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HOST MICROBE INTERACTIONS

Genetic Diversity of *Bartonella* spp. in Wild Mammals and Ectoparasites in Brazilian Pantanal

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Abstract

The present work aimed to investigate the genetic diversity of *Bartonella* in mammals and ectoparasites in Pantanal wetland, Brazil. For this purpose, 31 *Nasua nasua*, 78 *Cerdocyon thous*, 7 *Leopardus pardalis*, 110 wild rodents, 30 marsupials, and 42 dogs were sampled. DNA samples were submitted to a quantitative real-time PCR assay (qPCR). Positive samples in qPCR were submitted to conventional PCR assays targeting other five protein-coding genes. Thirty-five wild rodents and three *Polygenis (P.) bohlsi bohlsi* flea pools showed positive results in qPCR for *Bartonella* spp. Thirty-seven out of 38 positive samples in qPCR were also positive in cPCR assays based on *ftsZ* gene, nine in *nuoG*-cPCR, and six in *gltA*-cPCR. Concatenated phylogenetic analyses showed that two main genotypes circulate in rodents and ectoparasites in the studied region. While one of them was closely related to *Bartonella* spp. previously detected in Cricetidae rodents from North America and Brazil, the other one was related to *Bartonella alsatica*, *Bartonella pachyuromydis*, *Bartonella birtlesii*, *Bartonella acomydis*, *Bartonella silvatica*, and *Bartonella callosciuri*. These results showed that at least two *Bartonella* genotypes circulate among wild rodents. Additionally, the present study suggests that *Polygenis (P.) bohlsi bohlsi* fleas could act as possible *Bartonella* vectors among rodents in Pantanal wetland, Brazil.

Keywords Bartonellaceae · Wild carnivores · Fleas · qPCR · Wild rodents · Brazilian wetland

Introduction

Bartonella species comprise facultative, fastidious, gram-negative intracellular bacteria belonging to the alpha-2 class of Proteobacteria [1]. The transmission of these agents occurs mainly through blood-sucking arthropod vectors. Seventeen *Bartonella* species are known to be etiological agents of several diseases in animals and humans [2].

Rodents are considered as natural reservoirs for several *Bartonella* species. The association between rodents and *Bartonella* shows great importance, since these animals present persistent and subclinical bacteremia for long periods of time. Infections by a wide variety of *Bartonella* species have been reported in many different rodent species worldwide [3].

In Brazil, *Bartonella* spp. have been detected in synanthropic rodents in Salvador city, state of Bahia (Atlantic forest biome) [4] and in wild rodents from peri-urban areas from Mato Grosso do Sul and Pernambuco states [5, 6]. Recently, different *Bartonella* genotypes have been detected in wild and synanthropic rodents from different Brazilian biomes [7]. In addition to this, *Bartonella* spp. have been detected among cats [8], dogs [9], and wild carnivores [10, 11] in Brazil.

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Due to the lack of information about the epidemiology and transmission routes of *Bartonella* spp. among wildlife in Brazil, the present study aimed to investigate the occurrence of *Bartonella* in wild mammals, domestic dogs, and ectoparasites in Brazilian Pantanal wetland.

Materials and Methods

Study Area

The fieldwork was conducted at the Nhumirim ranch (56° 39′ W, 18° 59′ S), located in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul, central-western Brazil (Fig. 1). This region is characterized by a mosaic of semideciduous forest, arboreal savannas, seasonally flooded fields covered by grasslands with dispersed shrubs, and several temporary and permanent ponds. The Pantanal is the largest Neotropical floodplain, and it is well known for a rich biodiversity. Two well-defined seasons are recognized: a rainy summer (October to March) and a dry winter (April to September) [12–14].

Biological Sampling

Between the years of 2013 and 2015, four field expeditions were performed in August 2013, October 2013, August 2014, and March 2015. Free-ranging *Cerdocyon thous* (crab-eating fox), *Nasua nasua* (coati), and *Leopardus pardalis* (ocelot) were caught using a Zootech® (Curitiba, PR, Brazil) model wire box live trap (1 × 0.40 × 0.50 m), which was made with galvanized wire mesh and baited with a piece of bacon every afternoon. Twenty traps were placed on the ground at 2-km intervals, left open during 24 h and checked twice a day for 12 days. The animals were immobilized with an intramuscular injection of zolazepan and tiletamine at dosages of 8 mg/kg for ocelots and 10 mg/kg crab-eating foxes and coatis. Blood samples were collected by puncture of the cephalic vein stored in Vacutainer® containing EDTA and stored at − 20 °C until DNA extraction.

Small mammals (rodents and marsupials) were captured using live traps (Sherman®, H. B. Sherman Traps, Tallahassee, FL, USA, and Tomahawk® Tomahawk Live Traps, Tomahawk, WI, USA) baited with a mixture of banana, peanut butter, oat, and sardines. Traps were set up for seven consecutive nights along linear transects, placed on the ground at 10-m intervals and alternating between trap type in two field expeditions (August 2014 and March 2015). The total capture effort was 200 traps-nights, equally distributed in two expeditions (August 2014 and March 2015). The rodents and marsupials had to be euthanized in order to perform the identification of the animal's species, based on external and cranial morphological characters and karyological analyses. The animals were firstly anesthetized with an intramuscular injection of ketamine (10–30 mg/kg) with acepromazine (5–10 mg/kg) for rodents (proportion 9:1), or xylazine (2 mg/kg) for marsupials (1:1). After anesthesia, the animals were euthanized with potassium chloride, which doses ranged from 75 to 150 mg/kg. Spleen

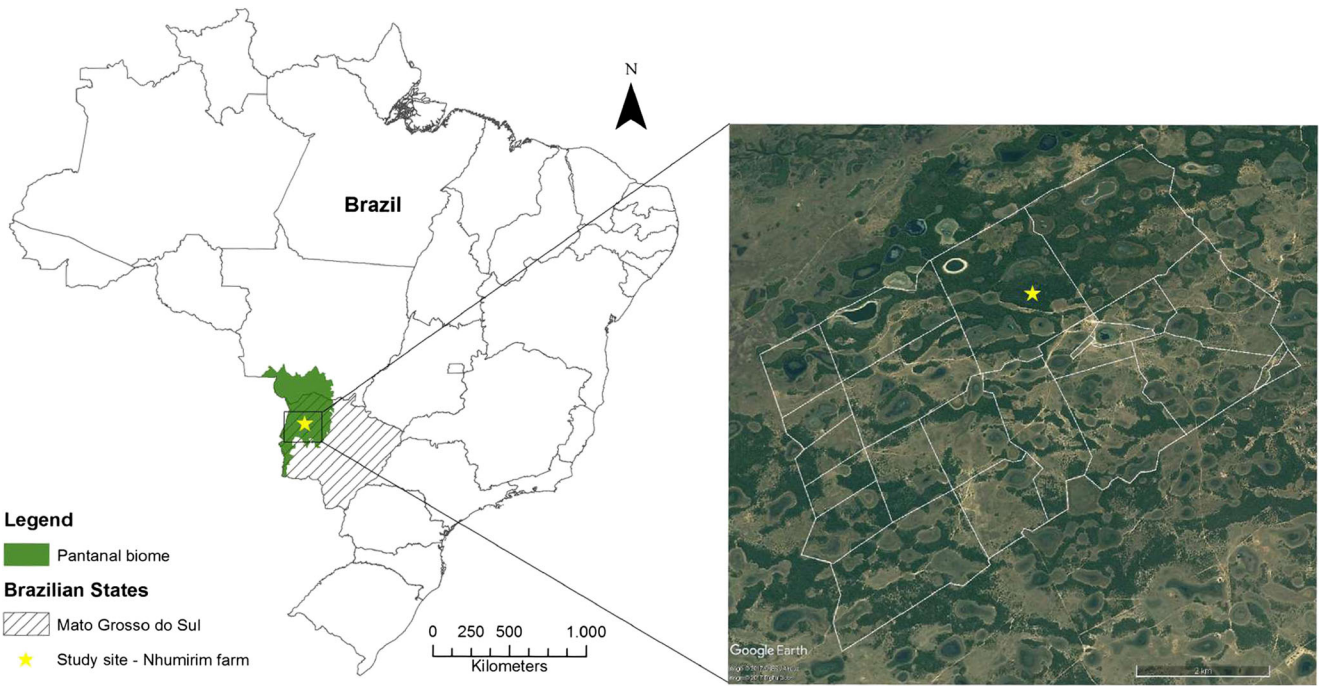

Fig. 1 Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study

samples were collected and stored in absolute alcohol (Merck®, Kenilworth, NJ, USA).

A total of 256 animals were captured, including 158 carnivores, including 78 *C. thous*, 31 *N. nasua*, 7 *L. pardalis*, 110 wild rodents (77 *Thrichomys fosteri*, 25 *Oecomys mamorae*, and 8 *Clyomys laticeps*), and 30 wild marsupials (14 *Thylamys macrurus*, 11 *Gracilinanus agilis*, 4 *Monodelphis domestica*, and 1 *Didelphis albiventris*). Additionally, 42 blood samples from domestic dogs cohabiting the same studied area were collected [12–14].

Ectoparasite Identification

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The identification was performed using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera [15, 16] and *Amblyomma* spp. nymphs [17]. *Amblyomma* spp. larvae could not be identified to the species level considering the insufficient literature available until now. The identification of fleas was performed following previously described taxonomic keys [18].

One thousand five hundred eighty-two ticks parasitizing the sampled mammals were collected, including 1033 (65.2% [115 adults and 918 nymphs]) *Amblyomma sculptum* Berlese specimens, 241 (15.2% [78 adults and 163 nymphs]) *Amblyomma parvum* Aragão specimens, 32 (2%) *Amblyomma ovale* Koch adults, 1 (0.06%) *Amblyomma tigrinum* Koch adult, 1 (0.06%) *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini) adult, 1 (0.06%) *Rhipicephalus sanguineus* s.l. (Latreille) adult, 4 (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae. Furthermore, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were also collected [12–14].

Molecular Analysis

DNA was extracted from 200 μL of each whole blood (158 wild carnivores and 42 domestic dogs) and 10 mg of spleen (140 small mammals) samples using the DNeasy Blood & Tissue Kit (Qiagen®, Valencia, CA, USA), according to the manufacturer's instructions. The total number of individual ticks and pools were 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae. DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), while the adults were processed individually. The collected ticks were pooled according to individual host animals. A total of 39 pooled fleas samples were submitted to DNA extraction. The fleas DNA extraction was also performed in pools consisting of up to five individuals. Ticks and fleas were macerated and submitted to DNA extraction, using the same kit before mentioned [12–14].

In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH) [19], ticks mitochondrial 16S rRNA [20], and fleas cytochrome-c oxidase subunit I (Cox-1) [21] genes were performed (Table 1). All 298 DNA animal samples amplified the predicted product for GAPDH gene. Out of 523 sampled ticks, 31 (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph, and 2 pooled *Amblyomma* larvae]) showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses [12–14].

Firstly, a previously described broad range quantitative real-time PCR (qPCR) protocol based on *nuoG* gene was used aiming to detect and quantify *Bartonella* spp. DNA copies (number of copies/μL) [8]. Serial dilutions were performed aiming to construct standard curves with different concentrations of plasmid DNA (pIDTSMART - Integrated DNA Technologies) (2.0×10^7 to 2.0×10^0 copies/μL), which encoded an 83-bp *Bartonella henselae-nuoG* gene fragment. The number of plasmid copies was determined in accordance with the formula (Xg/μL DNA/[plasmid size (bp) × 660]) × 6.022×10^{23}xplasmid copies/μL. Each qPCR assay was performed including duplicates of each DNA sample. All the duplicates showing Cq difference values higher than 0.5 were re-tested. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1}$/slope). To determine the limit of detection from the qPCR assay, the standard curves generated by 10-fold dilutions were used to determine the amount of DNA that could be detected with 95% of sensitivity. All positive samples in qPCR assays were submitted to previously described conventional PCR (cPCR) assays targeting five other protein-coding genes, namely *gltA* (750 bp) [22], *ftsZ* (600 bp) [24], *nuoG* (400 bp) [23], *ribC* (420 bp) [24], and *rpoB* (585 bp) [9] (Table 1). The reaction products were purified using Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific®, Waltham, MA, USA). Purified amplified DNA fragments from positive samples were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyzer – Applied Byosystem/Perkin Elmer).

Bioinformatics/Phylogenetic Analysis

The sequences obtained from positive samples were first submitted to a screening test using Phred-Phrap software version 23 [25] to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program was used to

Table 1 Oligonucleotides sequences, target genes and thermal conditions used in conventional PCR assays targeting GAPDH endogenous genes and *gltA*, *rpoB*, *nuoG*, *ftsZ*, and *ribC* gene fragments of *Bartonella* spp. in biological samples from wild mammals and domestic dogs, trapped and sampled, respectively, in Pantanal wetland, Brazil

Oligonucleotide sequences (5′–3′)	Gene	Product size (bp)	Cycling conditions	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/mammals	400	95 °C for 5 min; 35 cycles of 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s; and final extension of 72 °C for 5 min	[19]
16S+1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1 (CCGGTCTGAACTCAGATCAAGT)	16SrRNA/ticks	460	10 cycles of 92 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min, followed by 32 cycles of 92 °C for 1 min, 54 °C for 35 s and 72 °C for 1,35 min, and final extension of 72 °C for 7 min	[20]
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/fleas	710	95 °C for 1 min, 35 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 10 s, and final extension of 72 °C for 5 min	[21]
443f (GCTATGTCTGCATTCTATCA) 1210R (GATCYTCAATCATTTCTTTCCA)	*gltA/ Bartonella* spp.	750	94 °C for 5 min, 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, and final extension of 72 °C for 5 min.	[22]
1615s (ATYACYCATAARCGYCGTCTTTCTGCTCTGG) 2267as (GGATCTAAATCTTCYGTYGCACGRATACG)	*rpoB/ Bartonella* spp.	585	95 °C for 2 min, 55 cycles of 94 °C for 15 s, 62 °C for 15 s, and 72 °C for 15 s, and final extension of 72 °C for 5 min.	[9]
nuoGF (GGCGTGATTGTTCTCGTTA) nuoGR (CACGACCACGGCTATCAAT)	*nuoG/Bartonella* spp.	400	94 °C for 5 min, 35 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, and final extension of 72 °C for 5 min.	[23]
ftszF (CATATGGTTTTCATTACTGCYGGTATGG) ftszR (TTCTTCGCGAATACGATTAGCAGCTTC)	*ftsZ /Bartonella* spp.	600	94 °C for 5 min, 35 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min, and final extension of 72 °C for 5 min	[24]
ribcF (TYGGTTGTGTKGAAGATGT) ribcR (AATAATMAGAACATCAAAAA)	*ribC/Bartonella* spp.	420	94 °C for 5 min, 35 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 5 min	[24]

analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in GenBank international database. All sequences that showed appropriate quality standards and identity with *Bartonella* spp. were deposited in the GenBank international database. Samples showing positive results for more than one protocol had their sequences concatenated, using the fragment merger software, version 1 [26]. The sequences were aligned with sequences retrieved from GenBank using MAFFT software, version 7 [27].

The best evolutionary model was selected by the program jModelTest2 (version 2.1.6) on XSEDE [28], under the Akaike information criterion (AIC) [29]. Phylogenetic inference was based on Bayesian Inference (BI). The BI analysis was performed with MrBayes 3.1.2 [30]. Markov chain Monte Carlo (MCMC) simulations were run for 10^9 generations with a sampling frequency of every 100 generations and a burn-in of 25%.

Additionally, analysis of nucleotide polymorphisms of sequences obtained in the present study was performed. The sequences were aligned using Clustal/W [31]. The number of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi), and number of variable sites were determined using the program DnaSP 5, version 5.10.01 [32].

Results

Thirty-five (31.8%) wild rodents and three (7.8%) *Polygenis (P.) bohlsi bohlsi* flea pools (collected from three *T. fosteri* wild rodents) showed positivity in qPCR assays for *Bartonella* spp. based on the *nuoG* gene. None of sampled *C. thous*, *N. nasua*, *L. pardalis*, marsupials, domestic dogs, and ticks showed positivity in qPCR assays for *Bartonella* spp. based on the *nuoG* gene. Among *Bartonella*-positive rodents, 30 (85.7%) belonged to *T. fosteri* species and five (14.2%) belonged to *O. mamorae* species. The *Bartonella*-positive fleas in qPCR assays were not collected from any positive wild rodent.

The number of copies of *Bartonella-nuoG* fragment/μL ranged from 1.33×10^0 to 2.61×10^6. The efficiency and correlation coefficient of qPCR reactions ranged from 91.1 to 101.3% and 0.948 to 0.998, respectively. The quantification mean (copies of *Bartonella-nuoG* fragment/μL) of all reactions were 6.913×10^4, ranging from 1.33×10^0 to 2.61×10^6. The quantification mean between the groups of hosts were: *T. fosteri* = 3.73×10^2copies/μL (1.33×10^0–3.58×10^3), *O. mamorae* = 5.23×10^5copies/μL (1.57×10^1–2.61×10^6) and *Polygenis (P.) bohlsi bohlsi* = 3.11×10^1copies/μL (2.77×10^1–3.51×10^1).

Thirty-seven samples (97.3%) out of 38 qPCR-positive samples for *Bartonella* also showed positivity in cPCR based on the *ftsZ* gene, of which 29 belonged to *T. fosteri* species, 5 belonged to *O. mamorae* species, and 3 belonged to *Polygenis (P.) bohlsi bohlsi* flea species. Four *O. mamorae* and two flea pools positive samples in *ftsZ* cPCR also showed positivity in cPCR based on *gltA* gene. In addition to this, 9 *T. fosteri* positive samples in *ftsZ* cPCR also showed positivity in cPCR based on *nuoG* gene (Fig. 2). None of sampled animals or arthropods was positive in cPCR assays based on *rpoB* and *ribC* genes (Fig. 2). All *gltA*, *ftsZ*, and *nuoG* sequences obtained from positive animals and fleas were deposited in the GenBank international database under the following accession numbers: KX578719, KX827420, KY273622-KY273657, and KY304482-KY304486.

The BLAST analysis of a 750-bp fragment of *Bartonella gltA* gene obtained from four *O. mamorae* spleen samples showed 96% identity with the American isolated R-phy1 *Bartonella* sp. (Z70010). The *Bartonella* sp. *gltA* sequences obtained from flea samples showed 95% identity with a *Bartonella* spp. sequence obtained from a wild Brazilian rodent (KX086733). On the other hand, the *Bartonella ftsZ* sequences (600 bp) obtained from the same four *O. mamorae* spleen samples showed 91% identity with *B. vinsonii* subsp. *berkhoffii* (CP003124). Twenty-nine *Bartonella ftsZ* sequences obtained from *T. fosteri* spleen samples showed 94–96% identity with *B. alsatica* (AF467763). Three *Bartonella ftsZ* sequences obtained from flea pools showed 92% identity with a *Bartonella* spp. sequence obtained from a wild Brazilian rodent (KX036239). The BLAST analysis of a 400-bp fragment of *Bartonella nuoG* gene obtained from nine *T. fosteri* spleen samples showed 94–95% identity with *B. alsatica* (EF659935) (Table 2).

The concatenated phylogenetic analysis of *Bartonella* based on *gltA* and *ftsZ* genes showed that eight *Bartonella gltA* + *ftsZ* sequences obtained from *O. mamorae* wild rodents and four *Bartonella gltA* + *ftsZ* sequences obtained

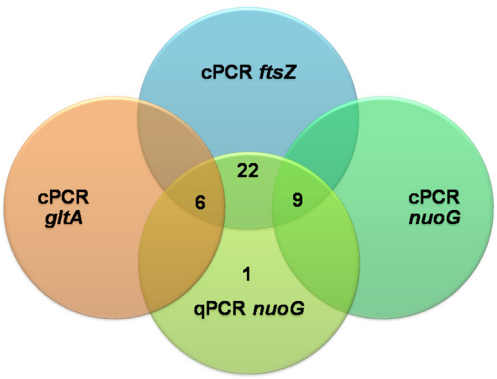

Fig. 2 Venn diagram showing the co-positivity between qPCR based on *nuoG* gene and cPCR assays based on *ftsZ*, *nuoG*, and *gltA* genes

from *Polygenis (P.) bohlsi bohlsi* flea pools were pooled in the same branch of *Bartonella* sequences obtained from Brazilian wild rodents previously deposited in the GenBank, with clade support of 100, based on BI analysis and GTR+G+I evolutionary model for both genes alignments (Fig. 3). Besides, the concatenated phylogenetic analysis of *Bartonella* based on *gltA* and *ftsZ* genes showed that *Bartonella* spp. sequences obtained from *O. mamorae* wild rodents and *Polygenis (P.) bohlsi bohlsi* pools from the present study were closely related to *Bartonella* genotypes detected in *Sigmodon hispidus* in the USA and *Bartonella* genotypes detected in Brazilian wild rodents previously deposited in the GenBank (Fig. 3). Additionally, the concatenated phylogenetic analysis of *Bartonella* based on *nuoG* and *ftsZ* genes showed that 18 *nuoG+ftsZ Bartonella* sequences obtained from *T. fosteri* wild rodents were grouped in the same clade of *B. alsatica*, *B. pachyuromydis*, *B. birtlesii*, *B. acomydis*, *B. silvatica*, and *B. callosciuri*, with high clade support of 99, based on BI analysis and GTR+G+I (for *ftsZ* aligment) and TrN+I+G (for *nuoG* aligment) evolutionary models (Fig. 4).

Among the six *Bartonella gltA* sequences analyzed, four different haplotypes were found, showing Pi = 0.02889, hd = 0.8667, and K = 16.6667. The haplotypes #2 and #4 were formed by two sequences each, while the haplotypes #1 and #3 were formed by only one sequence each. The haplotypes #1, #2, and #3 were formed only by *gltA* sequences obtained from *O. mamorae* and the haplotype #4 was formed by sequences obtained from fleas samples. Analyzing the alignment of 37 *Bartonella fstZ* sequences, four different haplotypes were also found, with Pi = 0.03523, hd = 0.413, and K = 9.3018. The haplotype#1 was formed by only one *Bartonella fstZ* sequence obtained from *T. fosteri*, the haplotype #2 was formed by 28 sequences also obtained from *T. fosteri*, the haplotype #3 was formed by five sequences obtained from *O. mamorae*, and the haplotype #4 was formed by three sequences obtained from fleas samples. The *Bartonella nuoG* alignment did not present variable sites, so it was not possible to observe any polymorphism or nucleotide diversity between the sequences of this gene.

Discussion

The present study showed the presence of *Bartonella* DNA in wild rodents' spleen samples and *Polygenis (P.) bohlsi bohlsi* fleas collected from rodents in Brazilian Pantanal wetland.

The found occurrence of *Bartonella* spp. among wild rodents sampled in the present study was higher (31.8%) than that found among synanthropic rodents (19.2%) in the city of Salvador, state of Bahia, northeastern Brazil [4] and in wild rodents (0%) sampled in the semi-arid region of Pernambuco [6], northeastern Brazil, but similar to that found in a previous recent study involving wild rodents (34.4%) sampled in southern Pantanal [7]. However, the occurrence of *Bartonella* infection among wild rodents in Pantanal biome in the present study was lower than that found in wild rodents (42.8%) in peri-urban areas from the same state where the present study was performed [5]. A closer contact among wild rodents from peri-urban areas with different host species (including domestic animals) and arthropods could explain the differences observed in prevalence rates.

Based on the phylogenetic analysis, a genotype closely related to *Bartonella* spp. (phylogenetic group A and strain R-phy1) detected in Cricetidae rodents from North America [1, 33] and Brazil [7] was detected in *O. mamorae* rodents, also belonging to Cricetidae family. On the other hand, based on the gene-sequence-based criteria for species definition in bacteriology [34], the genotype detected in this present study may correspond to a new *Candidatus*Bartonella species. A previous experimental study reported high host specificity of

Table 2 Maximum identity *of gltA*, *ftsZ*, and *nuoG Bartonella* spp. referring to the full length sequences detected in wild rodents and fleas sampled in Brazilian Pantanal, by BLAST analysis

Host	Biological sample	Number of sequences analyzed	Product size (bp)	Target gene	% identity by BLAST® analysis
O. mamorae	Spleen	4	679	*gltA*	*Bartonella* sp. strain R-phy1 (Z70010) 96%
Polygenis (P.) bohlsi bohlsi	Flea	2	670	*gltA*	Uncultured *Bartonella* sp. wild rodent Brazil (KX086733) 95%
O. mamorae	Spleen	4	550	*fts*Z	*B. vinsonii* subsp. berkhoffii (CP003124) 91%
T. fosteri	Spleen	30	545	*fts*Z	*B. alsatica* (AF467763) 94–96%
Polygenis (P.) bohlsi bohlsi	Flea	3	570	*fts*Z	*Bartonella* spp., wild rodent Brazil (KX036239) 96%
T. fosteri	Spleen	9	350	*nuoG*	*Bartonella alsatica* (EF659935) 94–95%

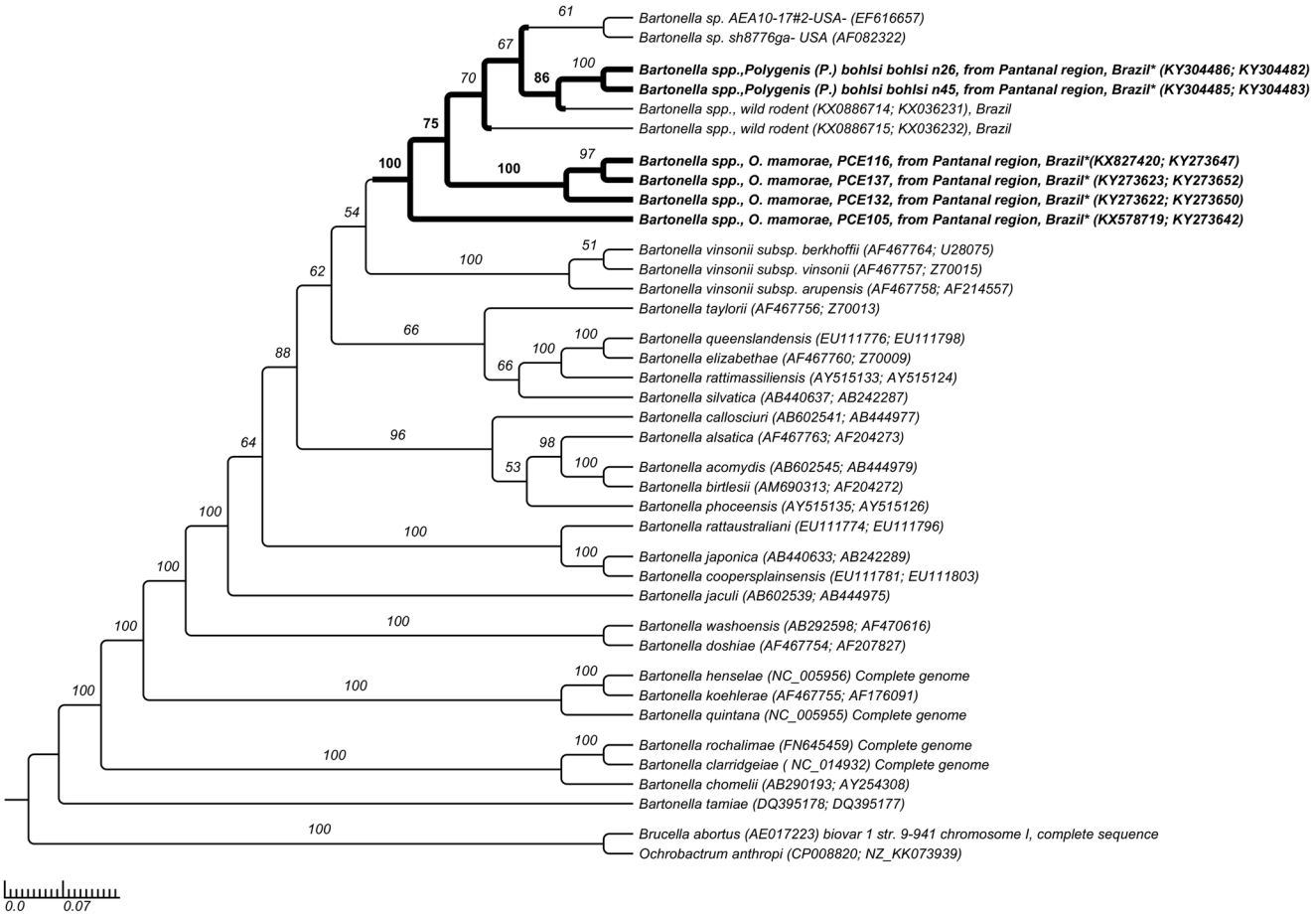

Fig. 3 Phylogenetic tree constructed with 1245-bp *Bartonella gltA+ftsZ* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Ochrobactrum anthropi* (CP008820, NZ_KK073939) and *Brucella abortus* (AE017223) as outgroups

Bartonella sp. belonging to the phylogenetic group A for Cricetidae rodents (*Sigmodon hispidus* and *Peromyscus leucopus*) when compared to BALB/c mice and Wistar rats [35]. These partial results suggest that a genotype closely related to strain R-phy1or a possible new *Candidatus* Bartonella species is circulating among wild rodents from Brazil.

Although a genotype closely related to *B. alsatica*, *B. pachyuromydis*, *B. birtlesii*, *B. acomydis*, *B. silvatica*, and *B. callosciuri* was detected among *T. fosteri* rodents in Brazil based on the *ftsZ* and *nuoG* concatenated phylogenetic analysis, the identity values of *ftsZ* gene sequences were considerably low, which also suggest that a possible new *Candidatus* Bartonella species could be also circulating among *T. fosteri* rodents, based on the criteria for species definition in bacteriology [34]. Although the zoonotic potential of *Bartonella* belonging to phylogenetic group A, *B. pachyuromydis*, *B. birtlesii*, *B. acomydis*, *B. silvatica*, and *B. callosciuri*are still unknown, it is known that *B. alsatica* causes lymphadenitis [36] and endocarditis in humans [37], highlighting the importance of future studies to elucidate the ecological pathways involving this *Bartonella* genotype among rodents and vectors in Brazilian Pantanal region.

Among the ectoparasites that infest wild rodents, fleas are considered the main vectors for *Bartonella* species [38]. Indeed, fleas are also considered important reservoirs for *Bartonella* [39]. Recently, *B. clarridgeiae* and *B. henselae* were detected in *Ctenocephalides felis felis* fleas collected from cats in the semi-arid region of Pernambuco, Brazil [6]. In the same study, *B. rochalimae* and *B. vinsoni berkhofii* were detected in *Pulex irritans* fleas collected from wild and domestic canids [6]. In the abovementioned study, although the sampled animals from which *Bartonella* positive-ectoparasites were collected showed to be negative in PCR assays, they were seropositive to *Bartonella* spp. [6]. In the present study, a *Bartonella* closely related to R-phy1 genotype was detected in both *O. mamorae* rodents and *Polygenis (P.) bohlsi bohlsi* fleas in Pantanal. Although *Bartonella*-positive fleas were collected from rodents showing negativity in PCR assays for *Bartonella*, the phylogenetic analysis showed that these two genotypes were closely related to each other. These results showed that the diagnosis of *Bartonella* spp. based only on PCR amplification of biological host's samples can show a lack of sensitivity, due to the fact the bacteremia found

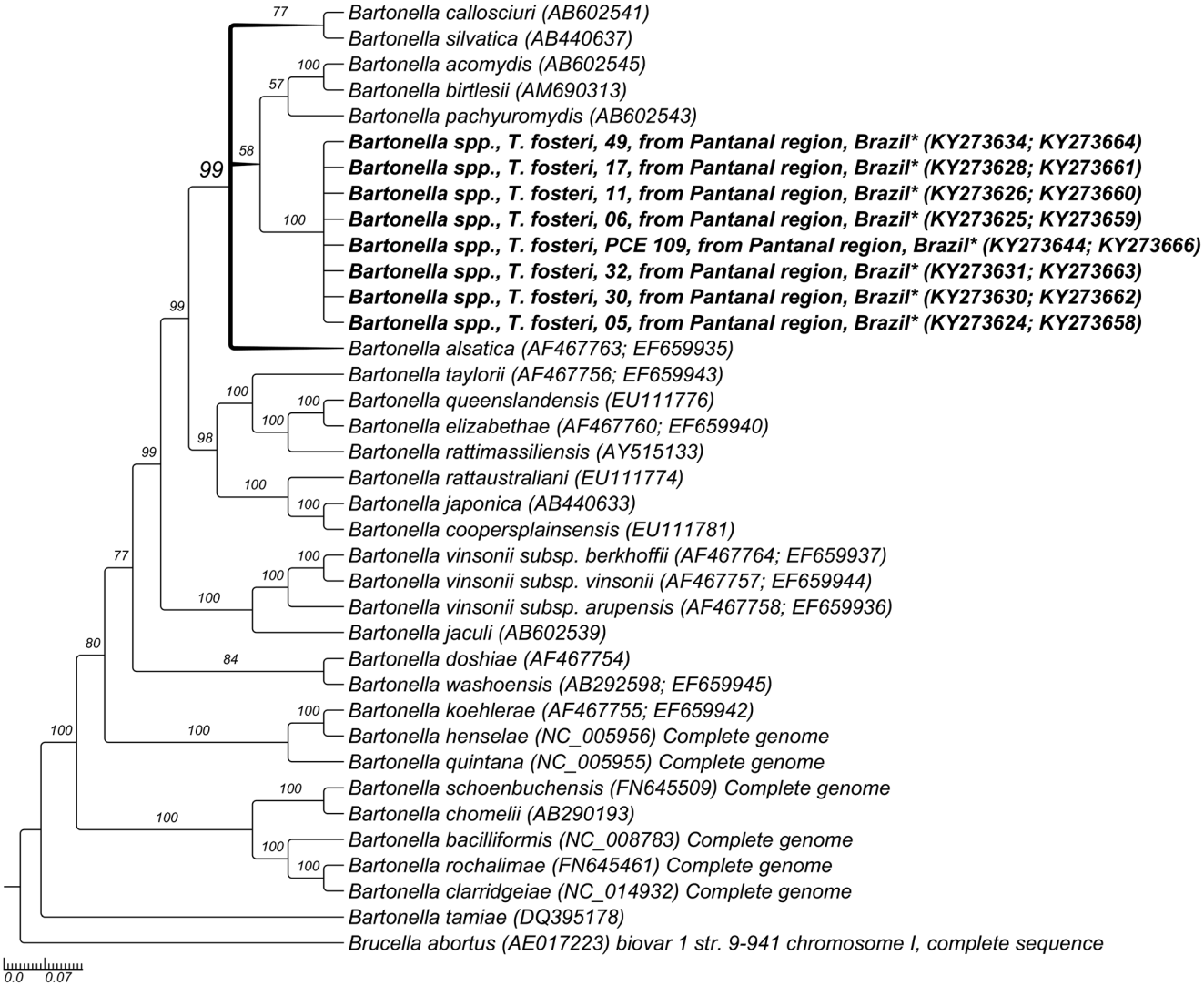

Fig. 4 Phylogenetic tree constructed with 820-bp *Bartonella nuoG+ftsZ* sequences, using Bayesian method and GTR+G+I and TrN+I+G evolutionary models. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Brucella abortus* (AE017223) as outgroup

is usually low. However, the hemoconcentration of host blood meal in arthropods digestive tract could be responsible for the improvement in the sensitivity of molecular diagnosis of *Bartonella.* Therefore, the molecular assays performed on arthropods collected from hosts seem to reflect the epidemiological status of the bacteria among wild animals in nature in a better way. In addition to this, further investigations are needed in order to verify the vector competence of this flea species aiming at elucidating the epidemiology of these new *Bartonella* genotypes (or *Candidatus* species) in Brazil.

Although a previous study demonstrated the vectorial competence of *Ixodes ricinus* ticks in transmitting *B. birtlesii* to rodents [40], the biological role of ticks in the transmission of *Bartonella* in the environment has not yet been confirmed, and its epidemiological role is considered secondary [41]. In a previous study conducted in Pernambuco state, northeastern Brazil [6], none of *A. auricularium*, *A. dubitatum*, *R. sanguineus*, and Argasidae tick specimens collected from wild and domestic animals showed positivity for *Bartonella* spp. in molecular assays. Considering that similar results were found in this present study, the importance of ticks in the bartonellosis epidemiology in Brazil remains unclear.

The *Bartonella* nucleotide diversity based on *gltA* sequences (Pi = 0.028, hd = 0.8667) found in the present study was similar to that found in previous studies among rodents sampled in different biomes in Brazil (Pi = 0.024) [7], Asia (Pi = 0.02154), and North America (Pi = 0.01427) [42], but higher than that found among *B. grahamii* genotypes detected in *Myodes rutilus*, *Microtus fortis*, and *Apodemus agrarius*-rodentsin China (Pi = 0.012) [42]. On the other hand, the found *gltA* diversity was lower than that found among *Bartonella* genotypes detected in *Myodes glareolus* rodents

sampled in Paris, France (Pi = 0.077) [43]. These findings reinforce the hypotheses that within the same gene (*gltA*), recombination and mutation events may happen, promoting distinct haplotypes diversity in a certain population. In addition to this, the *ftsZ* nucleotide diversity observed (Pi = 0.035) was also similar to that found among *Bartonella* genotypes detected in rodents in a previous study in Brazil (Pi = 0.037) [7], but higher than those previously reported among *B. grahamii* genotypes detected in rodents in France (Pi = 0.016) [43] and China (Pi = 0.010) [42]. These results reveal some degree of *Bartonella* genetic diversity among the populations of wild rodents in Brazil.

Previously, *Bartonella* spp. were molecularly detected in six different species of wild felids, namely *Leopardus wiedii, L. pardalis, Leopardus tigrinus, Leopardus geoffroyi, Puma yagouaroundi*, and *Panthera onca*, maintained in captivity in Paraná state, southern Brazil [10]. Additionally, antibodies to *B. clarridgeiae* and *Bartonella vinsonii* subsp. *berkhoffii* were detected in four different species of wild canids, namely *C. thous, Speothos venaticus, Canis lupus*, and *Lycalopex vetulus*, maintained in captivity in the states of São Paulo and Mato Grosso, Brazil [11]. In the present study, none of the sampled wild carnivores showed presence of *Bartonella* DNA in blood samples. In a previous study involving free-roaming domestic cats in a zoo environment in Brazil [44], *Bartonella* DNA was detected in 30% of the sampled cats. Considering that domestic cats are the main reservoirs for *B. henselae* and *B. clarridgeiae* [2], the presence of infected cats in a zoo environment may have facilitated the *Bartonella* transmission to the wild carnivores maintained in captivity, which probably did not happen among wild canids and felids sampled in the present study, since they live free and the contact with domestic cats is scarce.

None of the domestic dogs sampled in the present study showed to be positive for *Bartonella* in qPCR assays. In Brazil, a low occurrence of *Bartonella* was reported in dogs from São Paulo (2/198) and Pernambuco (0/109) states [6, 9]. Besides, the diagnosis of *Bartonella* spp. based only on PCR amplification shows lack of sensitivity, due to the fact the bacteremia found in dogs may be usually low. A previous study showed that a combined approach using isolation culture-based growth medium, such as BAPGM, followed by PCR amplification, can provide a substantial improvement in chances of detection of *B. henselae* and *B. vinsonii* (*berkhoffii*) in the blood of naturally infected dogs [45].

In conclusion, the present study revealed that wild rodents in southern Pantanal region, Brazil, are exposed, at least, to two different genotypes (or *Candidatus*) of *Bartonella* spp. Considering the fact that some *Bartonella* genotypes found in this present study showed to be closely related to *B. alsatica*, a well-known zoonotic *Bartonella* species, studies aiming at assessing the transmission routes among rodents, involved vectors and the zoonotic potential of the isolates are much needed in order to prevent human cases of bartonellosis. Lastly, this study provided an evidence of the possible participation of *Polygenis (P.) bohlsi bohlsi* in *Bartonella* transmission cycles among wild rodents in Brazil. However, in order to confirm the competence of *P. bohlsi* fleas to transmit *Bartonella*, experimental studies must be performed.

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Compliance with ethical standards All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license numbers 38145 and 38787-2) and was endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus Jaboticabal) no. 006772/13.

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