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RESEARCH

Molecular detection of piroplasmids in mammals from the Superorder Xenarthra in Brazil

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Abstract

Xenarthra mammals can be found from southern North America to southern South America, including all Brazilian biomes. Although it has been shown that Xenarthra mammals can play a role as reservoirs for several zoonotic agents, few studies investigate the diversity of piroplasmids (Apicomplexa: Piroplasmida) in this group of mammals. Taking into account that piroplasmids can cause disease in animals and humans, understanding the prevalence and diversity of piroplasmids in Xenarthra mammals would contribute to conservation efforts for this group of animals as well as to infer risk areas for transmission of emergent zoonosis. The present study aimed to investigate the occurrence and molecular identity of piroplasmids in free-living mammals of the Superorder Xenarthra from four Brazilian states (Mato Grosso do Sul, São Paulo, Rondônia, and Pará). For this, DNA was extracted from blood or spleen samples from 455 animals. A nested PCR based on the 18S rRNA gene was used as screening for piroplasmids. Of the 455 samples analyzed, 25 (5.5%) were positive. Additionally, PCR assays based on 18S rRNA near-complete, *cox-1*, *cox-3*, *hsp70*, *cytB*, *β-tubulin* genes and the ITS-1 intergenic region were performed. Five out of 25 positive samples also tested positive for ITS-1-based PCR. The phylogenetic analysis positioned three 18S rRNA sequences detected in *Priodontes maximus* into the same clade of *Babesia* sp. detected in marsupials (*Didelphis albiventris*, *Didelphis marsupialis*, and *Monodelphis domestica*) and *Amblyomma dubitatum* collected from opossums and coatis in Brazil. On the other hand, the 18S rRNA sequence obtained from *Dasypus novemcinctus* was closely related to a *Theileria* sp. sequence previously detected in armadillos from Mato Grosso State, grouping in a subclade within the *Theileria sensu stricto* clade. In the phylogenetic analysis based on the ITS-1 region, the sequences obtained from *Myrmecophaga tridactyla* and *Tamandua tetradactyla* were placed into a single clade, apart from the other piroplasmid clades. The present study demonstrated the molecular occurrence of Piroplasmida in anteaters and *Babesia* sp. and *Theileria* sp. in armadillos from Brazil.

Keywords Piroplasmids · Armadillos · Anteaters · Sloths

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Introduction

Diseases transmitted by arthropod vectors represent 22.8% of the current emerging diseases (Zanella 2016). Climate, environmental, and ecological changes favor the disruption of cycles of naturally established microorganisms and the consequent emergence of diseases, as well as the expansion of the distribution of vectors and greater proximity of wildlife with domestic animals and humans. Therefore, there is a greater interaction between reservoirs, vectors, and susceptible hosts, leading to serious problems for public and animal health (Shaw et al. 2001; André 2018).

The Superorder Xenarthra comprises the orders Cingulata and Pilosa. The first includes different species of armadillos, which are divided into the families Dasipodidae and Chlammyphoridae. The second order is composed of four families: Bradypodidae and Megalonychidae, which include sloths, and Cyclopedidae and Myrmecophagidae, which include the silk anteaters and anteaters (Gardner 2005). Currently, 38 species are registered, of which six species of sloths, seven species of silk anteater, three species of anteater, and 22 species of armadillo (ASASG 2023). They are distributed from southern North America to southern South America (Gardner 2005). In Brazil, these mammals are distributed throughout all of the country biomes. According to the IUCN (International Union for Conservation of Nature) red list of threatened species, the pygmy three-toed sloth (*Bradypus pygmaeus*) is classified as critically endangered. Maned three-toed sloth (*Bradypus torquatus*), giant anteater (*Myrmecophaga tridactyla*), giant armadillo (*Priodontes maximus*), and Brazilian three-banded armadillo (*Tolypeutes tricinctus*) are species classified as vulnerable to extinction (IUCN 2023).

Regarding the occurrence of vector-borne agents in this group of mammals, *Anaplasma marginale* (Guillemi et al. 2016), a new genotype of *Ehrlichia* (Soares et al. 2017), *Ehrlichia canis*, *Ehrlichia minasensis*, two putative novel *Anaplasma* ("*Candidatus* Anaplasma brasiliensis" and "*Candidatus* Anaplasma amazonensis") (Calchi et al. 2020a), a putative novel *Bartonella* ("*Candidatus* Bartonella washoensis subsp. Brasiliensis") (Calchi et al. 2020), and two new putative hemotropic *Mycoplasma* ("*Candidatus* Mycoplasma haematotetradactyla" and "*Candidatus* Mycoplasma haematomaximus") (de Oliveira et al. 2022) have been recently detected in Xenarthra mammals from South America.

Piroplasmids are tick-borne apicomplexan protozoa belonging to the order Piroplasmida, which is composed of the families Babesiidae (genera *Babesia* and *Rangelia*) and Theileriidae (genera *Theileria* and *Cytauxzoon*) (Solano-Gallego and Baneth 2011; Shock et al. 2012; França et al. 2014). These agents parasitize blood cells of vertebrate animals, such as erythrocytes, lymphocytes, monocytes, or endothelial cells, depending on the involved agent (Alvarado-Rybak et al. 2016). These protozoa can cause illnesses in domestic and wild animals (Hunfeld et al. 2008; Alvarado-Rybak et al. 2016) as well as humans (Homer et al. 2000; Kjemtrup and Conrad 2000; Zintl et al. 2003; Hunfeld et al. 2008; Leiby 2011).

Despite the worldwide occurrence of piroplasmids in wild animals, few are the reports about the occurrence and molecular identity of piroplasmids in mammals of the Superorder Xenarthra. In the past, two Piroplasmida species have been described, namely *Theileria brimont*, which was detected in pale-throated sloth (*Bradypus tridactylus*) from French Guiana (Léger and Mouzels 1917), and *Babesia choloepi,* detected in Linnaeus's two-toed sloth (*Choloepus didactylus*), in the same country (Dedet et al. 1988). In addition, *Theileria* sp. has been morphologically detected in nine-banded armadillo (*Dasypus novemcinctus*) from Pará state, Brazil (Laison et al. 1979). *Babesia* sp. has been detected in *Bradypus didactylus*, *Dasypus novemcinctus*, greater long-nosed armadillos (*Dasypus kappleri*), and silky anteaters (*Cyclopes didactlus*) from French Guiana (de Thoisy et al. 2000). All four descriptions were based only on the finding of intraerythrocytic parasites in blood smears. More recently, Soares et al. (2017) detected a new genotype of *Theileria* sp. in *D. novemcinctus* in the states of Pará and Mato Grosso, Brazil. In the phylogeny based on the partial 18S rRNA gene, the armadillo-associated sequences were grouped into a new clade along with a genotype detected in agouti (*Dasyprocta* sp.), closely related to the *Theileria sensu stricto* clade. Finally, piroplasmids were detected by blood smears and PCR assays based on the 18S rRNA gene in giant anteater (*Myrmecophaga tridactyla*) and southern tamandua (*Tamandua tetradactyla*) kept in rehabilitation centers in the states of Goiás and Minas Gerais, Brazil. However, no amplified product was sequenced (Fava et al. 2022).

Recently, the description of putative novel piroplasmids in Brazil (Ikeda et al. 2021; Gonçalves et al. 2021; Mongruel et al. 2022; de Oliveira et al. 2023) has contributed to the expansion of the Piroplasmida clades previously proposed by Jalovecka et al. (2019), emphasizing the need for additional studies aiming at untangling the complex diversity of this group of apicomplexan protozoa. The present study aimed to investigate the prevalence and diversity of piroplasmids in Xenarthra mammals sampled in four different Brazilian states.

Material and methods

Studied area and sampled mammal species

Between 2011 and 2022, a total of 455 biological samples (blood or spleen) were collected from 230 sloths (191

brown-throated three-toed sloths [*Bradypus variegatus*], 3 *Bradypus* sp., 5 Linnaeus's two-toed sloths [*C. didactylus*], 31 *Choloepus* sp.), 168 anteaters (37 southern tamandua [*T. tetradactyla*], 131 giant anteaters [*M. tridactyla*]), and 57 armadillos (4 southern naked-tailed armadillos [*Cabassous unicinctus unicinctus*], 10 nine-banded armadillos [*D. novemcinctus*], 11 six-banded armadillos [*Euphractus sexcinctus*], 32 giant armadillos [*Priodontes maximus*]). The samples were collected in four Brazilian states: São Paulo (SP), Mato Grosso do Sul (MS), Pará (PA), and Rondônia (RO) and covered three different biomes, namely Cerrado (SP and MS), Pantanal (MS), and Amazon (PA and RO) (Fig. 1). A small fragment of spleen was cut during necropsy of the animals and stored in DNAse/RNAse-free microtubes. Blood from live animals was collected in EDTA tubes. The collected samples were stored in a freezer at −20°C until use.

The biological samples from Mato Grosso do Sul state were represented by spleen samples (n=81) collected during necropsies of road-killed animals and blood samples (n=93) collected from free-living animals captured on site. All the samples were collected by the Anteaters & Highways Project and by the Giant Armadillo Conservation Program. In São Paulo state, only spleen samples (n=39) were collected from animals necropsied by the Wildlife Pathology Service of the School of Agricultural and Veterinarian Sciences. Finally, the blood samples obtained from the states of Pará (n=137) and Rondônia (n=105) are part of the DNA/tissues collection (TCC-USP) of the Laboratory of Phylogeny and Molecular Taxonomy of Trypanosomatids, Department of Parasitology, Institute of Biomedical Sciences (ICB).

DNA extraction and quality assessment

DNA was extracted from 10 mg of each spleen tissue and 200 µL of each blood sample using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. In order to verify the presence of amplifiable DNA and to discard the presence of PCR inhibitors, DNA samples were subjected to a conventional PCR (cPCR) assay targeting the mammalian endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (Birkenheuer et al. 2003). The positive samples were subjected to specific PCR assays for piroplasmids.

Molecular detection and characterization of piroplasmids

Firstly, DNA samples were screened for piroplasmids using a nested PCR (nPCR) assay targeting a fragment (~800 bp) of the 18S rRNA gene (Jefferies et al. 2007). Positive samples were subjected to further molecular characterization

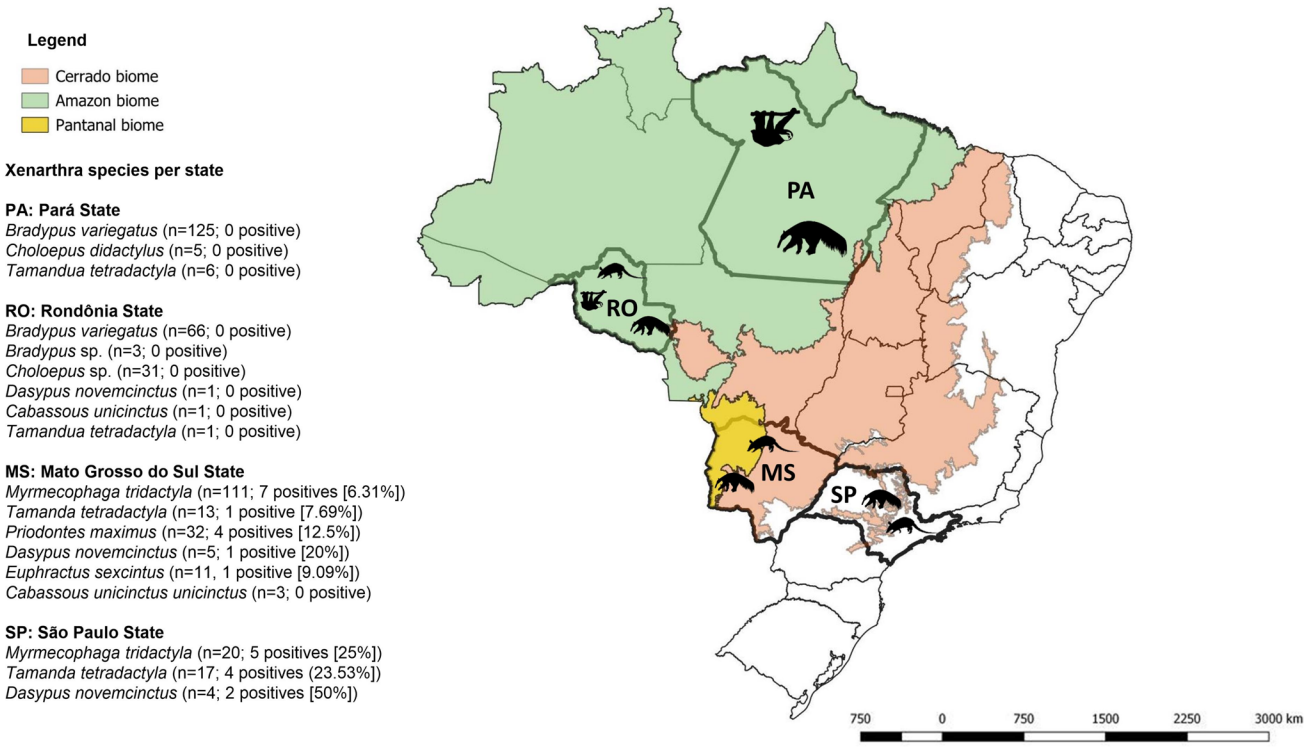

Fig. 1 Map of Brazil with the number of Xenarthra species sampled in the present study per state and the number and percentage of positive animals. The figures within the states represent which animals were collected in each state

using conventional PCR assays targeting different molecular markers: near-complete 18S rRNA gene (~1500 bp) (Kawabuchi et al. 2005; Merino et al. 2006; Quillfeldt et al. 2014; Yabsley et al. 2017; Greay et al. 2018), *cox-1* (~800 bp; Corduneanu et al. 2017), *cox-3* (~600 bp; Schreeg et al. 2016; Barbosa et al. 2019), *hsp70* (~700 bp; Soares et al. 2011), *cytB* (~1 kb; Barbosa et al. 2019), *β-tubulin* (~1214 bp; Zamoto et al. 2004), and ITS-1 (~450 bp; Shock et al. 2012). All primer sequences and thermal cycles used in the PCR assays are described in Table 1.

The assays were performed using 5 µL of the DNA samples in a mixture containing 0.75 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), PCR buffer (PCR buffer 10 X – 100 nM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM deoxynucleotides (dATP, dTTP, dCTP, and dGTP) (Invitrogen, Carlsbad, CA, USA), 1.5 mM of magnesium chloride (Invitrogen, Carlsbad, CA, USA), 0.5 µM of each primer (Invitrogen, Carlsbad, CA, USA), and sterile ultrapure water (Invitrogen, Carlsbad, CA, USA) comprising a total volume of 25 µL. In nPCR assays, 1 µL of the amplified product from the first PCR reaction was used as the target DNA in the second reaction. A DNA sample obtained from a dog experimentally infected with *Babesia vogeli* (Jaboticabal strain) (Furuta et al. 2009) and sterile ultrapure water were used as positive and negative controls, respectively, in all PCR assays for piroplasmids.

The products obtained in PCR assays were separated by horizontal electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA) in TEB running buffer pH 8.0 at 100 V/150 mA for 50 min. The gels were examined under ultraviolet light illumination using ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) and photographed using Image Lab Software v.4.1 (Bio-Rad, Hercules, CA, USA).

Sequencing, BLAST, and phylogenetic analyses

The amplified products that showed high band intensity on agarose gels were purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) and sequenced by Sanger's method using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the "Centro de Recursos Biológicos e Biologia Genômica (CREBIO -FCAV - UNESP).

The sequences obtained were submitted to a quality-screening test using Phred-Phrap software (version 23) (Ewing and Green 1998; Ewing et al. 1998) to evaluate the quality of the electropherograms and to obtain the consensus sequences from the alignment of the sense and antisense sequences. Consensus sequences were trimmed so that only nucleotides with Phred index above 20 were used. The BLASTn program (Altschul et al. 1990) was used to compare the obtained sequences with previously deposited sequences in the GenBank database (Benson et al. 2002). The sequences saved in "FASTA" format were aligned with other homologous sequences of each agent retrieved from the database (Genbank), using the MAFFT software (Katoh et al. 2019) and edited via Bioedit v. 7.0.5.3 (Hall 1999). W-IQ-Tree software was used for choosing the evolutionary model following BIC criterion as well as for phylogenetic analysis by the Maximum Likelihood method (available online: http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al. 2016). Clade support indices were evaluated through bootstrap analyses of 1000 repetitions. The phylogenetic trees were edited using Treegraph 2.0.56-381 beta software (Stover and Muller 2010). The choice of sequences and outgroups used in the phylogenetic analyses were based on the topology described by Jalovecka et al. (2019), and updated by works performed by Ikeda et al. (2021), Gonçalves et al. (2021), Mongruel et al. (2022), and de Oliveira et al. (2023).

Results

Molecular assays

All 455 DNA samples analyzed were positive for mammalian endogenous gene (*gapdh*) and subjected to a nPCR assay based on the 18S rRNA gene of piroplasmids. Of the 455 analyzed samples, 25 (5.49% [25/455]) were positive: 12 (9.16% [12/131]) giant anteaters, five (13.51% [5/37]) southern tamanduas, three (30% [3/10]) nine-banded armadillos, four (12.5% [4/32]) giant armadillos, and one (9.09% [1/11]) six-banded armadillo (Table 2). Due to the low intensity of the bands in agarose gel, only four samples (three giant armadillos and one armadillo) were subjected to sequencing (Table 3).

Out of the 25 samples positive in the nPCR for piroplasmids, 5 (20%; three southern tamanduas and two nine-banded armadillos) and 5 (20%; two southern tamanduas, two giant anteaters, and one nine-banded armadillo) were positive for the *cox-1* gene and the intergenic region (ITS-1), respectively (Table 3). Due to the low intensity of the obtained bands in agarose gel, only three ITS-1 amplicons were successfully sequenced. All 25 samples positive in the nPCR for piroplasmids were negative in the PCR assays based on the nearly complete 18S rRNA gene, *cox-3*, *hsp70*, *cytB*, and *β-tubulin* genes.

BLASTn analysis

In the BLASTn analysis of the 18S rRNA sequences obtained in this study, the three sequences from the giant armadillos were 100% identical to one sequence of *Babesia*

Table 1 Description of primers, amplicon sizes, and thermal sequences used in conventional and nested PCR assays

	Gene/ name primer	Primers sequences	Size (bp)	Thermal sequences	Reference
Endogenous gene	*gapdh* -GAPDHF -GAPDHR	5′TCCAACACCACC ACTGAGATCGGA C -3′ 5′- GTGAGAAGAAAT CGGACTGGCC3′	400	95°C for 5 min; 35 cycles: 95°C for 15s, 50°C for 30s, and 72°C for 30s; 72°C for 5 min	Birkenheuer et al. (2003)
Piroplasmids (screening)	18S rRNA -BTF1 -BTR1 -BTF2 -BTR2	5′GGCTCATTACAA CAGTTATAG-3′ 5′CCCAAAGACTTT GATTTCTCTC-3′ 5′CCGTGCTAATTG TAGGGCTAATAC-3′ 5′GGACTACGACGG TATCTGATCG-3′	800	94°C for 3 min, 58°C for 1 min, 72°C for 2 min; 45 cycles: 94°C for 30s, 58°C for 20s, and 72°C for 30s; 72°C for 7 min Annealing temperature 2nd round = 62°C	Jefferies et al. (2007)
Piroplasmids (characterization)	18S rRNA 1st reaction -Piro 0F -Piro 6R 2nd reaction -Piro 1F -Piro 5.5R	5′-GCCAGTAGTCAT ATGCTTGTGTTA-3′ 5′-CTCCTTCCTYTA AGTGATAAGGTT CAC-3′ 5′-CCATGCATGTCT WAGTAYAARCTT TTA-3′ 5′-CCTYTAAGTGAT AAGGTTCACAAA ACTT-3′	1500	95°C for 3 min; 33 cycles: 95°C for 1min, 59°C for 1 min, and 72°C for 2 min; 72°C for 10 min	Kawabuchi et al. 2005
Piroplasmids (characterization)	18S rRNA -NBAB1Bab -Hep1615R	5′-GGATAACCGTGC TAATTGT-3′ 5′-AAAGGGCAGGGA CGTAATC-3′	1484	95°C for 10 min; 40 cycles: 95°C for 30s, 55°C for 30s, and 72°C for 90s; 72°C for 10 min	Merino et al. (2006); Quillfeldt et al. (2014)
Piroplasmids (characterization)	18S rRNA 1st reaction -5.1 -B 2nd reaction – 1 -5.1 V2 -3.1 2nd reaction -2 -RLBF -RLBR	5′-CCTGGTTGATCC TGCCAGTAGT-3′ 5′-CCCGGGATCCAA GCTTGATCCTTC TGCAGGTTCACC TAC-3′ 5′-CATATGCTTGTC TTAAA-3′ 5′-CTCCTTCCTTTA AGTGATAAG-3′ 5′-GAGGTAGTGACA AGAAATAACAAT A-3′ 5′-TCTTCGATCCCC TAACTTTC-3′	~1700 (2nd reaction – 1) ~500 (2nd reaction – 2)	94°C for 1 min; 30 cycles: 94°C for 1 min, 48°C for 1 min, and 72°C for 2 min; 72°C for 5 min Annealing temperature 2nd round -1= 52°C Annealing temperature 2nd round -2= 50°C	Yabsley et al. (2017)
Piroplasmids (characterization)	18S rRNA -Nbab_1F -18SApiR	5′-AAGCCATGCATG TCTAAGTATAAG CTTTT-3′ 5′-GGATCACTCGAT CGGTAGGAG-3′	1500	95°C for 5 min; 50 cycles: 95°C for 45s, 60°C for 45s, and 72°C for 2min; 72°C for 5 min	Greay et al. (2018)
Piroplasmids (characterization)	*cox-1* -Bab_for1 -Bab_Rev1 -Bab_for2 -Bab_rev2	5′ATWGGATTYTAT ATGAGTAT3′ 5′ATAATCWGGWAT YCTCCTTGG3′ 5′TCTCTWCATGGW TTAATTATGATAT3′ 5′TAGCTCCAA TTGAHARWACA AAGTG3′	924	95°C for 1 min; 35 cycles; 95°C for 15s, 45°C for 30s, and 72°C for 1 min 72°C for 10 min Annealing temperature 2nd round = 49°C	Corduneanu et al. (2017)

Table 1 (continued)

	Gene/ name primer	Primers sequences	Size (bp)	Thermal sequences	Reference
Piroplasmids (characterization)	*cox-3* -Cox3F -Cox3R	5′-ACTGTCAGCTAA AACGTATC-3′ 5′-ACAGGATTAGAT ACCCTGG-3′	600	94°C for 5 min; 50 cycles: 94°C for 20s, 55°C for 30s, and 68°C for 45s; 72°C for 7 min	Schreeg et al. (2016); Barbosa et al. (2019)
Piroplasmids (characterization)	*hsp70* -Hsp70F1 -Hsp70R1	5′ CATGAAGCACTG GCCHTTCAA 3′ 5′ GCNCKGCTGATGGT GGTGTTGTA 3′	740	95°c for 5 min; 35 cycles: 95°C for 15s, 60°C for 30s, and 72°C for 30s; 72°C for 5 min	Soares et al. (2011)
Piroplasmids (characterization)	*CytB* -F -R	5′- TTAGTGAAGGAA CTTGACAGGT 3′ 5′-CGGTTAATCTTT CCTATTCCTTACG 3′	1Kb	94°C for 5 min; 50 cycles: 94°C for 20s, 56°C for 30s, and 68°C for 1 min; 72°C for 7 min	Schreeg et al. (2016); Barbosa et al. (2019)
Piroplasmids (characterization)	*B-tubulin* -Tubu-63F -Tubu-3	5′-CAAATWGGYGC-MAARTTYTGGGA-3′ 5′-TCGTCCATACCT TCWCCSGTRTAC CAGTG-3′	1214	95°C for 5 min; 50 cycles: 94°C for 40s, 55°C for 1min, and 72°C for 90s; 72°C for 5 min	Zamoto et al. (2004)
Piroplasmids (characterization)	ITS-1 -ITS13B -ITS15C -ITS13C -ITS15D	5′-CGATCGAGTGAT CCGGTGAATTA-3′ 5′-GCTGCGTCCTTC ATCGTTGTG-3′ 5′-AAGGAAGGAGAA GTCGTAACAAGG-3′ 5′-TTGTGTGAGCCA AGACATCCA-3′	450	94°C for 1 min; 34 cycles: 94°C for 30s, 52°C for 30s, and 72°C for 1 min. 72°C for 5 min Annealing temperature 2nd round = 49°C	Shock et al. (2012)

Table 2 Positive animals by state and type of sample analyzed

Species	Positive mammals per States of Brazil		Positives per type of sample	
	Mato Grosso do Sul	São Paulo	Blood samples	Spleen samples
Tamandua tetradactyla	0.57% (1/175)	9.76% (4/41)	0	4.13% (5/121)
Myrmecophaga tridactyla	4.00% (7/175)	12.19% (5/41)	0	9.92% (12/121)
Dasypus novemcinctus	0.57% (1/175)	4.88% (2/41)	0	2.48% (3/121)
Euphractus sexcinctus	0.57% (1/175)	0	0	0.83% (1/121)
Priodontes maximus	2.28% (4/175)	0	1.20 (4/334)	0
TOTAL	14	11	4	21

sp. previously detected in a white-eared opossum (*Didelphis albiventris*) from Brazil. The sequence detected in a nine-banded armadillo showed 91.4% identity with *Theileria* sp. detected in goats from China. The three ITS-1 sequences obtained from two giant anteaters and one southern anteater showed identity ranging from 94.2 to 94.4% to *Cytauxzoon felis* previously detected in *Dermacentor variabilis* from the USA (Table 4).

Phylogenetic analyses

The phylogenetic analysis based on an 805 bp alignment of the 18S rRNA gene and inferred by Maximum Likelihood method and TIM3+I+G evolutionary model positioned the piroplasmid sequences obtained from *P. maximus* into the same clade of *Babesia* sp. previously detected in marsupials (*Monodelphis domestica*, *Didelphis albiventris*, *Didelphis marsupialis*, and *Didelphis aurita*) and *Amblyomma dubitatum* ticks collected from opossums and coatis (*Nasua nasua*) from central-western Brazil, with a bootstrap of 100%. The sequence detected in *D. novemcinctus* was closely related to *Theileria* sp. detected in nine-banded armadillos in the state of Mato Grosso State, Brazil, forming a subclade within the *Theileria sensu stricto* clade with a bootstrap of 94% (Fig. 2).

The phylogenetic analysis based on a 937 bp alignment of the ITS-1 region and inferred by Maximum Likelihood method and HKY+G evolutionary model positioned the

Table 3 Positive animals in the PCR assays for piroplasmids performed in the present study

Xenarthra species (ID)	Location	18S rRNA gene	ITS-1
Tamandua tetradactyla (NEC16)	MS	NS	-
Myrmecophaga tridactyla (NEC 28)	MS	NS	-
Dasypus novemcinctus (NEC 20)	MS	S	-
Myrmecophaga tridactyla (NEC 24)	MS	NS	-
Myrmecophaga tridactyla (NEC 32)	MS	NS	-
Myrmecophaga tridactyla (NEC 34)	MS	NS	-
Myrmecophaga tridactyla (NEC 36)	MS	NS	-
Euphractus sexcinctus (NEC 52)	MS	NS	-
Myrmecophaga tridactyla (NEC 69)	MS	NS	-
Myrmecophaga tridactyla (NEC 71)	MS	NS	-
Priodontes maximus (DN02)	MS	NS	-
Priodontes maximus (TC11)	MS	S	-
Priodontes maximus (TC12)	MS	S	-
Priodontes maximus (TC16)	MS	S	-
Tamandua tetradactyla (33433)	SP	NS	+ (S)
Tamandua tetradactyla (34255)	SP	NS	-
Dasypus novemcinctus (35881)	SP	NS	+ (NS)
Dasypus novemcinctus (36563)	SP	NS	-
Myrmecophaga tridactyla (40088)	SP	NS	+ (S)
Myrmecophaga tridactyla (40766)	SP	NS	+ (S)
Tamandua tetradactyla (41060)	SP	NS	+ (NS)
Myrmecophaga tridactyla (41201)	SP	NS	-
Myrmecophaga tridactyla (41662)	SP	NS	-
Myrmecophaga tridactyla (42046)	SP	NS	-
Tamandua tetradactyla (42535)	SP	NS	-

MS, Mato Grosso do Sul state; *SP*, São Paulo state; *NS*, non-sequenced samples; *S*, sequenced samples

three sequences obtained from *T. tetradactyla* and *M. tridactyla* into a single clade, apart from the other piroplasmid clades, with a bootstrap of 95% (Fig. 3).

Discussion

The present study reported a positivity of 5.5% (25/455) for piroplasmids in mammals from the Superorder Xenarthra sampled in the states of São Paulo, Mato Grosso do Sul, and Rondônia e Pará. All animals from the states of Pará and Rondônia were negative for these agents. This positivity is in agreement with frequencies reported in previous studies conducted with Xenarthra from Brazil, which ranged from 0 to 11.76% (Soares et al. 2017; Silva et al. 2021; Fava et al. 2022).

Even though *Babesia* spp. and *Theileria* spp. have been morphologically described in xenarthrans from South America (Léger and Mouzels 1917; Dedet et al. 1988; Laison et al. 1979), piroplasmid 18S rRNA sequences from these mammals were first described in a study conducted in the Amazon biome, in the states of Amazonas and Mato Grosso. A putative novel piroplasmid genotype, which was positioned in a sister clade to the *Theileria sensu stricto* clade, was detected in *D. novemcinctus* (6.25%) (Soares et al. 2017).

The phylogenetic analysis based on 18S rRNA gene demonstrated the occurrence of two different genera of piroplasmids in Xenarthra. The sequences detected in *P. maximus* were positioned into the "South American Marsupialia group," a recently described clade that contains putative novel species of *Babesia* sp. detected in marsupials and *A. dubitatum* ticks from Brazil (Gonçalves et al. 2021; de Oliveira et al. 2023; Perles et al. 2023). The sequence detected in *D. novemcinctus* was positioned within the *Theileria sensu stricto* clade along with a *Theileria* sp. previously detected in armadillos (Soares et al. 2017). Despite several attempts, the amplification of the near-complete 18S rRNA was not successful, probably due to the low parasitemia in these animals or the poor quality or integrity of the DNA extracted. Additionally, the primer specificity might have prevented amplification of the DNA of piroplasmid species that occurs in Xenarthra, directly influencing the sensitivity of the test performed and precluding robust phylogenetic inferences about the Xenarthra-associated piroplasmids.

The phylogenetic analysis based on the ITS-1 positioned the sequences obtained from *M. tridactyla* and *T. tetradactyla* into a single clade, apart from the other ones containing known species of piroplasmids. This finding suggests the occurrence of putative novel piroplasmids in this group of mammals. Unfortunately, the existence of few piroplasmids ITS-1 sequences deposited in GenBank hindered further conclusions regarding the phylogenetic positioning of the obtained sequences. According to Gou et al. (2012), the ITS region is easily amplified in PCR assays for piroplasmids since it is present in multiple copies in the genome. Most likely, it was possible to obtain amplicons for this molecular marker despite the lack of amplification for the other genic regions.

The tick vectors involved in the transmission of piroplasmids among xenarthrans are yet to be described. Several studies conducted in Brazil have shown a wide variety of tick species parasitizing these animals (Labruna et al. 2002; Marques et al. 2002; Arzua et al., 2005; Dantas-Torres et al. 2010; Garcia et al. 2013; Sanches et al. 2014; Soares et al. 2015; Acosta et al. 2016; Kluyber et al. 2016; Luz et al. 2018; Szabó et al. 2019; Bernardes et al. 2022; Martins et al. 2023). The great diversity of life habits and behaviors among the different members of this group of mammals together with the wide geographical distribution and the diversified associated biomes may contribute to the tick diversity associated with xenarthrans. For instance, Desbiez and Kluyber (2013) demonstrated that the burrows built by giant armadillos are

Table 4 BLASTn results of the sequences obtained in the amplification of target genes for piroplasmids in Xenarthra blood or spleen samples

Specie/identification	Location	Target gene	Size (bp)	*Query-cover* (%)	*E-value*	Identity (%)	GenBank sequence (access number)
Priodontes maximus / TC11	MS	18S rRNA	728	100	0	100	*Babesia* sp., detected in *Amblyomma dubitatum* from Mato Grosso do Sul State, Brazil (MW342734)
Priodontes maximus / TC16	MS	18S rRNA	638	100	0	100	*Babesia* sp., detected in *Amblyomma dubitatum* from Mato Grosso do Sul State, Brazil (MW342734)
Priodontes maximus / TC12	MS	18S rRNA	709	99	0	100	*Babesia* sp., detected in *Amblyomma dubitatum* from Mato Grosso do Sul State, Brazil (MW342734)
Dasypus novemcinctus / NEC20	MS	18S rRNA	731	100	0	91.39	*Theileria* sp. detected in goat from China (MG930118)
Myrmecophaga tridactyla / 40088	SP	Região ITS-1	242	27	4 X10^{18}	94.20	*Cytauxzoon felis* detected in *Dermacentor variabilis* from the USA (KC119625)
Myrmecophaga tridactyla / 40766	SP	Região ITS-1	233	29	3X10^{19}	94.37	*Cytauxzoon felis* detected in *Dermacentor variabilis* from the USA (KC119625)
Tamandua tetradactyla / 33433	SP	Região ITS-1	138	48	4 X10^{18}	94.20	*Cytauxzoon felis* detected in *Dermacentor variabilis* from the USA (KC119625)

SP, São Paulo state; *MS*, Mato Grosso do Sul state

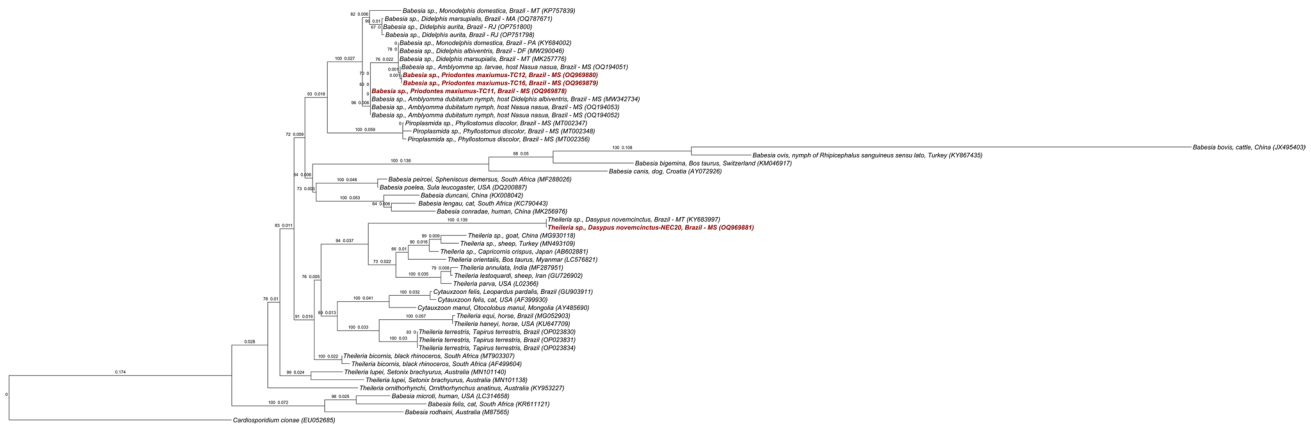

Fig. 2 Phylogenetic analysis of Piroplasmids 18S rRNA sequences inferred from 805 bp alignment generated by Maximum Likelihood and with TIM3+I+G evolutionary model. The bootstrap values and the branch lengths are represented at the nodes of each clade. *Cardiosporidium cionae* was used as outgroup

used by different animals, including other Xenarthra species. In addition, the authors described that giant armadillo burrows maintain a constant temperature (26°C) that can attract arthropod vectors, being considered a very relevant "hotspot" for pathogen transmission. The authors also reported having seen giant anteaters and other animals taking sand baths in the sand mounds outside the burrows, which may favor contact with ticks. Anthropization and deforestation of biomes can favor the spread of more generalist tick species, while decrease the number of ticks showing high host specificity (Ogrzewalska et al. 2011; Ramos et al. 2020). These facts may favor the exchange of ectoparasites and pathogens between animals and humans and may explain the occurrence of, at least, two different piroplasmids in armadillos. In addition, the behavior described above together with the diversity of ticks and vector-borne agents that these mammals can harbor highlights the potential role that Xenarthra mammals can play in the epidemiological cycles of piroplasmids. Further studies should be carried out to unravel the real molecular identity of piroplasmids that parasitize Xenarthra mammals,

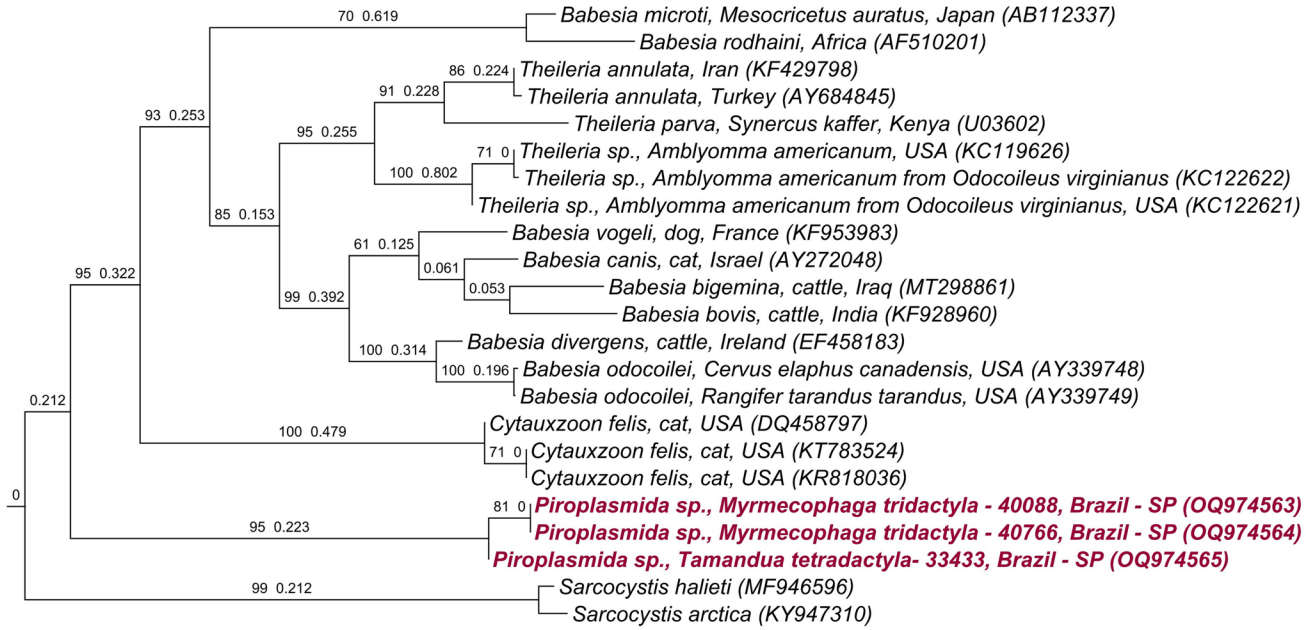

Fig. 3 Phylogenetic analysis of Piroplasmids ITS-1 sequences inferred from 937 bp alignment generated by Maximum Likelihood and with HKY+G evolutionary model. The bootstrap values and the branch lengths are represented at the nodes of each clade. *Sarcocystis halieti* and *Sarcocystis arctica* were used as outgroup

as well as the tick species associated with their transmission. Finally, studies aiming at investigating the role of Xenarthra mammals as hosts for piroplasmids that infect other wildlife species (e.g. opossums) and humans are much needed.

Conclusion

The present study demonstrated the molecular occurrence of piroplasmids in animals of the superorder Xenarthra from Brazil. This is the first work to report the presence of piroplasmids DNA in xenarthrans in the states of Mato Grosso do Sul and São Paulo. At least two different piroplasmid species parasitize armadillos, since the obtained sequences were positioned into two different clades. While the sequences detected in *P. maximus* were positioned within the South American Marsupialia clade, the sequence detected in *D. novemcinctus* was positioned along with another sequence detected in the same host species, forming a subclade within the *Theileria sensu stricto* clade. A putative novel Piroplasmida was detected in anteaters from Brazil. These results demonstrated the circulation of different species of piroplasmids in Xenarthra and their possible role in the maintenance and transmission of these agents. Further studies should be conducted to clarify the role of these agents in public and wildlife health.

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Data availability The datasets generated and analyzed during the current study are available in the NCBI GenBank Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers OQ969878-OQ969881 and OQ974563-OQ974565.

Declarations

Ethical approval Animal procedures and management protocols were approved by Institute Chico Mendes for Conservation of Biodiversity (SISBIO number 53798–5) and by the Ethics Committees on Animal

use of the ICB—USP (protocol number 98) and the School of Agricultural and Veterinarian Sciences (FCAV/UNESP) (protocol number 9412/2022). The DNA samples were registered under SISGEN #A4C38D5 ("Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado").

Consent to participate and consent for publication Not applicable.

Conflict of interest The authors declare no conflict of interest.

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