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Multi-locus sequencing reveals a novel *Bartonella* in mammals from the Superorder Xenarthra

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Abstract

The superorder Xenarthra consists of sloths, anteaters and armadillos, mammals originated from South America and currently distributed from the south of North America to the south of South America. The present study aimed to investigate the occurrence and genetic diversity of *Bartonella* spp. in blood and spleen samples from free-living Xenarthra mammals in the states of São Paulo (SP), Mato Grosso do Sul (MS), Rondônia (RO) and Pará (PA). Based on a quantitative real-time PCR (qPCR) assay, a *Bartonella* spp. *nuoG* gene fragment was detected in 1.51% (5/330) of the samples: 4 six-banded armadillos (*Euphractus sexcinctus*) sampled in the MS and 1 southern tamandua (*Tamandua tetradactyla*) sampled in the PA. Eight sequences (5 *ftsZ*, 2 *gltA* and 1 *rpoB*) were obtained in the conventional PCR assays. In both phylogenetic analyses based on Bayesian and distance (SplitsTree) methods, the obtained *ftsZ*, *gltA* and *rpoB* sequences were positioned in a distinct clade, but related to *B. washoensis*. The analysis of SplitsTree and genotype networks based on *B. washoensis* sequences from several hosts from various localities of the world showed that the sequences of the present study were allocated in a group separated from the other sequences, indicating that they probably originated from median vectors and large numbers of mutational events. Additionally, the analyses performed by BLAST showed low percentages of identities of the sequences obtained in the present study when compared to those previously deposited in GenBank. Therefore, we propose a new Candidatus to *Bartonella* occurring in Xenarthra in Brazil. The present study was the first to report the occurrence of *Bartonella* sp. in mammals of the superorder Xenarthra in the world, and it was the first to describe a new Candidatus related to *B. washoensis* in Brazil.

KEYWORDS

anteater, armadillo, bartonella, Brazil, genetic diversity, sloth

1 | INTRODUCTION

The superorder Xenarthra comprises a monophyletic group of mammals originated from South America. This superorder is composed of two orders, namely Cingulata and Pilosa, whose synapomorphy comprises the presence of additional joints between the lumbar vertebrae (Glass, 1985). The Cingulata order, composed of the Dasypodidae family, is characterized by the presence of osteoderms; it comprises 21 species of armadillos, from which 11 occur in Brazil. On the other hand, the Pilosa order encompasses animals with thick coat and absence or small development of teeth; it comprises four families, namely Bradypodidae (three-fingered sloths), Megalonychidae (two-fingered sloths), Cyclopedidae (Silky anteater) and Myrmecophagidae (anteaters), with 5 genera and 10 species, from which eight occur in Brazil (Gardner, 2005). These mammals are distributed from southern North America to the south of South America. In Brazil, these animals can be found in all biomes.

The genus *Bartonella*, belonging to the order Rhizobiales and the Bartonellaceae family, comprises alphaproteobacteria, Gram-negative, aerobic, fastidious and facultative intracellular bacteria, which mainly parasitize erythrocytes and endothelial cells of mammals. They can be transmitted by arthropod vectors, scratches, bites, as well as by contact with blood or fluids from infected animals (Birtles & Raoult, 1996; Breitschwerdt, 2014). Up to now, 33 *Bartonella* species have been validated (Buffet, Kosoy, & Vayssier-Taussat, 2013; Kosoy & Goodrich, 2019), from which 17 show zoonotic potential (Chomel et al., 2009; Maggi et al., 2011; Mascarelli, McQuillan, Harms, Harms, & Breitschwerdt, 2014).

Bartonella spp. has been detected in several species of wild animals. In Brazil, they have been described in rodents (Sousa et al., 2018; Gonçalves et al., 2016), wild carnivores (Filoni et al., 2012; Fleischman et al., 2014; Fontalvo et al., 2017; Guimarães et al., 2009), bats (André et al., 2019; Ferreira et al., 2018; Ikeda et al., 2017) and associated ectoparasites, such as fleas (Sousa et al., 2018; Schott et al., 2019) and bat flies (Do Amaral et al., 2018). To the best of authors' knowledge, there are no reports on the occurrence of these agents in mammals from the superorder Xenarthra so far.

Bartonella washoensis, an agent with zoonotic potential, has squirrels of the Sciuridae family as its natural reservoirs (Bai et al., 2008; Inoue et al., 2009, 2011; Jardine et al., 2005; Kosoy, Murray, Gilmore, Bai, & Gage, 2003; Osikowicz et al., 2016; Rubio et al., 2014; Stevenson et al., 2003). This pathogen was isolated from human patients with cardiac disease and meningitis in the USA (Kosoy et al., 2003; Probert et al., 2009). The transmission route of *B. washoensis* among rodents, humans and other animals is unclear. Although fleas have been incriminated as possible vectors *B. washoensis*, the participation of other arthropods in the transmission of this agent should not be rule out (Kosoy et al., 2003). Indeed, *B. washoensis* was molecularly detected in fleas (*Pulex* sp., *Oropsylla hirsuta* and *Oropsylla montana*) collected from squirrels from the Sciuridae family in the USA and Mexico (Gonzáles et al., 2016; Osikowicz et al., 2016; Stevenson et al., 2003). In the state of California, USA, this *Bartonella* species was detected in a human patient with meningitis, using isolation techniques in blood and chocolate agar and PCR assays (Probert et al., 2009). Also, *B. washoensis gltA* sequences identical to that found in a human patient were detected in blood samples from the rodent *O. beechevi* and associated fleas (*O. montana*) sampled in the patient's rural property.

The present study aimed to investigate the occurrence and genetic diversity of *Bartonella* spp. in Xenarthra mammals sampled in four different states in Brazil.

2 | MATERIAL AND METHODS

2.1 | Sampling and sampled species

Between 2017 and 2018, spleen samples were collected from 40 anteaters [33 giant anteaters (*Myrmecophaga tridactyla*) and 7 southern tamanduas (*Tamandua tetradactyla*)] and 16 armadillos [8 six-banded armadillo (*Euphractus sexcinctus*), 5 nine-banded armadillos (*Dasypus novemcinctus*), 1 giant armadillo (*Priodontes maximus*) and 2 southern naked-tailed armadillos (*Cabasous unicinctus*)]. These samples were collected by the team of the 'Bandeiras e Rodovias' Project, during necropsy of road-killed animals in the state of Mato Grosso do Sul, central-western Brazil. Additionally, between 2011 and 2018, spleen samples were collected during necropsy procedures from anteaters [n = 36 (20 giant anteaters and 16 southern tamanduas)], and 4 nine-banded armadillos, performed by the Wildlife Pathology Service of the School of Agricultural and Veterinarian Sciences (FCAV/UNESP, Jaboticabal, state of São Paulo, southeastern Brazil). The spleen fragments were stored in RNAse and DNAse-free microtubes (KASVI®) at −70°C for subsequent DNA extraction.

Additionally, 242 blood samples were collected from free-ranging sloths and anteaters during the filling process of a hydroelectric power plant in the states of Rondônia [n = 105 (68 brown-throated sloths (*Bradypus variegatus*), 31 *Choloepus* sp., 3 *Bradypus* sp., 1 southern anteater, 1 nine-banded armadillo and 1 southern naked-tailed armadillo)] and Para [n = 137 (126 brown-throated sloths, 5 two-toed sloths (*Choloepus didactylus*) and 6 southern anteaters)] in North Brazil. Blood samples were collected in tubes containing EDTA and stored in a freezer (−70°C) for subsequent DNA extraction.

In total, 338 biological samples were collected from Xenarthra mammals: 194 from *B. variegatus*, 3 from *Bradypus* sp., 5 from *C. didactylus*, 31 from *Choloepus* sp., 30 from *T. tetradactyla*, 53 from *M. tridactyla*, 3 from *C. unicinctus*, 10 from *D. novemcinctus*, 8 from *E. sexcinctus* and 1 from *P. maximus* (Figure 1).

2.2 | Molecular analysis

DNA was extracted from 10 mg from each spleen tissue and 200 μL from each blood sample using the DNeasy™ Blood & Tissue Kit (Qiagen™), according to the manufacturer instructions. The presence of amplifiable DNA was verified by a conventional PCR assay targeting the mammal endogenous glyceraldehyde-3-phosphate

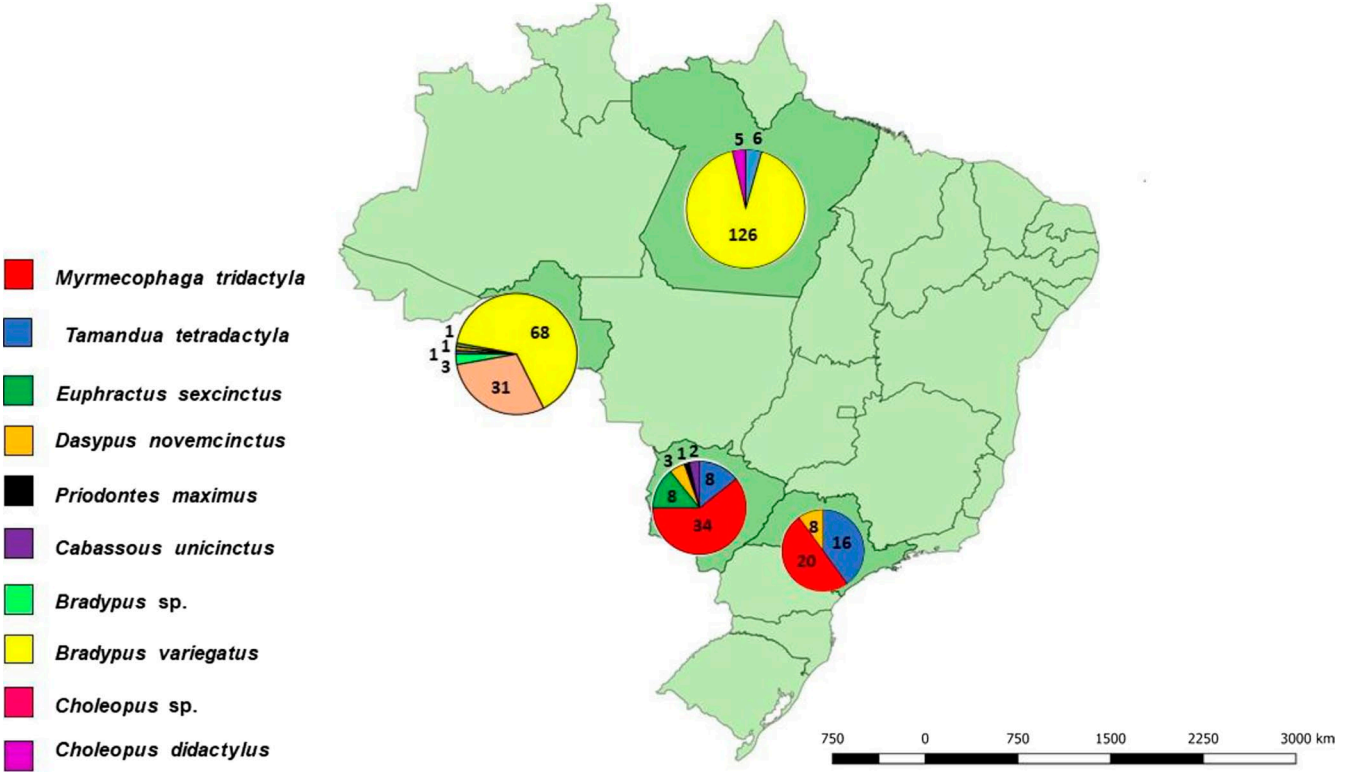

FIGURE 1 Number and origin of the mammals from the Superorder Xenarthra sampled in Brazil [Colour figure can be viewed at wileyonlinelibrary.com]

dehydrogenase (*gapdh*) gene (Birkenheuer, Levy, & Breitschwerdt, 2003). The positive samples in this assay were submitted to specific PCR assays for *Bartonella* spp.

A quantitative real-time PCR (qPCR) assay based on the *nuoG* gene was performed to detect and quantify the number of *Bartonella* DNA copies/μL in each sample. The assay was performed on a mixture with a final volume of 10 μL, containing 1.2 μM of each primer, i.e. F-Bart (5′-CAATCTTCTTTTGCTTCACC-3′) and R-Bart (5′-TCAGGGCTTTATGTGAATAC-3′) and hydrolysis probe TexasRed-5′ – TTYGTCATTTGAACACG - 3′(BHQ2a-Q), 5 μL of 2x buffer (GoTaq™ Probe qPCR Master Mix; Promega Corporation), 9 μL (q.s.p.) of sterilized ultrapure water (Nuclease-Free Water, Promega Corporation) and 1 μL of the DNA sample. The amplification conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 10 s and 52.8°C for 30 s (André et al., 2015). The qPCR was conducted in a low-profile multiplate unskirted PCR (BioRad™) using a CFX96 thermal cycler (BioRad™). Serial dilutions were performed to construct standard curves with different concentrations (2.0×10^7 to 2.0×10^0 copies) of a plasmid encoding a fragment of the 83 bp *nuoG* gene of *B. henselae* DNA (pIDTSMART; Integrated DNA Technologies). The number of plasmid copies was determined according to the formula (XG/μL DNA/[Plasmid tamanho (BP) × 660]) × 6, 22 × 10^{23} × plasmid copies/μL.

All DNA samples were initially tested in duplicates. All duplicates whose Cq difference was higher than 0.5 were retested in triplicate. Amplification efficiency (*E*) was calculated from the slope of the standard curve in each run using the following formula (*E* = 10−1/slope; Bustin et al., 2009).

For molecular characterization, the positive samples in qPCR were submitted to conventional PCR (cPCR) assays based on five genic regions: *gltA* (750 bp) (Billeter, Gundi, Rood, & Kosoy, 2011), *rpoB* (800 bp) (Paziewska, Harris, Zwolińska, Bajer, & Siński, 2011), *ftsZ* (600 bp) (Maggi, Duncan, & Breitschwerdt, 2005), *pap-31* (564 bp) (Maggi et al., 2005) and *ribC* (429 bp) (Johnson, Ayers, McClure, Richardson, & Tellier, 2003). *Bartonella henselae* DNA, detected in a naturally infected cat (André et al., 2015), and nuclease-free water (Promega Corporation) were used as the positive and negative controls, respectively.

The products obtained in all the cPCR assays were submitted to ethidium bromide (Life Technologies™)-stained agarose gels (1%) electrophoresis, at 100 V and 150 mA for 50 min. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System; Bio Rad™) using the Image Lab software, version 4.1.

2.3 | Purification of amplicons, sequencing and phylogenetic analysis

The amplified products were purified using the Silica Bead DNA Gel Extraction kit (Thermo Fisher Scientific) and were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific™) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems™; Sanger, Nicklen, & Coulson, 1997).

The sequences obtained were submitted to a quality-screening test using software Phred-Phrap version 23 (Ewing, Hillier, Wendl, & Green, 1998; Ewing & Green, 1998), in order to evaluate the quality

of the electropherogram and to obtain the consensus sequence from the alignment of the sense and antisense sequences (Ewing et al., 1998). The BLASTn program (Altschul, Gish, Miller, Myers, & Lipman, 1990) was used to compare the obtained nucleotide sequences with previous sequences deposited in GenBank database (Benson et al., 2017).

The consensus sequences obtained in this study and those retrieved from GenBank were aligned using the MAFFT software version 7 (Katoh & Standley, 2013). The best evolutionary model was chosen using the jModelTest2 software (version 2.1.6) on XSEDE (Darriba, Tab oada, Doallo, & Posada, 2012) via the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010). The phylogenetic analysis was based on the Bayesian inference method (BI), performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) via the CIPRES Science Gateway. Markov chain Monte Carlo (MCMC) simulations were run for 10^6 generations with a sampling frequency of every 100 generations and a burn-in of 25%.

Additionally, the same alignments for the phylogenetic analyses based on *ftsZ*, *gltA* and *rpoB* genes were used for distance analysis (Split-Network), using the SplitsTree program version 4.11.3 (Huson & Bryant, 2006). For this purpose, the 'Neighbour-Net' method was used. The support values were estimated under 100 'bootstrap' replicates.

2.4 | Genetic diversity and genealogies within the group of *Bartonella washoensis*

For the analysis of the genetic diversity among the different sequences of *B. washoensis*, the *ftsZ*, *gltA* and *rpoB* sequences obtained in this study were aligned with sequences of *B. washoensis* previously deposited in the GenBank (Table S1). These alignments were used to calculate the nucleotide diversity (π), polymorphism level (diversity of haplotypes [Dh], number of haplotypes [*h*] and the average number of nucleotide differences [*K*]), using DnaSP v5 software (Librado & Rozas, 2009). The nucleotide sequences were submitted to the TCS Network (Clement, Posada, & Crandall, 2000) and distance analysis based on Split-Network, inferred using the programs Population Analysis with Reticulate Trees (popART) and SplitsTree v 4.11.3 (Huson & Bryant, 2006; Leigh & Bryant, 2015), respectively.

3 | RESULTS

3.1 | Occurrence of *Bartonella* spp. in Xenarthra mammals sampled

Out of the 338 Xenarthra biological samples, 330 were positive in cPCR for the *gapdh* gene. Five out of the 330 samples (1.51%) were positive in the qPCR assay for *Bartonella* spp. based on the *nuoG* gene, including 4 spleen samples from *Euphractus sexcinctus* from MS state and 1 blood sample from *Tamandua tetradactyla* from PA state. The number of copies of a fragment of the *nuoG* gene of *Bartonella* spp. per microlitre ranged from 3.07×10^1 to 1.69×10^2, (Table 1), with an average of 7.126×10^1 copies/μL of DNA. The efficiency, R^2, slope and Y-intercept of the reactions ranged from 92.2% to 103.4% (mean = 99.70%), 0.960 to 0.980 (mean = 0.976), −3.545 to −3.241 (mean = −3.34) and 37.895 to 38.160 (mean = 38,004), respectively, following the recommendations of the MIQE guidelines (Bustin et al., 2009; Table 1).

All 5 positive samples in qPCR assay were also positive for at least one gene in the cPCR assays for *Bartonella* spp.: 5 (100%) positive for *ftsZ* gene, 2 (40%) for *gltA* gene, 2 (40%) for the *rpoB* gene and 3 (60%) for the *pap-31* gene. None was positive in cPCR based on the *ribC* gene (Table 1). Unfortunately, the 3 *pap-31* and 1 *rpoB* sequences obtained in the present study showed bad quality in Phred-Phrap analysis, due to the low intensity of the bands in electrophoresis agarose gel, precluding their use in the phylogenetic

TABLE 1 The results obtained in quantitative real-time (q)PCR and conventional (c)PCR assays for *Bartonella* spp. for spleen and blood samples from Xenarthra mammals sampled in Brazil

		qPCR (*nuoG*)					cPCR				
State	Species/ID	Mean quantification (copies/μL)	*E* (%)	R^2	Slope	Y-intercept	*gltA*	*ftsZ*	*rpoB*	*Pap-31*	*ribC*
Mato Grosso do Sul	*Euphractus sexcinctus*/NEC23	4.68×10^1	103.40	.98	−3.244	37.895	-	+	-	-	-
Mato Grosso do Sul	*E. sexcinctus*/NEC46	1.69×10^2	103.50	.96	−3.241	37.959	-	+	+	NS	-
Mato Grosso do Sul	*E. sexcinctus*/ NEC51	5.52×10^1	103.50	.96	−3.241	37.959	+	+	-	NS	-
Mato Grosso do Sul	*E. sexcinctus*/NEC57	4.54×10^1	103.50	.96	−3.241	37.959	+	+	-	NS	-
Pará	*Tamandua tetradactyla*/BM132	3.07×10^1	92.20	.973	−3.525	38.16	-	+	NS	-	-

Note: ID, Identification; E, Efficiency; R^2, determination coefficient; NS, positive samples in cPCR assays but not sequenced due to the low intensity of amplified products.

analyses. The sequences obtained were deposited to the international database GenBank under access numbers MN165706, MN165707, MN165708, MN165709, MN165710, MN165711, MN165712 and MN165713.

3.2 | BLASTn analysis and phylogenetic and distance inferences

The results of the analysis performed on the BLASTn platform for each obtained sequence are shown in Table 2. The two obtained *gltA* sequences showed 90.73%–91.33% identity with a sequence of *Bartonella* sp. (KJ816667) previously detected in bats from Costa Rica. Both sequences also showed 91% of identity with *B. quintana* obtained from a specimen of *Rhesus macaque* sampled in China (CP003784). On the other hand, the obtained *rpoB* and *ftsZ* sequences showed 95.15%–97.12% identity with *B. washoensis*.

The phylogenetic tree inferred by Bayesian analysis based on the *ftsZ* gene positioned the five sequences obtained from armadillos and anteater in a distinct clade, albeit related to *Bartonella* sp. detected in *Martes melampus* in Japan and *B. washoensis*, with 76% and 86% branches support, respectively (Figure 2). The same topology was observed in the phylogenetic analysis based on the *gltA* gene, in which two *Bartonella* sequences obtained from armadillos were

TABLE 2 Analysis of identity performed in BLAST for spleen and blood samples from mammals of the superorder Xenarthra positive for *Bartonella* spp

Species	Target gene	Query coverage	Identity/host/country/GenBank accession number
Euphractus sexcinctus NEC23	*ftsZ*	100%	96.83% *B. washoensis* from *Spermophilus dauricus* from Japan (AB519073)
E. sexcinctus NEC46	*ftsZ*	93%	97.12% *B. washoensis* from *Spermophilus dauricus* from Japan (AB519073)
	rpoB	100%	95.15% *Bartonella* sp. JM–1 from *Martes melampus* from Japan (AB611855)
E. sexcinctus NEC51	*ftsZ*	100%	96.72% *Bartonella* sp. JM–1 from *Martes melampus* from Japan (AB611851)
	gltA	100%	90.73% Unculture *Bartonella* sp. clone SJ126 from a bat from Costa Rica (KJ816667)
E. sexcinctus NEC57	*ftsZ*	100%	96.73% *B. washoensis* from *Spermophilus dauricus* from Japan (AB519073)
	gltA	99%	91.33% Unculture *Bartonella* sp. clone SJ126 from a bat from Costa Rica (KJ816667)
Tamandua tetradactyla BM132	*ftsZ*	100%	97.1% *B. washoensis* from *Spermophilus dauricus* from Japan (AB519073)

FIGURE 2 Phylogenetic analysis of *ftsZ* sequences (600 pb) based on the topology generated by the BI method with the TPM1uf+I+G evolutionary model. The numbers at the nodes correspond to bootstrap values accessed with 1,000 replicates. *Brucella abortus* and *Ochrobactrum anthropi* were used as outgroups [Colour figure can be viewed at wileyonlinelibrary.com]

allocated close to *B. washoensis*, with 95% branch support (Figure 3). The only *rpoB* sequence obtained from an armadillo was close to *Bartonella* sp. obtained from a specimen of *Jaculus orientalis* in Japan, with 60% branch support, and within a larger clade containing *B. washoensis* and *Bartonella* sp. detected in *M. melampus*, with 100% branch support (Figure 4).

The distance analysis obtained by the SplitsTree program corroborated the phylogenetic analyses, since all the sequences obtained in the current study clustered together, although they were inside the group composed of *B. washoensis* and *Bartonella* sp. detected in *M. melampus*, they separated themselves of the other sequences in a distinct manner (Figure 5).

3.3 | Distance and diversity analysis of genotypes in the group of *Bartonella washoensis*

Split-Network analyses performed for the three target genes showed that *B. washoensis* sequences were distributed according to the host species and associated ectoparasites (Figures 6a, 7a and 8a). In all Split-Network analyses, the sequences from the present study were allocated together and closer to the *Bartonella* sequence detected in a mustelid (*M. melampus*) from Japan than the other *B. washoensis* sequences previously detected in rodents.

Out of the 26 sequences *ftsZ* analysed (Figure 6b), 19 different genotypes were found, with π = 0.03264, hd = 0.969 and *K* = 13.12308 (Table 3). The genotype #3 comprised three *Bartonella* sequences found in *Tamiasciurus tamiasciurus* (American red squirrel). The genotype #6 comprises two *Bartonella* sequences found in *Spermophilus dauricus* (Daurian ground squirrel). The genotype #13 comprises three *Bartonella* sequences found in *C. ludovicianus*. The genotype #17 comprises two *Bartonella* sequences detected in the present study, one found in *T. tetradactyla* and the other in *E. sexcinctus*. The genotype #16, comprising the *Bartonella* sequence detected in *M. melampus* (Japanese marten) from Japan, showed to be the most related to sequences detected in the present study. The *B. washoensis* genotypes detected in the USA were positioned at the opposite end of the genotype network when compared to those detected in the present study.

The genotypes analysis based on 35 *gltA B. washoensis* sequences, including the two sequences obtained in the present study, indicated the presence of 27 genotypes, with π = 0.03712, hd = 0.970 and *K* = 11.35966 (Table 3). Four genotypes comprised more than one sequence (Figure 7b). The genotype #1 comprised six *Bartonella* sequences, two of which were found in *Cynomys cynomys* (black-tailed prairie dog) and four in *Oropsylla hirsute* fleas. The genotype #2 consisted of a *Bartonella* sequence found in *C. cynomys* and one of *O. hirsute*. The genotype #5 consisted of a *Bartonella*

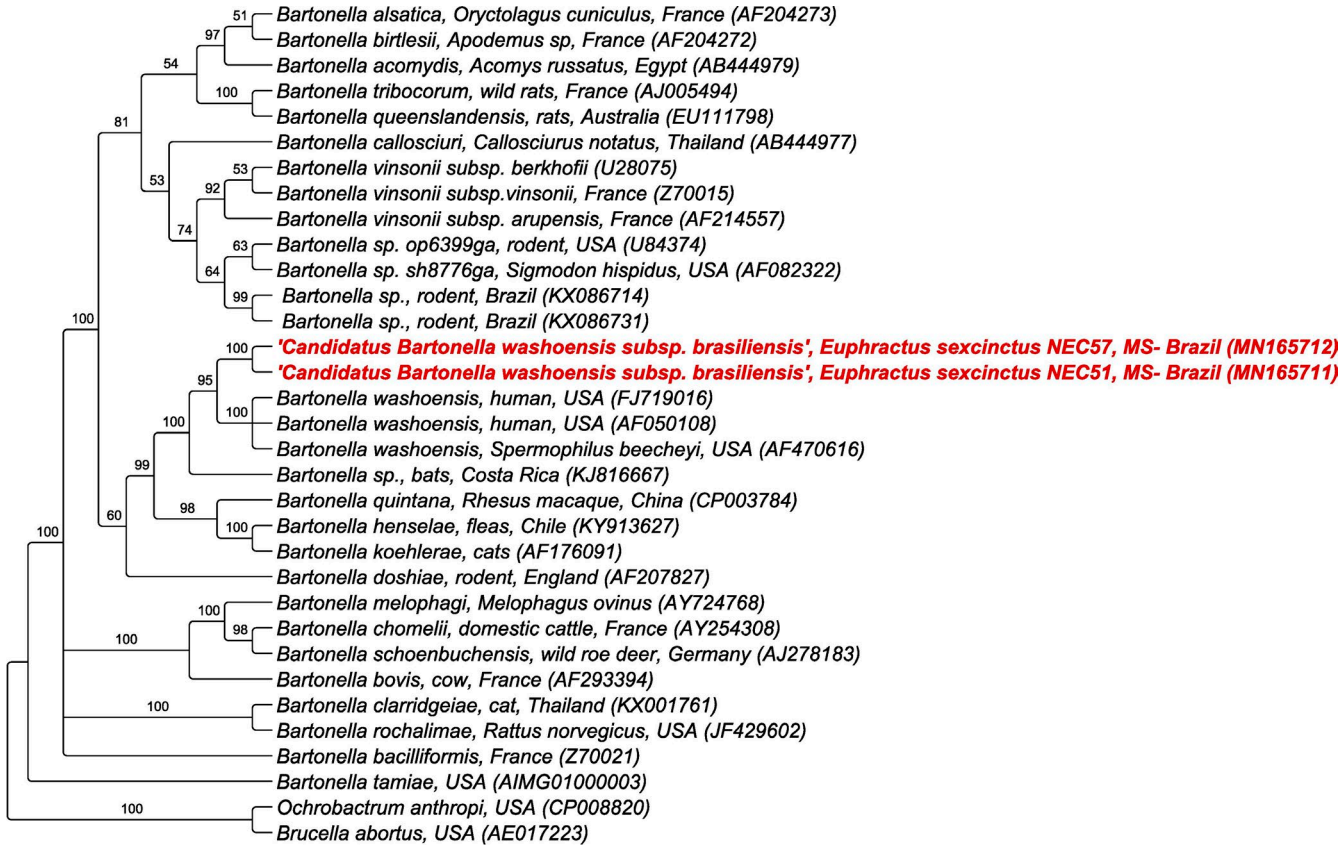

FIGURE 3 Phylogenetic analysis of *gltA* sequences (750 pb) based on the topology generated by the BI method with the TIM3+I+G evolutionary model. The numbers at the nodes correspond to bootstrap values accessed with 1,000 replicates. *Brucella abortus* and *Ochrobactrum anthropi* were used as outgroups [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 Phylogenetic analysis of sequences of *rpoB* sequences (800 pb) based on the topology generated by the BI method with the TIM1+I+G evolutionary model. The numbers at the nodes correspond to bootstrap values accessed with 1,000 replicates. *Brucella abortus* and *Ochrobactrum anthropi* were used as outgroups [Colour figure can be viewed at wileyonlinelibrary.com]

sequence detected in *Otospermophilus beecheyi* (California ground squirrel) and one detected in humans. The genotype #7 comprised two *Bartonella* sequences, including one detected in *C. cynomys* and the other in *Spermophilus tridecemlineatus* (thirteen-lined ground squirrel). Both genotypes detected in the present study (#26 and #27) and genotype #24 (detected in *Spermophilus spilosoma* [spotted ground squirrel] in Mexico and representing the closest genotype to sequences detected in the present study) originated from the same median vector. However, they were separated from each other by several mutational events and an additional median vector. Both the genotypes detected in the present study and the one detected in a spotted ground squirrel in Mexico were distinctly allocated from the other *B. washoensis* genotypes.

Out of the 25 *rpoB* sequences analysed (Figure 8b), 22 different genotypes were found, with π = 0.05206, hd = 0.987 and *K* = 28.99667 (Table 3). While the genotype #7 consisted of two *Bartonella* sequences detected in *Tamia sibiricus* (Siberian chipmunk), the genotype #20 comprised three *Bartonella* sequences found in *Jaculus orientalis* (Greater Egyptian jerboa). The genotypes #21 (Japan) and #22 (from the present study) originated from the same median vector and were closely related to each other.

Both the distance analysis and the genotype networks corroborated, since the spatial distribution of the genotypes in the genotype networks showed similar topology to that observed in the distance analysis, with the positioning of the sequences of *B. washoensis* in groups according to the host species (Figures 6–8).

The three gene fragments evaluated showed high genotype diversity (H ranged from 0.969 to 0.987), with no huge variations of diversity among them (π = 0.03712, 0.03264 and 0.05206 for *gltA*, *ftsZ* and *rpoB*, respectively; Table 3).

4 | DISCUSSION

The present study reported the occurrence of *Bartonella* sp. phylogenetically associated with *B. washoensis* in six-banded armadillos and southern anteater in Brazil. To the best of authors' knowledge, this is the first report of *Bartonella* sp. in mammals from the superorder Xenarthra in the world.

It is known that squirrels from the Sciuridae family are the natural reservoirs for *B. washoensis* (Bai et al., 2008; Inoue et al., 2009, 2011; Jardine et al., 2005; Kosoy et al., 2003; Osikowicz et al., 2016; Rubio et al., 2014; Stevenson et al., 2003). Additionally, genotypes closely related to *B. washoensis* were detected in rodents from the Cricetidae family, such as *Microtus maximowiczii* and *Myodes rutilus* in China (Li et al., 2015), and from the Heteromyidae family, such as *Dipodomys merriami* in Mexico (Rubio et al., 2014). Additionally, genotypes phylogenetically associated with *B. washoensis* were also detected in a specimen of *M. melampus* in Japan (Sato et al., 2012), in the heart valves from sea otters (*Enhydra lutris kenuoni*) in Alaska (Carrasco et al., 2014) and in a dog with vegetative endocarditis from the mitral valve in the USA (Chomel, Wey, & Kasten, 2003).

To the best of authors' knowledge, *B. washoensis* has been reported only in North America and Eurasia (Inoue et al., 2011), since the main species of wild reservoirs are geographically restricted to these continents. The present study reported, for the first time, the

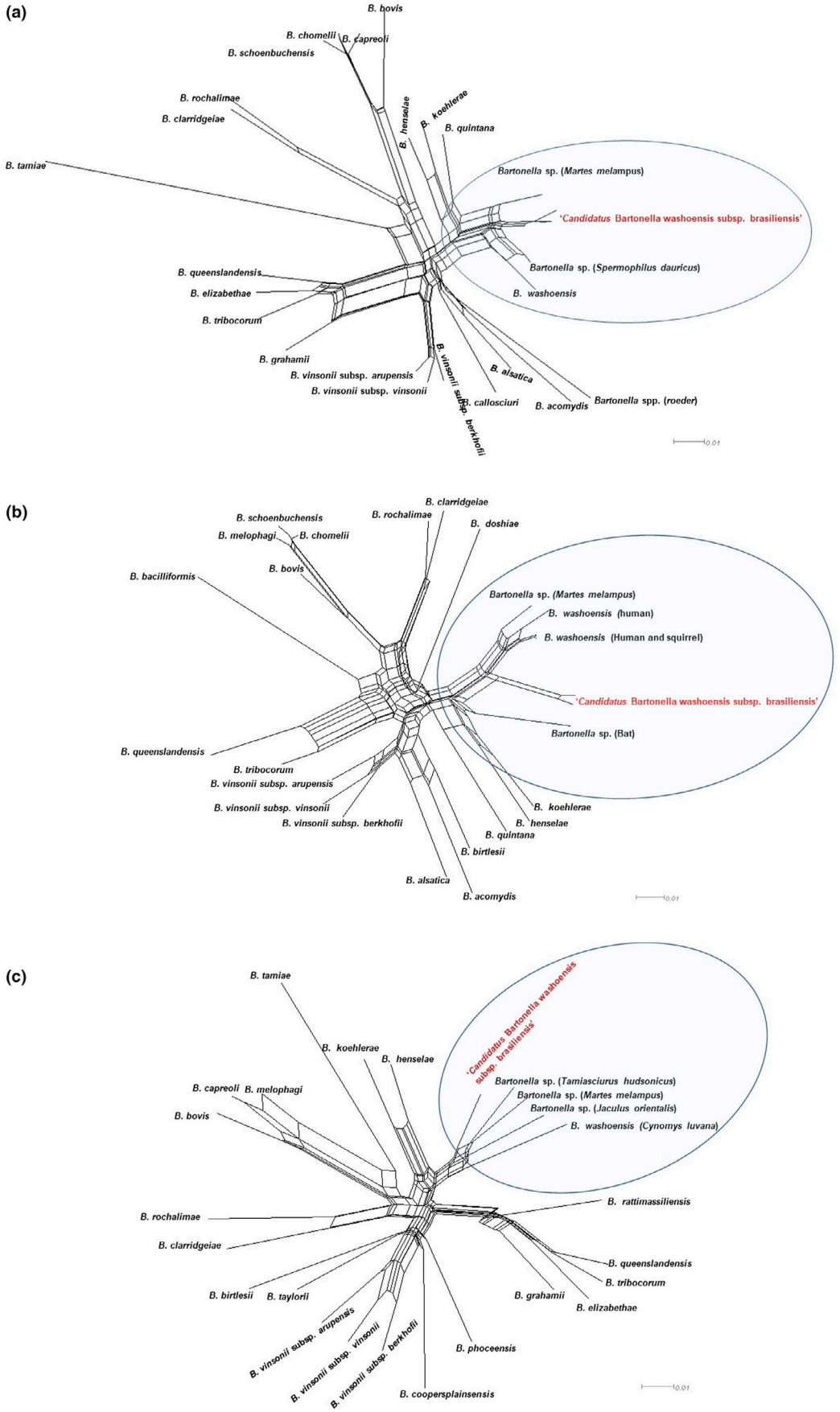

FIGURE 5 Split-Network based in distance from sequences from different *Bartonella* species. (a) *ftsZ* gene, (b) *gltA* gene and (c) *rpoB* gene [Colour figure can be viewed at wileyonlinelibrary.com]

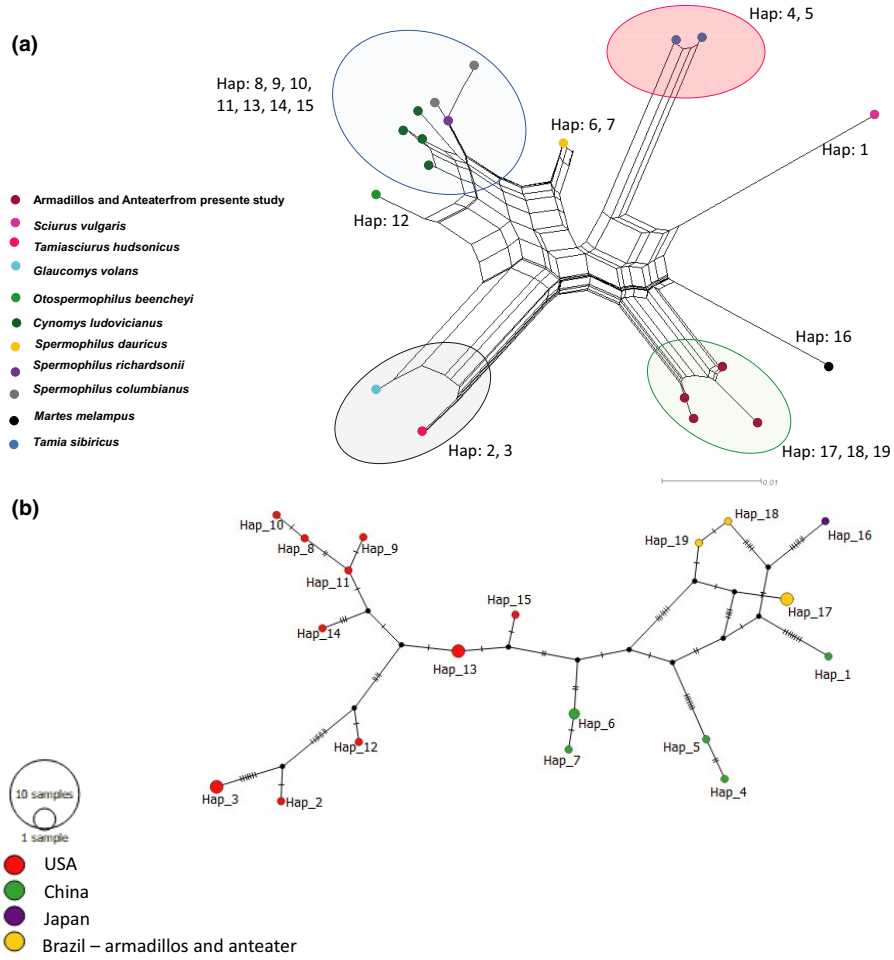

FIGURE 6 (a) Split-Network based in distance from *Bartonella washoensis ftsZ* sequences obtained from different hosts. (b) TCS network based on *B. washoensis ftsZ* sequences obtained in the present study and worldwide [Colour figure can be viewed at wileyonlinelibrary.com]

occurrence of *Bartonella* genotypes similar to *B. washoensis* in biological samples from armadillos and anteater. These findings highlight the possible circulation of this agent or some new phylogenetically related species in Brazil.

The phylogenetic analyses based on *ftsZ*, *gltA* and *rpoB* genes showed that *Bartonella* sequences detected in Xenarthra from the present study formed a distinct clade, albeit related to *B. washoensis*. Similarly, Bai et al. (2008) proposed a new subspecies, namely '*Candidatus* Bartonella washoensis subsp. cynomysii', when detected *Bartonella* sequences (*gltA*, 16S rRNA, *rpoB*, *ftsZ* and *ribC*) in prairie dogs (*C. ludovicianus*) sampled in Boulder County, Colorado, since they formed a subgroup within the clade of *B. washoensis*. Also, Inoue et al. (2011) demonstrated that, even though *Bartonella* sequences (*gltA*, 16S rRNA, *rpoB*, *ftsZ*, *ribC* and *groEL*) detected in squirrels belonging to five different genera (*Spermophilus*, *Tamiasciurus*, *Glaucomys*, *Tamias* and *Sciurus*) were allocated in the *B. washoensis* large clade, they formed five distinct subclades according to each squirrel genus. These findings suggest the occurrence of genetic diversity among *B. washoensis* isolates, depending on the genus and/or species of the vertebrate host (Inoue et al., 2011).

In front of the results obtained in the Bayesian phylogenetic inferences, additional distance and diversity analyses of genotypes were performed in order to obtain a better understanding of the distribution of *B. washoensis* genotypes and its relatedness with the sequences detected in this study. The Split-Network analysis demonstrated, as already suggested in the literature (Inoue et al., 2011), that the *B. washoensis* sequences are grouped according to the genus/species of their vertebrate hosts, since each group formed was constituted by *Bartonella* isolated from mammals belonging to the same species. In the distance analysis based on the *gltA* gene, the sequences found in Xenarthra mammals were allocated in a group apart from the others and closer to those previously detected in *Sciurus vulgaris* (Eurasian red squirrel). In fact, according to previous studies that performed phylogenetic analyses by concatenating six genetic markers, Eurasian red squirrels are the most basal host species of *B. washoensis* (Inoue et al., 2011). Such positioning of the *B. washoensis* isolates from *S. vulgaris* was also observed in the analyses of distance in the present study, once the *B. washoensis* genotype associated with this squirrel species was distantly allocated from the others in all three genetic markers analysed. On the other hand, the *Bartonella rpoB* and *ftsZ* sequences detected in Xenarthra mammals were closer to the *Bartonella* sequences detected in a mustelid (*Martes melampus*) sampled in Japan.

Genotype analyses showed a high genotypic diversity for the three genetic markers analysed. Each *Bartonella* sequence detected in Xenarthra formed a distinct genotype, except for *ftsZ* sequences, for which the genotype #17 comprised three sequences found in armadillos

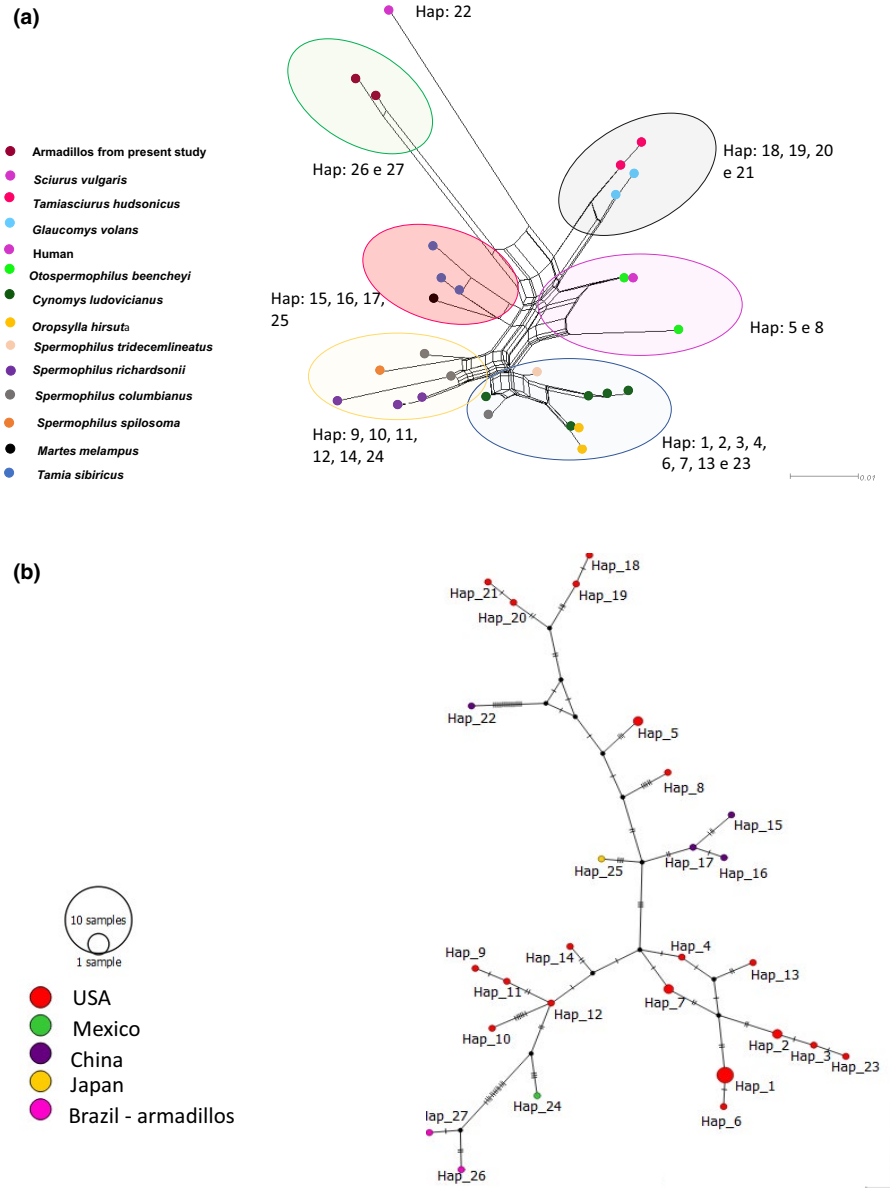

FIGURE 7 (a) Split-Network based in distance from *Bartonella washoensis gltA* sequences obtained from different hosts. (b) TCS network based on *B. washoensis gltA* sequences obtained in the present study and worldwide [Colour figure can be viewed at wileyonlinelibrary.com]

and anteater. The other Xenarthra sequences corresponded to the genotypes #26 and #27 (*gltA*); #18 and #19 (*ftsZ*) and #22 (*rpoB*). Remarkably, the *gltA*, *rpoB* and *ftsZ* sequences of the present study were more distantly allocated from the centre of the genotypic network, showed a greater number of mutational events when compared to the other sequences detected in the world and were originated from median vectors. These findings, along with those previously presented and regarding the Split-Network, strongly suggest the existence of a new genotype related to *B. washoensis* circulating in Xenarthra mammals in Brazil.

Corroborating the data presented in Bayesian, distance and genotype network analyses, the BLAST analysis showed low identity of the *gltA*, *rpoB* and *ftsZ* sequences obtained when compared to those previously deposited in the GenBank. Interestingly, although the *Bartonella ftsZ* and *rpoB* sequences from the sampled armadillos and anteater showed high identity with sequences of *B. washoensis*, the *gltA Bartonella* sequences detected in armadillos showed high identity with *B. quintana* sequences, albeit allocated in the clade of *B. washoensis* in the Bayesian phylogenetic analysis. In fact, when the *Bartonella gltA* sequences detected in armadillos were submitted to BLAST analysis comparatively to the *gltA* gene complete sequence retrieved from the *B. washoensis* whole genome (UAQI01000024), it was possible to verify that 55% of the nucleotides of the *Bartonella* sequences obtained from armadillos showed 93% identity with *B. washoensis* sequence (data not shown). On the other hand, these sequences showed 91% identity with *B. quintana*, which may explain the fact that these sequences were allocated in the clade of *B. washoensis*.

La Scola, Zeaiter, Khamis, and Raoult (2003) established criteria for the determination of new *Bartonella* species based on the percentage of identity with those previously described, depending on the genetic marker analysed. According to these criteria, *Bartonella gltA* sequences that present identity lower than 96% with those previously described could indicate the existence of a new species. Similarly, *Bartonella rpoB* and *ftsZ* sequences presenting identity percentages lower than 95.4% and 97.9%, respectively, with species already described could indicate

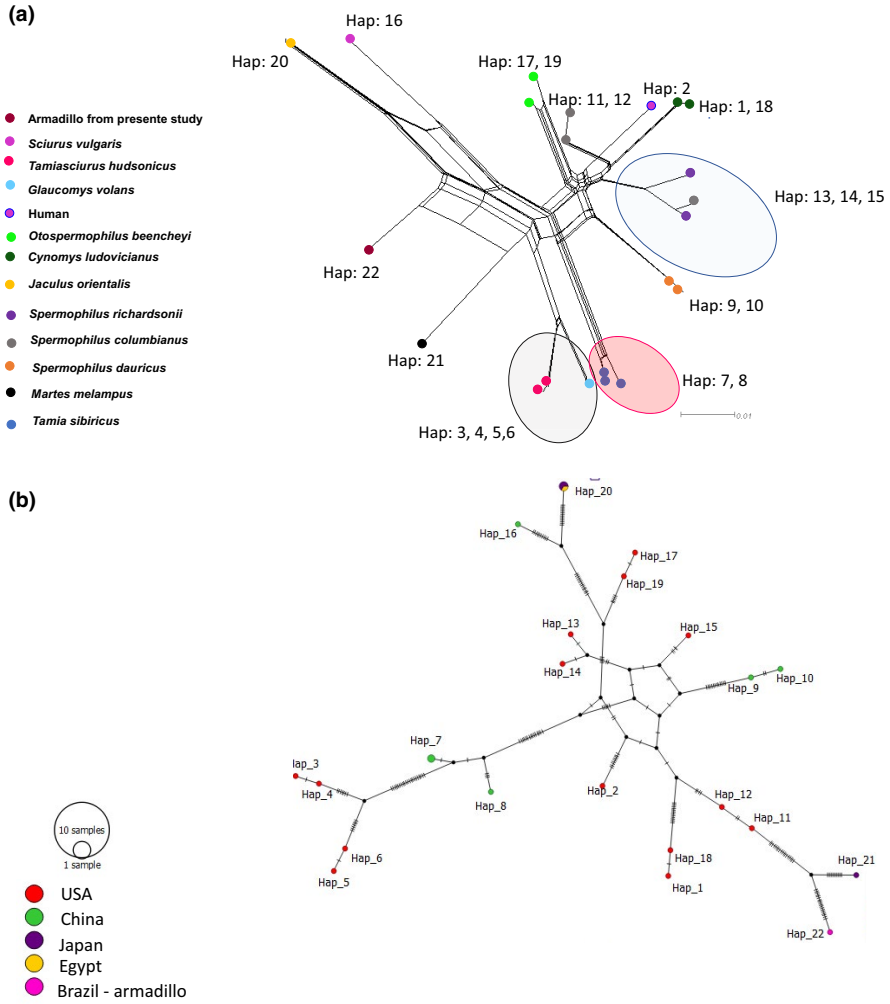

FIGURE 8 (a) Split-Network based in distance from *Bartonella washoensis rpoB* sequences obtained from different hosts. (b) TCS network based on *B. washoensis rpoB* sequences obtained in the present study and worldwide [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Genetic diversity and polymorphisms of the *gltA*, *ftsZ* and *rpoB* sequences of *Bartonella washoensis* detected in Brazil and worldwide

Gene	bp	*N*	VS	GC%	*h*	dh (mean ± *SD*)	π (mean ± *SD*)	*K*
ftsZ	516	26	48	44.6	19	0.969 ± 0.02	0.03264 ± 0.00231	13.12308
gltA	306	35	58	33.5	27	0.97 ± 0.02	0.03712 ± 0.00369	11.35966
rpoB	759	25	108	43.9	22	0.987 ± 0.017	0.05206 ± 0.00448	28.99667

Note: dh, diversity of genotypes; GC%, C + G content; h, number of genotypes; K, nucleotide difference number; *N*, number of sequences analysed; *SD*, standard deviation; π, nucleotide diversity (per site); VS, number of variable sites.

new species of the aforementioned agent. In the present study, all the found sequences presented percentages of identity lower than the values above discriminated for all three genes analysed. In front of that, the sequences found in the present study could be considered a new Candidatus to the *Bartonella* species, related to *B. washoensis*, which we named '*Candidatus* Bartonella washoensis subsp. brasiliensis'.

The results of the present study raised questions about new genotypes or a new subspecies of *B. washoensis* circulating in Brazil, in addition to presenting new vertebrate hosts for this agent. Further studies are needed in order to perform the isolation and whole-genome sequencing of this new Candidatus to *Bartonella*, its distribution, hosts and transmission routes, since its strict relatedness to *B. washoensis*, a zoonotic agent.

5 | CONCLUSION

The present study showed, for the first time, the occurrence of *Bartonella* sp. in mammals from the superorder Xenarthra in the world. Furthermore, this is the first study to describe a *Candidatus* related to *B. washoensis* in South America.

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ETHICAL APPROVAL

Animal procedures and management protocols were approved by Institute Chico Mendes for Conservation of Biodiversity (SISBIO N° 53798–5) and by the ethics committees on animal use of the ICB—USP (protocol number 98) and the School of Agricultural and Veterinarian Sciences (FCAV/UNESP) (protocol number 11.794).

CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in National Center for Biotechnology Information at [https://www.ncbi.nlm.nih.gov/], reference number [*rpoB*: MN165713; *gltA*: MN165711, MN165712; *ftsZ*: MN165706–MN165709].

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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