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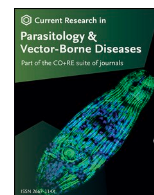

Diversity of *Ehrlichia* spp., *Anaplasma* spp. and *Neorickettsia* spp. in vampire bats

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ABSTRACT

Although bats (Mammalia: Chiroptera) act as natural reservoirs for many zoonotic pathogens around the world, few studies have investigated the occurrence of *Anaplasmataceae* agents in bats, especially vampire bats. The family *Anaplasmataceae* (order Rickettsiales) encompasses obligate intracellular bacteria of the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Neoehrlichia*, *Wolbachia*, and *Allocryptoplasma*. The present study aimed to investigate, using molecular techniques, the presence of species of *Anaplasma*, *Ehrlichia*, and *Neorickettsia* in vampire bats sampled in northern Brazil. Between 2017 and 2019, spleen samples were collected from vampire bats belonging to two species, *Desmodus rotundus* ($n = 228$) from the states of Pará ($n = 207$), Amazonas ($n = 1$), Roraima ($n = 18$) and Amapá ($n = 3$), and *Diaemus youngii* ($n = 1$) from Pará. Positivity rates of 5.2% (12/229), 3% (7/229), and 10.9% (25/229) were found in PCR assays for *Anaplasma* spp. (16S rRNA gene), *Ehrlichia* spp. (*dsb* gene) and *Neorickettsia* spp. (16S rRNA gene), respectively. The present study revealed, for the first time, the occurrence of *Anaplasma* spp. and different genotypes of *Ehrlichia* spp. in vampire bats from Brazil. While phylogenetic analyses based on the *dsb* and *ftsZ* genes of *Ehrlichia* and 16S rRNA of *Anaplasma* spp. revealed phylogenetic proximity of the genotypes detected in vampire bats with *Anaplasmataceae* agents associated with domestic ruminants, phylogenetic inferences based on the *gltA* and *groEL* genes evidenced the occurrence of genotypes apparently exclusive to bats. *Neorickettsia* sp. phylogenetically associated with *N. risticii* was also detected in vampire bats sampled in northern Brazil.

1. Introduction

The family *Anaplasmataceae* encompasses obligate intracellular alphaproteobacteria that are able to infect a wide variety of blood cell types, including erythrocytes, granulocytes, monocytes, macrophages, and platelets. The genera *Anaplasma* and *Ehrlichia* are among the most important tick-borne agents, which can infect both animals and humans (Doudier et al., 2010). These agents develop both in invertebrate hosts (ixodid ticks), which also act as vectors, and in vertebrate hosts, which consist of a wide variety of mammals and a limited number of birds (Dumler et al., 2001; Rymaszewska and Grenda, 2008; Hildebrandt et al., 2010; André, 2018). The genus *Neorickettsia*, in turn, encompasses digenetic trematode endosymbionts that can eventually cause diseases in humans and animals (Vaughan et al., 2012; Dittrich et al., 2015).

There are 181 species of bats in Brazil, which represents more than 10% of the total number of species worldwide (~1460) (Garbino et al., 2022; MDD, 2023). Chiropterans are adapted to a wide variety of environments and feeding strategies, being able to live in wild, peri-domestic or urban areas, which favors their contact with different animals and humans (Mühldorfer, 2013). Despite the importance of bats as reservoirs of pathogenic and/or zoonotic agents, studies focusing on the occurrence of *Anaplasmataceae* agents in these animals are still scarce. Further investigations are needed to fully elucidate the role of bats as hosts/reservoirs of these agents (Nunes et al., 2017).

In Poland, *Anaplasma phagocytophilum msp2* DNA was detected in 1.25% (1/80) blood samples from the insectivorous bat *Myotis myotis*

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(Szubert-Kruszyńska et al., 2019). Using the same PCR protocol, Afonso and Goydadin (2018) detected the presence of *A. phagocytophilum* DNA in the guano of the insectivorous bat *Rhinolophus hipposideros* from a maternity roost in France. Both authors emphasized the importance of these findings for public health, since *A. phagocytophilum* is a zoonotic agent and these bat species can live in peri-urban and urban environments. In Russia, Zabashta et al. (2019) reported the occurrence of *A. phagocytophilum* in brain samples from 14.28% (1/7) *Pipistrellus pipistrellus* and *Ehrlichia* sp. in brain samples from 47.36% (9/19) of *P. pipistrellus* and 16.66% (1/6) of *Pipistrellus kuhlii*. *Ehrlichia* sp., phylogenetically related to a 16S rRNA genotype detected in *Haemaphysalis* spp. ticks collected from dogs in Asia, was detected in blood samples from 16% (4/25) *Brachyphylla carvernarum* bats sampled in the Island of Saint Kitts (Reeves et al., 2016).

Bats, especially insectivorous bats, may be involved in the *Neorickettsia risticii* cycle through horizontal transmission (harboring adult trematodes that will produce eggs and contaminate the environment) by ingestion of infected intermediate hosts (arthropods or snails infected with metacercariae) (Gibson et al., 2005; Vaughan et al., 2012; Greiman et al., 2017). The bacteria can be released into the lumen of the vertebrate's gastrointestinal tract, invade and multiply in the epithelial cells of the colon, subsequently translocating to the bloodstream and infectious monocytes, mast cells, and macrophages (Lin et al., 2009). *Neorickettsia* spp. are known to affect multiple organs in their mammal hosts (Greiman et al., 2013). In bats, infections have been reported in different organs, such as spleen, liver, lungs, and intestine (Pusterla et al., 2003; Gibson et al., 2005; Cicuttin et al., 2013, 2017; Hornok et al., 2018; Ikeda et al., 2021; de Mello et al., 2023). *Neorickettsia risticii* has been reported in 8.2% (5/62) *Tadarida brasiliensis* from Argentina (Cicuttin et al., 2013, 2017), in 60% (3/5) *Myotis yumanensis* (Pusterla et al., 2003) and 44.7% (17/38) *Myotis lucifugus* and 40% (6/15) *Eptesicus fuscus* (Gibson et al., 2005) from the USA, and in 4/4 *Myotis dasycneme* from the Netherlands (Hornok et al., 2018).

In Brazil, there are few reports regarding the occurrence of *Anaplasmataceae* agents in bats. *Neorickettsia* DNA sequences, with a high degree of identity ($> 95\%$) to *N. risticii* and *N. findlayensis*, were detected in 13.6% (57/418) of the samples (34/133 blood samples, 22/135 spleen samples, and 1/19 larvae of *Ornithodoros hasei*) collected from 135 non-hematophagous bats belonging to 12 different species, using a nested (n) PCR based on the 16S rRNA gene (Ikeda et al., 2021). Additionally, these authors detected, using a PCR based on the 16S rRNA gene, *Anaplasma* spp. in 1.67% (7/418) of the samples (3 spleen samples from *Platyrrhinus lineatus*, 3 blood samples from *Phyllostomus discolor* and 1 *Ornithodoros hasei* larva). Furthermore, 11.96% (50/418) positivity for *Ehrlichia* spp. was detected in bats (*Artibeus planirostris*, *Artibeus lituratus*, *P. discolor*, *P. lineatus*, *Eptesicus furinalis*, *Carollia perspicillata* bats) and associated ectoparasites (*Megistopoda aranea*, *Trichobius costalimai*, *Strebla hertigi* and *O. hasei)*. Recently, *Neorickettsia* 16S rDNA was detected in liver samples (1.5%; 3/198) from vampire bats in Brazil (de Mello et al., 2023). The analysis of the bacterial microbiome of non-hematophagous bats and associated ectoparasites revealed the presence of *Anaplasma* spp. in oral swabs, as well as in the microbial composition of Spinturnicidae mites. In turn, *Ehrlichia* spp. was identified in the microbiome of Macronyssidae mites (André et al., 2023).

Since *Anaplasmataceae* comprises bacteria of public health significance and the real role of bats in the epidemiology of these agents remains to be elucidated, the present work aimed to detect and molecularly characterize *Anaplasmataceae* agents in spleen samples from vampire bats sampled in the Amazon Rainforest biome, northern region of Brazil.

2. Materials and methods

2.1. Sample collection

Spleen samples from vampire bats belonging to the species *Desmodus rotundus* ($n = 228$) and *Diaemus youngii* ($n = 1$) were kindly provided by the "Rabies Diagnostic Laboratory" of the Instituto Evandro Chagas, Belém, Pará. Carcasses of vampire bats were collected between 2017 and 2019, in the states of Pará (206 *D. rotundus* and 1 *D. youngii*), Roraima (18 *D. rotundus*), Amapá (3 *D. rotundus*) and Amazonas (1 *D. rotundus*) (Fig. 1).

2.2. DNA extraction

DNA was extracted from bat spleen samples (10 mg) using the BIOPUR Mini Spin Plus kit, following the manufacturer's recommendations. DNA concentration and absorbance ratio (260/280 nm) were measured by spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA).

2.3. PCR for endogenous mammalian gene

To verify the quality of the extracted DNA and avoid the presence of false negative results, spleen samples were tested by a conventional PCR (PCR) based on the mammalian *gapdh* (glyceraldehyde 3-phosphate dehydrogenase) gene (Birkenheuer et al., 2003).

2.4. Molecular screening and characterization for Ehrlichia spp.

The molecular screening for *Ehrlichia* spp. was performed using a nested PCR (nPCR) based on the *dsb* gene (~370 bp) (Almeida et al., 2013). Positive samples were subjected to the following PCR or nested PCR protocols for further molecular characterization: nearly-complete 16S rRNA (~1470 bp) (Greiman et al., 2014), *groEL* (~680 bp) and *gltA* (~650 bp) (Müller et al., 2018), *sodB* (~600 bp) (Victoria et al., 2015), ITS 23S-5S (~300 bp) (Rejmanek et al., 2012), *omp-1* (~700 bp) (Inayoshi et al., 2004), *ftsZ* (~462 bp) and *rpoB* (~581 bp) (Su et al., 2021). DNA from the Jaboticabal strain of *Ehrlichia canis* (maintained in DH82 cell culture) was used as a positive control. Sterile ultrapure water (Invitrogen®, Carlsbad, California, USA) was used as a negative control in the PCR assays.

2.5. Molecular screening and characterization for Anaplasma spp.

Molecular screening for *Anaplasma* spp. was performed using a nPCR based on the short 16S rRNA gene (~546 bp) (Massung et al., 1998). Samples positive in the screening PCR assays were subjected to PCR protocols for additional molecular characterization based on the following molecular markers: large 16S rRNA (~1406 bp) (Oh et al., 2014), *gltA* (~650 bp) (Müller et al., 2018), *groEL* (~340 bp) (Zhang et al., 2016), ITS 23S-5S intergenic region (~300 bp) (Rejmanek et al., 2012) and *msp1β* (Carelli et al., 2007), to confirm the identity obtained by BLASTn analysis. gBlocks® (Integrated DNA Technologies, Coralville, Iowa, USA) containing a fragment of the 16S rRNA gene from *Anaplasma phagocytophilum* as an insert and DNA samples from wild boars positive for *Anaplasma* spp. (Santana et al., 2022) were used as positive controls. Sterile ultrapure water (Invitrogen®, Carlsbad, California, USA) was used as a negative control in the PCR assays.

In order to obtain a large sequence of the 16S rRNA gene of *Anaplasma* sp., the positive samples in the protocol that amplify a ~1406 bp sequence (Oh et al., 2014) were subjected to pGEM-T Easy (Promega, Madison, WI, USA) cloning, according to the manufacturer's recommendations. Clones were selected from each positive sample according to the blue/white colony system. The colonies that had the gene fragment of interest confirmed by PCR were subjected to plasmid DNA extraction using the Wizard® Plus SV Minipreps DNA Purification Systems (Promega, Madison, Wisconsin, USA). Subsequently, plasmid DNA was submitted to sequencing with primers M13 F (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) and M13 R (5′-GTC ATA GCT GTT TCC TGT GTG A-3′) (Lau et al., 2010) that flank the multiple cloning sites of the pGEM-T Easy plasmid to include the target gene fragments. Sequencing

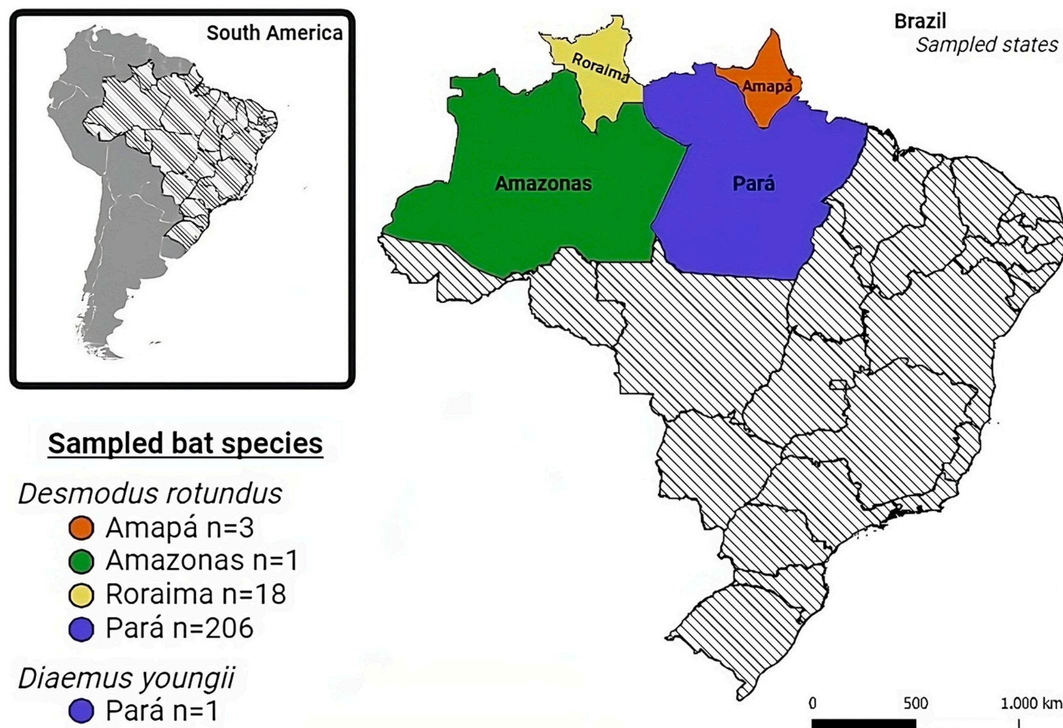

Fig. 1. Map showing the Brazilian states where vampire bats have been collected.

was carried out as described below.

2.6. Molecular screening and characterization for Neorickettsia spp.

Molecular screening for *Neorickettsia* spp. was performed using a nPCR based on the 16S rRNA gene (~527 bp) (Chae et al., 2003). Positive samples were subjected to an nPCR assay based on the *groEL* (~526 bp) and *p51* (~569 bp) (Barlough et al., 1998) genes. gBlocks® (Integrated DNA Technologies, Coralville, Iowa, USA) containing fragments of the 16S rRNA, *groEL* and *p51* genes from *N. risticii* were used as positive controls. Sterile ultrapure water (Invitrogen®, Carlsbad, California, USA) was used as a negative control in the PCR assays.

2.7. Sequencing and phylogenetic analyses

Amplicon sequencing was performed by the Sanger method (Sanger et al., 1977) using the ABI PRISM 3730 DNA Analyzer (Applied Biosystems) at the Human Genome and Stem Cell Research Center, Institute of Biosciences, University of São Paulo (USP), São Paulo, SP, Brazil. The electropherograms were subjected to a quality screening test using the Phred-Phrap software (v. 23) (Ewing and Green, 1998) in order to obtain consensus sequences. The BLASTn tool (Altschul et al., 1990) was used to compare the sequences obtained with those previously deposited in the GenBank database (Benson et al., 2013).

For phylogenetic inferences, sequences from the present study were aligned with sequences retrieved from GenBank using MAFFT v. 7 software (Katoh et al., 2019). The best evolutionary model was chosen using jModelTest2 software (Darriba et al., 2012) through the CIPRES portal (Miller et al., 2010). Maximum Likelihood (ML) phylogenetic analysis was inferred on the IQ-TREE online server (Trifinopoulus et al., 2016) using 10,000 replicates of UFBoot2 – Ultrafast Bootstrap (Hoang et al., 2018). Editing of the phylogenetic trees as well as outgroup rooting was performed using the software Treegraph 2.0.56–381 beta (Stöver and Müller, 2010).

3. Results

3.1. DNA quality

The average DNA concentration and absorbance ratios (260/280 nm and 260/230 nm) of the DNA samples extracted from the spleen of vampire bats were 81.3 ng/µl (SD ± 199.5), 1.94 nm and 2.25 nm (SD ± 3.87 and ±2.50), respectively. All DNA samples obtained from bat spleens ($n = 229$) were positive in PCR based on the endogenous mammalian gene (*gapdh*).

3.2. Ehrlichia spp.

Out of the 229 spleen samples, 7 (3.05%; 7/229) were positive in the screening nPCR for the *dsb* gene for *Ehrlichia* spp. [100% (7/7) samples from *D. rotundus* from Pará]. Out of these positive samples, four readable sequences were obtained. Regarding the other PCR assays, one sample (3.12%; 1/32) was positive for the *groEL* gene, one (3.12%; 1/32) for the *gltA* gene, two (6.25%; 2/32) for the *sodB* gene, two (6.25%; 2/32) for the 23S-5S ITS, two (6.25%; 2/32) for *ftsZ*, and none for the nearly-complete 16S rRNA, *omp-1* and *rpoB* genes. The number of sequences obtained, as well as the results of the BLASTn analysis, including sequence size, query coverage, E-value, percentage of identity and GenBank accession numbers are shown in Table 1.

Phylogenetic analysis based on the *dsb* gene (650 bp alignment), inferred by the ML method and TVM+G evolutionary model, grouped the four sequences obtained from *D. rotundus* in the same clade of *Ehrlichia* sp. sequences previously detected in cattle and *Rhipicephalus microplus* ticks from Brazil, *E. minasensis* sequences detected in cattle from Australia, *Rhipicephalus* sp. ticks from Pakistan, and in *R. microplus* and sloths (*Bradypus variegatus*) from Brazil (Fig. 2).

In the phylogenetic analysis based on the *ftsZ* gene (530 bp alignment), inferred by the ML method and GTR+I+G evolutionary model, the sequence obtained from bat #111 was positioned in a sister group, supported by a bootstrap value of 96%, with sequences from *Ehrlichia* sp. previously detected in *Haemaphysalis longicornis* ticks from Japan

Table 1
BLASTn results of *Ehrlichia* spp., *Anaplasma* spp. and *Neorickettsia* spp.sequences obtained from spleen samples from *Desmodus rotundus* in northern Brazil.

Sample ID (Location: GenBank ID)	Sequence size (bp)	Target gene	BLASTn result	Host	Query coverage (%)	E-value	Identity (%)	GenBank ID (Country)
Sample #172 (Pará: PP129555)	460	*groEL*	*Ehrlichia ewingii*	*Homo sapiens*	98	3e-179	92.11	AF195273 (USA)
Sample #172 (Pará: PP129554)	582	*gltA*	*Ehrlichia* sp.	*Phyllostomus discolor*	98	0.0	88.75	OK338001 (Brazil)
Sample #111 (Pará: PP159556)	334	*ftsZ*	*Ehrlichia* sp.	*Haemaphysalis longicornis*	96	4e-103	88.47	MT268180 (Japan)
Sample #8 (Pará: PP155038)	311	*dsb*	*Ehrlichia minasensis*	*Bos taurus*	100	1e-157	99.68	MH500007 (Australia)
Sample #44 (Pará: PP155039)	311	*dsb*	*Ehrlichia minasensis*	*Bos taurus*	100	2e-154	99.04	MH500007 (Australia)
Sample #107 (Pará: PP155040)	311	*dsb*	*Ehrlichia minasensis*	*Bos taurus*	100	1e-157	99.68	MH500007 (Australia)
Sample #223 (Pará: PP155041)	311	*dsb*	*Ehrlichia minasensis*	*Bos taurus*	100	1e-157	99.68	MH500007 (Australia)
Sample #73 (Pará: PP109409)	1164	16S rRNA	*Anaplasma marginale*	Cattle	99	0.0	99.91	CP023731 (Brazil)
Sample #137 (Roraima: PP124931)	503	16S rRNA	*Neorickettsia* sp.	*Equus caballus*	100	0.0	99.80	OR352967 (Brazil)
Sample #207 (Pará: PP124932)	501	16S rRNA	*Neorickettsia* sp.	*Equus caballus*	100	0.0	100	OR352967 (Brazil)

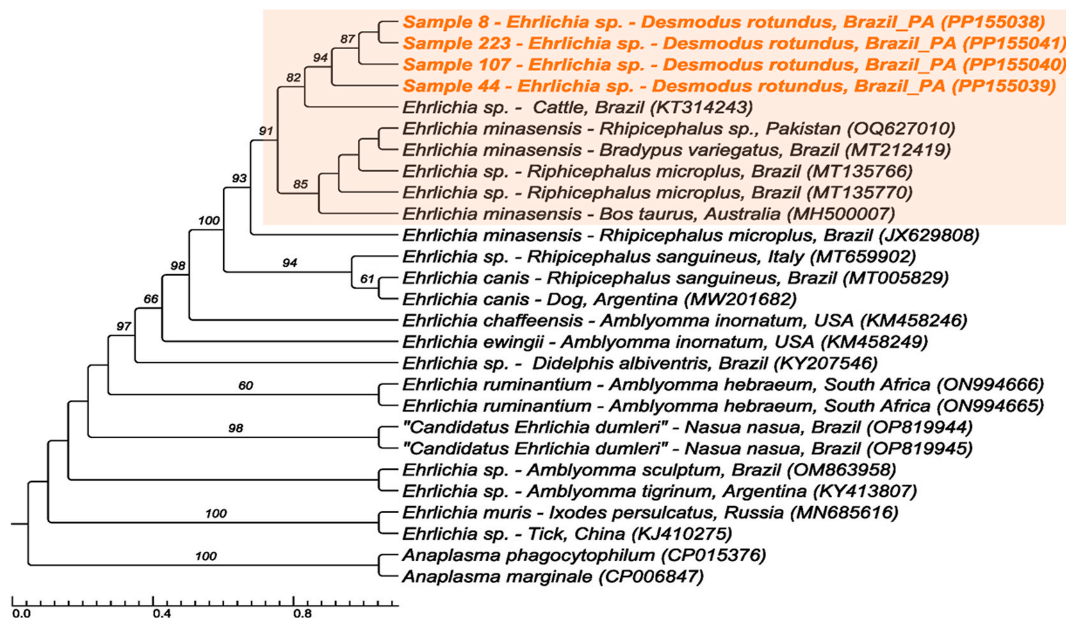

Fig. 2. Phylogenetic tree based on an alignment of *dsb* gene sequences (~650 bp) for *Ehrlichia* spp. using the Maximum Likelihood (ML) method and TVM+G as the evolutionary model. Sequences detected in the present study are highlighted in orange. *Anaplasma phagocytophilum* (CP015376) and *Anaplasma marginale* (CP006847) were used as outgroups.

(Fig. 3).

Readable sequences were obtained from sample #172 for the *gltA* and *groEL* genes. Both phylogenies were constructed using the Maximum Likelihood (ML) method. The evolutionary model GTR+I+G was used for the inference of the *gltA* alignment (~590 bp), while TnR+I+G was the evolutionary model used in the phylogenetic inference of the *groEL* gene alignment (~460 bp). In the *gltA* phylogenetic tree, sequence #172 was positioned as a sister group to *Ehrlichia* sp. previously detected in *Phyllostomus discolor* from Brazil (OK338001), supported by a bootstrap value of 100% (Fig. 4).

On the other hand, the phylogenetic tree based on the *groEL* gene positioned sequence #172 in a unique clade, sister to a clade containing *Ehrlichia* sp. sequences previously detected in *Amblyomma* ticks from Argentina (KY425416) and Japan (LC565631), "*Candidatus* Ehrlichia dumleri" detected in coatis from Brazil (OP819939), "*Candidatus* Ehrlichia occidentalis" detected in *Amblyomma triguttatum* ticks from Australia, and *Ehrlichia ruminantium* previously detected in cattle from Africa (GQ457107) (Fig. 5).

3.3. Anaplasma spp.

Twelve spleen DNA samples from vampire bats were positive in the PCR assay for *Anaplasma* spp.: 5.2% (12/229) based on the 16S rRNA gene (Massung et al., 1998). Two samples (16.7%; 2/12) were positive in the PCR based on a large fragment of the 16S rRNA gene and two in the PCR based on the ITS region (16.7%; 2/12). A readable sequence (1164 bp) was obtained after cloning the large fragment of the 16S rRNA gene based on the protocol established by Oh et al. (2014). The sequence was obtained from a spleen sample of *D. rotundus* from Pará and showed 99.91% identity (100% query coverage and E-value = 0) with a sequence of *Anaplasma marginale* previously detected in cattle from Brazil (CP023731) (Table 1). No samples were positive in PCR assays

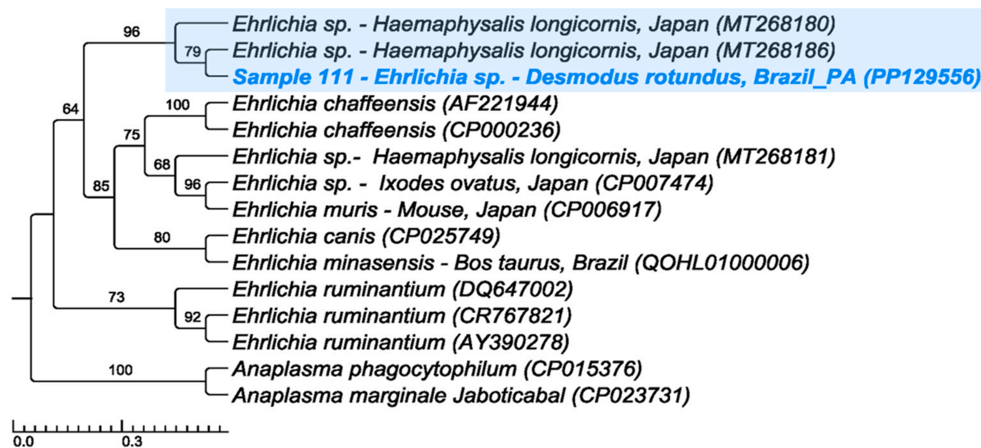

Fig. 3. Phylogenetic tree based on an alignment of *ftsZ* gene sequences (530 bp) for *Ehrlichia* spp. using the Maximum Likelihood (ML) method and GTR+I+G as the evolutionary model. Sequences detected in the present study are highlighted in blue. *Anaplasma phagocytophilum* (CP015376) and *Anaplasma marginale* (CP023731) were used as outgroups.

Fig. 4. Phylogenetic tree based on an alignment of *gltA* gene sequences (590 bp) for *Ehrlichia* spp. using the Maximum Likelihood (ML) method and GTR+I+G as the evolutionary model. Sequences detected in the present study are highlighted in purple. *Anaplasma phagocytophilum* (CP015376) and *Anaplasma marginale* (CP006847) were used as outgroups.

targeting the short 16S rRNA (Parola et al., 2000), *gltA* and *groEL* genes.

Phylogenetic analysis of the 16S rRNA gene of *Anaplasma* spp. (1400 bp alignment), inferred by the ML and GTR+G evolutionary model positioned sequence #73 in a sister clade containing *Anaplasma marginale* sequences detected in cattle from Brazil (CP023731) and South Africa (AF414873) (Fig. 6). The presence of *A. marginale* DNA in a spleen sample from *D. rotundus* was confirmed by a qPCR based on the *msp1β* gene.

3.4. Neorickettsia spp.

For *Neorickettsia* spp., 10.9% (25/229) spleen DNA samples [96% (24/25) samples from *D. rotundus* from Pará (20/24), Amapá (1/24) and Roraima (2/24) and 4% (1/25) from *D. youngii* from Pará] were positive in the nPCR screening based on the 16S rRNA gene. The three 16S rRNA gene sequences obtained revealed > 87.33% identity with *N. risticii* sequences previously detected in horses from Brazil. Out of those, 24% (6/25) were positive in the nPCR based on the *groEL* gene; however, no readable sequence was obtained. The sequences obtained for the 16S rRNA gene and the results of BLAST analyses, including sequence length, query coverage, E-value, percentage of identity and GenBank accession numbers are shown in Table 1.

Phylogenetic analysis of the 16S rRNA gene of *Neorickettsia* spp. (alignment of 720 bp) inferred by ML method using TIM2+I+G evolutionary model positioned the obtained sequences closely to *N. risticii* sequences previously detected in *Myotis lucifugus* bats from the USA (AY388503) and *Tadarida brasiliensis* bats from Argentina (KX001784) and with *Neorickettsia* sp. previously detected in *D. rotundus* and *D. ecaudata* bats (OQ366521, OQ366522), horses (MK760560) and coatis (OP980545) from Brazil (Fig. 7).

4. Discussion

The present study reports the occurrence of *Anaplasmataceae* agents

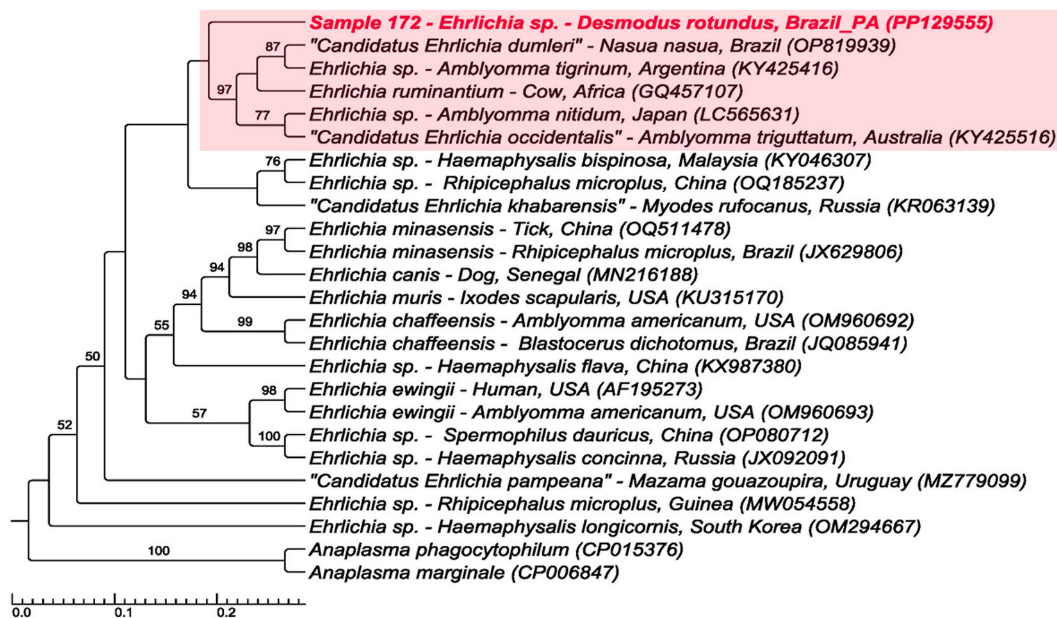

Fig. 5. Phylogenetic tree based on an alignment of *groEL* gene sequences (460 bp) for *Ehrlichia* spp. using the Maximum Likelihood (ML) method and TnR+I+G as the evolutionary model. Sequences detected in the present study are highlighted in red. *Anaplasma phagocytophilum* (CP015376) and *Anaplasma marginale* (CP006847) were used as outgroups.

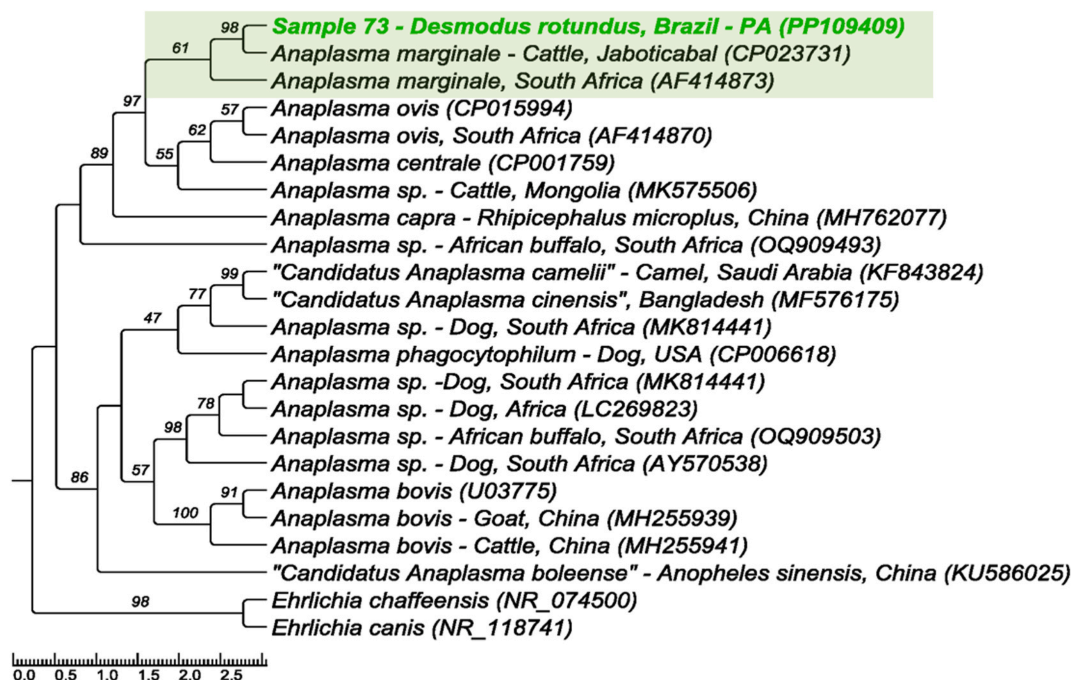

Fig. 6. Phylogenetic tree based on an alignment of 16S rRNA gene sequences (1400 bp) for *Anaplasma* spp. using the Maximum Likelihood (ML) method and GTR+G as the evolutionary model. Sequences detected in the present study are highlighted in green. *Ehrlichia chaffeensis* (NR_074500) and *Ehrlichia canis* (NR_118741) were used as outgroups.

in spleen samples from vampire bats from Brazil. Few studies have previously shown the occurrence of *Ehrlichia* sp. in non-hematophagous bats and associated ectoparasites. The occurrence of *Ehrlichia* spp. detected in the present study (3%; 7/229) was lower than that detected in blood samples (16%; 4/25) collected from *Brachyphylla caverum* bats in St. Kitts and Nevis (Reeves et al., 2016). The 16S rRNA sequences obtained revealed 95% identity with *Ehrlichia* sp. sequences previously detected in ticks collected from dogs in Asia (Reeves et al., 2016). In Russia, Zabashta et al. (2019) reported the occurrence of *Ehrlichia* spp. in 47.4% (9/19) of *P. pipistrellus* brain samples and 16.66% (1/6) of *P. kuhlii*, with a higher positivity rate when compared to that found in the present study. Bat-associated ectoparasites, such as *Argas vespertilionis*, which are ticks commonly associated with bats and their habitats, were found to be positive (4.2%; 5/120) for *Ehrlichia/Anaplasma* when collected from *P. pipistrellus* bats in England. The three 16S rRNA sequences obtained were grouped into a clade together with *Ehrlichia* spp. and *E. canis* (Lv et al., 2018). In the Ukraine, two specimens of *Carios vespetilionis* (4.7%; 2/43) collected from *Pipistrellus*

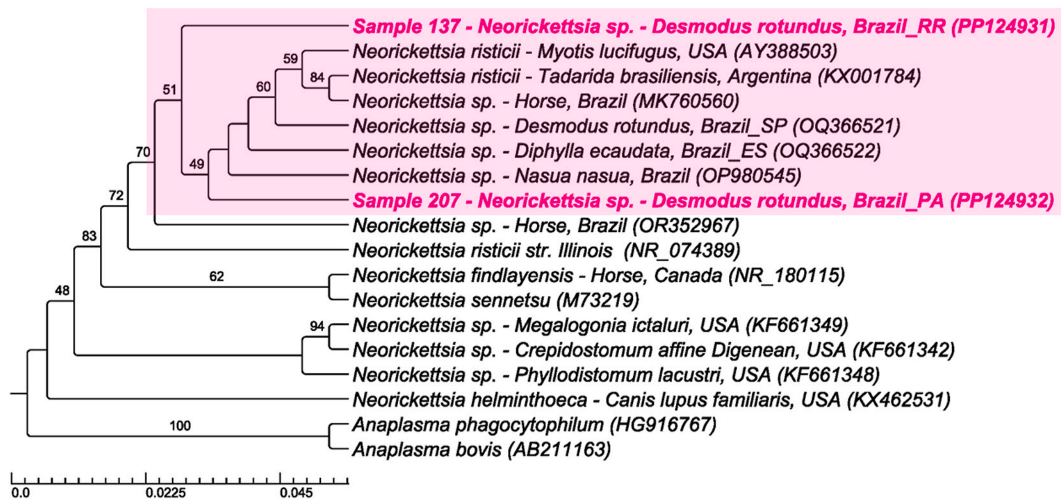

Fig. 7. Phylogenetic tree based on an alignment of 16S rRNA gene sequences (720 bp) for *Neorickettsia* spp. using the Maximum Likelihood (ML) method and TVM+G as the evolutionary model. Sequences detected in the present study are highlighted in pink. *Anaplasma phagocytophilum* (HG916767) and *Anaplasma bovis* (AB211163) were used as outgroups.

pygmaeus and *P. kuhlii* were positive for *Ehrlichia/Anaplasma*. The 16S rRNA sequence obtained was positioned in a clade together with "*Candidatus* Ehrlichia shimanensis" and *Ehrlichia* sp., close to *E. minasensis* (Vlaschenko et al., 2022).

In Brazil, Ikeda et al. (2021) reported an occurrence of *Ehrlichia* sp. of 11.96% (50/418) in blood samples (20%), spleen (16%) and associated ectoparasites 14% bat flies (*Megistopoda aranea* (n = 3), *Trichobius costalimai* (n = 2) and *Strebla hertigi* (n = 2)), three (6%) tick larvae (*O. hasei*), six (12%) Spinturnicidae mites (*Periglischrus* sp., *Periglischrus torrealbai* and *Periglischrus acutisternus*), and 19 (38%) Macronyssidae mites (*Steatonyssuss* sp.). Phylogenetic analysis based on the *gltA* gene positioned the sequence obtained from a blood sample of *P. discolor* in a single branch, close to *E. ruminantium*. In the present study, phylogenetic analysis based on the same gene, demonstrated that the sequence obtained from *D. rotundus* (sample #172) was closely related to a sequence of *Ehrlichia* sp. detected in *P. discolor* bats from the Brazilian midwest (Ikeda et al., 2021), supported by a sister clade containing a sequence identified in *H. bispinosa* from Malaysia. This finding may indicate the occurrence of genotypes of *Ehrlichia* spp. which are specific to bats in Brazil.

In the present study, sequences of the *dsb* gene from *Ehrlichia* sp., obtained from spleen samples of *D. rotundus*, were shown to be closely related to *E. minasensis*, the causative agent of a cattle disease, possibly transmitted by *R. microplus* (Aguiar et al., 2014, 2019; Cabezas-Cruz et al., 2016). The recent detection of *Ehrlichia dsb* gene sequences related to *E. minasensis* in Xenarthra mammals from Brazil (Calchi et al., 2020) and non-hematophagous bats (Ikeda et al., 2021) suggest that this species of *Ehrlichia* can infect a wider range of hosts than previously suggested. Although *R. microplus* mainly parasitizes cattle, larvae of this tick species were found parasitizing *Molossus rufus* bats in Brazil (da Silva et al., 2023). Up to now, there are no reports of *R. microplus* parasitizing vampire bats. It is known that *D. rotundus* preferentially feeds on cattle due to availability and ease of access when compared to other animals (Mayen, 2003). Future studies are needed to investigate the possible transmission of *E. minasensis* or a closely related genotype through hematophagy.

The sequence obtained from sample #111 of *D. rotundus* for the *ftsZ* gene showed to be closely related to *Ehrlichia* sp. sequences previously detected in *H. longicornis* ticks from Japan. This tick species can feed on a wide variety of domestic and wild hosts. In China, cattle are the main hosts of *H. longicornis* (Zhao et al., 2020). The phylogenetic proximity observed between *Ehrlichia* sp. sequences detected in cattle, their associated ectoparasites and vampire bats suggests that the *Ehrlichia* genotypes circulating in bats could be related to their feeding habits and might have been acquired from their prey during blood-feeding.

On the other hand, the phylogenic inference based on the *groEL* gene positioned the sequence obtained from sample #172 of *D. rotundus* in a unique clade, sister to a clade containing *Ehrlichia* sp. sequences detected in *Amblyomma* spp. ticks from Australia, Japan and Argentina, "*Ca.* Ehrlichia dumleri" detected in coatis from Brazil and *E. ruminantium* previously detected in cattle from Africa. The present study demonstrates, for the first time, by phylogenetic inferences based on different molecular markers that bats can harbor a diversity of *Ehrlichia* spp. genotypes.

The low occurrence of *Anaplasma* sp. observed in spleen samples analyzed in the present study (5.24%; 12/229) was lower than that observed by Afonso and Goydadin (2018), who reported an occurrence of 22.7% (63/278) for *A. phagocytophilum*, using a PCR based on the *msp2* gene, in fecal samples from insectivorous bats (*Rhinolophus hipposideros*) sampled in France. On the other hand, other studies revealed low positivity for *Anaplasma* spp. in bats or associated ectoparasites. In Poland, 1.7% (1/59) of *Myotis myotis* blood samples were positive for *A. phagocytophilum*, using a PCR based on the *msp2* gene (Szubert-Kruszyńska et al., 2019). Using PCR/qPCR assays based on the *msp2* gene, an occurrence of 4.3% (6/138) for *A. phagocytophilum* was detected among *I. simplex* ticks collected from bats in Hungary and Romania (Hornok et al., 2019) and 14.3% (1/7) in *Nyctalus noctula* bats from Russia (Zabashta et al., 2019). In midwestern Brazil, *Anaplasma* spp. DNA was detected, using nPCR based on the 16S rRNA gene, in spleen samples (42.8%; 3/7) of *Platyrrhinus lineatus*, blood samples (42.8%; 3/7) of *Phyllostomus discolor*, and larvae (14.3%; 1/7) of the tick *Ornithodoros hasei*, in Brazil. The sequence obtained from *O. hasei* showed 97.6% identity with *A. phagocytophilum* detected in a deer (*Hydropotes inermis*) from the Republic of Korea and 97.4% identity with *A. phagocytophilum* and *A. bovis* sequences detected in *H. longicornis* from South Korea (Ikeda et al., 2021). Recently, sequences from *Anaplasma* spp. were detected in the microbiome of oral swabs from non-hematophagous bats in central-western Brazil (André et al., 2023).

The phylogenetic positioning of the 16S rRNA gene sequence of *Anaplasma* sp. revealed close proximity to *Anaplasma marginale* sequences, forming a sister clade to *Anaplasma ovis*, *A. centrale*, and *Anaplasma* spp. sequences. The presence of *A. marginale* DNA in a spleen sample from *D. rotundus* was confirmed by a qPCR based on the *msp1β* gene. In the Ukraine, a 16S rRNA sequence from *Anaplasma* sp. obtained from *Carios vespertilionis* tick collected from *Pipistrellus* spp. was positioned in a clade with sequences from *Anaplasma* spp. and *Anaplasma*

ovis, previously detected in domestic ruminant-associated ticks (Vlaschenko et al., 2022), corroborating our findings. Considering that *R. microplus*, the biological vector of *A. marginale* among cattle in Brazil, has never been reported parasitizing vampire bats, the specimen of *D. rotundus* sampled herein might have acquired this *Anaplasmataceae* agent through its feeding habits.

Multilayer network analyses carried out to evaluate the interactions between vectors, bats and pathogens in Brazil, revealed interactions between *Anaplasma* sp., *P. discolor* and *Ornithodoros hasei*, since such a tick species was found parasitizing *P. discolor* and both were placed in the same module in the analyses performed. However, the low occurrence of *Anaplasma* sp. identified among the bats evaluated hampered further discussions regarding the transmission of these agents (Alcantara et al., 2022). For *Ehrlichia* sp., interaction networks revealed that blood-sucking arthropods might represent an important route of transmission between bats, as previously suggested in other studies (Dumler et al., 2015; Saito and Walker, 2016). *Steatonyssus* spp. mites (Macronyssidae) were related to *Ehrlichia* sp. in the interaction network with non-hematophagous bats from midwestern Brazil (Alcantara et al., 2022). Recently, *Anaplasma* spp. and *Ehrlichia* spp. sequences were detected in the microbiome of Spinturnicidae and Macronyssidae mites, respectively, collected from non-hematophagous bats in Brazil (André et al., 2023). Additional studies should be carried out to better understand the interactions between vectors-vampire bats-*Anaplasma/Ehrlichia*, given the proximity of bats to humans, the occurrence of these agents in domestic animals (Rar and Golovljova, 2011; Ismail and McBride, 2017; Silaghi et al., 2017; Rar et al., 2021) and the detection of new genotypes of these agents in wild animals in Brazil (Calchi et al., 2020; Vieira et al., 2022; Perles et al., 2022).

The occurrence of *Neorickettsia* sp. reported in the present study (10.9%; 25/229) was lower compared to those detected in *Myotis yumanensis* (60%; 3/5) (Pusterla et al., 2003), *Myotis lucifugus* 44.7% (17/38) and *Eptesicus fuscus* (40%; 6/15) (Gibson et al., 2005) from the USA, and *Myotis dasycneme* (100%; 4/4) from the Netherlands (Hornok et al., 2018). On the other hand, Ikeda et al. (2021) detected the presence of *Neorickettsia* sp. in blood (59.6%; 34/57) and spleen (38.6%; 22/57) samples collected from 12 species of non-hematophagous bats in midwestern Brazil. Conversely, the occurrence reported in the present study was higher than that detected in *Tadarida brasiliensis* bats in Argentina (8.2%; 5/62) (Cicuttin et al., 2013, 2017) and in vampire bats sampled in different Brazilian states (1.5%; 3/198) (de Mello et al., 2023).

Phylogenetic analysis based on the 16S rRNA gene from sequences #137 and #207 revealed close proximity to sequences from *N. risticii* detected in bats from the USA and Argentina, as well as sequences from *Neorickettsia* sp. previously detected in *D. rotundus* and *D. ecaudata* bats, horses and coatis (*Nasua nasua*) from Brazil. The detection of *N. risticii* in horse blood samples collected in the State of Rio de Janeiro (Paulino et al., 2020), as well as in *Heleobia* spp. collected in the State of Rio Grande do Sul (Coimbra et al., 2006), a region where an outbreak of equine monocytic neorickettsiosis had previously been reported (Dutra et al., 2001), reveals the presence of these agents in different regions of Brazil. Recently, *Neorickettsia* sp. was also detected in coatis in midwestern Brazil (Perles et al., 2023). The recent detections of *Neorickettsia* sp. in non-hematophagous bats (Ikeda et al., 2021) and hematophagous bats (de Mello et al., 2023) highlight the circulation of this agent among bats in Brazil. The detection of *Neorickettsia* spp. infecting digenetic trematodes found to parasitize bats (Greiman et al., 2017) emphasizes the contribution of these parasites in the maintenance of *Neorickettsia* spp. Additional studies must be carried out to better understand which bat-associated trematodes are involved in the epidemiology of *Neorickettsia* spp. among vampire bats in Brazil. Considering that *D. rotundus* can also feed on horses, the acquisition of this pathogen by blood-feeding cannot be ruled out. Furthermore, future studies aiming to isolate and analyze the complete genome of this agent are necessary to investigate the real molecular identity of *Neorickettsia* spp. in Brazil.

5. Conclusion

The present work revealed, by molecular methods, for the first time, *Anaplasma* sp. and different *Ehrlichia* genotypes in vampire bats from Brazil. While phylogenetic analyses based on the *dsb* and *ftsZ* genes of *Ehrlichia* and 16S rRNA of *Anaplasma* spp. revealed phylogenetic proximity of the genotypes detected in vampire bats with *Anaplasmataceae* agents associated with domestic ruminants, phylogenetic inferences based on the *gltA* and *groEL* genes evidenced the occurrence of genotypes apparently exclusive to bats. *Neorickettsia* sp. phylogenetically associated with *N. risticii* occurs in vampire bats sampled from northern Brazil.

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Ethical approval

All experimental procedures were approved by the “National Council for Animal Experimentation Control (CONCEA), Animal Use Committee - CEUA-FCAV/UNESP” and SISGEN under protocol numbers 015782/19 and A434E64, respectively.

CRediT authorship contribution statement

Victória Valente Califre de Mello: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Laryssa Borges de Oliveira:** Investigation, Methodology, Writing – review & editing. **Taciana Fernandes Souza Barbosa Coelho:** Investigation, Writing – review & editing. **Daniel Antonio Braga Lee:** Methodology, Writing – review & editing. **Lorena Freitas das Neves:** Methodology, Writing – review & editing. **Eliz Oliveira Franco:** Methodology, Writing – review & editing. **Anna Claudia Baumel Mongruel:** Methodology, Writing – review & editing. **Rosangela Zacarias Machado:** Investigation, Writing – review & editing. **Marcos Rogério André:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this article.

Data availability

The data supporting the conclusions of this article are included within the article. The newly generated sequences were submitted to the GenBank database under the accession numbers PP155038-PP155041, PP129554-PP129556, PP109409, PP124931 and PP124932.

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