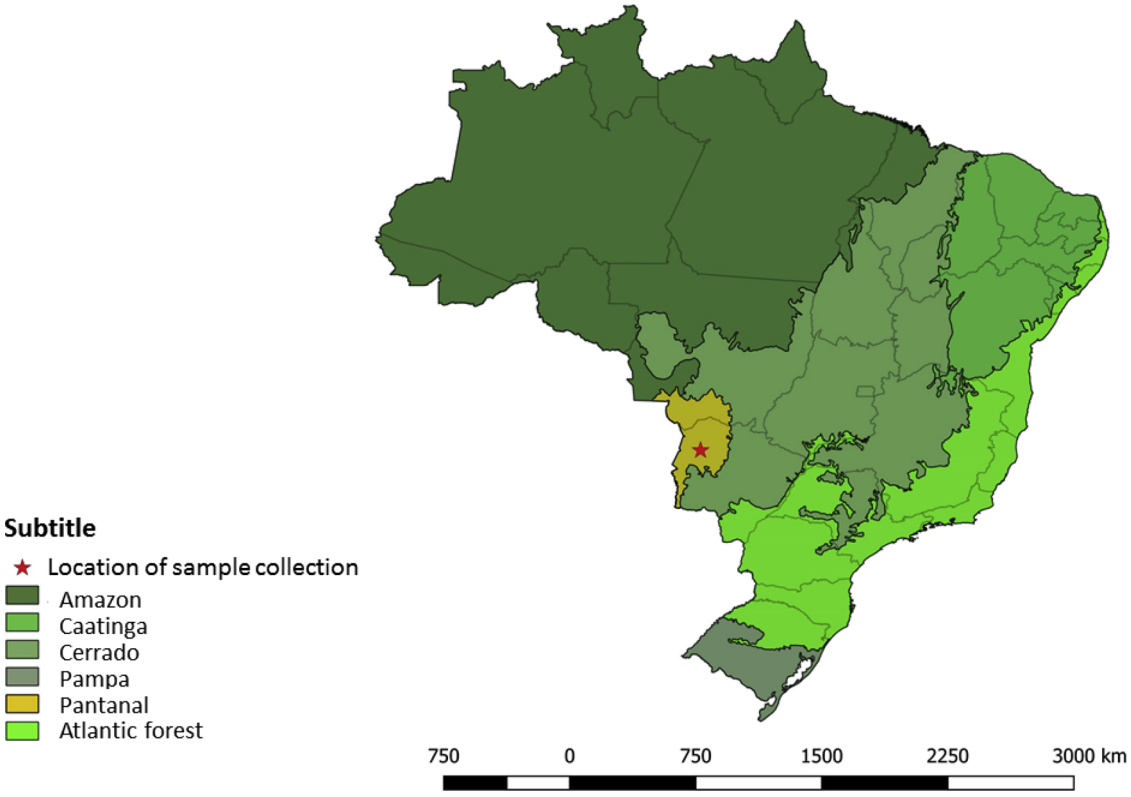

Fig. 1. Map of Brazil showing the Pantanal, focusing on the Nhecolândia subregion, in Corumbá, where the beef cattle blood was sampled.

presence of *Trypanosoma vivax* IgG antibodies in the studied area, following the protocol established by Aquino et al. [22], with modifications. The *T. vivax* total antigen used was produced in the Laboratory of Immunoparasitology of the Universidade Estadual Paulista (FCAV, UNESP, Jaboticabal), according to standard protocol [23]. The cutoff point was calculated as the mean absorbance of the negative controls times two and a half (2.5) [24].

2.3. DNA extraction from bovine blood samples

DNA from bovine blood samples was extracted using the DNA extraction protocol previously described [25]. The DNA samples were identified, their 260/280 and 260/230 ratios and concentrations were measured by spectrophotometry (Nanodrop - Thermo Scientific) and stored at −20 °C for subsequent PCR assays.

2.4. Endogenous PCR control

To avoid false negative results due to the presence of inhibitors and to verify the presence of amplifiable DNA in the samples, the DNA samples were submitted to a conventional Polymerase Chain Reaction (cPCR) to amplify the *glyceraldehyde-3-phosphate dehydrogenase endogenous gene* (*gapdh*) from mammals, following the previously described protocol [26].

2.5. Detection and characterization of hemoplasmas

2.5.1. cPCR for hemoplasmas targeting 16S rRNA gene

PCR positive samples for the endogenous gene were subsequently subjected to conventional PCR assays for detecting hemoplasmas. To this end, a 16S rRNA based PCR was performed [2]. Sterilized ultrapure water (Invitrogen®, Carlsbad, California, USA) was used as a negative control in all PCR assays. DNA from *M. wenyonii* and *'Candidatus* Mycoplasma haemobos' were kindly provided by Dr. Aline Girotto and used as positive controls in all amplification reactions.

2.5.2. cPCR for hemoplasmas targeting RNase P

After detection for hemoplasmas based on the 16S rRNA gene, the samples positive for said gene underwent cPCR targeting the RNase P gene [2]. All PCR assay products were subjected to horizontal electrophoresis in 1.0% agarose gel stained with Ethidium Bromide (0.5 µL/mL) in TEB run buffer pH 8.0 (44.58 M Tris-base, 0.44 M boric acid, 12.49 mM EDTA) for visualizing the results. Electrophoresis was performed at 90 V/150 mA for 60 min. The amplified products were determined using a molecular weight marker of 100 base pairs (Life Technologies®, Carlsbad, California, USA). The results were visualized and analyzed by an ultraviolet light transilluminator, coupled to a data analysis software (ChemiDoc MP Imaging System, BIO RAD®).

2.6. Purification and sequencing of amplified products

The PCR products based on the 16S rRNA and RNase P genes were purified using the "Silica Bead DNA Gel Extraction" Kit (Thermo Scientific, San Jose, CA, USA) following the manufacturer's recommendations. The purified amplified material was quantified, and its 260/230 and 260/280 ratios were also checked for determining the purity by reading each sample absorbance in the Nanodrop spectrophotometer (Thermo Scientific, San Jose, CA, U.S). The products amplified in the hemoplasma PCR assays were sequenced using an automated technique based on the dideoxynucleotide chain termination method [27] in the ABI PRISM 3700 DNA Analyzer sequencer (Applied Biosystems, Foster City, California, USA). The amplimers were sequenced at the Center for Biological Resources and Genomic Biology (CREBIO) of the Department of Technology of the College of Agrarian and Veterinary Sciences (FCAV, UNESP, Jaboticabal).

2.7. Sequence analysis

2.7.1. Phylogenetic inferences

The electropherograms generated in the sequencing were analyzed considering only the bases with quality higher than 20 by the PhredPhrap software [28]. The FASTA-saved sequences were aligned with other sequences of the same gene available on the GenBank database [29] in the MAFFT software [30]. The "FASTA" saved alignments were transformed into Nexus on the Alignment Transformation Environment site, so that the Bayesian analysis was performed in the MrBayes 3.2.2 software, XSEDE [31], via the CIPRES portal [32]. Bayesian analysis was performed with 10^8 generations and numbers of substitution classes varying according to the evolutionary model found for each data set. The evolutionary model was found based on the Akaike Information Criterion (AIC) in the jModelTest 2 software [33]. The phylogenetic trees' editing, including rooting (via an external group), was performed using Treegraph Software 2.0.56-381 beta [34].

2.7.2. Analysis of Mycoplasma spp. genotypes based on the 16S rRNA gene in the DnaSP software

The *Mycoplasma* spp. sequences were analyzed to determine the number of genotypes in the sequences using the DnaSP software version 5.10.1 [35].

2.7.3. Distance analysis by SplitsTree software

The 16S rRNA gene sequences (flanked by the HemMyco16S-41 s and HemMyco16S-938as oligonucleotides) obtained in this study, were analyzed by SplitsTree4 [36] to evaluate the distance relationships using the Neighbor-Net and Uncorrected p-distance.

2.7.4. Phylogeographic analysis by PopART software

The partial sequences of the 16S rRNA gene fragment obtained in this study and those of *M. wenyonii* and *'Candidatus* Mycoplasma haemobos' already in the GenBank were further submitted to PopART analysis (Population Analysis with Reticulate Trees) [37]. The sequences were grouped according to their spatial distribution in the DnaSP software version 5.10.1 [35] and saved in NEXUS format. After that, the NEXUS file was opened in PopART, by the Median-Joining genotyping network inference method [38] described previously.

2.8. Statistical analysis

The frequency of seropositive animals for *T. vivax* per category (cow versus calves; female versus male calves) was analyzed by the Chi-square test (X_2) and Fisher's exact test, using the software The R Project for Statistical Computing (R version 3.4.4). P values < 0.05 were considered as indicators for statistical differences in the number of positive animals in each state.

3. Results

3.1. Seropositivity to Trypanosoma vivax by the Indirect Immunoenzyme Assay (ELISA)

The ELISA serological test revealed the presence of *T. vivax* IgG antibodies in 98.5% (197/200) and 83,5% (167/200; 85,3% (93/109) males and 81,3% (74/91) females) of cows and calves, respectively, demonstrating the high endemism for this etiological agent in the studied region. Regarding the seropositivity for *T. vivax* according to the calves' gender, the Fisher and Chi-square values were 0.4525 and 0.57, respectively, showing no significant statistical difference. On the other hand, the value of Fisher's applied to seropositivity for *T. vivax* between cows and calves was 6,834 e^{-8}, showing a significant difference between the positivity of the sampled age group. The number of seropositive cows showed to be higher than the number of calves seropositive for *T. vivax*.

3.2. Quality of DNA from bovine blood samples and cPCR for the gapdh gene

The mean DNA concentration and the absorbance ratios (260/280 nm and 260/230 nm) of the DNA samples were 31.8 ng/μL (SD ± 10.2), 1.4 nm and 0.47 nm (SD ± 0.1 and ± 0.5), respectively. All 400 DNA samples (100%) analyzed were positive for the gapdh gene (endogenous control) in cPCR.

3.3. Detecting Mycoplasma spp. by 16S rRNA cPCR

From the 400 blood samples tested, 2.25% (9/400) were positive for hemoplasmas in 16S rRNA cPCR (using the HemMycop16S-41 s and HemMyco16S-938as primers). However, only the blood samples from calves were positive for *Mycoplasma* spp. while cows were all negative according to PCR assays.

3.4. Detecting hemoplasmas by RNase P cPCR

Out of the 9 samples previously positive in cPCR assays for hemoplasmas based on 16S rRNA gene, 66.67% (6/9) were positive in cPCR assay targeting RNase P gene.

3.5. Identity analysis of the sequences obtained by BLASTn

3.5.1. Analysis of Mycoplasma spp. sequences for the 16S rRNA gene

All nine sequenced positive samples had quality sequences of the 16S rRNA gene as determined by the quality analysis of the PhredPhrap software. BLASTn analysis of the obtained sequences revealed identity percentages ranging from 99 to 100% with the *'Candidatus* Mycoplasma haemobos' and *M. wenyonii* sequences previously deposited on the GenBank. Due to the low intensity of the band formed, it was not possible to obtain a quality sequencing related to the RNase P gene, making phylogenetic inferences based on this gene impossible.

3.6. Phylogenetic analysis

3.6.1. Phylogenetic analysis of Mycoplasma sp.

The Bayesian Inference (BI) method and the GTR + G evolutionary model generated a cladogram with 12 clades, supported by posterior probability values ranging from 51 to 100%. The obtained sequences were positioned in three clades (#1, #6 and #7), two in the *Mycoplasma suis* group, and the third in the *Mycoplasma haemofelis* group (Fig. 2).

The sequences S1 and S5 for samples B165 and B120, respectively, were positioned in the first clade, forming polytomies, together with *Candidatus* Mycoplasma haemobos sequences of cattle from Switzerland (EF616468), Taiwan (KJ883514; KJ883515) and China (EF460765). Among the polytomies generated in clade #1, there was also a branch comprising the 'CMh' and *Mycoplasma* sp. sequences that originated from buffaloes from Mozambique (MF992084) and China (EF424082), respectively. Also, a branch containing the 'CMh' sequences from bovines from Japan (EU367965) and Brazilian buffaloes (KY328834), phylogenetically positioned next to a sequence of *Mycoplasma* sp. from Swiss horses (FN421445). Sequences obtained from samples B145, B146, B152, B155 and B184, in turn, clustered in clade #6, being phylogenetically close to two *M. wenyonii* sequences detected in cattle from Germany (FN392885; FN392886), China (AY769937) and Japan (EU367934), supported by an ancestral Mw sequence detected in cattle in China (EF221880).

Sequences of samples B186 and B187 were positioned on a polytomic branch within clade #7, along with the *M. wenyonii* sequence detected in cattle from Mexico (KX171205). Among the polytomies generated, a branch was formed with the Mw sequences detected in cattle in Japan (EU367963) and Austria (KY412804), and in buffaloes from Mozambique (MF981947).

3.7. Analysis of identity between sequences

3.7.1. Analysis of Mycoplasma spp. genotypes based on the 16S rRNA gene in the DnaSP software

Six genotypes were identified among the nine sequences analyzed, with $\pi = 0.0889$, Dh = 0.917 and K = 51.55556 (Table 1). Genotype #1 included the S1 and S5, referring to the 'CMh', while the other genotypes (#2, #3, #4, #5 and #6) included the Mw sequences.

3.7.2. Genotype analysis based on the 16S rRNA gene from 'Candidatus Mycoplasma haemobos'

The distance analysis of the *'Candidatus* Mycoplasma haemobos' sequences revealed two genotypes. The genotype #1 included the largest number of sequences, coming from Switzerland, Japan, China, Mozambique, Taiwan, and the states of Maranhão and Paraná in Brazil, and those obtained in this study (S1 and S5) from the Pantanal region. It is inferred that the genotype #2 from Switzerland appeared/originated from #1. The network of genotypes obtained from the previously described Median-Joining inference method [38] can be observed in Fig. 3.

3.7.3. Genotype analysis based on the Mycoplasma wenyonii 16S rRNA gene

The distance analysis of the *M. wenyonii* sequences revealed seven genotypes. Genotype #5 included the largest number of sequences, from China, Austria, Germany, Turkey, Mozambique, Mexico, the United States, Japan, and Brazil, from Maranhão and from the Pantanal region in MS (S3 and S4) obtained in the present study. It is inferred that genotype #5 is a precursor of the other genotypes since mutational events that started with it (symbolized by line risks) generated genotypes #2 and #6. Genotype # 3 from Japan may have originated from genotype #2 or #6. Genotype #2, in turn, comprised the Pantanal, MS (S2, S6, and S7) sequences, from Germany and England. It is inferred that genotype #4, which encompasses sequences exclusively from the Pantanal, MS (S8 and S9), arose from genotype #2 since a greater number of mutational events occurred among them. Genotypes #1 and #7, as well as #4, may have derived from genotype #2. The network of genotypes obtained from the Median-Joining inference method [38] is shown in Fig. 4.

3.8. Phylogeographic analysis by SplitsTree

3.8.1. Phylogeographic analysis based on the 'Candidatus Mycoplasma haemobos' 16S rRNA gene

The distance analysis performed for the first 16S rRNA gene fragment of *'Candidatus* Mycoplasma haemobos' positioned its S1 and S5 sequences from B165 and B120 samples, respectively, close to each other. The sequence S5 positioned at the same point as the sequences of said agent detected in cattle from Japan (EU367965), and in Taiwanese dogs (KJ883514), demonstrating high conservation of the used gene fragment among them. In turn, the S1 sequence was positioned at the same point as the *'Candidatus* Mycoplasma haemobos' sequence previously detected in Taiwanese dogs (KJ883515), and both were strictly close to the sequence detected in buffaloes from Mozambique (MF992084). Additionally, the S5 sequence had a certain proximity to the buffalo sequence obtained in Maranhão, northeastern Brazil (KY328834). The phylogeographic relationships obtained by the SplitsTree4 software with the "Neighbor-Net" and "Uncorrected p-distance" parameters for the 16S rRNA gene of *'Candidatus* Mycoplasma haemobos' sequences can be seen in Fig. 5.

3.8.2. Phylogeographic analysis of Mycoplasma wenyonii

The distance analysis of the *Mycoplasma wenyonii* sequences positioned the sequences obtained in this study in two clades. In the first clade, sequences S3 and S4, for B186 and B187 samples, respectively, were pooled with sequences previously detected in cattle from Japan

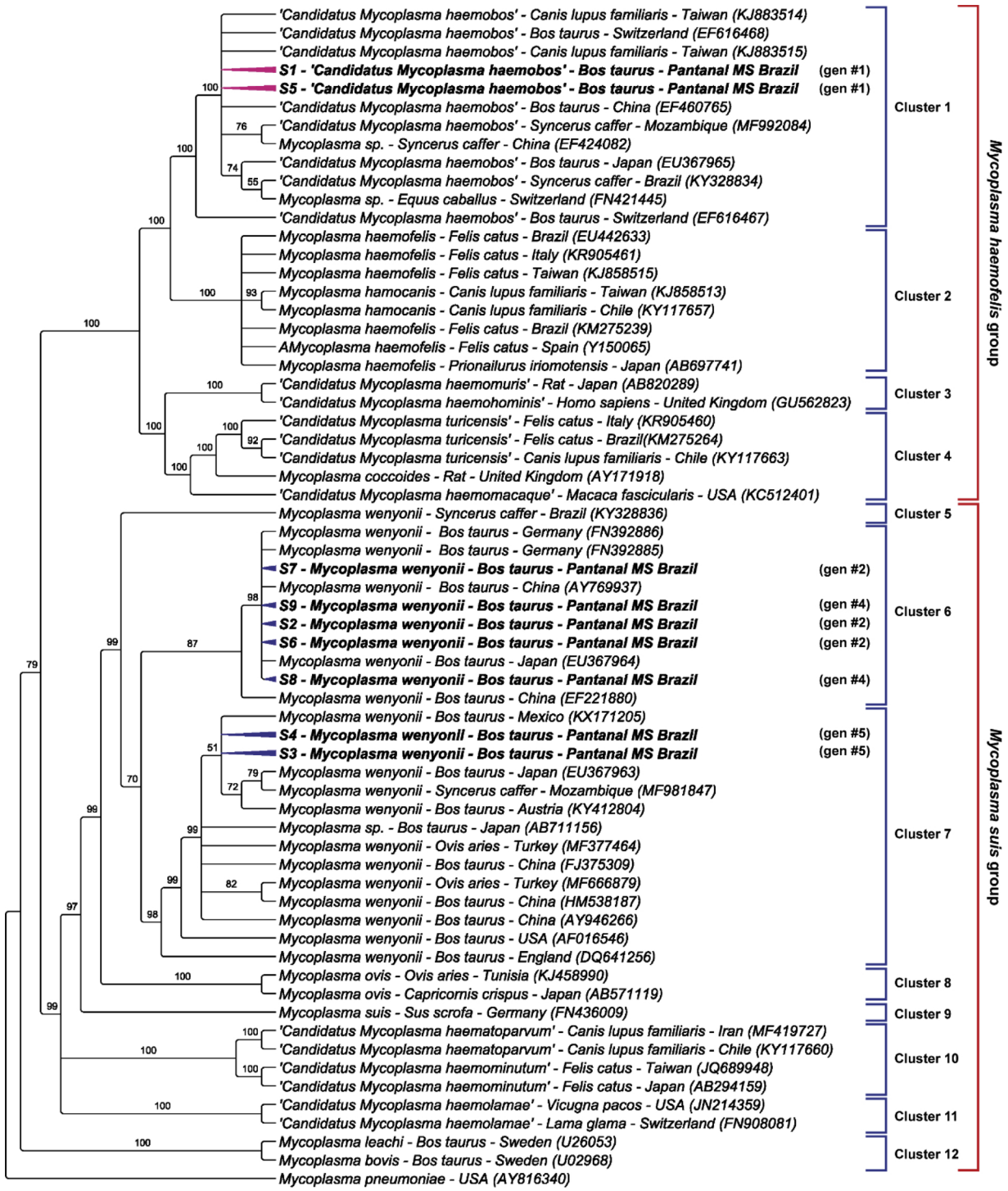

Fig. 2. The phylogenetic relationship within the genus *Mycoplasma* based on an approximately 800 bp fragment of the 16S rRNA gene. The phylogenetic tree was inferred from the Bayesian method and the GTR + G evolution model. The genotypes of each sequence obtained by the PopArt software are shown in parentheses. The numbers in each branch are the posterior probability values, greater than 51% accessed with a number of generations equal to 1,000,000. *Mycoplasma pneumoniae* was used as an outgroup.

(HM538187; AB711156), sheep from Turkey (MF666879; MF377464), and cattle from China (AY946266; FJ375309) and Mexico (KX171205). Sequences S2, S6, S7, S8 and S9 corresponding to samples B184, B145, B146, B152, and B155, respectively, were grouped in the second clade and showed greater proximity to sequences detected in cattle from China (AY769937), Japan (EU367964) and Germany (FN392886). There was no significant proximity of any sequence from Mato Grosso do Sul, with the *Mycoplasma wenyonii* sequence previously detected in buffaloes in Maranhão, Northeast Brazil (KY328836). The phylogeographic relationships obtained by the SplitsTree4 software with the "Neighbor-Net" and "Uncorrected p-distance" parameters for the sequences of *Mycoplasma wenyonii* 16S rRNA gene can be observed in Fig. 6.

Table 1
Polymorphisms of *Mycoplasma* sp. 16S rRNA sequences detected in beef cattle sampled in the Pantanal Sul-Matogrossense.

	~ (bp)	N	S	GC%	h	Dh (mean ± SD)	π (mean ± SD)	K
16S rRNA Gene	800	9	131	0,452	6	0,917 ± 0,073	0,08889 ± 0,032	51,55556

N - number of analyzed sequences; S - number of variable sites; GC - G + C content; h - number of genotypes; Dh - diversity of genotypes; SD - standard deviation; π - nucleotide diversity (per site); K - nucleotide difference number.

4. Discussion

This study showed, for the first time, the occurrence and molecular characterization of *M. wenyonii* and *'Candidatus* Mycoplasma haemobos' in a beef cattle population in Brazil. To the best of our knowledge, although several studies have suggested that *Mycoplasma* spp. has co-evolved with several animal species, including humans [2], little is known about the origin, evolution, transmission, and dispersion of hemoplasmas. In fact, the epidemiology of hemotropic mycoplasmas that parasitize domestic and wild animals, as well as humans in Brazil is still little known.

The occurrence of hemoplasmas detected in beef cattle (2.25% [9/400], 77.7% [7/9] for *M. wenyonii* and 22.22% [2/9] for *Candidatus* Mycoplasma haemobos') in this study was lower than those found in dairy cattle in Rondônia (64.7% [207/320]) [14]; Paraná (61% [264/433]) [13], and Santa Catarina (40.9% [9/22]) [39], in Brazil. The percentage of positive animals was lower than those reported for cattle in other countries such as Japan [40], Switzerland [12], China [41], and England [9].

Since the transmission mechanisms of hemoplasmas have not yet been fully elucidated and no biological vectors have been identified so far, arthropod vectors such as ticks, flies and mosquitoes may possibly play a role only in the mechanical transmission of these agents [5,42]. In addition, the impossibility of *in vitro* culture of hemoplasmas makes it difficult to perform vector competence studies [43,44].

Recently, ticks of the *Rhipicephalus (Boophilus) microplus* species have been incriminated as mechanical vectors of hemoplasmas [45]. Thus, the low occurrence of hemoplasmas in the sampled beef cattle may reflect the higher resistance to ectoparasites of zebu cattle (*Bos indicus*) compared to taurine (*Bos taurus*). Several studies have demonstrated that Nellore cattle are highly resistant to some pathogens and ectoparasites, especially ticks [46]. Such resistance may be associated with some factors such as bovine self-cleaning behavior, fur characteristics [47], genes expressing lipocalin and keratin [48], among others. In fact, most of the studies conducted around the world have evaluated the occurrence of hemoplasmas in dairy cattle (taurine) (England [9], Japan [10], China [11], Switzerland [12], among others).

The 'CMh' and *Mw* were detected by PCR assays targeting the 16S rRNA gene, which is responsible for encoding the minor subunit of ribosomal RNA (SSU rRNA). Present in all bacteria, this gene function remained the same throughout evolution, being a conserved gene that is large enough (~1500 bp and with approximately 50 functional domains) to allow molecular typing analyzes [49]. This gene is observed in multiple copies in the bacterial genome, unlike other genes that present only one copy. The presence of several copies may be associated with the high levels of gene transcription, necessary to supply the demand of the RNA molecules for assembly of ribosomes [50]. Even with the multiplicity of copies of such gene, the cPCR detected a low level of hemoplasmas in the present study. Alternatively, the occurrence could be possibly higher if qPCR protocols had been used, due to a higher sensitivity of this technique.

Herein, serology confirmed the endemicity for *T. vivax* in the sampled region, a fact previously observed by other researchers [17,18]. Even though the seropositivity for *T. vivax* did not differ between

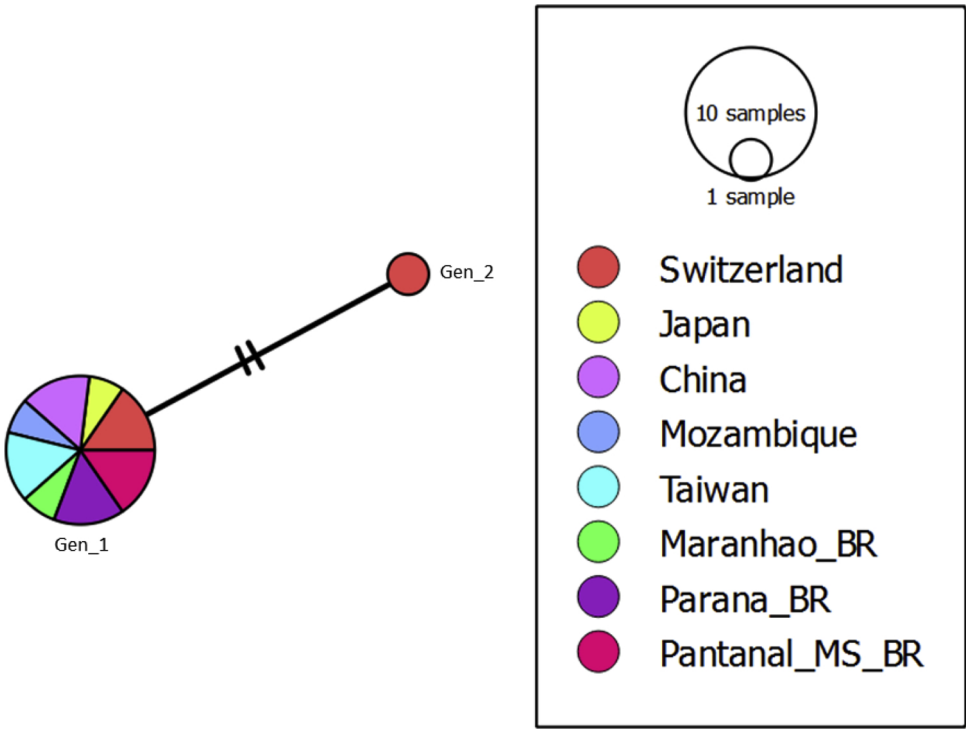

Fig. 3. A network of genotypes formed between the '*Candidatus* Mycoplasma haemobos' sequences.

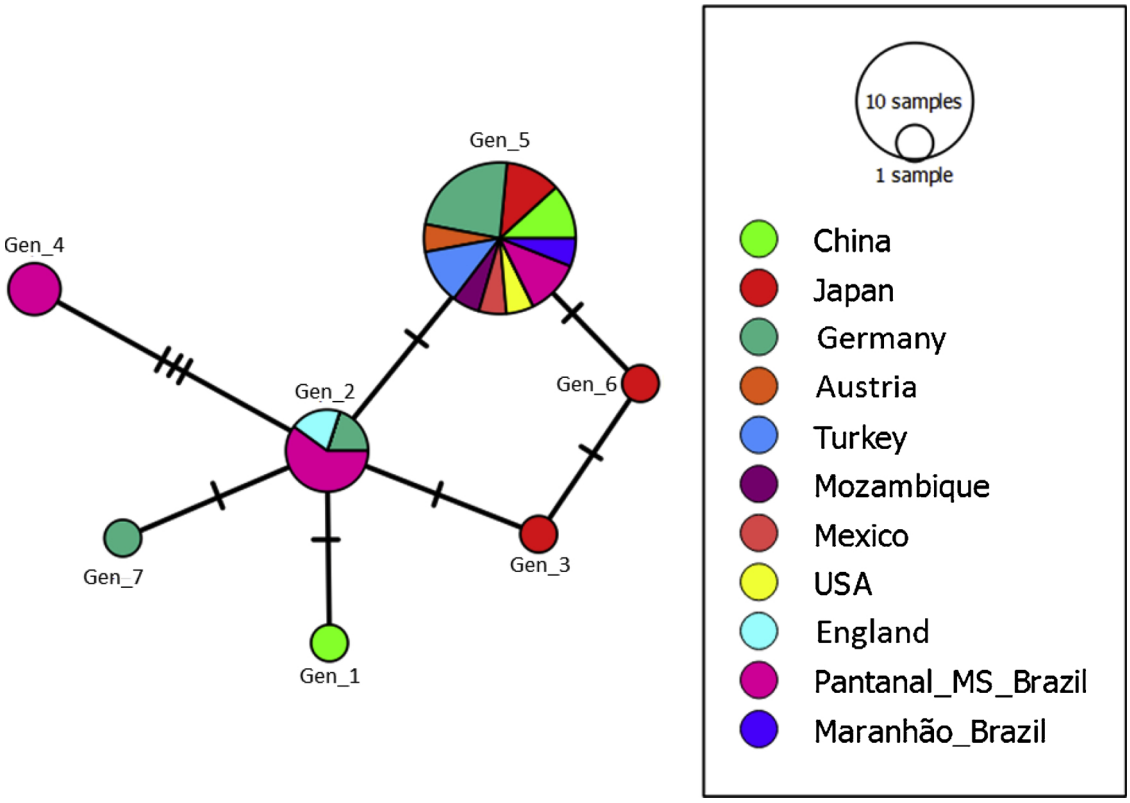

Fig. 4. Genotype network formed for the *Mycoplasma wenyonii* sequences.

female and male calves, a higher seropositivity was found among cows when compared to calves, as previously reported [51]. This is mainly due to the permanence of cows for a long time in the analysed farms, favoring their exposure to infected mechanical arthropod hematophagous vectors or contaminated fomites. Immunosuppression has been associated with *T. vivax* infections, predisposing the host to coinfections and even vaccine failures against other pathogens [52–54]. Of the 9 calf blood samples positive for hemoplasmas in the PCR, 88.89% (8/9) were seropositive for *T. vivax*. Co-infection between hemoplasmas and *T. vivax* has been described in the literature [19]. The trypanosomiasis pathogenesis varies and clinical signs are nonspecific, represented by fever, anemia, weakness, abortion, among others, similarly to those found in hemoplasmosis. The animal age is one of the risk factors for hemoplasma infection, according to Messick [42]. This supports our results since only the calves were positive for hemoplasmas by molecular methods, which might be related to the immune competence and the ability to fight infection by adult animals. Although hemoplasmosis hardly leads to death, such agents may intensify the virulence of co-infections [55].

The topology of phylogenetic analyses targeting the 16S rRNA gene fragments commonly divides the hemoplasmas into these two subgroups [2]. The same phylogenetic pattern could be observed in studies with hemoplasmas in Brazilian cattle [14], wild buffalo (*Syncerus caffer*) in Mozambique, Africa [56], small ruminants in Turkey [57], cattle in Germany, Switzerland and Japan [58,12,10], and reindeer (*Rangifer tarandus*) in the United States [59].

Distance analyses based on the 16S rRNA gene were performed for the sequences of the studied gene. Such analyses allow visualizing relationships between sequences that are not seen by other phylogenetic inference methods, observing complex events that occurred along the evolution, such as duplication and loss of genes, horizontal transfer, among others [36]. Thus, the SplitsTree software grouped the *Mw* sequences (S3 and S4) into a clade close to sequences from Mexico (KX171205), China (FJ375309; AY946266), Japan (HM538187; AB711156) and Turkey (MF666879; MF377484), while the others (S2, S6, S7, S8, and S9) were clustered in another group, closely related to sequences from China (AY769937), Japan (EU357964) and Germany (FN392885). The positioning of the S3 and S4 sequences and the Mexico sequence (KX171205) corroborated the BI analysis, as well as the analysis of the other sequences (S2 and S6-S9), which were positioned close to the sequences from China (AY769937), Japan (EU367964) and Germany (FN392886).

The distance analysis of the 'CMh' sequences (S1 and S5) indicated greater proximity with sequences from Taiwan (KJ883515; KJ883514), Japan (EU367965) and Mozambique (MF992084), as observed in the BI analysis, in which they were positioned in the same clade. The sequence from Switzerland (EF616468), with which they also shared a common ancestor in the phylogenetic analyses, was a little more distant from the 'CMh' S1 and S5 sequences from the Brazilian Pantanal, showing a high number of mutational events among them throughout evolution, resulting in this small distance.

The PopART results, in turn, exploit population genetic data through a network of genotypes, allowing visualization of biogeography, genealogical relationships and population history [37]. The genotype network formed with the 'CMh' sequences (S1 and S5) obtained in the present study and taken from GenBank revealed the presence of two genotypes. The genotype #1, containing the largest number of sequences, included those from the Brazilian Pantanal (S1 and S5), Switzerland and Taiwan, which were close in the BI and distance analyses, indicating that they represent the same genotype.

On the other hand, the network of *Mw* genotypes revealed 7 genotypes. The sequences from the Brazilian Pantanal were positioned in three of them (Gen #2, Gen #4, and Gen #5). In contrast, the distance analysis indicated that the S8 and S9 sequences were isolated, more distant from the others. Such positioning corroborates the PopART analyses, whereby such sequences corresponded to genotype #4, which included only of these two sequences. These findings indicate that both belong to the same genotype and diverge from the others, although they

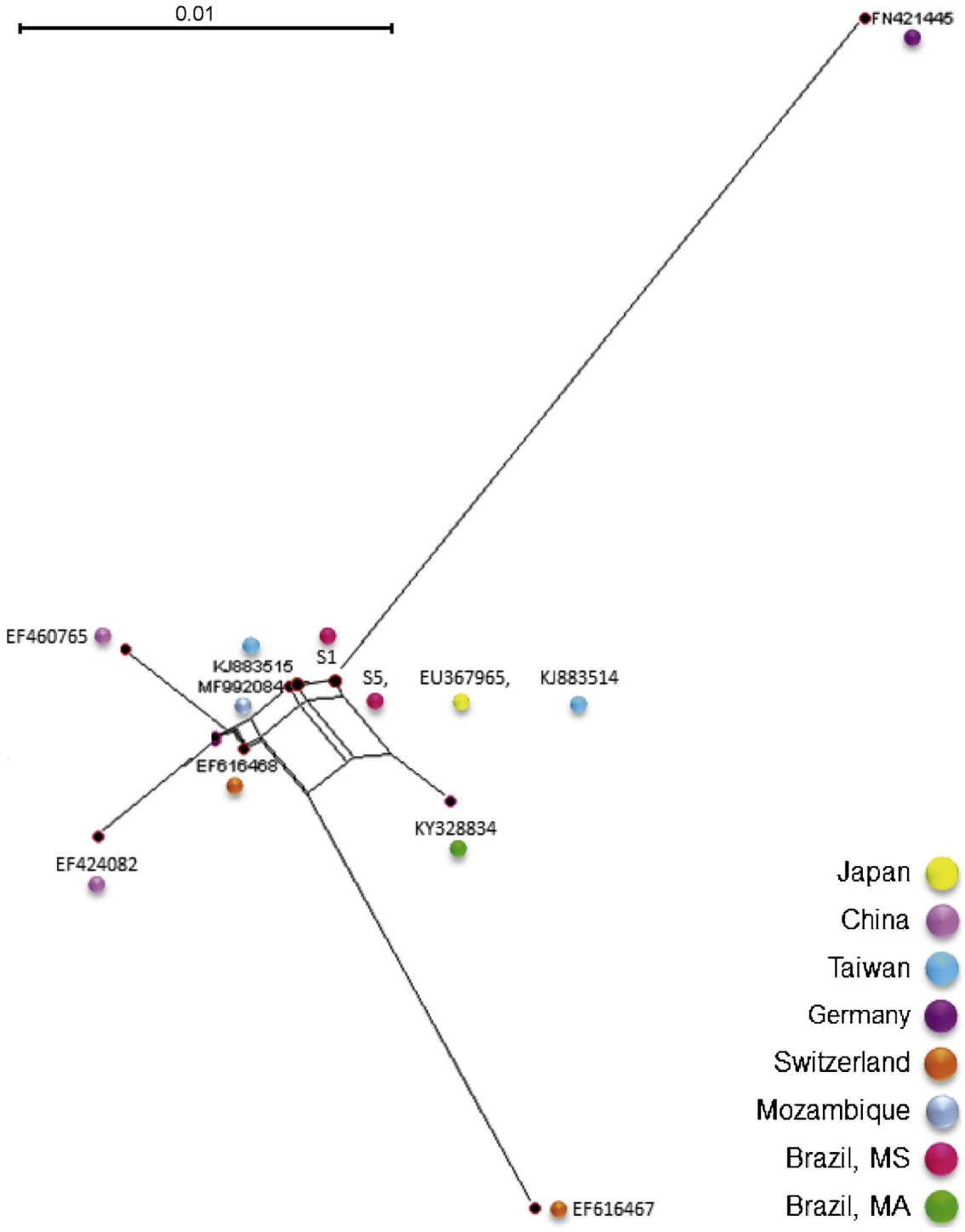

Fig. 5. Phylogeography of sequences related to the 16S rRNA gene of *'Candidatus* Mycoplasma haemobos' from Brazil and the world.

shared the same ancestor of the S2, S6 and S7 sequences, which were classified as genotype #2 and also included the sequences from Germany and England. Despite sharing the same genotype, the S2, S6, and S7 sequences positioned far from the sequence detected in England by the distance and BI analyses, whose results indicated greater phylogenetic proximity (BI) with those from China, Japan, and Germany.

The *Mw* S3 and S4 sequences comprised genotype #5 along with sequences from China, Japan, Germany, Austria, Turkey, Mozambique, the United States, Mexico, and Brazil. The sequence from Mexico shared the same common ancestor as the sequences S3 and S4 in the phylogenetic analyses, and they were positioned close to each other in the distance analysis, corroborating each other. Also, it is important to emphasize that while 'CMh', Mw and other species of *Mycoplasma* spp. sequences were used in the Bayesian analysis, only 'CMh', Mw and *Mycoplasma* sp. sequences were used in distance and genotype analyses. Despite some divergence between the sequences, the analyses carried out corroborated each other.

Finally, the results of this study indicated a low occurrence of hemoplasmas in beef cattle in the Brazilian Pantanal, even in the presence of endemism for *T. vivax,* a protozoan that has been previously associated with immunosuppression in infected animals. Further studies are needed to elucidate the transmission mechanisms of hemotropic

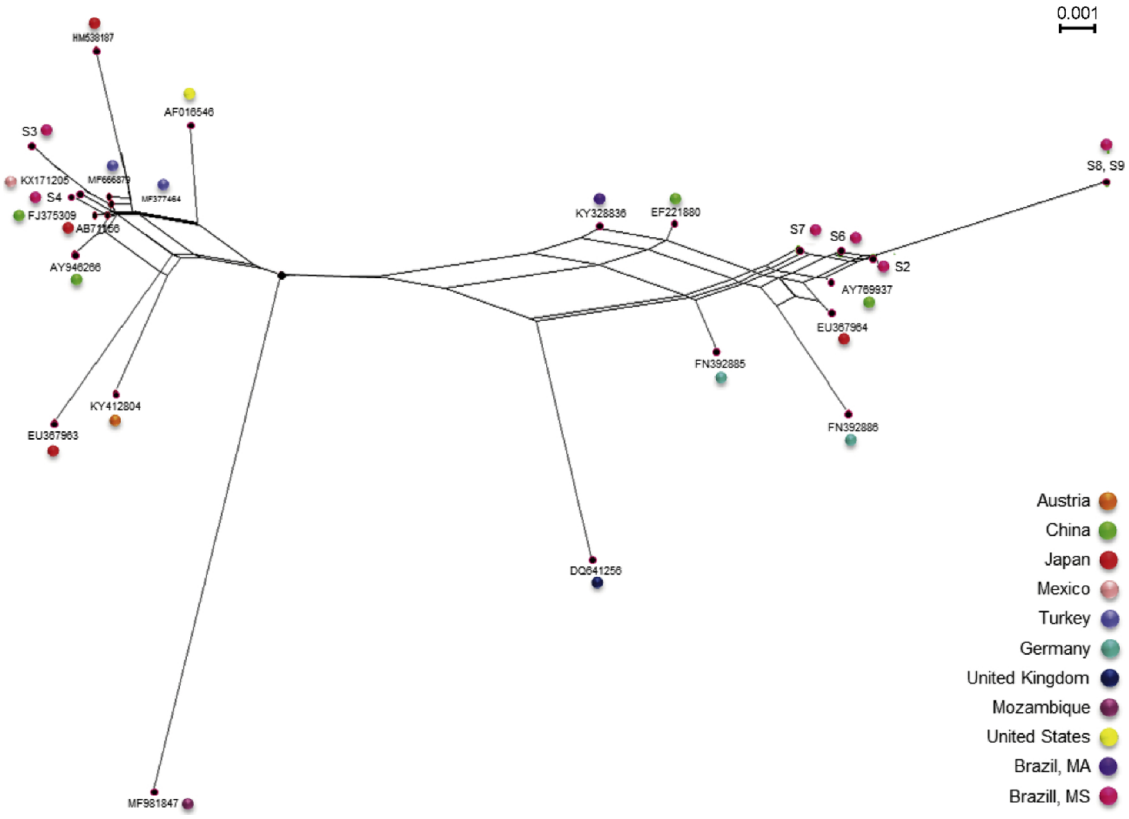

Fig. 6. Phylogeography of sequences related to the *Mycoplasma wenyonii* 16S rRNA gene from Brazil and the world.

mycoplasmas and their role as an anemia-causing agent in single infections and co-infections with other hemoparasites.

5. Conclusion

The low occurrence of *Mycoplasma wenyonii* and *'Candidatus* Mycoplasma haemobos' was verified in beef cattle from the Brazilian Pantanal, an endemic area for trypanosomiasis. The present work reported, for the first time, the molecular occurrence of 'CMh' and *M. wenyonii* in beef cattle in Latin America. Despite the conservation of the 16S rRNA gene, considerable diversity of hemoplasma genotypes was observed infecting beef cattle in Pantanal wetland.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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