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Short Communication

Post mortem protozoan hemoparasites detection in wild mammals from Mato Grosso state, Midwestern Brazil

Detecção *post mortem* de hemoparasitas protozoários em mamíferos selvagens do estado de Mato Grosso, Centro-Oeste do Brasil

Victoria Luiza de Barros Silva[1]; Sayanne Luns Hatum de Almeida[1]; Maerle Oliveira Maia[2]; Tarcísio Ávila Santos[3]; Lucas Avelino Dandolini Pavelegini[3]; Gabriela Brocco Zaffalon[3]; Arlei Marcili[4]; Thaís Oliveira Morgado[5]; Valéria Dutra[2]; Luciano Nakazato[2]; Richard de Campos Pacheco[1]*

[1]Laboratório de Parasitologia Veterinária e Doenças Parasitárias dos Animais Domésticos e Silvestres, Hospital Veterinário – HOVET, Faculdade de Medicina Veterinária – FAVET, Universidade Federal de Mato Grosso – UFMT, Cuiabá, MT, Brasil
[2]Laboratório de Microbiologia e Biologia Molecular Veterinária, Hospital Veterinário – HOVET, Faculdade de Medicina Veterinária – FAVET, Universidade Federal de Mato Grosso – UFMT, Cuiabá, MT, Brasil
[3]Laboratório de Patologia Veterinária, Hospital Veterinário – HOVET, Faculdade de Medicina Veterinária – FAVET, Universidade Federal de Mato Grosso – UFMT, Cuiabá, MT, Brasil
[4]Programa de Pós-graduação em Medicina e Bem-Estar Animal, Universidade de Santo Amaro – UNISA, São Paulo, SP, Brasil
[5]Setor de Animais Silvestres, Hospital Veterinário – HOVET, Faculdade de Medicina Veterinária – FAVET, Universidade Federal de Mato Grosso – UFMT, Cuiabá, MT, Brasil

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Abstract

To a better insight into the epidemiology and genetic diversity of protozoan hemoparasites infections in wild mammals, this study aimed to the post mortem detection of DNA from species of the order Piroplasmida (*Babesia* sp., *Cytauxzoon* sp., and *Theileria* sp.) and suborder Adelorina (*Hepatozoon* sp.) using polymerase chain reaction based on the 18S rRNA gene followed by genetic sequencing of blood and spleen samples collected from carcasses of 164 free-ranging and captive wild mammals from Mato Grosso state. Among them, one *Leopardus pardalis*, three *Panthera onca*, two *Puma concolor* were positive for *Cytauxzoon* sp., and six *Tapirus terrestris* tested positive for Piroplasmida, while one *L. pardalis* was positive for *Hepatozoon* sp. Furthermore, an uncharacterized piroplasmid genetically related to *Theileria* sp. previously detected in cats from Brazil was described in lowland tapirs. Despite the controversy regarding the epidemiological threat of these protozoa, the detection of these tick-borne agents in wild free-living and captive mammals, even when asymptomatic, demonstrates the importance of monitoring, particularly in hotspots such as the state of Mato Grosso, to verify the circulation and genetic diversity, to anticipate the possible emergence of diseases, and even their consequences to other animals as well as humans.

Keywords: *Cytauxzoon* sp., *Hepatozoon* sp., *Theileria* sp., PCR.

Resumo

Para uma melhor compreensão da epidemiologia e diversidade genética das infecções por hemoprotozoários em mamíferos selvagens, este estudo teve como objetivo a detecção *post mortem* de DNA de espécies da ordem Piroplasmida (*Babesia* sp., *Cytauxzoon* sp. e *Theileria* sp.) e subordem Adelorina (*Hepatozoon* sp.), utilizando-se a reação em cadeia pela polimerase, baseada no gene 18S rRNA, seguido de sequenciamento genético de amostras de sangue e baço, coletadas de 164 carcaças de mamíferos selvagens de vida livre e cativos do estado de Mato Grosso. Entre eles, um *Leopardus pardalis*, três *Panthera onca*, dois *Puma concolor* foram positivos para *Cytauxzoon* sp., e seis *Tapirus terrestris* testaram positivos para Piroplasmida, enquanto um *L. pardalis* foi positivo para *Hepatozoon* sp. Além disso, foi descrito em antas, um piroplasmídeo não caracterizado geneticamente, relacionado à *Theileria* sp., previamente detectado em gatos do Brasil. Apesar da controvérsia quanto à ameaça epidemiológica desses protozoários, a detecção desses agentes em mamíferos silvestres e cativos, mesmo quando

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*Corresponding author: Richard de Campos Pacheco. E-mail: richard@ufmt.br

assintomáticos, demonstra a importância do monitoramento, principalmente em *hotspots*, como no estado de Mato Grosso, para verificar a circulação e a diversidade genética, a fim de antecipar o possível surgimento de doenças e, até mesmo, suas consequências para outros animais, bem como os humanos.

Palavras-chave: *Cytauxzoon* sp., *Hepatozoon* sp., *Theileria* sp., PCR.

Living beings interact, and these interactions are the result of natural selection and the activities of evolution. Among these relationships, parasitism is one of the most successful, and one of the main factors responsible for modulating communities (Timi & Poulin, 2020) and reducing host fitness in various ways. Significant environmental changes and consequent increases in the interaction between domestic and wild animals (André et al., 2015) can increase the emergence of new diseases in new hosts. Molecular studies of hemoparasite genera belonging to the Apicomplexa have demonstrated the wide distribution and variety of hosts and vectors (Wang et al., 2017; van As et al., 2020), however, much remains to be elucidated.

Considering the expanse of Brazil and the great biodiversity of mammals in the state of Mato Grosso, there are currently few studies on apicomplexan protozoans and their relationship with large wild mammals in this region (André et al., 2010; Furtado et al., 2017b). Therefore, polymerase chain reaction (PCR)-based methods were used to investigate the diversity and occurrence of the apicomplexan parasites *Babesia*, *Cytauxzoon*, *Hepatozoon*, and *Theileria* in blood and spleen samples collected from carcasses of wild mammals in the state of Mato Grosso, Brazil.

From December 2019 to July 2021, tissue samples (blood and spleen) were collected from carcasses of road-killed free-roaming and captive wild animals from six municipalities in the State of Mato Grosso, Brazil, attended at Veterinary Hospital and sent for routine necropsy at the Veterinary Pathology sector of the Federal University of Mato Grosso, located in the Cuiabá municipality, as depicted in Table 1 and shown in Figure 1.

Procedures in this study were previously approved by the Ethics Committee on Animal Research of the Federal University of Mato Grosso (CEUA protocol no. 23108.015878/2019-65) and "Instituto Chico Mendes de Conservação da Biodiversidade" (ICMBio permit no. 55104-1).

DNA extraction from blood and tissue samples (spleen) was performed using the DNA extraction PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. To verify the success of extraction, an initial PCR targeting a fragment of the mammalian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was performed as previously described (Birkenheuer et al., 2003). Extracted DNA samples were then subjected to further PCR assays to amplify fragments of the small ribosomal DNA subunit 18S of Piroplasmida members (*Babesia* sp., *Cytauxzoon* sp., and *Theileria* sp.) and *Hepatozoon* spp. Negative controls (nuclease-free water) and appropriate positive controls for each PCR assay were included, as follows: *Babesia caballi* (GenBank accession number MG906574) and *Hepatozoon canis* (GenBank accession number MG496257) from blood of naturally infected horse and dog, respectively. DNA samples were subjected to nested PCR (nPCR) to amplify a fragment (~800 base pairs - bp) gene for Piroplasmida (*Babesia* sp., *Cytauxzoon* sp., and *Theileria* sp.), as previously described (Jefferies et al., 2007). Furthermore, biological samples were screened using a previously described conventional PCR (cPCR) protocol (Ujvari et al., 2004) by targeting a fragment (~600 bp) of the 18S rDNA region of the *Hepatozoon* spp. The PCR products were resolved on 1.5% agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium Inc, Fremont, CA, USA) and visualized using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Amplicons of the expected sizes were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and prepared for sequencing according to the instructions provided in the BigDye™ kit (Applied Biosystems, Foster, CA, USA). An ABI PRISM 3500 Genetic Analyzer (ABI DNA Model 3500 Series Genetic Analyzer, Applied Biosystems, Inc., Foster City, CA, USA) was employed to conduct the sequencing procedures using the same primers used for the PCR. The obtained sequences were then subjected to BLAST analyses to determine the closest identities by comparison to organisms available in GenBank.

Sequences of the 18S rRNA gene generated in this study and homologue sequences retrieved from GenBank were used to construct alignments for *Theileria* spp. representatives. The selected sequences were aligned using Clustal X (Thompson et al., 1997), and manually adjusted with GeneDoc (Nicholas et al., 1997). Two phylogenetic inferences were performed for alignment. Inferences by maximum parsimony were constructed according to their implementation in PAUP version 4.0b10 (Swofford, 2002), using a heuristic search with 1000 replicates, 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences), and tree bisection and reconnection (TBR) branch swapping. MrBayes v3.1.2 was used to perform Bayesian analyses (Huelsenbeck & Ronquist, 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generations. The first 25% of trees represented burn-in, and the remaining trees were

Table 1. Molecular detection by polymerase chain reaction (PCR) of Piroplasmida (genera *Babesia*, *Cytauxzoon*, and *Theileria*) and *Hepatozoon* spp. in blood (B) and spleen (S) samples of free living (FL) and captive (C) wild mammals from Mato Grosso state, Brazil, during 2019-2021.

Species (n. of individuals when >1)	Popular name	Origin	Municipality[a]	Piroplasmida		*Hepatozoon* spp.	
				Nº. of tissue samples positive by PCR (%)			
				B	S	B	S
ORDER CARNIVORA							
Family Procyonidae							
Nasua nasua (4)	South American Coati	FL	4	0	0	0	0
Nasua nasua (2)	South American Coati	C	4	0	0	0	0
Potus flavus (3)	Kinkajou	FL	4; 5	0	0	0	0
Procyon cancrivorus (2)	Crab-eating Raccoon	FL	6	0	0	0	0
Family Mustelidae							
Lutrinae sp. (3)	Otter	FL	6	0	0	0	0
Pteronura brasiliensis (3)	Giant Otter	FL	6	0	0	0	0
Family Canidae							
Cerdocyon thous (12)	Crab-eating Fox	FL	3; 4	0	0	0	0
Chrysocyon brachyurus (2)	Maned Wolf	C	4	0	0	0	0
Family Felidae							
Herpailurus yagouaroundi (2)	Jaguarundi	FL	6	0	0	0	0
Leopardus pardalis (4)	Ocelot	FL	6	0	1 (25%)[b]	1 (25%)[c]	0
Leopardus pardalis (2)	Ocelot	C	4	0	0	0	0
Pantera onca (2)	Jaguar	FL	6	0	0	0	0
Pantera onca (6)	Jaguar	C	4	0	3 (50%)[b]	0	0
Puma concolor (8)	Puma	FL	6	2 (25%)[b]	0	0	0
Puma concolor (3)	Puma	C	4	0	0	0	0
ORDER RODENTIA							
Cavia aperea (2)	Brazilian Guinea Pig	FL	6	0	0	0	0
Dasyprocta sp. (4)	Azara's Agouti	FL	6	0	0	0	0
Hydrochoerus hydrochaeris (9)	Capybara	FL	4; 6	0	0	0	0
Sciurus aestuans	Brazilian squirrel	FL	6	0	0	0	0
ORDER LAGOMORPHA							
Sylvilagus brasiliensis	Tapeti	FL	6	0	0	0	0

[a]Municipality as shown in Figure 1, as follow: 1. Barão de Melgaço; 2. Cáceres; 3. Chapada dos Guimarães; 4. Cuiabá; 5. Nova Xavantina; 6. Poconé; [b]Results refer to samples testing positive for *Cytauxzoon* sp.; [c]Results refer to samples testing positive for *Hepatozoon* sp.; [d]Results refer to samples testing positive for *Theileria* sp.

Table 1. Continued...

Species (n. of individuals when >1)	Popular name	Origin	Municipality[a]	Piroplasmida		*Hepatozoon* spp.	
				Nº. of tissue samples positive by PCR (%)			
				B	S	B	S
ORDER ARTIODACTYLA							
Blastocerus dichotomus	March Deer	FL	6	0	0	0	0
Mazama americana	Red Brocket	FL	6	0	0	0	0
Mazama gouazoubira (5)	Gray Brocket	FL	6	0	0	0	0
Pecari tajacu	Collared peccary	C	4	0	0	0	0
Tayassu pecari	White-lipped Peccary	FL	6	0	0	0	0
ORDER PERISSODACTYLA							
Tapirus terrestris (17)	Lowland tapir	FL	2; 3; 4; 6	3 (17.64%)[d]	4 (25.52%) [d]	0	0
Tapirus terrestris (4)	Lowland tapir	C	4	0	0	0	0
ORDER PILOSA							
Myrmecophaga tridactyla (10)	Giant Anteater	FL	1; 6	0	0	0	0
Myrmecophaga tridactyla (9)	Giant Anteater	C	4	0	0	0	0
Tamandua tetradactyla (7)	Southern tamandua	FL	2; 4; 6	0	0	0	0
ORDER CINGULATA							
Dasypodidae novemcinctus (3)	Nine-banded armadillo	FL	6	0	0	0	0
Euphractus sexcinctus	Yellow armadillo	FL	6	0	0	0	0
ORDER DIDELPHIDAE							
Didelphis albiventris (3)	White-eared opossum	FL	4	0	0	0	0
ORDER PRIMATES							
Aotus infulatus (9)	Azara's Night Monkey	FL	4	0	0	0	0
Alouatta caraya (2)	Black-and-gold Howler Monkey	FL	4	0	0	0	0
Mico melanurus (11)	Black-tailed Marmoset	FL	4	0	0	0	0
Sapajus apella (4)	Black-capped Capuchin	FL	4	0	0	0	0
Total (164)							

[a]Municipality as shown in Figure 1, as follow: 1. Barão de Melgaço; 2. Cáceres; 3. Chapada dos Guimarães; 4. Cuiabá; 5. Nova Xavantina; 6. Poconé; [b]Results refer to samples testing positive for *Cytauxzoon* sp.; [c]Results refer to samples testing positive for *Hepatozoon* sp.; [d]Results refer to samples testing positive for *Theileria* sp.

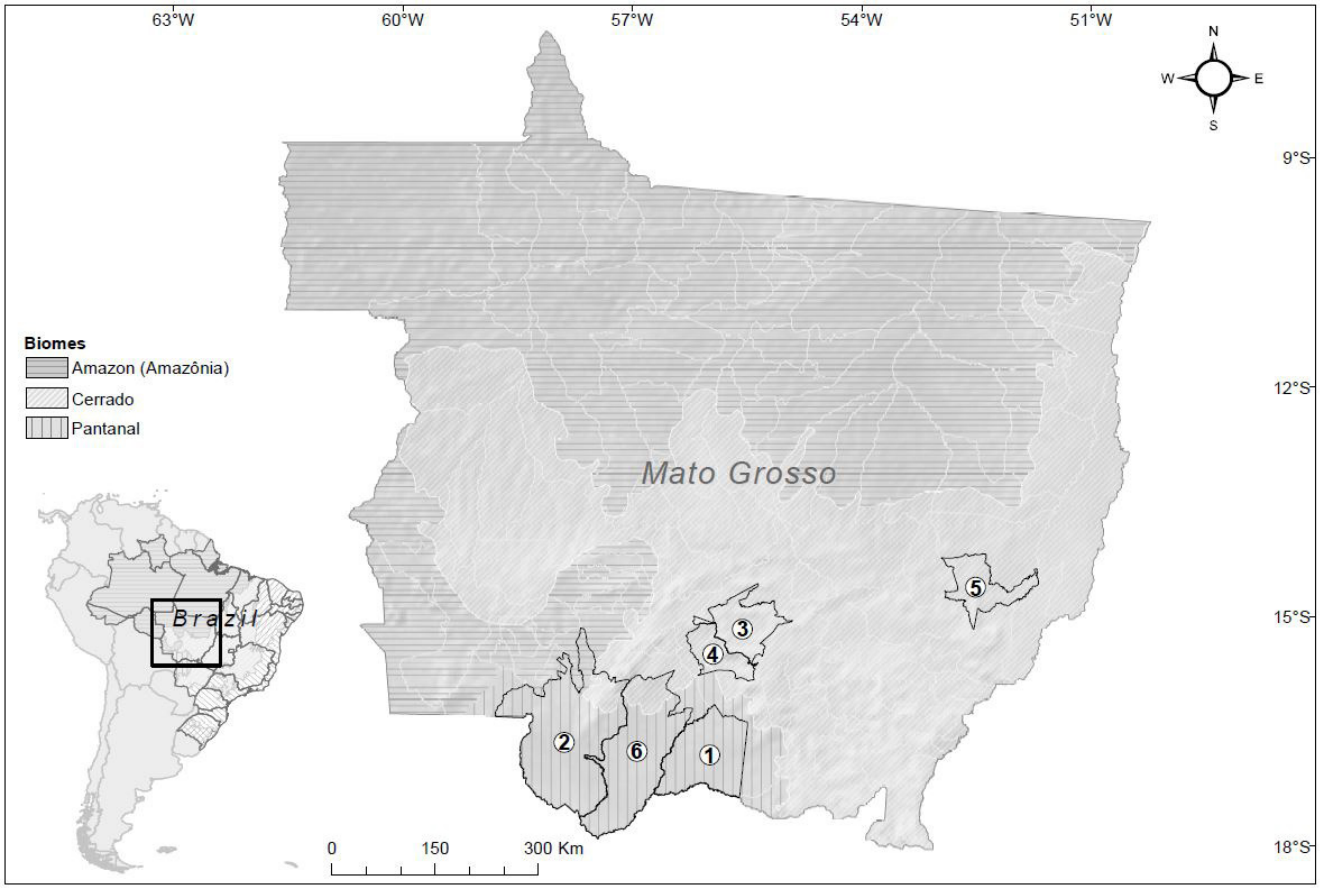

Figure 1. Map of the municipalities within the State of Mato Grosso, Brazil, where blood and tissue samples (spleen) of wild mammals were collected for molecular detection by polymerase chain reaction (PCR) of Piroplasmida (genera *Babesia*, *Cytauxzoon*, and *Theileria*) and *Hepatozoon* spp., during 2019–2021. 1. Barão de Melgaço; 2. Cáceres; 3. Chapada dos Guimarães; 4. Cuiabá; 5. Nova Xavantina; 6. Poconé.

used to calculate the Bayesian posterior probability. GTR+I+G was the standard model used in MrBayes software. The tree was rooted in *Toxoplasma gondii* as an out-group. GenBank accession numbers for all sequences used for the phylogenetic analyses were embedded in each tree.

A total of 159 blood and 160 spleen samples from 164 specimens of wild mammals belonging to at least 31 different species were subjected to DNA extraction. DNA from all samples tested for *gapdh* internal control amplified the predicted product. Table 1 provides a list of all tested animals and the results of their molecular analyses grouped according to species, origin, and locality.

Amplicons for Piroplasmida were detected in four species of wild mammals (*Leopardus pardalis*, *Panthera onca*, *Puma concolor*, and *Tapirus terrestris*). Among the animals that exhibited positive results for this protozoa, one *L. pardalis* showed a positive spleen sample, and three *P. onca* and two *P. concolor* individuals had positive results in the spleen and blood, respectively. Among the six specimens of *T. terrestris*, Piroplasmida DNA was present in the blood and spleen samples from one animal, two specimens were positive for Piroplasmida in the blood, and three specimens had positive results in the spleen, as determined by the nPCR assay.

Partial sequences of the 18S rRNA gene from *L. pardalis*, *P. onca*, and *P. concolor* were identical to each other and 100% (721/721 bp) identical with sequences of *Cytauxzoon felis* (MT904037, AF399930, and AY679105). Since the present study relied on a small fragment of the 18S rRNA gene, herein we referred to as *Cytauxzoon* sp. isolate MT (MZ489665). Furthermore, partial sequences of the 18S rRNA gene obtained from six specimens of *T. terrestris* yielded two different haplotypes with 99% (743/747 bp) similarity with sequences of the *Theileria* genera (KP410271, KP410272, and KP410273) detected in free-roaming domestic cats in Midwestern Brazil. The GenBank accession numbers for the partial sequences generated for the *Theileria* isolates in the present study, herein designated *Theileria* sp. isolate tapir MT1 and *Theileria* sp. isolate tapir MT2 are MZ490586 and MZ491096, respectively.

The phylogenetic analyses of a partial 18S rDNA obtained from wild felids (*L. pardalis*, *P. onca*, and *P. concolor*) show *Cytauxzoon* sp. isolate MT clustered in a clade with other *C. felis* sequences. Furthermore, the phylogenetic analyses of partial 18S rDNA sequences obtained from *T. terrestris* indicate that *Theileria* sp. isolate tapir MT1 and *Theileria* sp. isolate tapir MT2 formed a clade with other *Theileria* spp. recently detected in domesticated and stray cats in the states of Mato Grosso Sul and São Paulo, Brazil (Figure 2), and genetically related to *Theileria equi* (6.38% of divergence).

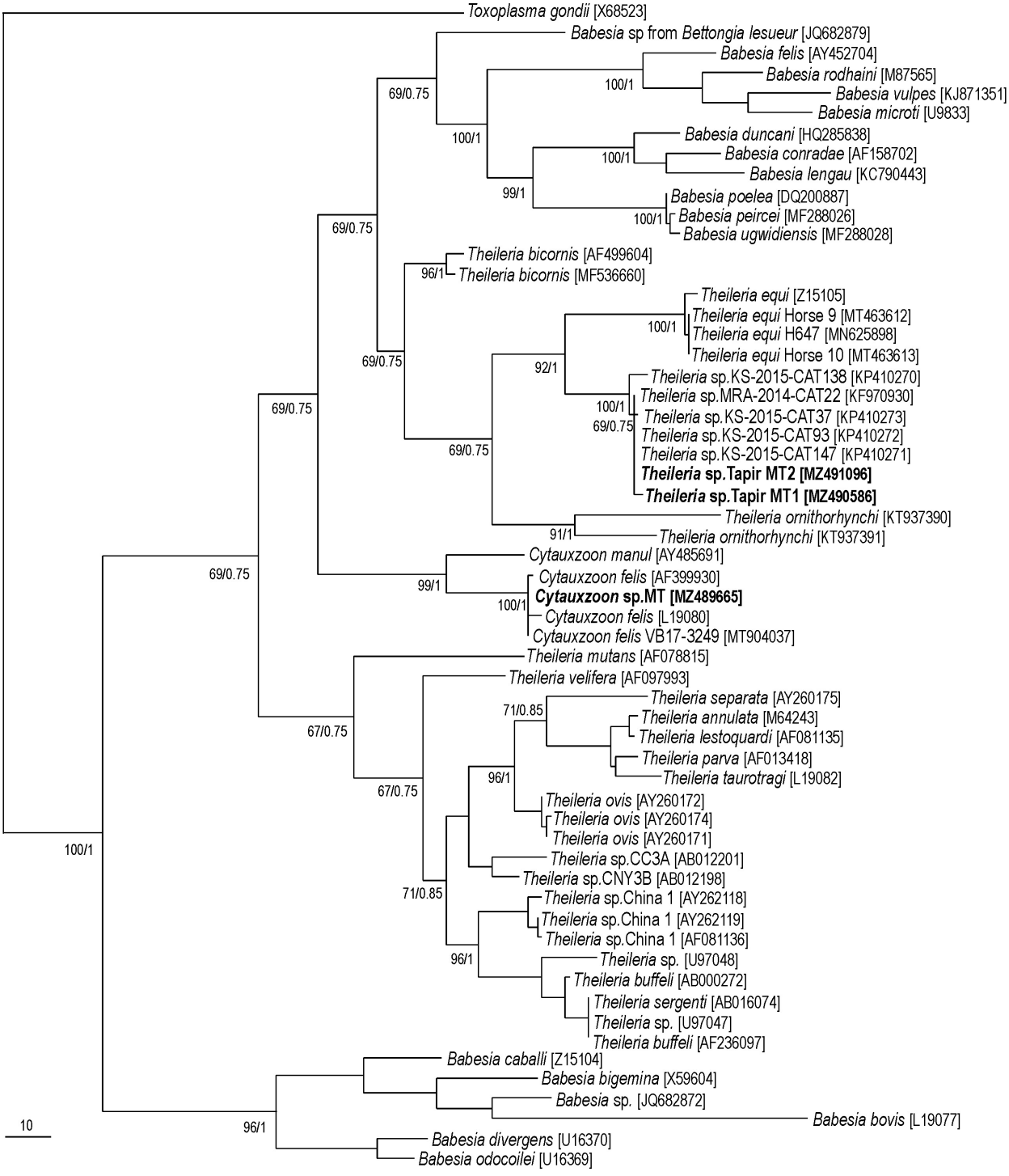

Figure 2. Maximum parsimony and Bayesian tree constructed for an alignment of sequences of *Cytauxzoon* spp. and *Theileria* spp. species using 18S rRNA gene sequences. Numbers at nodes are the support values for the major branches (bootstrap over 500 replicates). The sequence obtained in this study is highlighted in bold. Numbers in brackets correspond to GenBank accession numbers.

Among the mammal samples molecularly tested for *Hepatozoon* spp., only one *L. pardalis* yielded amplicons in blood samples after the 18S rRNA-based cPCR that revealed 100% (554/554 pb) identity with *Hepatozoon felis* (AB771570, AB771562). However, considering the small size of the fragment sequenced from 18S rRNA gene, as described above, herein we referred to as *Hepatozoon* sp. isolate ocelot MT (MZ490540). Co-infection with *Hepatozoon* and piroplasmid agents was not detected among the samples tested.

The present study demonstrated the presence of apicomplexan parasites in blood and/or spleen samples from wild mammals from the state of Mato Grosso, Midwestern Brazil. Infections found in *L. pardalis*, *P. onca*, and *P. concolor* by *Cytauxzoon* sp. corroborate previous studies that demonstrated a high occurrence of this agent in wild felids in Brazil (de Sousa et al., 2018; Furtado et al., 2017b; Santos et al., 2021).

In North America, bobcat (*Lynx rufus*) is the most common natural host for *C. felis,* with both *Amblyomma americanum* and *Dermacentor variabilis* ticks as suitable vectors (Wang et al., 2017). Despite the pathogenicity of the genotypes that circulate in domestic cats and wild felids in the country is still unknown (André et al., 2015), one case of death of cytauxzoonosis has been described in Brazil in lions from Rio de Janeiro State (Peixoto et al., 2007). In Brazil, high infection rates in *P. onca* incriminate this felid as a possible reservoir host for *Cytauxzoon* (Furtado et al., 2017b), although this assumption should be verified, along with the role of other neotropical felid species serving as natural reservoirs (André et al., 2009), as well as possible clinical signs of infection in wild Brazilian felines. Finally, little is known about the natural vectors of Brazilian isolates of *Cytauxzoon* sp.. It is possible that ticks of the genus *Amblyomma* are responsible for maintaining and transmitting this pathogen (Furtado et al., 2017b).

Tapirus terrestris (lowland tapir) is a wide-ranging herbivore ungulate of the order Perissodactyla, that is found in all biomes in Brazil and is highly susceptible to anthropogenic threats, including cohabitation and increased exposure to domestic and wild animal pathogens (Medici, 2011). In this study, a natural infection is described with *Theileria* genus positioned by phylogenetic analysis with *Theileria* sp. detected in cats (André et al., 2014, 2015). Piroplasms have been described infecting domestic cats worldwide, being a major veterinary concern mainly in South Africa, with animals presenting severe clinical abnormalities (Penzhorn & Oosthuizen, 2020). In Brazil, molecular occurrences (11.9-19%) of *Babesia*/*Theileria* spp., genetically related to *Theileria* molecularly detected in the present study, were described in clinically health domesticated and stray cats (André et al., 2014, 2015), suggesting that cats are able to maintain the infection with no discernible untoward effects (Penzhorn & Oosthuizen, 2020).

Regardless the natural infections with *Theileria* (molecularly identified as *Theileria equi*) have previously been reported in lowland tapirs (Gonçalves et al., 2020), neither clinical manifestation nor the impact were described of these infections in tapirids with this protozoan. Furthermore, there was no evidence of lowland tapirs as possible natural reservoirs or even potential vectors for *Theileria* sp., since these mammals have the greatest richness of tick species in South America (Labruna et al., 2021).

The genus *Hepatozoon* can infect a wide range of domestic and wild animals, including avians, mammals, and reptiles worldwide, and despite its frequent presence in wild and domestic felids (van As et al., 2020), few studies on *Hepatozoon* infection have been conducted with neotropical felines (Metzger et al., 2008; André et al., 2010; Furtado et al., 2017a; de Sousa et al., 2018). At least four species of *Hepatozoon* are capable of infecting domestic and wild felines worldwide, as follows: *H. felis* was described in Spain (*Felis catus*), India (*Panthera tigris tigris*, *Panthera leo persica*, and *Panthera pardus fusca*), Brazil (*Felis catus*), and Thailand (*Panthera leo persica*); *Hepatozoon silvestris* was detected in *Felis silvestris silvestris* from Bosnia and Herzegovin; *Hepatozoon ingwe* and *Hepatozoon luiperdjie* were detected in *Panthera pardus pardus* in South Africa (van As et al., 2020). A putative new species of *Hepatozoon* sp. have been detected in wild felids from Brazil (André et al., 2010). Despite several tick genera, including *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus* have been reported to harbor *H. felis,* the vectors of this protozoan species remain unknown (Bhusri et al., 2017). The infection with *H. felis* has previously been described in *L. pardalis* from Brazil (Metzger et al., 2008; Santos et al., 2021). Although *Hepatozoon* infections are often subclinical in wild felids (Metzger et al., 2008; van As et al., 2020), studies have demonstrated that it can be fatal in domestic cats, particularly immunocompromised animals or those with concomitant infections (Wang et al., 2017). Considering that some Brazilian felids are threatened, especially keystone species such as *P. onca*, studies are recommended, especially based on postmortem investigations, due to the inherent difficulties to obtain samples from wild animals, to clarify whether *Hepatozoon* or other pathogens infections represent a risk to these animals.

The present study showed that *Cytauxzoon* sp. and *Hepatozoon* sp. circulate among wild felids, while an uncharacterized piroplasmid genetically related to *T. equi* was detected in lowland tapirs in the state of Mato Grosso. Despite controversy regarding the epidemiological threat of these protozoan infections, the detection in free-living and captive wild mammals demonstrates the importance of monitoring, especially in regions of conservation

interest, such as the state of Mato Grosso, to verify the circulation and genetic diversity of these agents in order to anticipate the possible emergence of diseases, and even their consequences. Furthermore, although clinical disease does not always develop in wildlife, mammals can be important reservoirs by contributing to the spread of the disease to other wild and domestic animals, as well as humans.

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