Check for updates

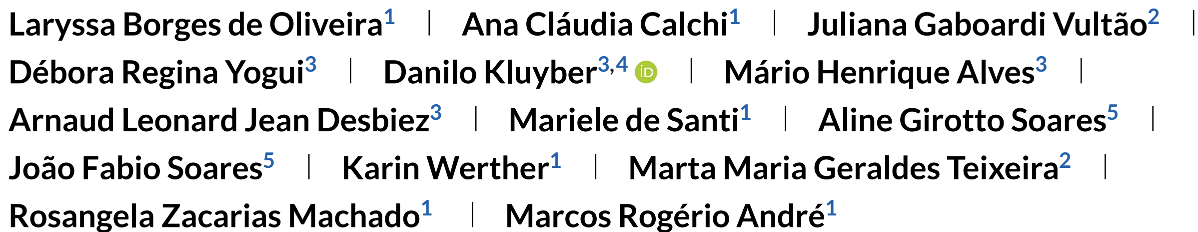
Received: 7 January 2022 | Revised: 5 March 2022 | Accepted: 14 March 2022

DOI: 10.1111/tbed.14523

Transboundary and Emerging Diseases WILEY

ORIGINAL ARTICLE

Molecular investigation of haemotropic mycoplasmas and *Coxiella burnetii* in free-living Xenarthra mammals from Brazil, with evidence of new haemoplasma species



Laryssa Borges de Oliveira[1] | Ana Cláudia Calchi[1] | Juliana Gaboardi Vultão[2] | Débora Regina Yogui[3] | Danilo Kluyber[3,4] | Mário Henrique Alves[3] | Arnaud Leonard Jean Desbiez[3] | Mariele de Santi[1] | Aline Girotto Soares[5] | João Fabio Soares[5] | Karin Werther[1] | Marta Maria Geraldes Teixeira[2] | Rosangela Zacarias Machado[1] | Marcos Rogério André[1]

[1]UNESP, Faculdade de Ciências Agrárias e Veterinárias/Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil

[2]Instituto de Ciências Biomédicas (ICB), Universidade de São Paulo, São Paulo, Brazil

[3]Instituto de Conservação de Animais Silvestres (ICAS), Campo Grande, Mississippi, Brazil

[4]Pesquisador associado do Naples Zoo at the Caribbean Gardens, Naples, Florida, USA

[5]Laboratório de Protozoologia e Rickettsioses Vetoriais, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

Correspondence

Marcos Rogério André, Laboratório de Imunoparasitologia, Departamento de Patologia, Reprodução e Saúde Única, Faculdade de Ciências Agrárias e Veterinárias Júlio de Mesquita Filho (UNESP), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP: 14884-900, Jaboticabal, São Paulo, Brazil.

Email: mr.andre@unesp.br

Funding information

Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Numbers: FAPESP #2018/02753-0, #2020/12037-0; Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: CNPq #303701/2021-8; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Grant/Award Numbers: CAPES #88887, 464146, /2019-00

Abstract

Although mammals of the superorder Xenarthra are considered hosts of a wide range of zoonotic agents, works aiming at investigating the role of these animals as hosts for bacteria with zoonotic potential are rare. The present study aimed to investigate the occurrence and molecularly characterize *Coxiella burnetii* and haemoplasma (haemotropic mycoplasmas) DNA in blood and spleen samples from 397 free-living Xenarthra mammals (233 sloths, 107 anteaters and 57 armadillos) in five Brazilian states (Mato Grosso do Sul, São Paulo, Pará, Rondônia and Rio Grande do Sul). All biological samples from Xenarthra were negative in the qPCR for *Coxiella burnetii* based on the *IS1111* gene. The absence of *C. burnetii* DNA in blood and spleen samples from Xenarthra suggests that these mammals may not act as possible hosts for this agent in the locations studied. When performed conventional PCR assays for the endogenous (*gapdh*) mammalian gene, 386 samples were positive. When screened by molecular assays based on the 16S rRNA gene of haemoplasmas, 81 samples were positive, of which 15.54% (60/386) were positive by conventional PCR and 5.44% (21/386) were positive by real-time PCR; three samples were positive in both assays. Of these, 39.74% (31/78) were also positive for the 23S rRNA gene and 7.69% (6/78) for the haemoplasma *RNAse P* gene. Among the samples positive for haemoplasmas, 25.64% (20/78) were obtained from anteaters (*Tamandua tetradactyla* and *Myrmecophaga tridactyla*), 39.74% (31/78) from sloths (*Bradypus tridactylus*, *Bradypus* sp. and *Choloepus* sp.) 34.61% (27/78) from armadillos (*Priodontes maximus*, *Euphractus sexcinctus* and *Dasypus novemcinctus*). A haemoplasma 16S rRNA sequence closely related and showing high identity (99.7%) to *Mycoplasma wenyonii* was detected, for the first time, in *B. tridactylus*. Based on the low identity and phylogenetic positioning of 16S rRNA and 23S rRNA sequences of haemoplasmas detected in anteaters and armadillos, the present study showed, for the first time, the occurrence of putative new

 © 2022 Wiley-VCH GmbH

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Candidatus haemotropic *Mycoplasma* spp. ("*Candidatus* Mycoplasma haematotetradactyla" and "*Candidatus* Mycoplasma haematomaximus") in Xenarthra mammals from Brazil.

KEYWORDS
anteaters, armadillos, haemoplasmas, Q-fever, sloths

1 | INTRODUCTION

Mammals of the superorder Xenarthra are considered reservoirs for different zoonotic agents. Although recently Kluyber et al. (2020) detected *Trypanosoma cruzi* and *Toxoplasma gondi* in free-living armadillos in the state of Mato Grosso do Sul, there are still few studies that investigated the occurrence of bacterial agents transmitted by arthropod vectors in this group of animals. An *Ehrlichia* sp. genotype related to *Ehrlichia ruminantium* was detected in a three-toed sloth (*Bradypus tridactylus*) in Pará state, northern Brazil (Soares et al., 2017). More recently, a new *Bartonella* Candidatus ("*Candidatus* Bartonella washoensis subspecies brasiliensis"), two new *Anaplasma* Candidatus ("*Candidatus* Anaplasma brasiliensis" and "*Candidatus* Anaplasma amazonensis") and two *Ehrlichia* species (*Ehrlichia canis* and *Ehrlichia minasensis*) have been described in free-living Xenarthra from Brazil (Calchi, Vultão, Alves, Yogui, Desbiez, Amaral, et al., 2020; Calchi, Vultão, Alves, Yogui, Desbiez, Santi, et al., 2020).

Haemotropic mycoplasmas (haemoplasmas), bacteria belonging to the class Mollicutes, family Mycoplasmataceae, and genus *Mycoplasma*, are characterized by lacking cell wall, high plasticity in the outer membrane (Edward & Freundt, 1967; Razin et al., 1998; Tratchenberg, 2005) and ability to adhere to the surface of erythrocytes of different vertebrate species (Messick, 2004). These agents emerge as important pathogens in human and veterinary medicine (Dos Santos et al., 2008; Hornok et al., 2011; Maggi, Compton, et al., 2013; Maggi, Mascarelli, et al., 2013; Steer et al., 2011; Yuan et al., 2009). In Brazil, there is an increasing number of research related to the detection of haemoplasmas in wild animals, which has allowed the discovery of new Candidatus species of haemotropic mycoplasmas in different hosts (Pontarolo et al., 2020; Vieira et al., 2021).

Coxiella burnetii is a γ-proteobacteria, Gram-negative, obligate intracellular, belonging to the order Legionellales and family Coxiellaceae. It is responsible for causing the zoonotic disease known as Q-Fever (Eldin et al., 2017). It has a cosmopolitan distribution, being reported worldwide except in New Zealand (Eldin et al., 2017). This aetiologic agent parasitizes different cell types, such as macrophages, monocytes, fibroblasts and epithelial cells, albeit showing higher tropism for phagocytes (Van Schaik et al., 2013). Its transmission occurs by inhalation of aerosols, ingestion of contaminated milk and dairy products and contact between infected individuals (Bouvery et al., 2003). In addition, haematophagous ectoparasites also present great importance in its transmission (Eldin et al., 2017). Regarding the detection of *C. burnetii* in wild animals from Brazil, the agent has been molecularly detected in spleen of rodents (Rozental et al., 2017) and bats (Ferreira et al., 2018). Also, Zanatto et al. (2019) reported serological evidence of exposure to this agent in free-living wild cervids. In Xenarthra, the agent was molecularly detected in faecal and spleen samples from a three-toed sloth (*B. tridactylus*) in French Guiana (Davoust et al., 2014). Studies aiming at assessing the health status of Xenarthra mammals are needed, since they can bring information for future projects for conservation of this group of animals, as well as for epidemiological surveillance and decision making regarding diseases control and prevention. Additionally, given the zoonotic potential of these bacteria, the detection and molecular characterization of such agents in wild animals contribute to increase the knowledge on the genetic diversity of these groups of pathogens. The present study aimed to investigate the molecular occurrence and characterize DNA of *Coxiella burnetii* and haemoplasmas in free-living Xenarthra mammals sampled in five Brazilian states.

2 | MATERIAL AND METHODS

2.1 | Ethics committee

The Xenarthra mammals sampling procedures were previously approved by the Chico Mendes Institute for Biodiversity Conservation (SISBIO no. 53798-5; no. 27587-12; no. 53798-13; no. 64752-2) and by the Ethics Committees on Animal Use (CEUA) of both ICB - USP (no. 98) and the Faculty of Agricultural and Veterinary Sciences – UNESP Jaboticabal Campus (no. 11. 794).

2.2 | Biological samples and Xenarthra species samples

The Xenarthra mammals' biological samples were collected in five Brazilian states: Mato Grosso do Sul, Pará, Rio Grande do Sul, Rondônia and São Paulo. In total, 397 animals were sampled: 194 Pale-throated sloth (*B. tridactylus*), 3 *Bradypus* sp., 5 Linnaeus's two-toed sloth (*Choloepus didactylus*), 31 *Choloepus* sp., 40 collared anteaters (*Tamandua tetradactyla*), 67 giant anteaters (*Myrmecophaga tridactyla*), 4 southern naked-tailed armadillos (*Cabassous unicinctus*), 14 long-nosed armadillos (*Dasypus novemcinctus*), 14 Six-banded armadillos (*Euphractus sexcinctus*) and 25 giant anteaters (*Priodontes maximus*) (Figure 1).

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

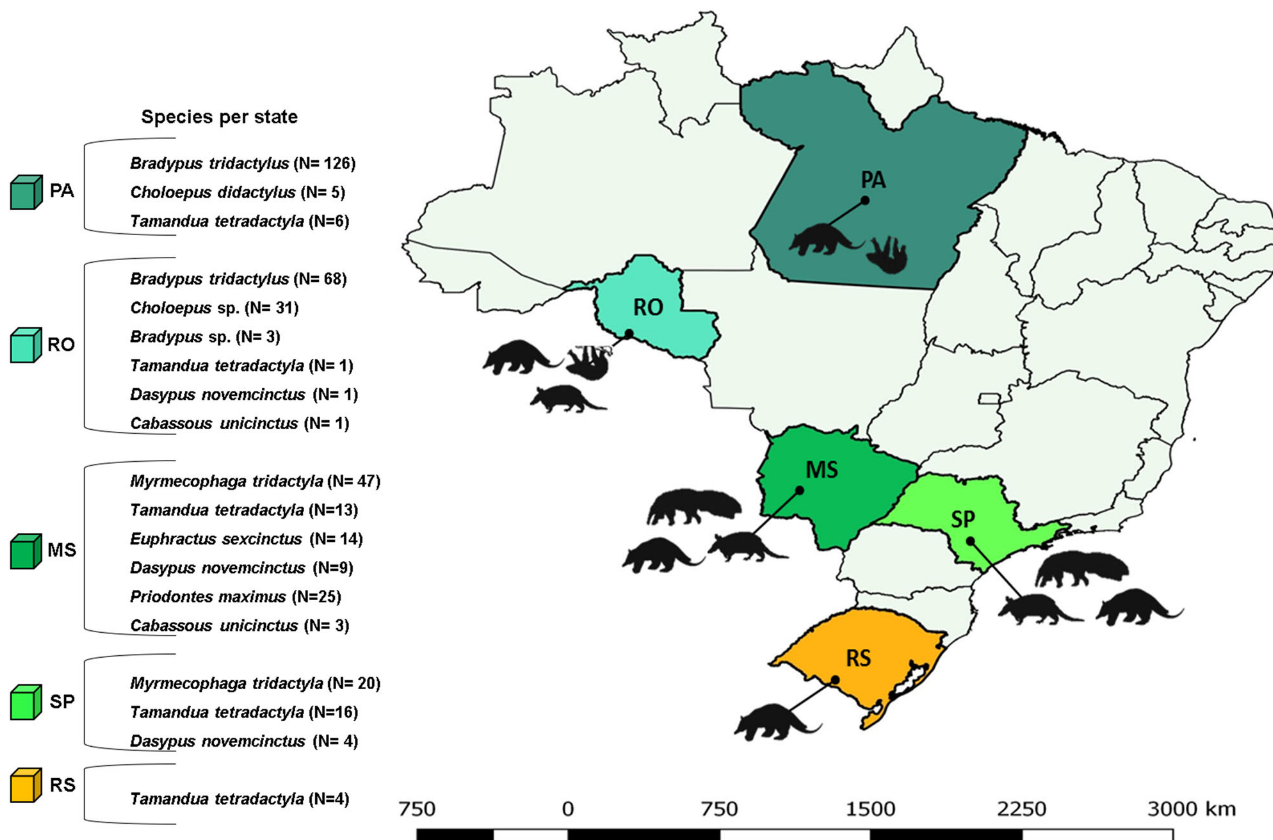


FIGURE 1 Map of Brazil with the number and origin of Xenarthra mammal species sampled in five Brazilian states

Spleen and blood samples were obtained during necropsy of animals victims of vehicular collision on highways in the states of Mato Grosso do Sul, Rio Grande do Sul and São Paulo. The collection of these samples in the state of Mato Grosso do Sul was performed by the Wild Animal Conservation Institute (Flags and Roads Project and the Giant Armadillo Conservation Program), by the Wildlife Pathology Service of the Faculty of Agricultural and Veterinary Sciences and Paulista State University (FCAV/UNESP) in the state of São Paulo and by the Laboratory of Protozoology and Vector Rickettsioses of Veterinary School of the Federal University of Rio Grande do Sul (UFRGS) in the state of Rio Grande do Sul. Additionally, blood samples were obtained from live animals captured in a wildlife rescue area due to the filling of hydroelectric dams in the states of Pará and Rondônia by the Institute of Biomedical Sciences of the University of São Paulo (USP).

Between the years 2015 and 2020, in the state of Mato Grosso do Sul, 76 spleen samples were collected (47 giant anteaters, 13 collared anteaters, 8 six-banded armadillos, 5 long-nosed armadillos, 1 giant armadillo and 2 southern naked-tailed armadillos) in the Cerrado biome by the Flags and Roads Project, and 35 blood samples in the Pantanal, by the Conservation Program of the Tatu-Canastra in the Nhecolândia region (24 giant armadillos, 6 six-banded armadillos, 1 southern naked-tailed armadillo and 4 long-nosed armadillos). In São Paulo, during the period from 2011 to 2018, the Wildlife Pathology Service obtained 40 spleen samples from Xenarthra species (20 giant anteaters, 16 collared anteaters and 4 long-nosed armadillos). In the year 2019, in the state of Rio Grande do Sul, spleen samples were collected from four collared anteaters by the Laboratory of Protozoology and Vector Rickettsioses. Between the years 2010 and 2013, 242 samples of sloths, anteaters and free-living armadillos were collected by the Institute of Biomedical Sciences during the filling process of a hydroelectric power plant in the states of Rondônia (no. = 105 [68 sloths, 31 *Choloepus* sp., 3 *Bradypus* sp., 1 collared anteater, 1 long-nosed armadillo and 1 southern naked-tailed armadillo]) and Pará (no. = 137 [126 sloths, 5 southern two-toed sloths, 6 collared anteaters]).

The spleen fragments and blood samples were conditioned in RNAse and DNAse-free microtubes (Kasvi®) and stored in freezer −70°C for later DNA extraction.

2.3 | Molecular analyses

DNA was extracted from 10 mg of each spleen fragment and from 200 μl of each blood sample using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. To assess the presence of amplifiable DNA and absence of PCR inhibitors, a conventional PCR (cPCR) assay based on the mammalian endogenous glyceraldehyde-3-phosphatidodesidrogenase (*gapdh*) gene (Birkenheuer et al., 2003) was performed. Samples positive in this assay were submitted to specific PCR assays for *C. burnetii* and haemoplasmas.

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.4 | Quantitative real-time PCR assay for *C. burnetii*

Samples positive in the PCR assays for the *gapdh* gene were subjected to a quantitative real-time PCR assay (qPCR) assay for *C. burnetii* based on the *IS1111* gene, according to the protocol of Klee et al. (2006). The qPCR assays were performed in 10 μl of full volume, containing 1 μl of DNA sample, 0.3 μM of each primer oligonucleotide (Cox-For [5′-GTCTTAAGGTGGGCTGCGTG-3′]; Cox-Rev '[5′-CCCCGAATCTCATTGATCAGC-3′]) and TaqMan hydrolysis probe (Cox-TM = FAM 5′-AGCGAACCATTGGTATCGGACGTT-TAMRA-TATGG-3′) (Table S1), 5, 0 μ; of 2 × Master Mix buffer (GoTaq™ Probe qPCR Master Mix, Promega Corporation, Madison, WI, USA), and ultra-pure sterile water (Nuclease Free, Promega Corporation) q. s.p. 3.1 μl. The standard curve was constructed with dilutions of Gblock (gBlocks® – Integrated DNA Technologies, Coralville, IA, USA) (2.0×10^7–2.0×10^0 copies/ml), which has a 295 bp insert of the *IS1111* gene. The G-block copy number was determined according to the formula: (XG/μL of DNA/[gBlocks size (bp) × 660]) × 6, 22 × 10^{23} × Gblock copies/μl). All DNA samples were tested in duplicate.

2.5 | qPCR and conventional PCR assays for haemoplasmas

To detect and quantify haemoplasma DNA, DNA samples positive in PCR assays for the *gapdh* gene were submitted to a qPCR assay based on 16S rRNA gene using Sybr Green as DNA intercalant, following the protocol established by Willi et al. (2009). For this, the primers SYBR-For(5′-AGCAATRCCATGTGAACGATGAA-3′) and SYBR-Rev (5′-TGGCACATAGTTWGCTGTCACTT −3′) were used (Table S1). Each DNA sample was subjected to this qPCR in duplicate. Amplification efficiency was calculated from the standard curve in each run using the following formula ($E = 10^{-1/slope}$). All analyses were performed according to the standards established by MIQE (Minimum Information for Publication of Quantitative real-time PCR Experiments) (Bustin et al., 2009). Sterile ultrapure water was purchased from Nuclease-Free Water (Promega®, Madison, WI, USA).

Additionally, all samples were subjected to a conventional PCR assay for haemoplasmas as described by Maggi, Chitwood, et al. (2013) based on the 16S rRNA gene, using the primers HemMycop16S-41s (5′-GYATGCMTATAAYACATGCAAGTCGARCG-3′) and HemMyco16S-938as (5′-CTCCACCACTTGTTCAGGTCCCCGTC-3′), which flank a 1380 bp fragment (Table S2). The positive samples in this cPCR assay were subjected to a cPCR assay also based on the 16S rRNA gene, which amplifies a ~900 bp fragment, overlapping to the previous fragment. The primers HemMycop16S-322s (5′-GCCCA TATTCCTACGGGAAGCAGCAGT-3′) and HemMycop16S-1420as (5′-GTTTGACGGGCGGTGTGTACAAGACC-3′) were used to amplify this second fragment of the 16S rRNA gene (Table S2).

In order to obtain amplicons with higher band intensity for purification, the positive samples in the previous assays were submitted to a semi-nested PCR based on the 16S rRNA gene, according to the protocol described by Di Cataldo et al. (2020). This assay amplifies approximately 1400 bp and was performed using the primers HemoF1 (5′-AGTTTGATCCTGGCTCAG-3′), HemoR2 (5′-TACCTTGTTACGACTTAACT-3′) in the first reaction, and HemoF2 (5′-ATATTCCTACGGGAAGCAGC-3′) and HemoR2 in the second reaction. Samples positive in qPCR and conventional PCR assays based on the 16S rRNA gene were subjected to PCR assays based on the 23S rRNA and *RNaseP* genes, which amplify fragments of 800 bp and 165 bp, respectively. The reagent concentrations used were the same as those described for the 16S rRNA gene-based PCR assays for haemoplasmas. The PCR assay based on the 23S rRNA gene was performed using the primers 23S_Haemo_F (5′-TGAGGGAAAGAGAGCCCAGAC-3′) and 23S_Haemo_R (5′-GGACAGAATTTACCTGACAAGG-3′) (Mongruel et al., 2020). In the PCR based on the *RNaseP* gene, the primers HemoMyco*RNaseP*30S (5′-GATKGTGYGAGYATYATATAAAAATAAARCTCRAC-3′) and HemoMyco*RNaseP*200as (5′-GMGGRGTTTACCGCGTTTCAC-3′) were used (Table 2) (Maggi, Compton, et al., 2013). *Mycoplasma suis* DNA (Gatto et al., 2019) and ultrapure autoclaved sterile water were used as positive and negative controls, respectively, in all PCR assays for haemoplasmas.

The amplified products in the cPCR assays were subjected to horizontal electrophoresis on 1.0% agarose gel stained with ethidium bromide (0.5 μl/ml) in TEB pH 8.0 running buffer (44.58 M Tris-base; 0.44 M boric acid; 12.49 mM EDTA). Electrophoresis was performed at 90 V/150 mA for 60 min. A 100 base pair molecular weight marker (Life Technologies®) was used to determine the amplified products. The results were visualized and analysed using an ultraviolet light transilluminator (ChemiDoc MP Imaging System, BIORAD®).

2.6 | Purification of amplicons and sequencing

The amplified products in the PCR assays were purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA), according to the manufacturer's recommendations. Quantification of total genomic material of the purified product was performed in a spectrophotometer (Nanodrop, Thermo Scientific®).

The sequencing of the amplified products was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) and conducted in the sequencer ABI 3730 DNA Analyzer, a 48 capillary DNA analysis system with Life Technologies – Applied Biosystems technology in the Center for Human Genome and Stem Cell Studies at USP and in the sequencer ABI PRISM 3700 DNA Analyzer (Applied Biosystems) in the Center for Biological Resources and Genomic Biology (CREBIO-FCAV/UNESP).

2.7 | Phylogenetic analyses

Analyses of the electropherograms generated by the Sanger sequencing were performed by observing the quality of the peaks corresponding to each sequenced base using Bioedit v. 7.0.5.3 software

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

(Hall, 1999). To build consensus sequences, the program Phred-Phrap version 23 (Ewing & Green, 1998; Ewing et al., 1998) was used to analyse both "forward" and "reverse" strands sequenced from the same sample, respecting the minimum quality value of 20 for each nucleotide to determine the nucleotide sequence. After building the consensus sequence of each sample, it was submitted to the BLAST analysis (Altschul et al., 1990), in order to compare with those sequences previously deposited in GenBank database (http://www.ncbi.nlm.nih.gov/genbank).

The sequences saved "FASTA" format were aligned with other homologous sequences of the same sequenced gene retrieved from GenBank using the Clustal/W software (Thompson et al., 1994) via Bioedit v. 7.0.5.3 (Hall, 1999). They were then transformed in Nexus and Phylip mode by the "Alignment Transformation Environment" website (Glez-Peña et al., 2010). Analysis methods for phylogeny were maximum likelihood, which was performed using W-IQ-Tree software (available online: http://iqtree.cibiv.univie.ac.at/) (Nguyen et al., 2015; Trifinopoulos et al., 2016). Clade supports for the maximum likelihood analyses were evaluated using bootstrap analyses (Felsenstein, 1985) of 1000 repetitions, with the evolutionary model TIM3+F+I+G4. Editing of the phylogenetic trees, as well as rooting (via external group), was performed using Treegraph 2.13.0 beta software (Stover & Muller, 2010).

2.8 | SplitsTree distance analysis

The software SplitsTree4 provides a framework for computing phylogenetic networks through mathematical concepts, for more complex models of evolution that are eventually not evaluated by other phylogenetic inference methods. In these approaches, the evolution of a gene is modelled through events such as speciation and mutations. SplitsTree allows for inference, based on distance analysis, of more complex events that have occurred throughout evolution, such as gene loss and duplication, horizontal transfer and others (Huson & Bryant, 2006). For this purpose, the parameters "Neighbor – Joining" and "Uncorrected p-distance" were used.

3 | RESULTS

3.1 | Conventional PCR for the endogenous mammalian *gapdh* gene

Among the 397 samples collected, 386 (97.22%) were cPCR positive for the endogenous *gapdh* gene.

3.2 | qPCR for *C. burnetii* based on the *IS1111* gene

The efficiency of the qPCR for *C. burnetii* ranged from 100.7% to 104.3% (mean = 102.5%), the r^2 from 0.978 to 0.984, the slope from −3.305 to −3.224 and Y-intercept ranged from 44.267 to 45.247. All samples were negative in the qPCR assays based on the *IS1111* gene of *C. burnetii*.

3.3 | qPCR for haemoplasmas based on the 16S rRNA gene and sequencing of the obtained amplicons

The qPCR parameters ranged from: 90.1% to 103% for efficiency; 0.966 to 0.993 for r^2; −3.245 to −3.586 for slope and 29.680 to 33.108 for Y-intercept. In qPCR analyses for haemoplasmas, 5.44% (21/386) of samples were positive (Table 1) but not amenable to quantification due to the low amount of initial agent DNA in the tested sample ("Monte Carlo effect") (Bustin et al., 2009). A variation in the melting temperature (T_m) of the positive samples varied according to the different haemoplasma species detected in sloths (T_m = 79°C) and armadillos (T_m = 77°C). After the evaluation of the electropherograms of the amplicons obtained in qPCR, few were the 16S rRNA sequences that presented good quality.

A haemoplasma 16S rRNA sequence obtained by qPCR and detected in a blood sample from a sloth (*Choloepus* sp.) that had a melting temperature of 79.5°C showed identity of 87.59% (68% query coverage; E-value of 0) with sequences of haemotropic *Mycoplasma* spp. detected in a crab-eating fox (*Cerdocyon thous*) (KY00267676) and a coati (*Nasua nasua*) (KY002668) from the Pantanal wetlands, state of Mato Grosso do Sul, central-western Brazil (De Sousa et al., 2017). Also, the haemoplasma 16S rRNA sequence detected by qPCR in a blood sample from a giant armadillo (*P. maximus*) blood that presented a melting temperature of 77°C showed identity of 83.39% (50% query coverage; E-value of 1–72) with a sequence of haemotropic *Mycoplasma* spp. detected in a coati (KY002672) from the Brazilian Pantanal (De Sousa et al., 2017).

3.4 | Conventional PCR for haemoplasmas

Out of the 386 DNA samples positive in the PCR for the endogenous *gapdh* gene, 60 (15.54%) were positive in the cPCR assay for haemoplasmas based on the 16S rRNA gene and using the primers HemMycop16S-41s and HemMyco16S-938as. Of these, 18 were from the species *B. tridactylus*, 1 *Bradypus* sp., 8 *T. tetradactyla*, 4 *Choloepus* sp., 12 *M. tridactyla*, 14 *P. maximus*, 2 *E. sexcinctus* and 1 *D. novemcinctus*. Of the 60 positive samples, 19.23% (15/78) were positive in the PCR assay using the primers HemMycop16S-322s and HemMycop16S-1420as, which amplifies a 900 bp fragment of the 16S rRNA gene, with an overlapping to the first amplified fragment. Of these, four samples belonged to animals of the species *T. tetradactyla*, one *Choloepus* sp., one *E. sexcinctus*, one *D. novemcinctus* and eight *M. tridactyla* (Table 2).

Additionally, out of the 78 samples positive in the qPCR and cPCR screening assays based on the 16S rRNA gene, 37.17% (29/78) were positive in the semi-nested PCR assay based on the 16S rRNA gene. Among the positive samples, 2 were from *M. tridactyla*, 1 *E. sexcinctus*, 6 *B. tridactylus*, 5 *T. tetradactyla* and 15 *P. maximus*.

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Melting temperature (T_m) of haemoplasma 16S rRNA gene fragments detected in Xenarthra blood samples in SYBR® green-based qPCR assays

ID	Species	Biological sample	Localization	T_m*	Mean of *Cq*	Average copy quantification of a fragment of the 16S rRNA gene of haemoplasmas/µl
BM1	*Bradypus tridactylus*	Blood	PA	79.50	30.39	4.411×10^0
BM24	*Bradypus tridactylus*	Blood	PA	79.50	29.80	4.981×10^0
BM58	*Bradypus tridactylus*	Blood	PA	79.50	30.01	9.262×10^1
BM84	*Bradypus tridactylus*	Blood	PA	79.50	29.91	1.782×10^0
BM100	*Bradypus tridactylus*	Blood	PA	79.50	32.07	1.474×10^0
BM111	*Bradypus tridactylus*	Blood	PA	79.00	30.48	3.848×10^0
PV40	*Bradypus tridactylus*	Blood	RO	79.50	29.38	1.327×10^0
PV175	*Bradypus tridactylus*	Blood	RO	79.50	22.51	1.348×10^2
PV642	*Bradypus tridactylus*	Blood	RO	79.50	31.10	5.881×10^1
PV938	*Choloepus* sp.	Blood	RO	79.50	32.06	3.053×10^1
PV1252	*Bradypus* sp.	Blood	RO	79.50	32.39	4.056×10^1
135	*Priodontes maximus*	Blood	MS	77.00	21.07	4.096×10^2
140	*Priodontes maximus*	Blood	MS	77.00	18.90	1.84×10^3
142	*Priodontes maximus*	Blood	MS	77.00	17.66	4.37×10^3
143	*Priodontes maximus*	Blood	MS	77.00	22.29	1.75×10^2
151	*Priodontes maximus*	Blood	MS	77.00	16.76	8.21×10^3
155	*Priodontes maximus*	Blood	MS	77.00	18.97	1.75×10^3
156	*Priodontes maximus*	Blood	MS	77.00	17.35	5.43×10^3
157	*Priodontes maximus*	Blood	MS	77.00	23.03	1.04×10^2
160	*Priodontes maximus*	Blood	MS	77.00	20.61	5.62×10^2
161	*Priodontes maximus*	Blood	MS	77.00	23.16	9.52×10^1

Abbreviations: MS, Mato Grosso do Sul; PA, Pará; RO, Rondônia; *T_m, Melting temperature

We obtained 39.74% (31/78) positive samples in the cPCR assay based on the 23S rRNA gene, including 8 *M. tridactyla*, 1 *E. sexcinctus*, 3 *B. tridactylus*, 4 *T. tetradactyla* and 15 *P. maximus* (Figure 2). When subjected to cPCR based on the *RNAseP* gene, 7.69% (6/78) of the samples were positive, including 2 *T. tetradactyla*, 1 *B. tridactylus*, 1 *Choloepus* sp., 1 *D. novemcinctus* and 1 *M. tridactyla* (Table 3). One sample of *T. tetradactyla* from Pará was positive for all cPCR assays (three fragments of the 16S rRNA gene, one fragment of the 23S rRNA gene and one of the *RNAseP* gene).

3.5 | BLASTn analysis of sequences obtained from cPCR assays for haemoplasmas

Sequencing was performed for samples showing high intensity bands in the conventional and semi-nested PCR assays. We obtained 4 sequences for the 16S rRNA gene and 1 for the 23S rRNA gene from collared anteaters, 2 sequences for the 16S rRNA gene and 4 for the 23S rRNA gene from giant armadillos, and only 1 sequence for the 16S rRNA gene from a sloth. BLAST analysis was performed with sequences obtained from two fragments of 16S rRNA (~1380 bp and ~1400 bp) and 23S rRNA (800 bp) (Table 4). All 16S rRNA sequences obtained from the Xenarthra mammals were deposited into Genbank under the following accession numbers: OK584814, OK584815, OK584816, OK584817, OK584818, OK584819 and OK584820. Also, the obtained 23S rRNA sequences were deposited under the following accession numbers: OK569852, OK569853, OK569854, OK569855 and OK569856.

3.6 | Phylogenetic analyses

Phylogenetic analyses were performed by the Maximum Likelihood method and TIM3+F+I+G4 evolutionary model based on two genes, namely 16S rRNA and 23S rRNA, of *Mycoplasma* spp. In the phylogeny based on the 16S rRNA gene (990 bp alignment), the haemoplasma sequences detected in *T. tetradactyla* were positioned into a single clade, sister to the clade comprising haemoplasma sequences

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Results obtained in cPCR assays for haemoplasmas based on two overlapping fragments of the 16S rRNA gene in spleen and blood samples from Xenarthra mammals sampled in Brazil

Positive cPCR screening for the ~900 bp fragment of the 16S rRNA gene (primers HemMycop16S-41s e HemMyco16S-938as)			Positivity in the cPCR for the ~900 bp fragment of the 16S rRNA gene (primers HemMycop16S-322s e HemMycop16S-1420as)[a]		
Species	**Biological sample**	**State (no. of positives/total)**	**Species**	**Biological sample**	**State (no. of positives/total)**
B. tridactylus	Blood	PA (13/126)	*B. tridactylus*	Blood	PA (0/13)
	Blood	RO (5/68)		Blood	RO (0/5)
Bradypus sp.	Blood	RO (1/3)	*Bradypus* sp.	Blood	RO (0/1)
	Blood	PA (2/6)		Blood	PA (1/2)
T. tetradactyla	Blood	MS (3/7)	*T. tetradactyla*	Blood	MS (3/3)
	Blood	RO (1/1)		Blood	RO (0/1)
	Spleen	SP (1/16)		Spleen	SP (0/1)
	Spleen	RS (1/4)		Spleen	RS (0/1)
M. tridactyla	Spleen	MS (8/33)	*M. tridactyla*	Spleen	MS (7/8)
	Blood	MS (2/14)		Blood	MS (0/2)
	Spleen	SP (2/20)		Spleen	SP (1/2)
Choloepus sp.	Blood	RO (4/31)	*Choloepus* sp.	Blood	RO (1/4)
E. sexcinctus	Spleen	MS (1/8)	*E. sexcinctus*	Spleen	MS (1/1)
	Blood	MS (1/6)		Blood	MS (0/1)
D. novemcinctus	Spleen	SP (1/4)	*D. novemcinctus*	Spleen	SP (1/1)
P. maximus	Blood	MS (14/24)	*P. maximus*	Blood	MS (0/14)

[a]This protocol was used only for the samples previously positive in the 16S rRNA gene-based screening cPCR (primers HemMycop16S-41s and HemMyco16S-938as).

Abbreviations: MS, Mato Grosso do Sul; PA, Pará; RO, Rondônia; RS, Rio Grande do Sul; SP, São Paulo.

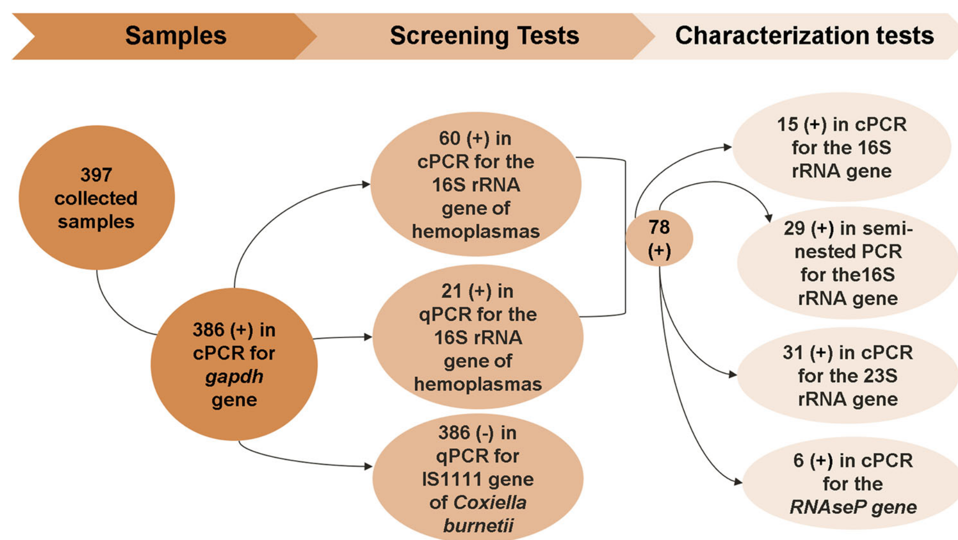


FIGURE 2 Flowchart with the number of positive and negative animals obtained in the PCR assays for screening and molecular characterization of *Coxiella burnetii* and haemotropic mycoplasmas

obtained in the present study in *P. maximus* and a sequence previously detected in *P. maximus* from Brazil (OK093397; unpublished data), with a clade support of 99. Genetic distance analyses showed a variation of 3.6% to 31.3% between sequences amplified in the present study and those previously deposited in GenBank, reinforcing the hypothesis that the amplified haemoplasma sequences belong to a new species (Table S3). The haemotropic *Mycoplasma* sp. sequence obtained from *B. tridactylus* was closely related to *Mycoplasma*

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Results obtained in semi-nested and conventional PCR assays for haemoplasmas based on the 16S rRNA, 23S rRNA and RNAseP genes in biological samples from Xenarthra mammals previously positive in 16S rRNA gene-based screening cPCR assays

	Positivity in semi-nested PCR based on 16S rRNA gene		Positivity on cPCR based on 23S rRNA gene	Positivity in cPCR based on *RNAse P* gene
Species	**Biological sample**	**State (No. of positives/total)**	**State (No. of positives/total)**	**State (No. of positives/total)**
B. tridactylus	Blood	PA (6/13)	PA (3/ 13)	PA (0/13)
	Blood	RO (0/5)	RO (0/5)	RO (1/5)
T. tetradactyla	Blood	PA (2/2)	PA (1/2)	PA (1/2)
	Blood	MS (2/2)	MS (1/2)	MS (0/2)
	Spleen	SP (0/1)	SP (1/1)	SP (1/1)
		RS (1/1)	RS (1/1)	RS (0/1)
M. tridactyla	Blood	MS (2/14)	MS (2/14)	MS (0/14)
	Spleen	MS (0/8)	MS (6/8)	MS (0/8)
	Spleen	SP (0/2)	SP (0/2)	SP (1/2)
E. sexcinctus	Spleen	MS (0/1)	MS (1/1)	MS (0/1)
	Blood	MS (1/6)	MS (0/6)	MS (0/6)
D. novemcinctus	Spleen	SP (0/2)	SP (0/2)	SP (1/2)
Choloepus sp.	Blood	RO (0/4)	RO (0/4)	RO (1/4)
P. maximus	Blood	MS (15/24)	MS (15/15)	MS (0/15)

Abbreviation: MS, Mato Grosso do Sul; PA, Pará; RO, Rondônia; RS, Rio Grande do Sul; SP, São Paulo.

wenyonii (EU367964) previously detected in cattle from Japan (Figure 3).

The 16S rRNA haemoplasma sequences detected in anteaters from the state of Pará showed an identity of 100% (E-value of 0 and Query coverage of 99%) to each other. When compared the 16S rRNA sequences obtained from anteaters from the states of Mato Grosso do Sul and Rio Grande do Sul, identity values of 100% (E-value 0 and Query coverage of 95%) and 99.87% (E value 0 and Query coverage of 86%), respectively, were found. 16S rRNA haemoplasma sequences detected in armadillos showed 100% identity (E-value 0 and Query coverage of 100%) to each other. The only 16S rRNA haemoplasma sequence obtained from a sloth showed identity of 93% (E-value 0 and Query coverage of 93%) to the haemoplasma sequences detected in anteaters from the states of Mato Grosso do Sul and Pará; on the other hand, it showed identity of 92% (E-value 0 and Query coverage of 79%) with the haemoplasma sequence detected in a anteater from Rio Grande do Sul. When comparing the haemoplasma sequences detected in armadillos from Mato Grosso do Sul with the haemoplasma sequence detected in a sloth from Pará, an identity of 92% (E-value 0 and query coverage of 91%) was found.

In 23S rRNA gene-based phylogenetic inference (810 bp alignment), the sequence obtained from a *T. tetradactyla* was positioned in a single clade, close to the sequences obtained from *P. maximus* in the present study, with a clade support of 92. Genetic distances ranging from 7.9% to 42.9% were observed between the 23S rRNA haemoplasma sequences detected in Xenarthra in the present study to those previously detected in other wild animals (Table S4). The sequences obtained from Xenarthra were closely related to haemoplasma genotypes previously detected in marsupials from Brazil (MN442084; MN442081; MN442082; MN442085; MN442083), with a clade support of 77 (Figure 4). The armadillo-associated 23S rRNA haemoplasma sequences from Mato Grosso do Sul state showed 100% identity (E-value of 0 and Query coverage of 100% and 94%) with each other and 93% (E-value 0 and Query coverage of 96%) with the anteater-associated haemoplasma sequence from Pará state.

3.7 | SplitsTree distance analysis

In the SplitsTree distance analysis, the 16S rRNA haemoplasma sequences detected in anteaters were positioned closely to each other. In turn, the sequences obtained from giant armadillos were positioned in the same branch as the sequence of haemotropic *Mycoplasma* sp. previously detected in an armadillo from Brazil (OK093397; unpublished). Anteater and armadillo-associated sequences were closely related to *M. suis* (AB610849). Additionally, the sloth-associated haemoplasma sequences showed to be closely related to *M. wenyonii* detected in cattle from Japan (EU367964) (Figure 5).

The 23S rRNA haemoplasma sequences detected in giant armadillos and anteater grouped in two unique clades, albeit close to "*Ca.* Mycoplasma haematoalbiventris" detected in *D. albiventris* from Brazil (M442085) (Figure 6).

4 | DISCUSSION

Currently, several studies have shown infection by haemoplasmas and *C. burnetii* in different wild animal species (Millan et al., 2020; Pontarolo

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 4 BLASTn results of the haemotropic *Mycoplasma* spp. sequences obtained in the semi-nested and conventional PCR assays based on the 16S rRNA and 23S rRNA genes, respectively

ID	Species/state	Gene	Size (bp)	QC (%)	E-value	Identity (%)	Host/location/Genbank accession number
BM 179	*T. tetradactyla*/PA	16S rRNA	∼1380	100%	0.0	96.49	*Mycoplasma* sp. em Tatu-canastra, Brasil (OK093397)
BM 43	*T. tetradactyla*/PA	16S rRNA	∼1400	100	0.0	96.30	*Mycoplasma* sp. em Tatu-canastra, Brasil (OK093397)
BM 51	*B. tridactylus*/PA	16S rRNA	∼1400	100	0.0	99.79	*Mycoplasma wenyonii* em búfalos e bovinos de leite, Cuba (MG948624)
67	*T. tetradactyla*/RS	16S rRNA	∼1400	100	0.0	96.09	*Mycoplasma* sp. em Tatu-canastra, Brasil (OK093397)
153	*P. maximus*/MS	16S rRNA	∼1400	100	0.0	100	*Mycoplasma* sp. em Tatu-canastra, Brasil (OK093397)
108	*T. tetradactyla*/MS	16S rRNA	∼1400	100	0.0	96.5	*Mycoplasma* sp. em Tatu-canastra, Brasil (OK093397)
159	*P. maximus*/MS	16S rRNA	∼1400	100	0.0	100	*Mycoplasma* sp. em Tatu-canastra, Brasil (OK093397)
BM 179	*T. tetradactyla*/PA	23S rRNA	∼800	96	0.0	90.58	*Mycoplasma haemomalae* Alpacas e Lhamas, EUA (NR_076983)
154	*P. maximus*/MS	23S rRNA	∼800	99	0.0	89.01	*Mycoplasma suis*, EUA (NR_103970)
138	*P. maximus*/MS	23S rRNA	∼800	100	0.0	88.45	*Mycoplasma suis*, EUA (NR_103970)
156	*P. maximus*/MS	23S rRNA	∼800	98	0.0	89.51	*Mycoplasma suis*, EUA (NR_103970)
160	*P. maximus*/MS	23S rRNA	∼800	100	0.0	89.05	*Mycoplasma suis*, EUA (NR_103970)

et al., 2020; Vieira et al., 2021; Zanatto et al., 2019). Such works highlighted the importance of these animals in the maintenance of such pathogens in the environment and in the emergence of haemoplasmosis and Q fever.

Herein, all biological samples from Xenarthra mammals were negative in the qPCR assay for *C. burnetii* based on the *IS1111* gene. Indeed, blood and spleen samples might not have been the ideal biological samples for performing the screening for *C. burnetii* in Xenarthra. Nonetheless, Davoust et al. (2014) detected *C. burnetii* in stool samples from a three-toed sloth in French Guyana, using the same quantitative real-time PCR protocol. On the other hand, *C. burnetii* was detected in spleen samples from wild rodents (Rozental et al., 2017) and bats (Ferreira et al., 2018) by nested PCR assays based on the *IS1111* gene.

Although the results of the present study suggest that Xenarthra mammals may not participate in the epidemiological cycle of Q fever in Brazil, studies aiming to detect *C. burnetii* in other biological samples (e.g., faeces) from Xenarthra in other geographical regions are needed. The role of wild animals in the epidemiology of this agent should be better investigated, since molecular and serological evidence of *C. burnetii* infection has been reported in humans from Brazil (Brenner et al., 2021; Davoust et al., 2014; Lamas et al., 2016).

Free-living wild animals show a higher prevalence of haemoplasmas when compared to those kept in captivity, due to a higher exposure to potential arthropod vectors, frequent involvement in disputes with other animals and aggressive behaviour (Willi et al., 2007). Although little is known about the origin, evolution, transmission and dispersion of haemoplasmas in the environment, several studies have shown their presence in different animal species as well as in humans (Maggi, Compton, et al., 2013; Millan et al., 2020).

The present study demonstrated the presence of DNA of haemotropic mycoplasmas in blood and spleen samples of Xenarthra mammals from different Brazilian states. For the first time, the occurrence of haemoplasma DNA was reported in anteaters, armadillos and sloths.

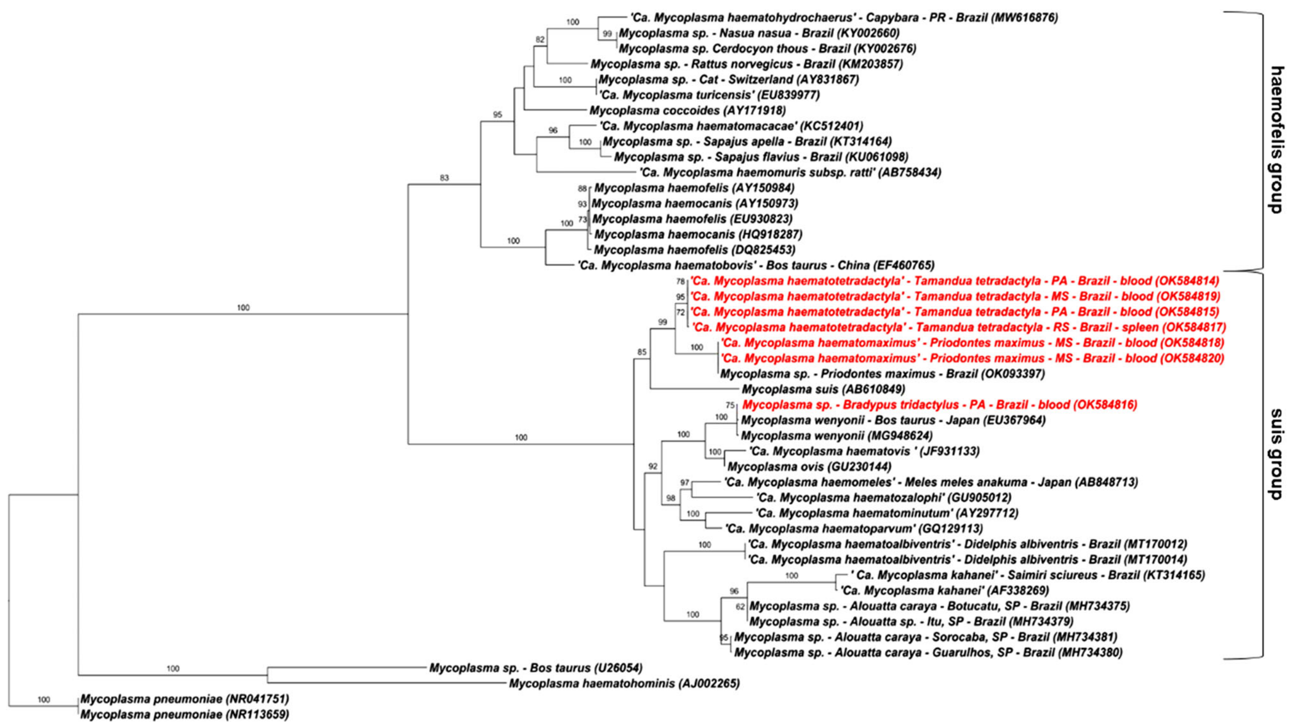


FIGURE 3 Phylogenetic analysis of 16S rRNA sequences of *Mycoplasma* spp. (990 bp alignment) generated by the Maximum Likelihood method and TIM3+F+I+G4 evolutionary model. *Mycoplasma pneumoniae* was used as an outgroup

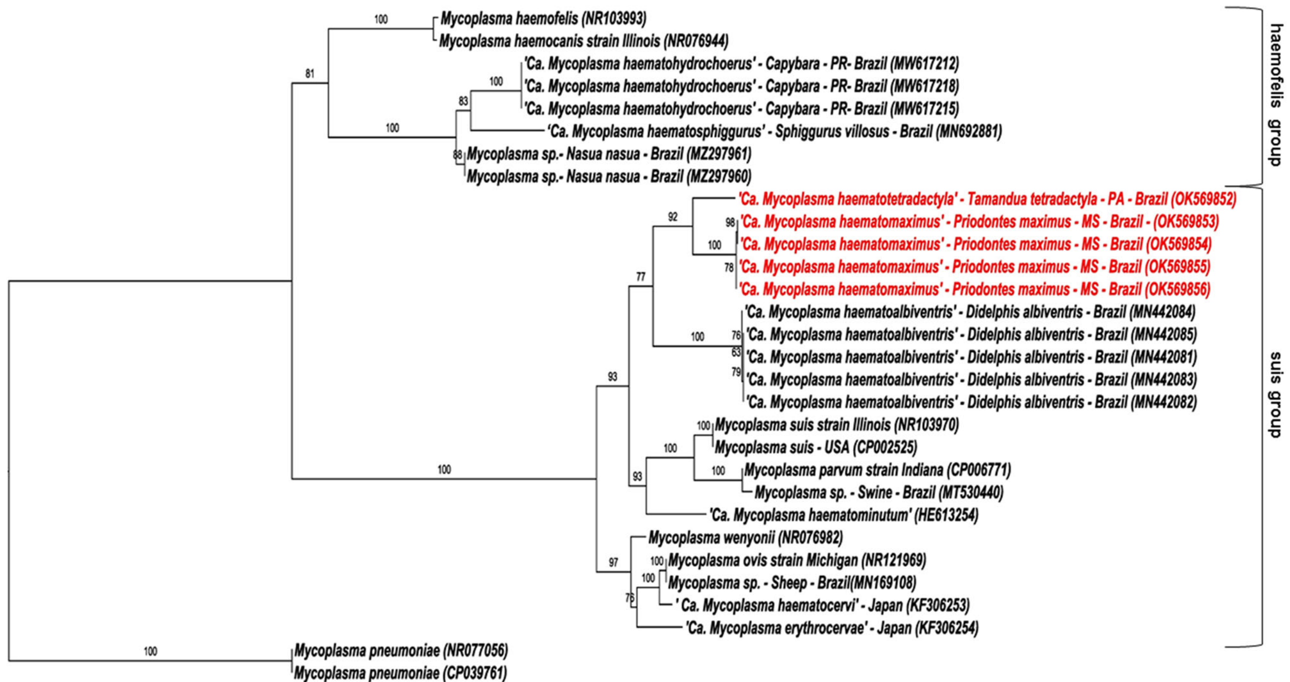


FIGURE 4 Phylogenetic analysis of 23S rRNA sequences of *Mycoplasma* spp. (810 bp alignment) generated by the Maximum Likelihood method and TIM3+F+I+G4 evolutionary model. *Mycoplasma pneumoniae* was used as an outgroup

In conventional PCR analyses based on the 16S rRNA gene, 15.54% of the sampled animals were positive for haemoplasmas. The low intensity of the amplified products verified in the present study, and consequently, the difficulty in sequencing these samples, is probably related to the chronic nature of haemoplasma infections in wild animals, which generally present very low or undetectable bacteraemia. This low bacteraemia might be related to the low pathogenicity of the involved haemoplasmas in this group of animals, or even to the more effective

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

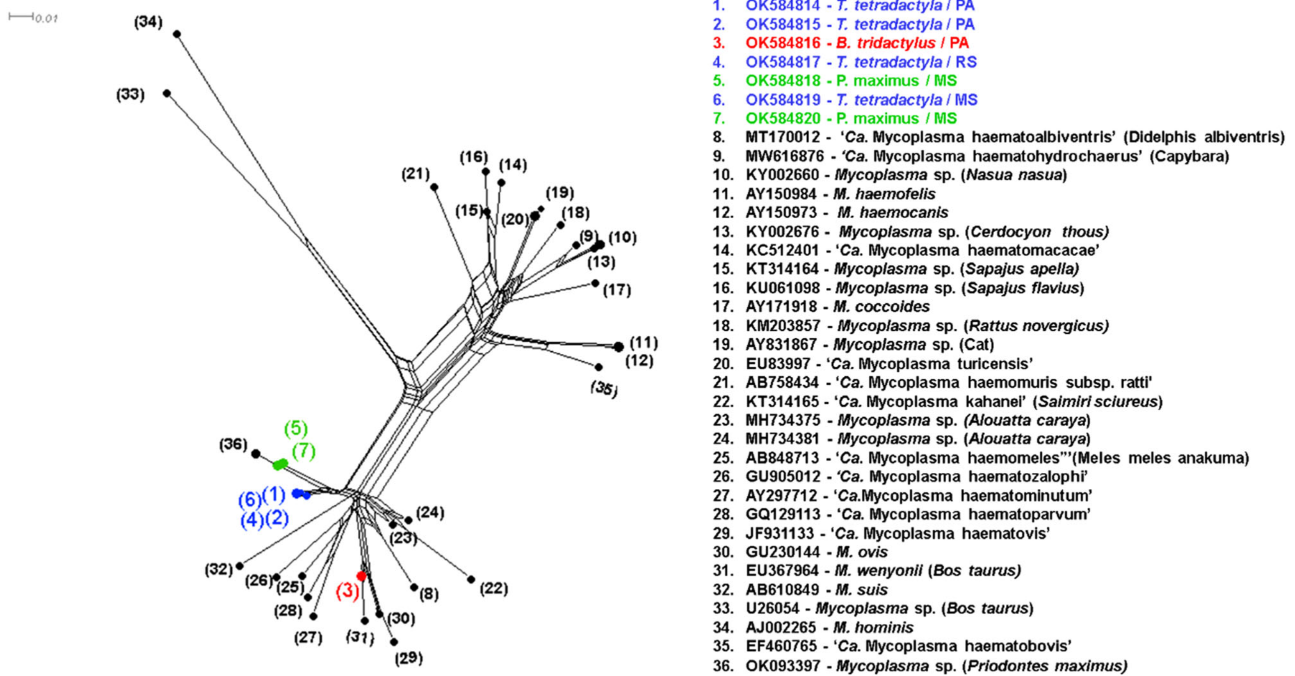


FIGURE 5 Distance analysis by SplitsTree4 software with the parameters "Neighbor – Net" and "Uncorrected p-distance" for the 16S rRNA gene

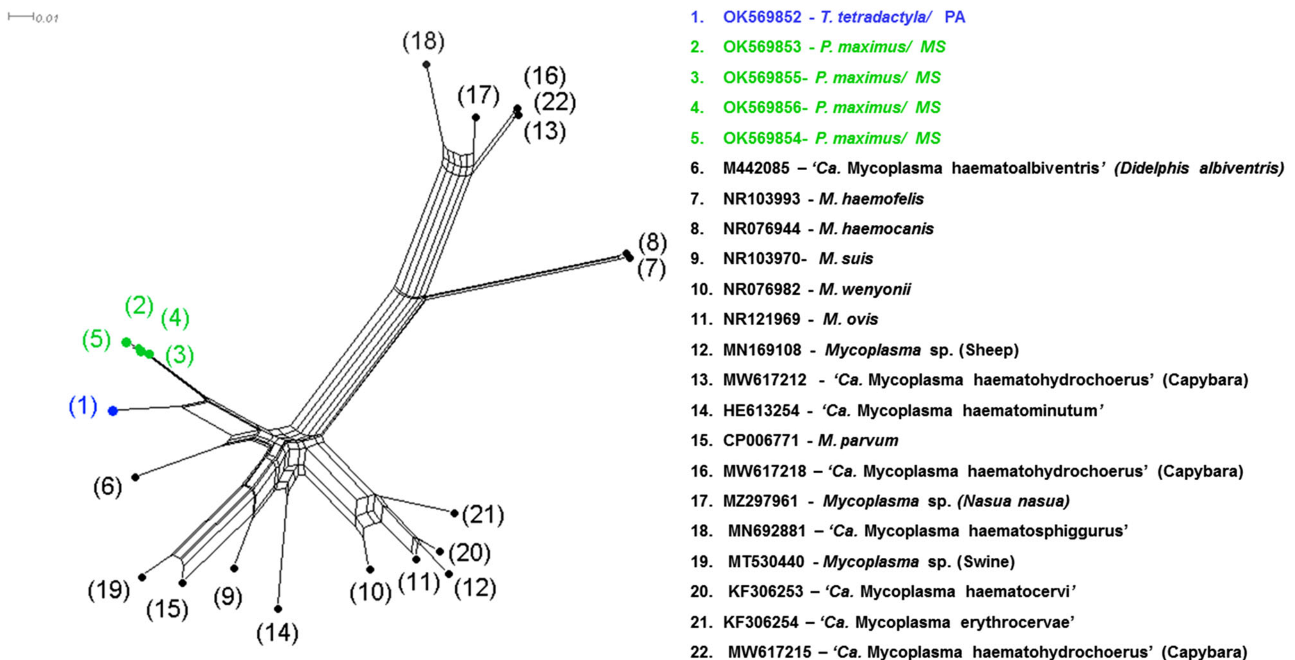


FIGURE 6 Distance analysis by SplitsTree4 software with the parameters "Neighbor – Net" and "Uncorrected p-distance" for the 23S rRNA gene

immune response of this group of animals to haemotropic mycoplasmas. Such difficulty has already been reported in the molecular detection and sequencing of haemoplasmas in wild carnivores and bats (Carneiro et al., 2020; Ikeda et al., 2021).

The phylogenetic positioning of both 16S rRNA and 23S rRNA haemoplasma sequences detected in the present study associated with the low identity values obtained by BLASTn analysis of the obtained 16S rRNA sequences (<97%) (Drancourt & Raoult, 2005;

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Hanage et al., 2006) suggests the circulation of possible new haemotropic *Mycoplasma* species in *T. tetradactyla* and *P. maximus*. The provisional names "*Candidatus* Mycoplasma haematotetradactyla" and "*Candidatus* Mycoplasma haematomaximus" are proposed herein. Nonetheless, future studies aiming at fully characterized these putative novel species should be performed using Whole genome sequencing approaches in order to compare the average nucleotide identity (ANI) of these novel putative species with closely related taxa (Goris et al., 2007; Jain et al., 2018). Interestingly, the 16S rRNA haemoplasma sequence detected in *B. tridactylus* showed high identity (99.7%) to *M. wenyonii*. Considering the cut-off proposed for 16S rRNA sequence similarities for species discrimination by Drancourt and Raoult (2005) associated to the lack of phylogenetic inferences based on other gene sequences and ANI comparison (Hanage et al., 2006; Goris et al., 2007; Jain et al., 2018), it is unclear the real identity of the haemoplasma detected in *B. tridactylus*.

Herein, the obtained 16S rRNA sequences were phylogenetically positioned within the large "suis" clade and grouped into three different clusters, one for each Xenarthra species (anteater, armadillo and sloth) and supported by high bootstrap values. A single clade was observed for 16S rRNA sequences obtained from collared anteaters, separated from giant armadillo-associated haemoplasmas with 99% support. The giant armadillo sequences were also positioned in a single clade and grouped to a *Mycoplasma* spp. sequence previously detected in an armadillo from Brazil (OK093397; unpublished data). *M. suis* showed to be an ancestral group for the large clade comprising giant armadillo and collared anteater-associated haemoplasma sequences, with support of 85%. The haemoplasma 16S rRNA sequence detected in a sloth was closely positioned to *M. wenyonii* previously detected in cattle from Japan (EU367964) (Tagawa et al., 2008).

The 23S rRNA sequences obtained in the present study also clustered within the large "suis" clade, corroborating with the phylogenetic findings for the 16S rRNA gene. Based on the 23S rRNA gene fragment, two unique clades were found: one for the collared anteater-associated haemoplasma sequence and the other sister clade for the giant armadillo haemoplasma sequences, with a bootstrap of 92% separating these two clades. Sequences of "*Candidatus* Mycoplasma haematoalbiventris" previously detected in *Didelphis albiventris* from Brazil (MN442084) (Pontarolo et al., 2020) were closely positioned to the sequences obtained in the present study, albeit separated under 77% bootstrap. SplitsTree distance analyses corroborated the findings of the phylogenetic analyses based on the Maximum Likelihood method.

The qPCR protocol for haemoplasmas based on the 16S rRNA gene using SYBR® Green as DNA intercalant uses universal primers for haemoplasmas, which were designed to detected several haemoplasma species. However, such a technique shows lower specificity when compared to TaqMan PCR assays (Willi et al., 2009). Although qPCR assays are considerably more sensitive than conventional PCR assays (Sykes, 2010), the present study found a high number of positive samples by conventional PCR assays. Such a finding may be due to the inability of the primers used in the qPCR assay to amplify DNA from genetically diverse haemoplasmas, such as those found in the present study. Thus, we suggest caution in the use of this assay in the identification and screening for haemoplasmas when it comes to biological samples from wild animals.

Melting curve analysis showed different melting temperature (T_m) values for Xenarthra-associated haemoplasmas: while a $T_m = 79.5°C$ was observed for haemoplasmas detected in sloths' biological samples, a $T_m = 77.0°C$ was observed for haemoplasmas detected in armadillos' biological samples. In fact, Willi et al. (2009) found different T_m values for different haemoplasma species, which ranged from 73.0°C to 77.5°C. Besides that, nonspecific amplification was found to occur due to the primers annealing to host DNA, with T_m ranging from 80°C to 83.05°C (data not shown). Therefore, positive results obtained in this qPCR should be confirmed by sequencing in order to rule out the occurrence of false-positive results due to nonspecific amplification. Vieira et al. (2015) suggest that specific TaqMan PCR assays for newly described species should be designed. Furthermore, PCR assays based on at least two molecular markers followed by sequencing and phylogenetic analyses have proven to be a valuable method in the identification of new haemoplasma species circulating in wild animals from Brazil (Collere et al., 2021; Pontarolo et al., 2020).

Given the zoonotic potential of haemoplasmas, the occurrence of these agents in wild animals should be better investigated, so that control and prevention measures can be established. The recent detection of *Mycoplasma haematohominis*, the causative agent of fever and haemolytic anemia in humans (Hattori et al., 2020; Steer et al., 2011) in bats in New Caledonia (Descloux et al., 2021), emphasizes the need for studying the genetic diversity of haemoplasmas in wild reservoirs.

5 | CONCLUSION

Based on the low identity and phylogenetic positioning of 16S rRNA and 23S rRNA sequences of haemoplasmas detected in anteaters and armadillos, the present study showed the occurrence of putative novel haemotropic "*Candidatus* Mycoplasma spp." ("*Candidatus* Mycoplasma haematotetradactyla" and "*Candidatus* Mycoplasma haematomaximus") in free-living mammals of the superorder Xenarthra in Brazil. A haemoplasma 16S rRNA sequence closely related and showing high identity (99.7%) to *M. wenyonii* was detected, for the first time, in *B. tridactylus*. On the other hand, the absence of *C. burnetii* DNA in blood and spleen samples from Xenarthra suggests that these mammals may not act as hosts for this agent in the studied locations.

ACKNOWLEDGEMENTS

The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo for the financial support (FAPESP #2018/02753-0 and #2020/12037-0), the Conselho Nacional de Desenvolvimento Científico e Tecnológico for the M.R.A. Productivity grant (CNPq #303701/2021-8), to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for the M.R.A. scholarship granted (CAPES #88887. 464146/2019-00), to the Laboratory of Evolutionary Biology and Conservation, Institute of Biomedical Sciences at USP, to the Institute for Wild Animal Conservation (ICAS) (Flags and Roads Project and the Giant Armadillo Conservation Program), the Service of Pathology

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

of Wild Animals (SEPAS) of the Faculty of Agricultural Sciences and Veterinary Sciences of the Paulista State University (FCAV-UNESP) and the Laboratory of Protozoology and Vector-borne Rickettsioses, Federal University of Rio Grande do Sul (UFRGS).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The Xenarthra mammals sampling procedures were previously approved by the Chico Mendes Institute for Biodiversity Conservation (SISBIO no. 53798-5; no. 27587-12; no. 53798-13; no. 64752-2) and by the Ethics Committees on Animal Use (CEUA) of both ICB - USP (no. 98) and the Faculty of Agricultural and Veterinary Sciences – UNESP Jaboticabal Campus (no. 11. 794).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article. Accession numbers of the generated sequences are available at Genbank database.

ORCID

Danilo Kluyber https://orcid.org/0000-0002-8964-566X

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Birkenheuer, A. J., Levy, M. G., & Breitschwerdt, E. B. (2003). Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. *Journal of Clinical Microbiology*, *41*, 4172–4177. https://doi.org/10.1128/JCM.41.9.4172-4177.2003

Bouvery, N. A., Souriau, A., Lechopier, P., & Rodolakis, A. (2003). Experimental *Coxiella burnetii* infection in pregnant goats: Excretion routes. *Veterinary Research*, *34*(4), 423–433.

Brenner, A. E., Muñoz-Leal, S., Sachan, M., Labruna, M. B., & Raghavan, R. (2021). *Coxiella burnetii* and related tick endosymbionts evolved from pathogenic ancestors. *Genome Biology and Evolution*, *13*(7), evab108. https://doi.org/10.1093/gbe/evab108

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *American Association of Clinical Chemists*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Calchi, A. C., Vultão, J. G., Alves, M. H., Yogui, D. R., Desbiez, A. L. J., Amaral, R. B., Santi, M., Teixeira, M. M. G., Werther, K., Machado, R. Z., & André, M. R. (2020). Multi-locus sequencing reveals a novel *Bartonella* in mammals from the superorder Xenarthra. *Transboundary and Emerging Diseases*, *67*(5), 2020–2033. https://doi.org/10.1111/tbed.13545

Calchi, A. C., Vultão, J. G., Alves, M. H., Yogui, D. R., Desbiez, A. L. J., Santi, M., Santana, M. S., da Silva, T. M. V., Werther, K., Teixeira, M. M. G., Machado, R. Z., & André, M. R. (2020). *Ehrlichia* spp and *Anaplasma* spp. in Xenarthra mammals from Brazil, with evidence of novel '*Candidatus* Anaplasma spp.' *Scientific Reports*, *10*, 12615. https://doi.org/10.1038/s41598-020-69263-w

Carneiro, F. T., Scalon, M. C., Amorim, G., Silva, W. A. C., Honorato, S. M., Pereira, F., Silva, L. H. R., Aquino, L. C., & Paludo, G. R. (2020). Hemoplasma infection in wild captive carnivores. *Brazilian Journal of Veterinary Research*, *40*(4), 293–299. https://doi.org/10.1590/1678-5150-PVB-6407

Collere, F. C. M., Delai, R. M., Ferrari, L. D. R., da Silva, L. H., Fogaça, P. L. C., Rodrigues, A. N., Gonçalves, D. D., Baggio, R. A., Moraes, M. F. D., Hoppe, E. G. L., André, M. R., Vieira, T., & Vieira, R. F. C. (2021). '*Candidatus* Mycoplasma haematonasua' and tick-borne pathogens in ring-tailed coatis (*Nasua nasua* Linnaeus, 1976) from the Iguaçu National Park, Paraná State, southern Brazil. *Transboundary and Emerging Diseases*, *68*(6), 3222–3229. https://doi.org/10.1111/tbed.14311

Davoust, B., Marié, J. L., de Santi, V. P., Berenger, J. M., Edouard, S., & Raoult, D. (2014). Three-toed sloth as putative reservoir of *Coxiella burnetii*, Cayenne, French Guiana. *Emerging Infectious Diseases*, *20*(10), 1760–1761. https://doi.org/10.3201/eid2010.140694

Descloux, E., Mediannikov, O., Gourinat, A. C., Colot, J., Chauvet, M., Mermoud, I., Desoutter, D., Cazorla, C., Klement-Frutos, E., Antonini, L., Levasseur, A., Bossi, V., Davoust, B., Merlet, A., Goujart, M. A., Oedin, M., Brescia, F., Laumond, S., Fournier, P. E., & Raoult, D. (2021). Flying Fox Hemolytic Fever, Description of a New Zoonosis Caused by Candidatus Mycoplasma haemohominis. *Clinical Infectious Diseases*, *73*(7), e1445–e1453. https://doi.org/10.1093/cid/ciaa1648.

De Sousa, K. C. M., Herrera, H. M., Secato, C. T., Oliveira, A. V., Santos, F. M., Rocha, F. L., Barreto, W. T. G., Macedo, G. C., Pinto, P. C. E. A., Machado, R. Z., Costa, M. T., & André, M. R. (2017). Occurrence and molecular characterization of hemoplasmas in domestic dogs and wild animals in a Brazilian wetland. *Acta Tropica*, *171*, 172–81. https://doi.org/10.1016/j.actatropica.2017.03.030

Di Cataldo, S., Hidalgo-Hermoso, E., Sacristán, I., Cevidanes, A., Napolitano, C., Hernández, C., Esperón, F., Moreira-Arce, D., Cabello-Stom, J., Müller, A., & Millán, J. (2020). Hemoplasmas are endemic and cause asymptomatic infection in the endangered Darwin's fox (*Lycalopex fulvipes*). *Applied and Environment Microbiology*, *86*(12), 779–820. https://doi.org/10.1128/AEM.00779-20

Dos Santos, A. P., dos Santos, R. P., Biondo, A. W., Dora, J. M., Goldani, L. Z., de Oliveira, S. T., Guimarães, A. M. S., Timenetsky, J., de Morais, H. A., González, F. H. D., & Messick, J. B. (2008). Hemoplasma infection in HIV-positive patient, Brazil. *Emerging Infectious Diseases*, *14*(12), 1922–1924. https://doi.org/10.3201/eid1412.080964

Drancourt, M., & Raoult, D. (2005). Sequence-based identification of new bacteria: A proposition for creation of an orphan bacterium repository. *Journal of Clinical Microbiology*, *43*, 4311–4315. https://doi.org/10.1128/JCM.43.9.4311-4315

Edward, D. G., & Freundt, E. A. (1967). Proposal for Mollicutes as name of the class established for the order Mycoplasmatales. *International Journal of Systematic Bacteriology*, *17*(3), 267–268. https://doi.org/10.1099/00207713-17-3-267

Eldin, C., Melenotte, C., Mediannikov, O., Ghigo, E., Million, M., Edouard, S., Mege, J. L., Maurin, M., & Raoult, D. (2017). From Q fever to *Coxiella burnetii* infection: A paradigm change. *Clinical Microbiology Reviews*, *30*(1), 115–190. https://doi.org/10.1128/CMR.00045-16

Ewing, B., & Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Research*, *31*(12), 186–194. https://doi.org/10.1101/gr.8.3.186

Ewing, B., Hillier, L., Wendl, M. C., & Green, P. (1998). Base-calling of automated sequencer traces using phred. I Accuracy assessment. *Genome Research*, *8*(3), 175–185. https://doi.org/10.1101/gr.8.3.175

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution; International Journal of Organic Evolution*, *39*(4), 783–791.

Ferreira, M. S., Guterres, A., Rozental, T., Novaes, R. L. M., Vilar, E. M., De Oliveira, R. C., Fernandes, J., Forneas, D., Junior, A. A., Brandão, M. L., Cordeiro, J., Del Valle Alvarez, M. R., Althoff, S. L., Moratelli, R., Cordeiro-Estrela, P., Silva, R., & Lemos, E. (2018). *Coxiella* and *Bartonella* spp. in bats (Chiroptera) captured in the Brazilian Atlantic Forest biome. *BMC Veterinary Research*, *14*(1), 279. https://doi.org/10.1186/s12917-018-1603-0

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Gatto, I. R. H., Sonalio, K., Amaral, R. B., Mores, N., Costa, O. A. D., André, M. R., & Oliveira, L. G. (2019). High frequency and molecular characterization of porcine hemotrophic mycoplasmas in Brazil. *Veterinary Microbiology*, *231*, 33–39. https://doi.org/10.1016/j.vetmic.2019.02.024

Glez-Peña, D., Gómez-Blanco, D., Reboiro-Jato, M., Fdez-Riverola, F., & Posada, D. (2010). ALTER: Program-oriented conversion of DNA and protein alignments. *Nucleic Acids Research*, *38*, 14–18. https://doi.org/10.1093/nar/gkq321

Goris, J., Konstantinidis, K. T., Klappenbach, J. A., Coenye, T., Vandamme, P., & Tiedje, J. M. (2007). DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology*, *57*, 81–91. https://doi.org/10.1099/ijs.0.64483-0

Guimarães, A. M. S., Toth, B., Santos, A. P., Do Nascimento, N. C., Kritchevsky, J. C., & Messick, J. B. (2012). Genome sequence of "*Candidatus* mycoplasma haemolamae" strain purdue, a red blood cell pathogen of alpacas (*Vicugna pacos*) and llamas (*Lama glama*). *Journal Bacteriology*, *194*(22), 6312–6313. https://doi.org/10.1128/JB.01557-12

Hall, T. A. (1999). BioEdit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hanage, W. P., Fraser, C., & Spratt, B. G. (2006). Sequences, sequence clusters and bacterial species. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *361*, 1917–1927. https://doi.org/10.1098/RSTB.2006.1917

Hattori, N., Kuroda, M., Katano, H., Takuma, T., Ito, T., Arai, N., Yanai, R., Sekizuka, T., Ishii, S., Miura, Y., Tokunaga, T., Watanabe, H., Nomura, N., Eguchi, J., Hasegawa, H., Nakamaki, T., Wakita, T., & Niki, Y. (2020). *Candidatus* Mycoplasma haemohominis' in Human, Japan. *Emerging Infectious Diseases*, *26*(1), 11–19. https://doi.org/10.3201/eid2601.190983

Hornok, S., Micsutka, A., Meli, M. L., Lutz, H., & Hofmann-Lehmann, R. (2011). Molecular investigation of transplacental and vector-borne transmission of bovine haemoplasmas. *Veterinary Microbiology*, *152*, 411–414. https://doi.org/10.1016/j.vetmic.2011.04.031

Huson, D. H., & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, *23*(2), 254–67.

Ikeda, P., Torres, J. M., Lourenço, E. C., Albery, G. F., Herrera, H. M., Oliveira, C. E., Machado, R. Z., & André, M. R.(2021). Molecular detection and genotype diversity of hemoplasmas in non-hematophagous bats and associated ectoparasites sampled in peri-urban areas from Brazil. *Acta Tropica*, *225*, 106203. https://doi.org/10.1016/j.actatropica.2021.106203

Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T., & Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nature Communications*, *9*, 5114. https://doi.org/10.1038/s41467-018-07641-9

Klee, S. R., Tyczka, J., Ellerbrok, H., Franz, T., Linke, S., Baljer, G., & Appel, B. (2006). Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. *BMC Microbiology*, *6*, 2. https://doi.org/10.1186/1471-2180-6-2

Kluyber, D., Desbiez, A., Attias, N., Massocato, G., Gennari, S., Soares, H., Baglagli, E., Bosco, S., Garces, H., Ferreira, J., Fontes, A., Suffys, P., Meireles, L., Luna, E., Jansen, A., & Roque, A. (2020). Zoonotic parasites infecting free-living armadillos from Brazil. *Transboundary and Emerging Diseases*, *68*(3), 1639–1651. https://doi.org/10.1111/tbed.13839

Lamas, C. C., Fournier, P. E., Zappa, M., Brandão, T. J., Januário-da-Silva, C. A., Correia, M. G., Barbosa, G. I., Golebiovski, W. F., Weksler, C., Lepidi, H., & Raoult, D. (2016). Diagnosis of blood culture-negative endocarditis and clinical comparison between blood culture negative and blood culture-positive cases. *Infection*, *44*, 459–466. https://doi.org/10.1007/s15010-015-0863-x

Maggi, R. G., Chitwood, M. C., Kennedy-stoskopf, S., & Deperno, C. S. (2013). Novel hemotropic *Mycoplasma* species in white-tailed deer (*Odocoileus virginianus*). *Comparative Immunology, Microbiology and Infectious Diseases*, *36*(6), 607–611. https://doi.org/10.1016/j.cimid.2013.08.001

Maggi, R. G., Compton, S. M., Trull, C. L., Mascarelli, P. E., Mozayeni, B. R., & Breitschwerdt, E. B. (2013). Infection with hemotropic Mycoplasma species in patients with or without extensive arthropod or animals contact. *Journal of Clinical Microbiology*, *51*(10), 3237–3241. https://doi.org/10.1128/JCM.01125-13.

Maggi, R. G., Mascarelli, P. E., Havenga, L. N., Naidoo, V., & Breitschwerdt, E. B. (2013). Coinfection with *Anaplasma platys*, *Bartonella henselae* and "*Candidatus* Mycoplasma haematoparvum" in a veterinarian. *Parasites & Vectors*, *6*, 103. https://doi.org/10.1186/1756-3305-6-103

Marrie, T. J., Schlech, W. F., Williams, J. C., & Yates, L. (1986). Q fever pneumonia associated with exposure to wild rabbits. *The Lancet*, *327*(8478), 427–429. https://doi.org/10.1016/S0140-6736(86)92380-9

Messick, J. B. (2004). Hemotrophic mycoplasmas (hemoplasmas): A review and new insights into pathogenic potential. *Veterinary Clinical Pathology*, *33*(1), 2–13. https://doi.org/10.1111/j.1939-165x.2004.tb00342.

Millán, J., Di Cataldo, S., Volokhov, D. V., & Becker, D. J. (2020). Worldwide occurrence of haemoplasmas in wildlife: Insights into the patterns of infection, transmission, pathology and zoonotic potential. *Transboundary and Emerging Diseases*, *68*(6), 3236–3256. https://doi.org/10.1111/tbed.13932

Mongruel, A. C., Spanhol, B., Valente, V. C., Porto, J. D. M., Ogawa, P. P. Otomura, L., & Vieira, R. F. C. (2020). Survey of vector-borne and nematode parasites involved in the etiology of anemic syndrome in sheep from Southern Brazil. *Brazilian Journal of Veterinary Parasitology*, *29*(3), e007320. https://doi.org/10.1590/S1984-29612020062

Nguyen, L. T., Schmidt, H. A., Von haeseler, A., & Minh, B. Q. (2015). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology and Evolution*, *32*(1), 268–274. https://doi.org/10.1093/molbev/msu300

Pontarolo, G. H., Kühl, L. F., Pedrassani, D., Campos, M., Figueiredo, F. B., Valente, J. D., de Barros, R., & Filho, I. (2020). 'Candidatus Mycoplasma haemoalbiventris', a novel hemoplasma species in white-eared opossums (*Didelphis albiventris*) from Brazil. *Transboundary and Emerging Diseases*, *68*(2), 565–572. https://doi.org/10.1111/tbed.13716

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology*, *62*(4), 1094–1156.

Rozental, T., Ferreira, M. S., Guterres, A., Mares-Guia, M. A., Teixeira, B. R., Gonçalves, J., Bonvicino, C. R., D'Andrea, P. S., & Lemos, E. R. S. (2017). Zoonotic pathogens in Atlantic Forest wild rodents in Brazil: *Bartonella* and *Coxiella* infections. *Acta Tropica*, *168*, 64–73. https://doi.org/10.1016/j.actatropica.2017.01.003

Sanger, F., Nicklen, S., & Coulson, A. R. (1997). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, *74*(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Soares, H. S., Marcili, A., Barbieri, A. R. M., Minervino, A. H. H., Malheiros, A. F., Gennari, S. M., & Labruna, M. B. (2017). Novel *Anaplasma* and *Ehrlichia* organisms infecting the wildlife of two regions of the Brazilian Amazon. *Acta Tropica*, *174*, 82–87. https://doi.org/10.1016/j.actatropica.2017.07.006

Steer, J. A., Tasker, S., Barker, E. N., Jensen, J., Mitchell, J., Stocki, T., Chalker, V. J., & Hamon, M. (2011). A novel hemotropic Mycoplasma (hemoplasma) in a patient with hemolytic anemia and pyrexia. *Clinical Infectious Diseases*, *53*(11), 147–151. https://doi.org/10.1093/cid/cir666

Stover, B. C., & Muller, K. F. (2010). TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics [Electronic Resource]*, *11*(7), 01–09. https://doi.org/10.1186/1471-2105-11-7

Sykes, J. E. (2010). Feline hemotropic mycoplasmas. *Journal of Veterinary Emergency and Critical Care*, *20*, 62–69. https://doi.org/10.1111/j.1476-4431.2009.00491.

Tagawa, M., Matsumoto, K., & Inokuma, H. (2008). Molecular detection of *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haemobos' in cattle in Hokkaido, Japan. *Veterinary Microbiology*, *132*(1–2), 177–180. https://doi.org/10.1016/j.vetmic.2008.05.006

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680. https://doi.org/10.1093/nar/22.22.4673

Tratchenberg, S. (2005). Quick guide: Mollicutes. *Current Biology*, *15*(13), 483–484. https://doi.org/10.1016/j.cub.2005.06.049

Trifinopoulos, J., Nguyen, L. T., Von haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, *44*, 232–235. https://doi.org/10.1093/nar/gkw256

Van Schaik, E. J., Chen, C., Mertens, K., Weber, M. M., & Samuel, J. E. (2013). Molecular pathogenesis of the obligate intracellular bacterium *Coxiella burnetii*. *Nature Reviews Microbiology*, *11*(8), 561–573. https://doi.org/10.1038/nrmicro3049

Vieira, R. F. C., Santos, N. J. R., Valente, J. D. M., Santos, L. P., Lange, R. R., Duque, J. C. M., Ferrari, M. V., Barros Filho, I. R., Collere, F. C. M., Ferrrari, L. D. R., Gonçalves, L. R., Sanches, G. S., André, M. R., & Vieira, T. (2021). 'Candidatus Mycoplasma haematohydrochoerus', a novel hemoplasma species in capybaras (*Hydrochoerus hydrochaeris*) from Brazil. *Infection, Genetics and Evolution*, *93*, 104988. https://doi.org/10.1016/j.meegid.2021.104988

Vieira, R. F. C., Vidotto, O., Vieira, T., Guimaraes, A. M. S., Santos, A. P., Nascimento, N. C., Santos, N. J. R., Martins, T. F., Labruna, M. B., Marcondes, M., Biondo, A. W., & Messick, J. B. (2015). Molecular investigation of hemotropic mycoplasmas in human beings, dogs and horses in a rural settlement in southern Brazil. *Journal of the Sao Paulo Institute of Tropical Medicine*, *57*(4), 353–7. https://doi.org/10.1590/S0036-46652015000400014

Willi, B., Boretti, F. S., Tasker, S., Meli, M. L., Wengi, N., Reusch, C. E., Lutz, H., & Hofmann-Lehmann, R. (2007). From Haemobartonella to hemoplasma: Molecular methods provide new insights. *Veterinary Microbiology*, *125*, 197–209.

Willi, B., Meli, M. L., Luthy, R., Honegger, H., Wengi, N., Hoelzle, L. E., Reusch, C. E., Lutz, H., & Hofmann-Lehmann, R. (2009). Development and application of a universal hemoplasma screening assay based on the SYBR Green PCR principle. *Journal of Clinical Microbiology*, *47*(12), 4049–4054. https://doi.org/10.1128/JCM.01478-09

Yuan, C. L., Liang, A. B., Yao, C. B., Yang, Z. B., Zhu, J. G., Cui, L., Yu, F., Zhu, N. Y., Yang, X. W., & Hua, X. G. (2009). Prevalence of *Mycoplasma suis* (*Eperythrozoon suis*) infection in swine and swine-farm workers in Shanghai, China. *American Journal of Veterinary Research*, *70*(7), 890–894. https://doi.org/10.2460/ajvr.70.7.890

Zanatto, D. C. S., Duarte, J. M. B., Labruna, M. B., Tasso, J. B., Calchi, A. C., Machado, R. Z., & Andre, M. R. (2019). Evidence of exposure to *Coxiella burnetii* in neotropical free-living cervids in South America. *Acta Tropica*, *197*, 105037. https://doi.org/10.1016/j.actatropica.2019.05.028

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: de Oliveira, L. B., Calchi, A. C., Vultão, J. G., Yogui, D. R., Kluyber, D., Alves, M. H., Desbiez, A. L. J., de Santi, M., Soares, A. G., Soares, J. F., Werther, K., Teixeira, M. M. G., Machado, R. Z., & André, M. R. (2022). Molecular investigation of haemotropic mycoplasmas and *Coxiella burnetii* in free-living Xenarthra mammals from Brazil, with evidence of new haemoplasma species. *Transboundary and Emerging Diseases*, *69*, e1877–e1891. https://doi.org/10.1111/tbed.14523

18651682, 2022, 5, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/tbed.14523 by UNIVERSIDADE ESTADUAL PAULISTA, Wiley Online Library on [02/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License