



Molecular detection of piroplasmids in synanthropic rodents, marsupials, and associated ticks from Brazil, with phylogenetic inference of a putative novel *Babesia* sp. from white-eared opossum (*Didelphis albiventris*)

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Abstract

The order Piroplasmida encompasses tick-borne pathogens of veterinary and medical importance positioned in two main families: Babesiidae and Theileriidae. Even though previous studies carried out in Brazil recorded the occurrence of piroplasmid species circulating in small mammals, 18S rRNA gene sequences were only partially sequenced, preventing the assessment of their phylogenetic positioning. The current study aimed to detect and characterize, using morphological, molecular, and bioinformatic approaches, piroplasmids from wild mammals and associated ticks sampled in Central-Western Brazil. Out of 67 *Didelphis albiventris* sampled, 22 (16.4%) were positive for piroplasmids by PCR. In contrast, none of the 48 small rodents and 14 capybaras (*Hydrochoerus hydrochaeris*) was PCR-positive. Four *Amblyomma dubitatum* ticks—one from *Rattus rattus*, one from *H. hydrochaeris*, and two from *D. albiventris*—out of 114 *Amblyomma* spp. DNA samples were positive for piroplasmids by PCR. The phylogenetic inference performed using the near-complete 18S rRNA gene positioned the putative novel piroplasmid species detected in *D. albiventris* and associated *A. dubitatum* ticks near to *Babesia* sensu lato clade (Western group—cluster III) and distant from the Australian marsupial-associated piroplasms. Phylogenetic inferences based on two additional molecular markers, namely *hsp-70* and *cox-1*, supported the near-complete 18S rRNA gene phylogenetic inference. Finally, the partial 18S rRNA gene sequences detected in ticks from rodents (*R. rattus* and *H. hydrochaeris*) showed 97.2–99.4% identity with the Piroplasmida previously detected in a capybara from Brazil, raising evidence that a still uncharacterized piroplasmid species has been identified in the capybara, the largest rodent species from South America.

Keywords *Babesia* spp. · Capybara · Opossum · Rodents

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Introduction

Environmental changes, such as deforestation and urbanization, caused by anthropic actions may alter the transmission dynamics of vector-borne agents, precipitating the emergence of infectious diseases and the spillover of pathogens from humans and domestic animals to wildlife and vice versa (Chomel 2007; Price et al. 2019). In urban and peri-urban areas, the frequency of contact between humans, their pets, and wildlife ranges from occasional encounters to permanently sharing sites, hence increasing the chance of pathogens spillover (Mackenstedt et al. 2015).

Piroplasmids, haemoprotozoan parasites belonging to the order Piroplasmida (Phylum Apicomplexa), encompass tick-borne pathogens of veterinary and medical importance distributed worldwide. These organisms are positioned into two main families (Babesiidae and Theileriidae) and taxonomically grouped into three main genera: *Babesia*, *Theileria*, and *Cytauxzoon* (Schnittger et al. 2012; Jalovecka et al. 2018; 2019).

Traditional parasitological methods such as microscopy are insufficient for identification and classification of piroplasmids found in wild vertebrate hosts. In contrast, molecular approaches have overcome some issues in the piroplasmids taxonomy (Schnittger et al. 2012; Jalovecka et al. 2019). Currently, based on molecular and phylogenetic data, ten clades of piroplasmids are distinguished (Jalovecka et al. 2019).

In contrast to studies carried out in Brazil targeting piroplasmids in domestic animals, the data regarding this group of protozoans in wild mammals is still incipient. For instance, Criado-Fornelio et al. (2009) detected a Piroplasmida genotype—showing 90% of identity with *Theileria equi*—in one out of 14 (7.14%) capybaras (*H. hydrochaeris*) sampled in Pelotas, State of Rio Grande do Sul, extreme south of Brazil. In addition, Wolf et al. (2017) reported two Piroplasmida genotypes in wild rodents (*Thrichomys pachyurus* [27.2%; 3/11]) and marsupials (*Monodelphis domestica* [50%; 1/2]) trapped in Poconé municipality, State of Mato Grosso, Central-Western Brazil. In the Amazon forest, Soares et al. (2017) reported *Theileria* spp. in one agouti (*Dasyprocta* sp. [$n=2$]) and four lowland pacas (*Cuniculus paca* [$n=32$]). In addition, the authors detected Piroplasmida DNA in one common opossum (*Didelphis marsupialis* [$n=19$]). Besides, Sousa et al. (2018) reported two Piroplasmida genotypes closely related to *Babesia vogeli* (6.5%; 5/77) and *T. equi* (1.3%; 1/77) in wild rodents (*Trychomys fosteri*) in Pantanal biome. Recently, Colle et al. (2019) reported a Piroplasmida genotype in two out of 31 (6.45%) *D. marsupialis* trapped in Sinop, State of Mato Grosso. This Piroplasmida genotype was identical to that one previously detected in common opossum sampled in Pará State, Northern Brazil (Soares et al. 2017).

Although some studies have been carried out aiming at performing the molecular characterization of piroplasmids in rodents and marsupials in Brazil, all of them were performed using a partial fragment of the 18S rRNA gene, precluding the accurate phylogenetic positioning of the detected piroplasmid species.

In light of the current scenario, the present study aimed to (i) investigate the occurrence and molecularly characterize piroplasmids in Rodentia and Didelphiomorpha mammals and their associated ticks in urban and urban forest fragments from Brazil; and (ii) morphologically and molecularly characterize a piroplasmid species infecting *D. albiventris* from Central-Western Brazil, by light microscopy and phylogenetic assessment based on three different molecular markers.

Material and methods

Ethical statement

The animal captures were in accordance with the licenses obtained from the “Instituto Chico Mendes de Conservação da Biodiversidade” (license number 56912–2), Imasul (license number 001/2017) and endorsed by the Ethics Committee of FCAV/UNESP under the number: 01952/18.

Study sites, mammals trapping, and blood and ectoparasite sampling

Between May 2017 and August 2018, 105 mammals belonging to four different species were sampled in different sites of Campo Grande municipality (–20° 42' 30" S, –54° 61' 60" W), State of Mato Grosso do Sul, Central-Western Brazil. In Campo Grande, 48 small rodents (*Rattus rattus* [$n=39$] and *Mus musculus* [$n=9$]) were trapped in urban areas (four sites) and urban forest fragments (four sites). Additionally, 14 capybaras (*H. hydrochaeris*) and 43 marsupials (*D. albiventris*) were trapped in three and six urban forest fragments, respectively, in Campo Grande. All capture procedures and blood sample collection were performed as previously described (Nantes et al. 2019; Gonçalves et al. 2020).

The sampled animals were checked for the presence of ticks. Once collected, the arthropods were placed in microtubes containing absolute ethanol (Merck®) and maintained at –20 °C until morphological identification and DNA extraction. The morphological identification was performed using previously described taxonomic keys (Onofrio et al. 2005; Martins et al. 2010; Linard et al. 2014; Anholt et al. 2014; Pereira et al. 2017).

Additionally, between May 2019 and January 2020, 24 blood samples from marsupials (*D. albiventris*) were obtained from a rescue center (“Centro de Triagem de Animais Silvestres do Distrito Federal”—CETAS—DF) in the Federal District. The animals were from urban areas of Brasilia (15° 47' 38" S 47° 52' 58" O), Distrito Federal (DF), and were sampled by convenience, independently of age, gender, or clinical status.

Giemsa-stained blood smears

Blood smears were performed using peripheral blood samples collected from wild Rodentia and Didelphiomorpha mammals, fixed with methanol (Merck®, Darmstadt, Germany) and stained with Giemsa (Giemsa stain, modified, Sigma-Aldrich®, St. Louis, MO, USA). The blood smears were then observed at 1000× magnification and images of the parasites recorded using a Olympus BX-43 microscope coupled to a camera (Olympus DP73). The piroplasmid dimensions detected in *D. albiventris*' blood smears were measured using the Olympus CellSens™ microscope imaging standard software.

DNA extraction and quality assessment

DNA was extracted from 10 mg of each small rodent spleen tissue, and from 200 µL of each blood sample from marsupials and capybaras, using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, California, USA), according to the manufacturer's instructions. Furthermore, the sampled arthropods were submitted to DNA extraction individually and/or in pools of up to three tick nymphs or seven tick larvae from the same host.

In order to discard the presence of PCR inhibitors, all extracted mammal DNA samples were used as a template in an internal control PCR targeting the mammal *gapdh* gene (Birkenheuer et al. 2003). Likewise, all arthropod DNA samples were submitted to an internal control PCR assay targeting the 16S rRNA (Black and Piesman 1994). Internal control-PCR positive samples were submitted to a nested PCR assay targeting a fragment of the 18S rRNA gene of piroplasmids.

Molecular detection and characterization of piroplasmids in mammals and associated ticks

Firstly, DNA samples were screened for piroplasmids DNA using a nested PCR assay targeting a small fragment (~ 800 bp) of the 18S rRNA gene as previously described (Jefferies et al. 2007). Additionally, the positive samples were subjected to further molecular characterization using conventional PCR assays

targeting seven molecular markers, namely near-complete 18S rRNA gene (1500 bp—Greay et al. 2018), *cox1* (~ 800 bp—Corduneanu et al. 2017), *hsp70* (~ 700 bp—Soares et al. 2011), *β-tubulin* (600 bp—Zamoto et al. 2004), ITS1 (~ 450 bp BROWN et al. 2009), *cytB* (~ 1 kb—Barbosa et al. 2019), and *cox3* (~ 600 bp Barbosa et al. 2019). Additionally, a qPCR assay targeting a *lsu* region (130 bp) was performed (Qurollo et al. 2017). Conventional PCR assays were carried out in 25 µL reaction volume containing 10× PCR buffer, 1.0 mM MgCl₂, 0.8 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U Taq DNA Polymerase (Life Technologies®), 0.3 µM of each primer, and 5 µl of DNA—used as a template.

A qPCR assay was carried out for marsupial blood samples DNA using the primers B-*lsu*-F (ACCTGTCAARTT CCTTCACTAAMTT) and B-*lsu*-R (TCTTAACCCAAC TCACGTACCA) as previously described (Qurollo et al. 2017). Briefly, the amplification reaction was performed using the CFX96 thermal cycler (Bio-Rad, CA, USA) real-time system. The qPCR assays were performed with a final volume of 10 µL containing 5 µl of 2xqPCR SYBRBIO (PCR Biosystems™, London, UK), 0.6 µM of each primer, and 1 µL of each DNA sample. The amplification protocol used was as follows: 3 min at 98 °C, followed by 40 cycles of 15 s at 98 °C, 15 s at 60 °C, and 15 s at 72 °C. The melting curves were acquired using 0.5 °C steps, withholds of 5 s, from 65 to 88 °C. The results were assessed through observation of amplification curves using a CFX96 thermal cycler. Serial dilutions were performed with the aim of constructing standard curves with different concentrations of Gblock DNA (Gblock; Integrated DNA Technologies, USA) (2.0×10^7 to 2.0×10^0 copies/µL), which encoded a 135-bp *B. bovis* *Isu* fragment.

DNA of *B. bovis* (Matos et al. 2017) and ultra-pure water were used as positive and non-template controls, respectively, in all (q)PCR assays.

The 18S rRNA gene (large fragment) positive amplicons were submitted to pGEM-T Easy vector cloning (Promega® Madison, WI, USA), following the manufacturer's recommendations. One clone from each positive sample was selected for sequencing, according to the blue/white colonies system. Finally, the identified clones were submitted to plasmid DNA extraction using the Illustra® PlasmidPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) and to a PCR assay targeting the multiple cloning sites of the pGEM T-Easy plasmid (Lau et al. 2013).

The obtained amplicons were purified using the EXOSAP-IT® (Applied Biosystems) and submitted to sequencing in an automatic sequencer (ABI Prism 310 Genetic Analyser—Applied Biosystem/Perkin Elmer). Consensus sequences were obtained by Phred-Phrap program with Phred quality score established at ≥ 20 (Ewing et al. 1998).

BLAST, phylogenetic analyses, and generation of distance matrices

Identity, query coverage, and e-values were assessed by BLASTn tool (using default parameters), available in the NCBI GenBank database (Altschul et al. 1990). The obtained sequences were aligned with other sequences retrieved from GenBank using MAFFT software, version 7 (Katoh et al. 2019), using default gap penalty on EMBL-EBI analysis tools (Madeira et al. 2019). The model “best of fit” was selected by the program jModelTest2 (version 2.1.6) on 11 XSEDE19, under the Akaike information criterion (AIC) (Darriba et al. 2012). The Bayesian inference (BI) analysis was performed for both large and short 18S rRNA gene sequences with MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003). Markov chain Monte Carlo (MCMC) simulations were run for 10^7 generations with a sampling frequency of every 100 generations and a burn-in of 25% using the CIPRES Science Gateway (Miller et al. 2010). The number of generations was selected based on the value of the average standard deviation of split frequencies (<0.02 , MrBayes version 3.2 Manual) (Ronquist and Teslenko 2012). Maximum likelihood tree inference was performed with IQ-TREE software (Trifinopoulos et al. 2016) for *hsp-70* and *cox1* sequences. The phylogenetic tree edition and rooting (outgroup) were performed using the Treegraph 2.0 beta software. The genetic distances were calculated using the p-distance method in MEGA X using the models previously selected in the phylogenetic analysis.

Results

Ticks and DNA extraction quality

Ticks were obtained from 5 out of 48 (10.4%) small rodents sampled in Campo Grande, MS. Moreover, 71.4% (10/14) of sampled capybaras were infested by ticks. Likewise, ticks were observed in 14 out of 43 (32.5%) of the trapped *D. albiventris* in Campo Grande. The identification of the tick species sampled is shown in Table 1. Ticks were not collected from *D. albiventris* sampled in Brasilia.

All but three of the tick samples and all of the mammal blood/tissue samples were positive in PCR assays targeting endogenous genes. The three tick samples collected from capybaras negative in arthropod-16S rRNA PCR assay were excluded from subsequent analyses.

Microscopic detection of Piroplasmids

Intra-erythrocytic oval ring-shaped organisms similar to piroplasmid merozoites were detected in blood smears from four and six *D. albiventris* from Campo Grande and Brasilia, respectively. These merozoites were encircled by basophilic-staining membrane of variable width around a less densely stained pale center. Also, this peripheral basophilic-staining membrane frequently showed more than a single dense chromatin. Merozoites in different stages of development ranged from 1.33 to 3.22 μm (mean = 2.25 ± 0.48) in length, and 1.07 to 2.63 μm (mean = 1.92 ± 0.53) in width (Fig. 1). Intra-erythrocytic structures resembling piroplasmids were neither found in capybaras nor in small rodents' blood smears.

Table 1 Occurrence of Piroplasmida DNA in the marsupials, rats, capybaras and their related ticks sampled in Campo Grande and Brasilia, Mid-western Brazil

Animal species	Sample type	No. of sampled animals	Occurrence of piroplasmids % (no.)	Ectoparasite species	No. of sampled ectoparasites	Occurrence of piroplasmids % (no.)
Mammals				Arthropods		
Rodentia				Ixodida		
<i>R. rattus</i>	DNA from spleen tissues	39	0% (0/39)	<i>Amblyomma</i> sp. ^a	62	11.1% (1/9) ^b
<i>M. musculus</i>	DNA from spleen tissues	9	0% (0/9)	–	–	–
<i>H. hydrochaeris</i>	DNA from whole blood	14	0% (0/14)	<i>A. dubitatum</i>	42	2.38% (1/42)
				<i>A. sculptum</i>	36	0% (0/33) ^c
				<i>Amblyomma</i> sp. ^a	2	0% (0/2)
Didelphimorphia						
<i>D. albiventris</i>	DNA from whole blood	43	25.5% (11/43)	<i>A. dubitatum</i>	70	7.14% (2/28) ^b

^a*Amblyomma* sp. refers to larvae sampled—in these specimens, only the genus was reported

^bEctoparasites-DNA pool samples

^cThree out of 36 *Amblyomma*-DNA samples were negative to the endogenous control (16S rRNA)

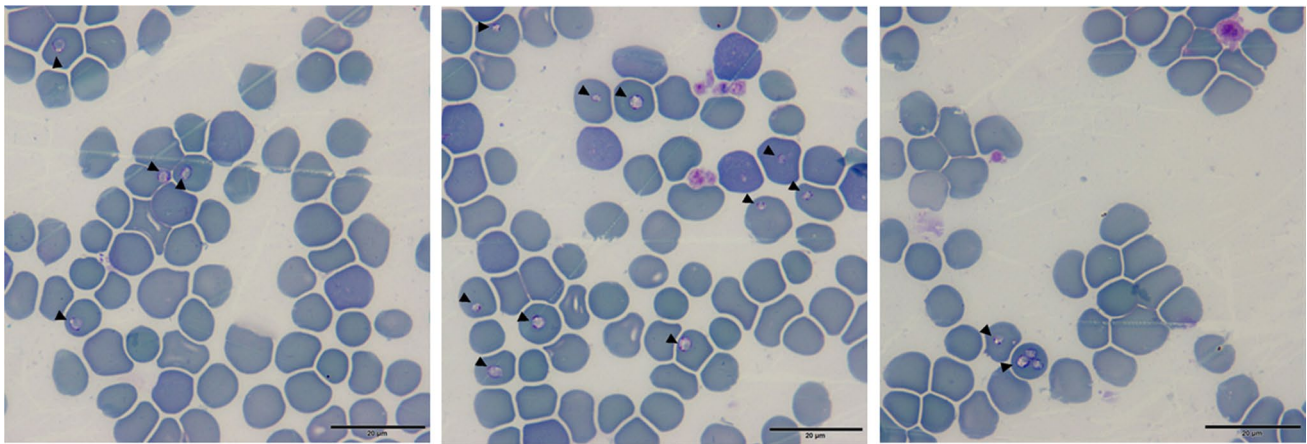


Fig. 1 Intra-erythrocytic ring-shaped merozoites of *Babesia* sp. in blood smears from a marsupial (*Didelphis albiventris*) captured in the city of Campo Grande, state of Mato Grosso do Sul, Central-Western Brazil

Occurrence of piroplasmids DNA in synanthropic mammals and ticks

None of 48 small rodents sampled in Campo Grande were positive in the nPCR assay for piroplasmids targeting a small fragment (~750 bp) of the 18S rRNA gene. On the other hand, one *A. dubitatum* nymph collected from a specimen of *R. rattus* was positive in the nPCR for piroplasmids targeting 18S rRNA gene. The amplified sequence (~750 bp) shared 99.4% identity (E-value = 0.0; query-coverage = 98%) with *Babesia* sp. (EF222255) detected in a capybara captured in Rio Grande do Sul, Southern Brazil. Similarly, none out of 14 capybara-DNA samples showed positivity in the nPCR assay. Among the 77 tick-DNA samples obtained from capybaras, one female adult specimen of *A. dubitatum* collected from a capybara was positive for piroplasmids. The sequence (~750 bp) shared 97.2% identity (E-value = 0.0; query-coverage = 100%) to *Babesia* sp. (EF222255) previously reported in a capybara.

In addition, 11 out of 43 (25.5%) *D. albiventris* sampled in Campo Grande were positive to piroplasmids in the nPCR targeting the 18S rRNA gene. Two (nymphs) out of 28 (7.14%) *A. dubitatum* samples obtained from *D. albiventris* were also positive. Likewise, 11 out of 24 (45.8%) *D. albiventris* sampled in Brasília were positive to piroplasmids targeting the 18S rRNA gene region in the screening protocol (S1 Table).

Molecular characterization of the detected piroplasmids

Aiming to investigate the identity of the detected piroplasmids, the positive samples were submitted to additional PCR assays targeting seven different molecular markers. One (obtained from *D. albiventris*) out of four 18S

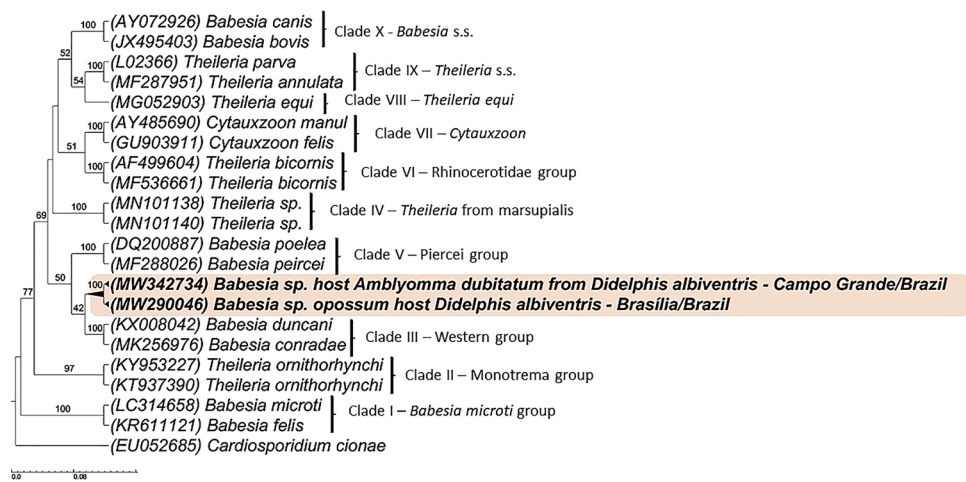
rRNA-A. *dubitatum* positive samples was also positive in a PCR assay targeting a large fragment of the 18S rRNA gene. Unfortunately, these tick DNA-samples were negative in the PCR assays targeting the other additional molecular targets, except for the tick-DNA samples obtained from *D. albiventris* ($n = 2$) and *R. rattus* ($n = 1$) that were positive in the qPCR assay targeting the *lsu* region. Another large fragment of 18S rRNA gene was obtained from a *D. albiventris* DNA blood sample. In addition, three *hsp70* and two *cox1* sequences were amplified and sequenced from *D. albiventris*. Out of the 43 and 24 *D. albiventris* from Campo Grande and Brasília tested, six and 12 were positive, respectively, in the qPCR targeting *lsu* region (S1 Table). The range of the melting temperature for *lsu* fragment ranged from 75 to 75.5 °C. Despite attempts—three times—sequences were not obtained for the other target genic regions.

Phylogenetic analyses

Two 18S rRNA gene large sequences were successfully obtained, one detected in a *D. albiventris* and another one in an *A. dubitatum* tick. The ML analysis based on an alignment of the ~1.3 kb 18S rRNA gene positioned these piroplasmid sequences in an exclusive clade, near to cluster III (*B. duncani* and *B. conrade*) previously established by Jalovecka et al. (2019), but with a low bootstrap value (42%) (Fig. 2). In addition, genetic distance ranging from 3.5 to 7.2% was observed between the amplified sequences and those belonging to cluster III (S2 Table). These findings support the assumption that the amplified piroplasmid sequences represent a distinct yet unknown species that likely does not belong to clade III.

Three *hsp-70* DNA sequences were successfully amplified and used in the phylogenetic analyses. Similar to the 18S rRNA phylogenetic analysis, the two sequences

Fig. 2 Phylogenetic tree based on an alignment of 1.3 kb of 18S rRNA sequences using maximum likelihood (ML) with GTR + I + G evolutionary model. Bootstrap supports were estimated by 1000 replicates and are presented at nodes. Sequences of piroplasmids detected in the present study are highlighted in bold. *Cardiosporidium cionae* was used as outgroup



obtained from *D. albiventris* captured in Brasília and one sequence amplified in *D. albiventris* from Campo Grande

were positioned in an exclusive clade, near to *Babesia* (WA1–DQ007005)—formally *Babesia duncani*—in ML

Fig. 3 Phylogenetic tree based on an alignment of 575 bp of *hsp-70* sequences using maximum likelihood (ML) with GTR evolutionary model. Bootstrap supports were estimated by 1000 replicates and are presented at nodes. Sequences of piroplasmids detected in the present study are highlighted in bold. *Plasmodium falciparum* was used as outgroup

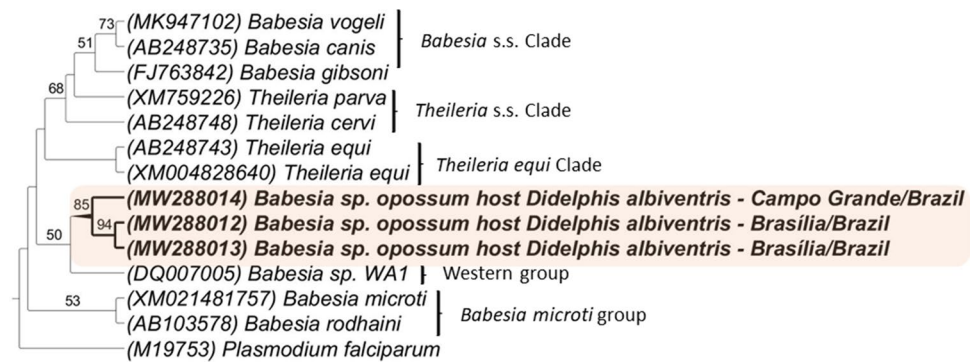
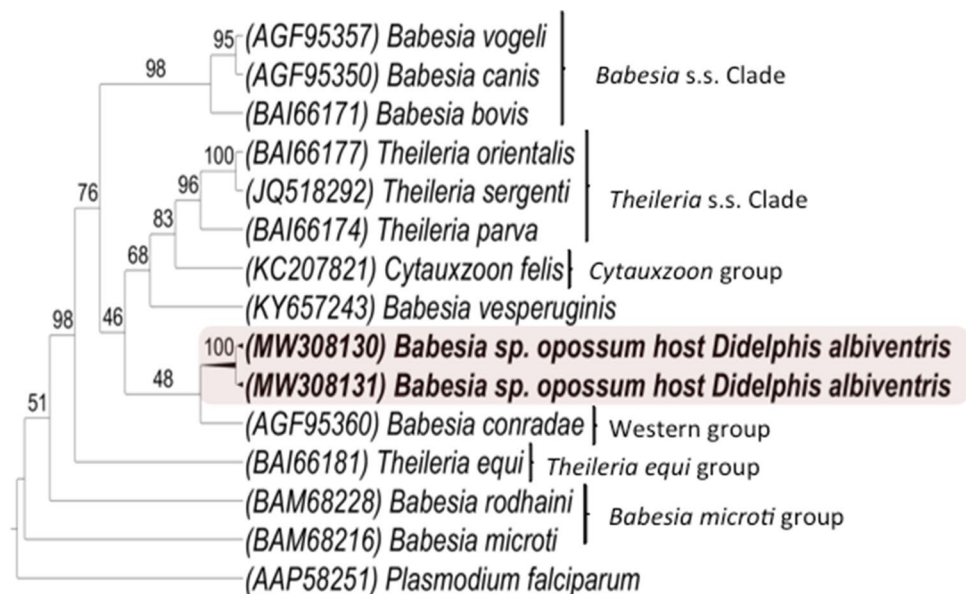


Fig. 4 Phylogenetic tree based on an alignment of 708 bp of *cox1* sequences translated into amino acids using maximum likelihood (ML) with TVM evolutionary model. Bootstrap supports were estimated by 1000 replicates and are presented at nodes. Sequences of piroplasmids detected in the present study are highlighted in bold. *Plasmodium falciparum* was used as outgroup



analysis but with a low support (50%) (Fig. 3). Despite the phylogenetic positioning, the amplified sequences showed a relatively high genetic distance (15%) when compared to the abovementioned *B. duncani* sequence (S3 Table).

Additionally, two *cox-1* DNA sequences were obtained from *D. albiventris* trapped in Brasília and submitted to ML analyses. Similar to previous trees, the two sequences were grouped near to the cluster III (*Babesia conradae*), but supported by a low bootstrap value (48%) (Fig. 4). Genetic distances were observed between the amplified *cox-1* sequences and *B. conradae* (group III—33.1%) as well as *Theileria* sensu stricto (group IX—30.2 to 31.2%) species (S4 Table).

Due to inconsistency in the phylogenetic tree based on small fragment of the 18S rRNA gene, only genetic distances were assessed for this target. Genetic distances ranging from 0 to 0.8% at the 18S rRNA gene were observed between the amplified piroplasmid sequences in *Didelphis* spp. and associated ticks in the present study and those previously detected in *Didelphis* spp. from Brazil (S5 Table), suggesting they represent the same species. Conversely, evolutionary distances of 8.8 to 9.7% were observed between the sequences detected in marsupials in the present study and those detected in Australian marsupials (S5 Table), evidencing they are distinct species. Additionally, two piroplasmid 18S rRNA gene sequences amplified from *A. dubitatum* ticks—from capybara and another one from the black rat (*R. rattus*)—grouped with a *Babesia* 18S rRNA gene sequence previously detected in a capybara sampled in southern Brazil. Genetic distance ranging from 0.8 to 3.4% was observed between these amplified haplotypes and that one reported in a capybara from Brazil, suggesting they may represent a novel species.

Discussion

In the present study, we report the occurrence and molecular characterization of a Piroplasmida species circulating in marsupials and their ticks from Brazil. In addition, piroplasmid sequences showing 97.2 to 99.4% identity with a sequence previously detected in a capybara from south Brazil was detected in *A. dubitatum* ticks collected from *H. hydrochaeris* and *R. rattus*, respectively. These results expand our knowledge on the phylogenetic positioning of piroplasmids from marsupials and add novel epidemiological data related to these hemoparasites in Brazil.

Herein, 34.3% (23/67) of all *D. albiventris* analyzed were found positive for piroplasmids. This prevalence was substantially higher than those previously reported in synanthropic and wild marsupials sampled in Brazil (0–5.8%) (Wolf et al. 2016; Soares et al. 2017; de Sousa et al. 2018; Colle et al. 2019). This finding may be attributed to different

aspects, including but not limited to the marsupial species analyzed, sampling sites, fauna composition, environmental conservation status, presence of ectoparasites, and PCR assay used in the screening. Although previous studies carried out in Brazil have sampled different marsupial species, only in a single case a species not belonging to the genus *Didelphis* (*Monodelphis*—Wolf et al. 2016) was positive for Piroplasmida, suggesting a potential specificity of this piroplasm to the genus *Didelphis* (Serra Freire 1979). However, we should keep in mind that species of *Didelphis* are more often captured and studied because it is a synanthropic animal and abundant in anthropized areas whereas *Monodelphis* comprises small marsupials with low densities that inhabit pristine areas (Olfifiers et al. 2005; Herrera et al. 2007).

Opposite to studies performed in Brazil that detected piroplasmid DNA in small rodents (Wolf et al. 2016; de Sousa et al. 2018), no piroplasmid DNA was found in samples of the black rat. Similar results were reported regarding the capybara DNA samples analyzed. Thus, the role of these rodents as reservoirs for Piroplasmida in Brazil may be limited. However, since few rodent samples were obtained and screened for piroplasmid DNA, these results should be analyzed with caution. Moreover, once piroplasm DNA was detected in *A. dubitatum* ticks collected from *R. rattus* and capybaras, the role of these mammals in the epidemiological cycle of this piroplasm must be further addressed.

Morphological identification of piroplasmids (previously named as *Nuttallia brasiliensis*/*Theileria brasiliensis*/*Babesia ernestoi*, and currently *Babesia brasiliensis*) in marsupials circulating in Brazil was performed virtually 100 years ago (Regendanz and Kikuth 1928). However, the molecular characterization of this Piroplasmida species has not been performed, precluding a robust comparison with the piroplasmids detected in the current study. More recently, the molecular detection of piroplasmids infecting marsupials has been performed in central-western and Northern Brazil (Wolf et al. 2016; Soares et al. 2017; Colle et al. 2019). However, the abovementioned studies targeted a small fragment of piroplasm-18S rRNA gene, precluding an accurate phylogenetic positioning of the piroplasmid species infecting *Didelphis* spp.

Unlike previous studies performed in Brazil, the molecular characterization of the piroplasmid species infecting South American marsupials and associated ticks is based on the near-complete 18S rRNA, *cox-1*, and *hsp-70* genes. The phylogenetic inference based on a large fragment of the 18S rRNA gene showed that the Piroplasmida detected in *D. albiventris* was positioned near to *Babesia* sensu lato (Western group—cluster III, comprising the species *B. duncani*, *B. lengau*, and *B. conradae*) as a sister taxon but with low support (42%). However, the sequences detected in the present study showed a considerable genetic distance from Western group, the most closely related Piroplasmida

species. Phylogenetic reconstructions using two mitochondrial genes (*cox-1* and *hsp-70*) were carried out using the piroplasmid sequences detected in marsupials from the present study. These analyses, associated to pairwise distances observed among *cox-1* and *hsp-70* sequences, provided supporting evidence for the uniqueness of this piroplasmid and corroborate the phylogenetic position as determined by 18S rRNA gene analysis of this protozoan species.

These findings demonstrated that the piroplasmids circulating in marsupials from Australia and Brazil are genetically distant, which is likely expected due to the origin, dispersion, and evolutionary history of these animals (Mitchell et al. 2015). The Australian marsupials are phylogenetically and immunologically more closely related between them than they are to any American marsupial. This finding suggests that they originated from a single ancestral stock that would have reached Australia in the beginning of the Tertiary (64 mya), coming from South America via Antarctica (Clements, 1968; Svartman, 2009). Thus, the piroplasmids lineages may have been isolated with their hosts in Australia, providing a most recent date of origin for the piroplasmids recorded in Australia, i.e., 64 mya.

BLAST analysis of a short 18S rRNA sequences (~750 bp) identified in *A. dubitatum* ticks collected from *R. rattus* and *H. hydrochaeris* showed 97.2–99.4% identity with a Piroplasmida previously detected in capybaras from south Brazil. Since little is known about this piroplasmid, further studies using molecular and morphological approaches are necessary to better characterize this protozoan. Considering that *A. dubitatum* is a three-host tick and *R. rattus* and *H. hydrochaeris* were captured in the same locality, it is possible that the specimen of *A. dubitatum* containing DNA of a capybara-associated piroplasmid might have previously acquired the parasite from capybaras found in that region.

Finally, since *A. dubitatum* is a tick species commonly identified in marsupials (Massini et al. 2019; Ueno et al. 2020) and capybaras (Queirogas et al. 2012) from Brazil, the role of this arthropod in the capybara and marsupial-associated piroplasmids' life cycles must be further investigated.

Conclusions

The present study showed the occurrence and characterized a putative novel piroplasmid species infecting *D. albiventris* and associated ticks in Central-Western Brazil. Accordingly, the near-complete 18S rRNA-based phylogenetic inference suggests that although the analyzed piroplasmid sequence seems to be most closely related with the Western group – cluster III, it cannot be associated with any of the recognized ten clades, supporting that it is a novel species. Phylogenetic inferences based on two additional molecular

markers, namely *hsp-70* and *cox-1*, supported the near-complete 18S rRNA gene phylogenetic inference. Furthermore, a partial 18S rRNA gene sequence detected in *A. dubitatum* ticks collected from *R. rattus* and *H. hydrochaeris* suggests that a still non-characterized piroplasmid species occurs in the largest rodent species from South America.

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Declarations

Conflict of interest The authors declare no competing interests.

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