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Evidence and molecular characterization of *Bartonella* spp. and hemoplasmas in neotropical bats in Brazil

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SUMMARY

The order Chiroptera is considered the second largest group of mammals in the world, hosting important zoonotic virus and bacteria. *Bartonella* and hemotropic mycoplasmas are bacteria that parasite different mammals' species, including humans, causing different clinical manifestations. The present work aimed investigating the occurrence and assessing the phylogenetic positioning of *Bartonella* spp. and *Mycoplasma* spp. in neotropical bats sampled from Brazil. Between December 2015 and April 2016, 325 blood and/or tissues samples were collected from 162 bats comprising 19 different species sampled in five states of Brazil. Out of 322 bat samples collected, while 17 (5·28%) were positive to quantitative PCR for *Bartonella* spp. based on *nuoG* gene, 45 samples (13·97%) were positive to cPCR assays for hemoplasmas based on 16S rRNA gene. While seven sequences were obtained for *Bartonella* (*nuoG*) ($n = 3$), *gltA* ($n = 2$), *rpoB* ($n = 1$), *ftsZ* ($n = 1$), five 16S rRNA sequences were obtained for hemoplasmas. In the phylogenetic analysis, the *Bartonella* sequences clustered with *Bartonella* genotypes detected in bats sampled in Latin America countries. All five hemoplasmas sequences clustered together as a monophyletic group by Maximum Likelihood and Bayesian Inference analyses. The present work showed the first evidence of circulation of *Bartonella* spp. and hemoplasmas among bats in Brazil.

Key words: Bartonellaceae, Chiroptera, hemotrophic mycoplasmas, phylogenetic analyses, South America.

INTRODUCTION

The order Chiroptera is considered the second largest group of mammals in the world, composed by ~20% of mammals with more than 1200 species that are present in all continents, except Antarctica [1]. These animals are known to be hosts of important zoonotic virus (e.g. lyssavirus and hantavirus) and bacteria (e.g. *Leptospira* spp. and *Pasteurella* spp.) [2].

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The genus *Bartonella* spp., belonging to the order Rhizobiales, comprises Gram-negative facultative intracellular α-proteobacteria that parasite mammals' erythrocytes and endothelial cells [3]. These re-emerging agents have been identified in a wide variety of mammals and incriminated as important cause of diseases in humans and animals, causing different clinical manifestations ranging from self-limiting to potentially fatal syndromes [4–6].

Hemotropic mycoplasmas (hemoplasmas) are Gram-negative bacteria belonging to the order Mycoplasmatales, family Mycoplasmataceae, genus *Mycoplasma* [7]. These pathogens are known to cause manifestations ranging from asymptomatic and chronic disease to severe hemolytic anemia due to the capacity of adhesion to the erythrocyte surface, causing indentation or deformation of the target-cell membrane. Acute infected animals can also present anorexia, fever, icterus, and hypoglycemia, depending on the species involved [8–10]. These agents have also been detected in different mammals' species, including humans [11–13].

Bartonella species can be transmitted by arthropod vectors, scratch, or by direct contact with blood or bodily fluids from animals [14]. Similarly, hemoplasmas are supposed to be transmitted mainly by blood-sucking arthropod transmission or aggressive interactions between animals [8, 9].

Bartonella spp. and *Mycoplasma* spp. have been detected in a variety of wild animals all over the world. In Brazil, these agents have been reported in several wild mammals, such as wild carnivores [15–17], deer [18], peccary [19], non-human primates [20, 21], and rodents [22–26].

The presence of *Bartonella* spp. in Chiroptera has been reported in bats sampled in the UK [27], Kenya [4], Taiwan [28], Peru [29], Nigeria [30], Puerto Rico [31], Finland [32], Madagascar [33], Costa Rica [34], Guatemala [35, 36], French Guiana [37], Gana [38], Algeria [39], and South Africa [40]. On the other hand, *Mycoplasma* spp. have already been molecularly detected in bats in the USA [13] and Spain [14].

The present work aimed to investigate the occurrence and assessing the phylogenetic positioning of *Bartonella* spp. and *Mycoplasma* spp. in bats sampled from Brazil.

MATERIAL AND METHODS

Between December 2015 and April 2016, tissue and/or blood samples were collected from 162 bats, comprising 19 species belonging to four different families, namely Vespertillionidae, Phyllostomidae, Mollossidae, and Natalidae, sampled in five states in Brazil (Mato Grosso, Pará, Paraná, São Paulo, and Tocantins) (Fig. 1).

The bat captures were performed in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license numbers 48306–1 and 10080–2), and were endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho', Câmpus Jaboticabal) n° 8189/15. The captures were performed as previously described [41, 42] using mist net (Zootech™, Curitiba, Paraná, Brazil) with variable sizes (3 m × 6 m, 3 m × 9 m, 3 m × 12 m). The nets were placed before sunset and kept closed until nightfall. Once opened, they were checked every 30 min until reaching sample effort of eight animals or until midnight. The animals were carefully removed from the mist nets with leather gloves, taken to the field laboratory to weight, determine species and gender, and then taken to the university's laboratory for euthanasia, necropsy procedures, and tissue samples collections.

DNA was extracted from 10 mg of spleen tissue and 25 mg of liver and heart tissues using the DNeasy™ Blood & Tissue Kit (Qiagen™, Valencia, California, USA), according to the manufacturer's instructions. The DNA quality was evaluated by concentration and 260/280 and 260/230 nm absorbance ratios using a spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, Delaware, USA), in which the purity for DNA samples is considered when ratios are ~1·8 and ~2·0, respectively. Also, a conventional PCR (cPCR) assay, based on a 400 pb fragment of GAPDH gene [43], was performed in order to evaluate the absence of inhibitors in DNA-extracted samples. Positive samples in the above-mentioned cPCR assay were submitted to additional *Bartonella* spp. and hemoplasmas PCR assays. All the cPCR assays were performed in a T100™ Thermal Cycler (BioRad™, Hercules, California, USA).

A previously described quantitative PCR (qPCR) protocol based on *nuoG* gene [45] was used aiming to detect and quantify *Bartonella* spp. DNA copies (number of copies/µl) in bats' biological samples. The qPCR assays were performed in 10 µl final volume reaction mixtures, containing 1 µl of DNA sample, 1·2 µM of each primer F-Bart (5′ – CAATCTTCTTTTGCTTC ACC – 3′), R-Bart (5′ – TCAGGGCTTTATGTGAAT AC – 3′), and hydrolysis probe TexasRed-5′ – TT YGTCATTTGAACACG-3′(BHQ2a-Q) – 3′, Master

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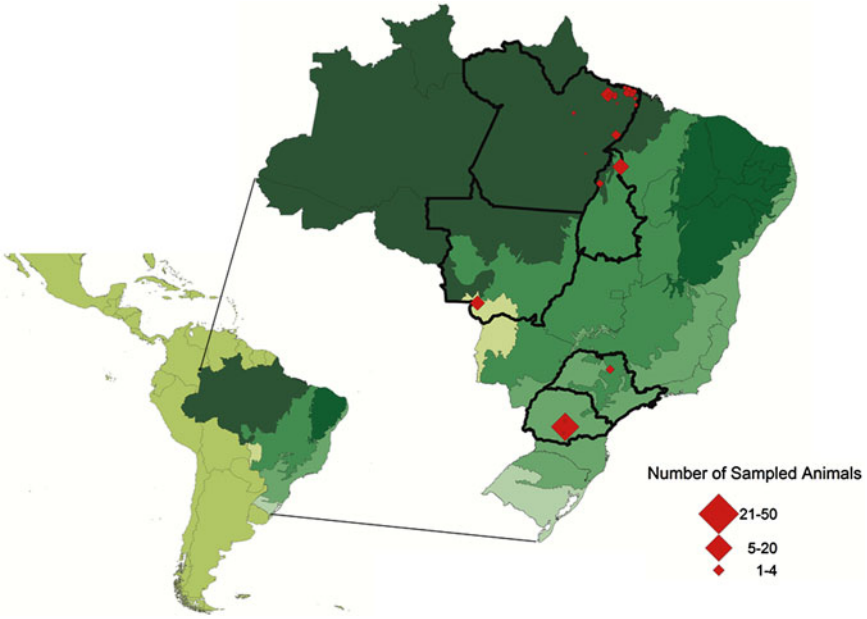

Fig. 1. Locations where bats were sampled in the present study. Each state is outlined in the map and the number of sampled animals is represented by a diamond symbol.

Mix 2× buffer (GoTaq™ Probe qPCR Master Mix, Promega Corporation, Madison, Wisconsin, USA) and ultra-pure sterilized water (Nuclease-Free Water, Promega Corporation, Madison, Wisconsin, USA) q.s.p. 10 µl. 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 [44]. PCR amplifications were conducted in low-profile multiplate unskirted PCR plates (BioRad™, Hercules, California, USA), using a CFX96 Thermal Cycler (BioRad™, Hercules, California, USA). Standard curves were constructed with serial dilutions of plasmid DNA (pIDTSMART – Integrated DNA Technologies, Coralville, Iowa, USA) ($2{\cdot}0 \times 10^7$–$2{\cdot}0 \times 10^0$ copies/µl), which encoded an 83 bp *Bartonella henselae-nuoG* gene fragment. The number of plasmid copies was determined in accordance following the formula:

$$\left(\frac{xg/\mu l\ \text{DNA}}{\text{plasmid length in bp} \times 660g/\text{mol}}\right) \times 6{\cdot}022 \times 10^{23}\ \text{copies/mol} = Y\ \text{copies/}\mu l.$$

Each qPCR assay was performed including duplicates of each bat DNA sample and plasmids. All duplicates showing *Cq* values difference higher than 0·5 were retested in triplicates. Amplification efficiency (*E*) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/\text{slope}}$). The standard curves generated by 10-fold dilutions were used to determine the amount of DNA that could be detected with 95% of sensitivity [45].

In order to perform the molecular characterization of *Bartonella* spp., DNA samples from positive bats in qPCR reactions were submitted to previously described cPCR assays targeting eight different genes, namely *nuoG* (400 bp) [46], *ribC* (420 bp) [47], *gltA* (750 bp) [48], *rpoB* (800 bp) [49], the intergenic region 16S–23SrRNA ITS (453–717 bp) [50], *groEL* (752 bp) [49, 51], *fstZ* (600 bp) [49], 16SrRNA (400 bp) [49], and *pap-31* (564 bp) [52]. *B. henselae* DNA obtained from a naturally infected cat [45] and sterilized ultrapure water (Nuclease-Free Water, Promega™, Madison, Wisconsin, USA) were used as positive and negative controls, respectively.

In order to amplify *Mycoplasma* spp. DNA, two cPCR assays based on 16SrRNA gene were performed, using two sets of primers, namely HemMycop16S-41s (5′-GYATGCMTAAYACATGCAAGTCGARCG-3′) and HemMyco16S-938as (5′ – CTCCACCACTTGTT CAGGTCCCCGTC – 3′) (fragment of ∼800 bp), and HemMycop16S-322s (5′ – GCCCATATTCCTACGG GAAGCAGCAGT – 3′) and HemMycop16S-1420 as (5′ – GTTTGACGGGCGGTGTGTACAAGACC – 3′) (fragment of ∼800 bp) [53]. Five microliters of DNA were used as a template in 25 µl reaction mixtures containing 10× PCR buffer, 1·0 mM $MgCl_2$, 0·8 mM deoxynucleotide triphosphate mixture, 1·5 U Taq Platinum DNA Polymerase (Life Technologies™, Carlsbad,

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California, USA), and 0·3 µM of each primer. *Mycoplasma haemofelis* DNA obtained from a naturally infected cat [54] and ultra-pure sterile water (Nuclease-Free Water, Promega™, Madison, Wisconsin, USA) were used as positive and negative controls, respectively. PCR amplifications were performed at 94 °C for 2 min followed by 55 repetitive cycles of 94 °C for 15 s, 68 °C for 15 s, and 72 °C for 18 s, followed by a final extension at 72 °C for 1 min. The 16S rRNA-*Mycoplasma* spp. positive samples were additionally submitted to an RNaseP gene-*Mycoplasma* sp. (165 bp) cPCR assay using the oligonucleotides HemoMycoRNaseP30s (5′-GATKGT GYGAGYATATAAAAAATAAARCTCRAC – 3′) and HemoMyco RNaseP200as (5′ – GMGGRGTTT ACCGCGTTTCAC – 3′). The conditions of amplification were the same as described above, except for the annealing temperature (59 °C) [53].

The products obtained in all the cPCR assays were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, California, USA) under 100 V/150 mA for 50 min. The gels were imaged under ultraviolet light (ChemiDoc MP Imaging System, Bio Rad™, Hercules, California, USA) using the Image Lab Software version 4.1.

Amplified products were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific™, Waltham, Massachusetts, USA) and submitted to sequencing, which was performed using the BigDye™ Terminator v3·1Cycle Sequencing Kit (Thermo Fisher Scientific™) and ABI PRISM 310DNA Analyzer (Applied Biosystems™, Foster City, California, USA) [55].

The sequences obtained from positive samples were first submitted to a screening test using Phred-Phrap software version 23 [56, 57] to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program [58] was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences from an international database (GenBank) [59]. The consensus sequences obtained in the present study and those retrieved from GenBank were aligned using the Clustal/W software [60] via Bioedit version 7.0.5.3 [61]. Phylogenetic inference was based on Bayesian Inference (BI) and Maximum Likelihood (ML) methods. The BI analysis was performed with MrBayes 3·1·2 [62] via CIPRES Science Gateway [63]. 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%. The ML analysis was inferred with the W-IQ-Tree tool available online (http://iqtree.cibiv.univie.ac.at/) [64, 65] using 1000 bootstrapping replicates. The best model of evolution was selected by the program jModelTest2 (version 2.1.6) on XSEDE [66], under the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) [67]. All the trees were examined in Treegraph 2·0·56–381 *β* [68].

RESULTS

A total of 122 liver tissue samples, 107 spleen tissue samples, 56 heart tissue samples, and 40 blood samples collected from 162 bats in different states of Brazil were used in this study. So, out of 325 bats' biological samples, 322 samples were positive in cPCR for GAPDH gene (Table 1). The mean and standard deviation of the values of concentration and absorbance ratios (260/280 and 260/230 nm) are shown in Table 2.

Seventeen (5·28%) out of 322 samples were positive for *Bartonella* spp. in qPCR assays based on the *nuoG* gene, including four samples collected from three bats from the state of Paraná (*Sturnira lilium* ($n = 3$)); six from the state of Pará (*Phyllostomus hastatus* ($n = 1$), *Carollia perspicillata* ($n = 4$), and *Natalus espiritosantensis* ($n = 1$)); and seven from the state of Tocantins (*C. perspicillata* ($n = 2$) and *Glossophaga soricina* ($n = 5$)). The efficiency, R^2, slope, and *Y*-intercept of reactions ranged from 90·8% to 98·6% (mean = 95·09%), 0·987 to 1 (mean = 0·995), −3·355 to −3·566 (mean = −3·447), and 37·284 to 38·871 (mean = 38·067), respectively. The quantification positive *nuoG Bartonella* spp. ranged from $4{\cdot}4 \times 10^3$ to $6{\cdot}95 \times 10^3$ copies/µl (Table 3).

Thirteen (76·47%) out of 17 positive samples in the qPCR were also positive for at least one target gene in cPCR assays for *Bartonella* spp. Four (30·77%) of them were positive for the *ftsZ* gene, three (23·07%) for the *nuoG* gene, three (23·07%) for the *ribC* gene, two (15·38%) for the *groEL* gene, and one (7·69%) was positive for the *rpoB* gene. None of them were positive for *pap-31* and *16SrRNA* genes and for the intergenic region 16S–23S rRNA (ITS). Due to the low intensity of some amplified products, which precluded high-quality sequencing, only seven *Bartonella* spp. sequences were obtained (*nuoG* ($n = 3$), *gltA* ($n = 2$), *rpoB* ($n = 1$), *ftsZ* ($n = 1$)). The sequences obtained were deposited to the GenBank under accession numbers KY356752–KY356758.

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Table 1. *Number of bat species positive for Bartonella spp. and hemoplasmas in Brazil, according to locality and tissue sampled*

State	Species	Number of sampled animals	Tissue sample	Total number of samples	Number of positive samples for *Bartonella* spp.	Number of positive samples for hemoplasmas
Paraná	*Sturnira lilium*	24	Liver, spleen and heart	150	04	13
	Molossus molossus	20			–	18
	Eptesicus sp.	05			–	02
	Sturnira tildae	01			–	–
Pará	*Carollia perspicillata*	26	Liver and spleen	117	04	–
	Artibeus planirostris	08			–	03
	M. molossus	07			–	02
	Glossophaga soricina	06			–	–
	Artibeus lituratus	03			–	–
	Molossus rufus	03			–	–
	Eumops glaucinus	02			–	–
	S. lilium	02			–	–
	Artibeus cinereus	01			–	–
	Phyllostomus discolor	01			01	–
	Phyllostomus hastatus	01			–	–
	Myotis nigricans	01			–	01
	Natalus espiritosantensis	01			01	–
	Uroderma bilobatum	01			–	–
	Micronycteris minuta	01			–	–
	Eumops auripendulos	01			–	01
	Mimon crenulatum	01			–	–
Tocantins	*G. soricina*	19	Whole blood	24	05	04
	C. perspicillata	5			02	–
São Paulo	*M. molossus*	03	Liver, spleen and heart	18	–	–
	Artibeus sp.	02			–	–
	Myotis sp.	01			–	–
Mato Grosso	*M. rufus*	15	Whole blood	16	–	01
	G. soricina	01			–	–
			TOTAL	325	17	45

Table 2. *Mean and standard deviation values of concentration and absorbance ratios (260/280; 260/230 nm) of DNA samples extracted from each type of bats' biological sample*

	Whole blood	Liver tissue	Spleen tissue	Heart tissue
Concentration (ng/μl)	5·03 ± 5·40	211·91 ± 140·35	139·06 ± 168·89	55·09 ± 24·02
260/280	2·49 ± 2·32	1·91 ± 0·27	1·86 ± 1·10	1·99 ± 0·07
260/230	0·52 ± 0·25	1·69 ± 0·44	2·30 ± 2·91	1·67 ± 0·39

Regarding the occurrence of hemoplasmas among bats, while 45 samples (13·97%) were positive for the first 16S rRNA protocol (using HemMycop16S-41s and HemMyco16S-938 as primers), 14 samples (4·34%) were positive for the second 16S rRNA protocol (using HemMycop16S-322s and HemMycop16S-1420 as primers). Among positive animals, 33 samples were collected from 18 bats in the state of Paraná (*Molossus molossus* ($n = 10$), *S. lilium* ($n = 8$), *Eptesicus* spp. ($n = 1$)); seven samples were collected from bats in the state of Pará (*Artibeus planirostris* ($n = 3$), *M. molossus* ($n = 2$), *Eumops auripendulos* ($n = 1$), *Myotis nigricans* ($n = 1$)); four from bats in the state of Tocantins (*G. soricina* ($n = 4$)); and one from a bat in the state of Mato Grosso (*Molossus rufus*). Twelve (26·66%) out of 45 samples positive for hemoplasmas based on 16S rRNA gene were also positive at RNAaseP gene-cPCR assay. A total of five (1·55%) samples were positive for the three performed protocols. Unfortunately, only five 16S rRNA hemoplasmas sequences (Paraná ($n = 4$), Pará ($n = 1$)), detected in specimens of *M. molossus*, were obtained due to the low intensity of some amplified products, which precluded high-quality sequencing. The sequences were deposited to the GenBank data under the accession numbers KY356747–KY356751. Only one bat sampled in Tocantins state belonging to *G. soricina* species was positive for both *Bartonella* sp. and *Mycoplasma* sp.

Based on BLAST analysis, the found *Bartonella nuoG* sequences ($n = 3$) showed 89–90% identity to *B. taylorii* (GenBank accession number EF659943) and 92% to *B. koehlerae* (GenBank accession number EF659942). Two *Bartonella gltA* sequences showed 98–100% identity to *Bartonella* sp. from bats sampled in Costa Rica (GenBank accession numbers KJ816674 and KJ816690). One *Bartonella rpoB* sequence showed 89% identity to *Bartonella* sp. Khabarovsk from Asian mammals (GenBank accession number AB779537). Finally, one *Bartonella ftsZ* sequence showed 98% identity to *Bartonella* sp. Honshu from deer sampled in Japan (GenBank accession number AB703117). The query coverage ranged from 96% to 100% in all BLAST analyses run for *Bartonella* sequences. In the phylogenetic inferences based on ML and BI methods (Figs 2–5), the *Bartonella* sequences obtained in the present study were mainly closely related to other *Bartonella* sequences obtained from New World bats. When present, *Bartonella* sequences obtained from Old World bats clustered separately with high branch support.

The concatenated phylogenetic assessment of positive sequences for both *nuoG* (400 pb) and *gltA* (750 pb) genes showed a very similar topology in BI and ML methods, forming two different clusters. The sequences from bats detected in a specimen of *S. lilium* sampled in the state of Paraná was closely related to *Bartonella* sequences detected in a Hippoboscidae bat fly (*Aspidoptera delatorrei*) from Costa Rica and in a specimen of *C. perspicillata* from Guatemala with 100/100% of branch support. A *Bartonella* genotype detected in a specimen of *C. perspicillata* sampled in Tocantins was positioned in the same cluster (with 59% and 71% of support in BI and ML analyses, respectively) than genotypes detected in bats from Peru and Guatemala, rodents from Brazil and the USA, and from an ectoparasite (*Polygenis gwyni*) collected from rodents also in the USA. Moreover, a *Bartonella* sequence obtained in a specimen of *G. soricina* sampled in Tocantins state was positioned in a more basal clade and closely related to *Bartonella* genotypes detected in specimens of *Carollia sowelli* and its Streblidae dipteran *Strebla guajiro* from Costa Rica (with branch support of 100%) and genotypes detected in bats from Peru and Guatemala with 66% in both analyses (ML and BI) (Fig. 2).

The *Bartonella rpoB* sequence obtained in a specimen of *S. lilium* sampled in the state of Paraná was positioned alone in a branch, but closely related to two *Bartonella* genotypes detected in wild rodents from Asia (AB779537; AB290276) and *B. taylorii* (AF165995), with a branch support of 85% in ML and 94% of probability in BI analyses (Fig. 3).

The *Bartonella ftsZ* sequence obtained in a specimen of *C. perspicillata* sampled in the state of Pará was positioned alone in a branch, but closely related

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Table 3. *Positive samples for Bartonella sp. in qPCR assays based on nuoG gene with the reactions parameters*

Sample	Species	Biological sample	Locality	*Cq* (mean)	Number of copies/µl	Efficiency (%)	R^2	Slope	*Y*-int
38019 B	*Phyllostomus hastatus*	Spleen	Viseu, Pará	34·23	$8{\cdot}208 \times 10^{0}$	98·1	0·998	−3·368	37·252
38197 B	*Carollia perspicillata*	Spleen	Castanhal, Pará	33·05	$1{\cdot}8035 \times 10^{1}$	98·1	0·998	−3·368	37·252
38295 B	*C. perspicillata*	Spleen	Belém-Mosqueiro, Pará	33·9	$9{\cdot}33 \times 10^{0}$	98·1	0·998	−3·368	37·252
38296 F	*C. perspicillata*	Liver	Belém-Mosqueiro, Pará	28·97	$4{\cdot}3945 \times 10^{2}$	93·7	1·000	−3·482	38·482
38706 F	*C. perspicillata*	Liver	Cachoeira do Piriá, Pará	32·69	$2{\cdot}2575 \times 10^{1}$	98·1	0·998	−3·368	37·252
37987 F	*Natalus espiritosantensis*	Liver	Belém-Outeiro, Pará	33·51	$1{\cdot}292 \times 10^{1}$	98·1	0·998	−3·368	37·252
45 B	*Sturnira lilium*	Spleen	Parque das Araucárias, Guarapuava, Paraná	32·69	$2{\cdot}22615 \times 10^{1}$	98·1	0·998	−3·368	37·252
48 B	*S. lilium*	Spleen	Parque das Araucárias, Guarapuava, Paraná	34·02	$1{\cdot}876 \times 10^{2}$	93·6	0·997	−3·486	38·444
49 B	*S. lilium*	Spleen	Parque das Araucárias, Guarapuava, Paraná	31·21	$1{\cdot}1875 \times 10^{2}$	93·6	0·997	−3·486	38·444
49 F		Liver		34·30	$7{\cdot}623 \times 10^{0}$	98·1	0·998	−3·368	37·252
#30	*C. perspicillata*	Blood	Arapoema, Tocantins	24·31	$6{\cdot}9575 \times 10^{3}$	98·1	0·998	−3·368	37·252
# 42	*C. perspicillata*	Blood	Arapoema, Tocantins	30·67	$2{\cdot}1435 \times 10^{2}$	92·5	0·997	−3·515	38·871
M5	*Glossophaga soricina*	Blood	Aguiarnopolis, Tocantins	36·65	$4{\cdot}4065 \times 10^{0}$	92·5	0·997	−3·515	38·871
M16	*G. soricina*	Blood	Aguiarnopolis, Tocantins	33·07	$1{\cdot}9385 \times 10^{1}$	98·6	0·988	−3·555	37·389
M21	*G. soricina*	Blood	Aguiarnopolis, Tocantins	32·66	$2{\cdot}6425 \times 10^{1}$	98·6	0·988	−3·555	37·389
M23	*G. soricina*	Blood	Aguiarnopolis, Tocantins	33·40	$1{\cdot}3905 \times 10^{1}$	98·1	0·998	−3·368	37·252
M41	*G. soricina*	Blood	Aguiarnopolis, Tocantins	35·12	$4{\cdot}7855 \times 10^{0}$	98·6	0·988	−3·555	37·389

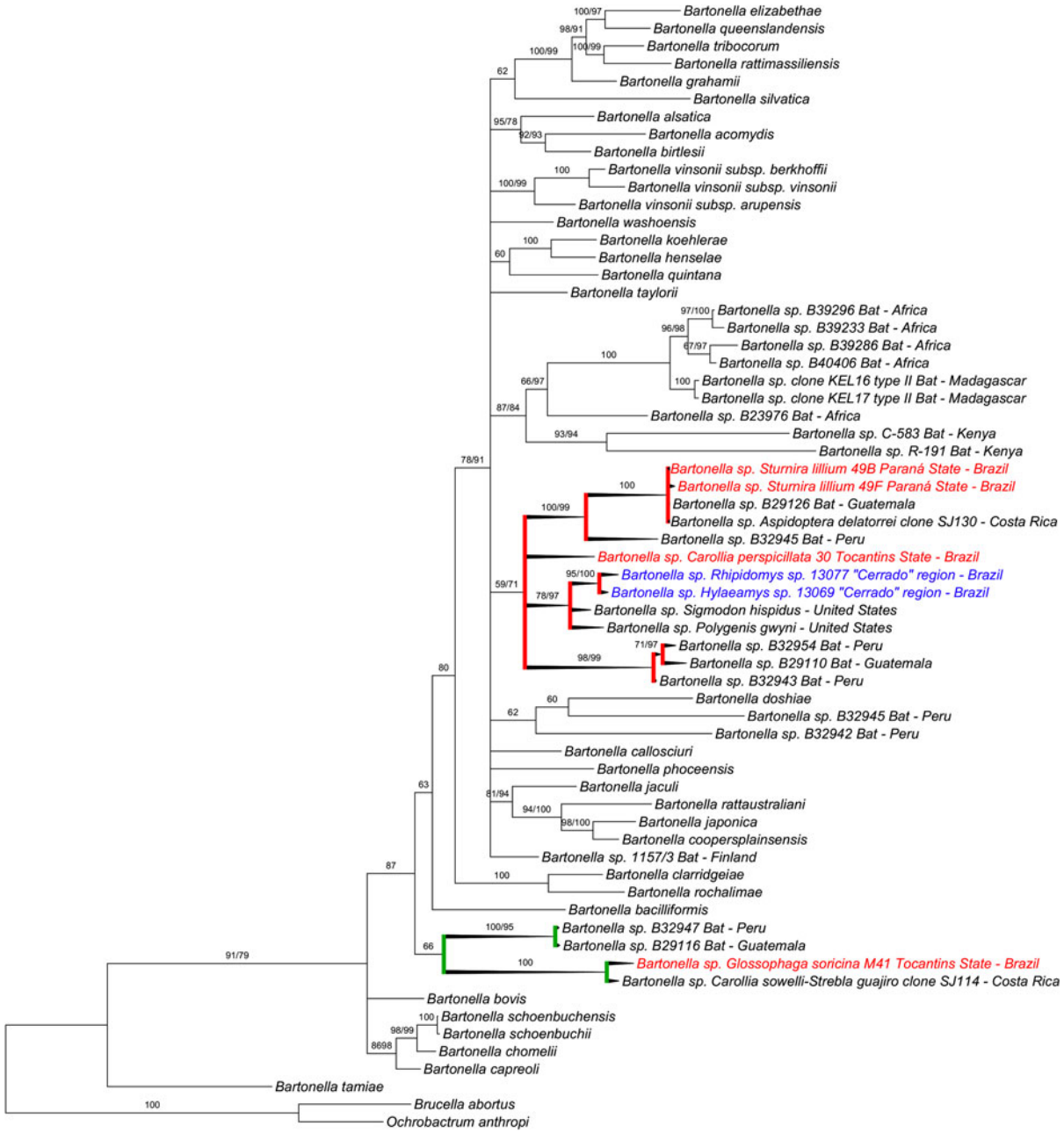

Fig. 2. Concatenated phylogenetic analysis of *Bartonella nuoG* and *gltA* sequences (3370 bp after alignment) based on the topology generate on the BI method. The values of support of posterior probability/bootstrap higher than 50% are shown in each branch. The sequences of the present work were highlighted in red composing two different clusters. One comprising three sequences of the present study (red branches) clustering with sequences obtained from bats from Guatemala and Peru and from rodents sampled in Brazil (sequences highlighted in blue) and in the USA with a 59/71% of probability. The other cluster (green branches) comprises one sequence obtained in the present study with sequences obtained from bats sampled in Costa Rica, Peru, and Guatemala, with 66% of probability. *Brucella abortus* and *Ochrobactrum anthropi* were used as outgroups.

(100% of branch support) to a *Bartonella* genotype detected in a deer in Japan (AB703117), *Bartonella chomelii* (KM215689/AB290193) and *Bartonella schoenbuchensis* (AF467765) (Fig. 4).

All five 16S rRNA hemoplasmas sequences detected in specimens of *M. molossus* sampled in the states of Paraná and Pará shared identity (93–96%) with *Mycoplasma coccoides* (AY171918), with query

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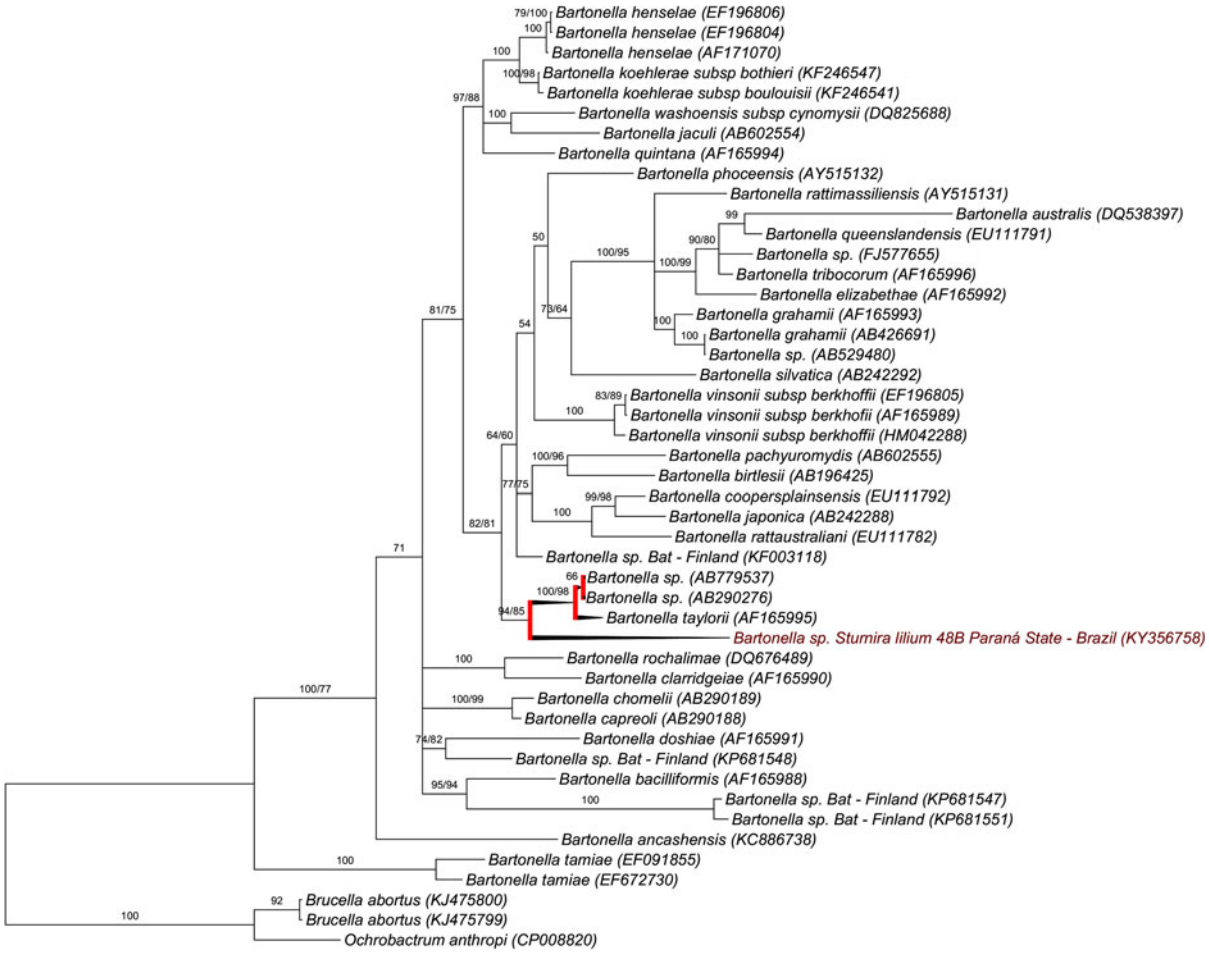

Fig. 3. Phylogenetic analysis of *Bartonella rpoB* sequences (1500 bp after alignment) based on the topology generate on the BI method. The values of support of posterior probability/bootstrap higher than 50% are shown in each branch. The sequence of the present work is highlighted in red. The sequence was positioned alone in one branch and clustered with *Bartonella* sp. from wild mammals from Asia with 94/85% of probability (red branches). *Brucella abortus* and *Ochrobactrum anthropi* were used as outgroups.

coverage ranging from 87% to 100%. Additionally, the found hemoplasma sequences showed 92–96% identity with an uncultured *Mycoplasma* sp. detected in an *Akodon* rodent from Brazil (KT215636), with query coverage ranging from 87% to 99%. All the hemoplasmas sequences obtained in the present study clustered together as a monophyletic group with branch support of 95/100% by ML and BI analyses. This group of sequences was positioned between *M. coccoides* (AY171918) and hemoplasma genotypes detected in rodents from Brazil (KM203857; KT215636). The hemoplasmas sequences detected in bats in the present study were positioned in the *Haemofelis* group (Figure 5).

DISCUSSION

The present work reported the occurrence and molecular characterization of *Bartonella* spp. and *Mycoplasma* spp. among bat's population sampled in five different states of Brazil. This group of mammals has been incriminated as potential reservoirs for several pathogens due to their high mobility, worldwide distribution, and social behavior [35].

The *Bartonella* occurrence found in the present study (5·28%) was similar to that found in bats (3·38% (13/384)) sampled in Swaziland and South Africa, Africa [40]. Overall there is a relatively higher occurrence of *Bartonella* in bats sampled in the Old World, such as in Algeria (60% (6/10) by qPCR) [39], Nigeria (51·35% (76/148) by qPCR and 15·54% (23/148) by culture) [30], Kenya (32·02% (106/331) by culture) [4], and Madagascar (44·68% (21/47) by PCR) [33]. In neotropical species, *Bartonella* species have been already detected by solid culture followed by cPCR or only by PCR in bats sampled from Guatemala, Peru, and Costa Rica, whose

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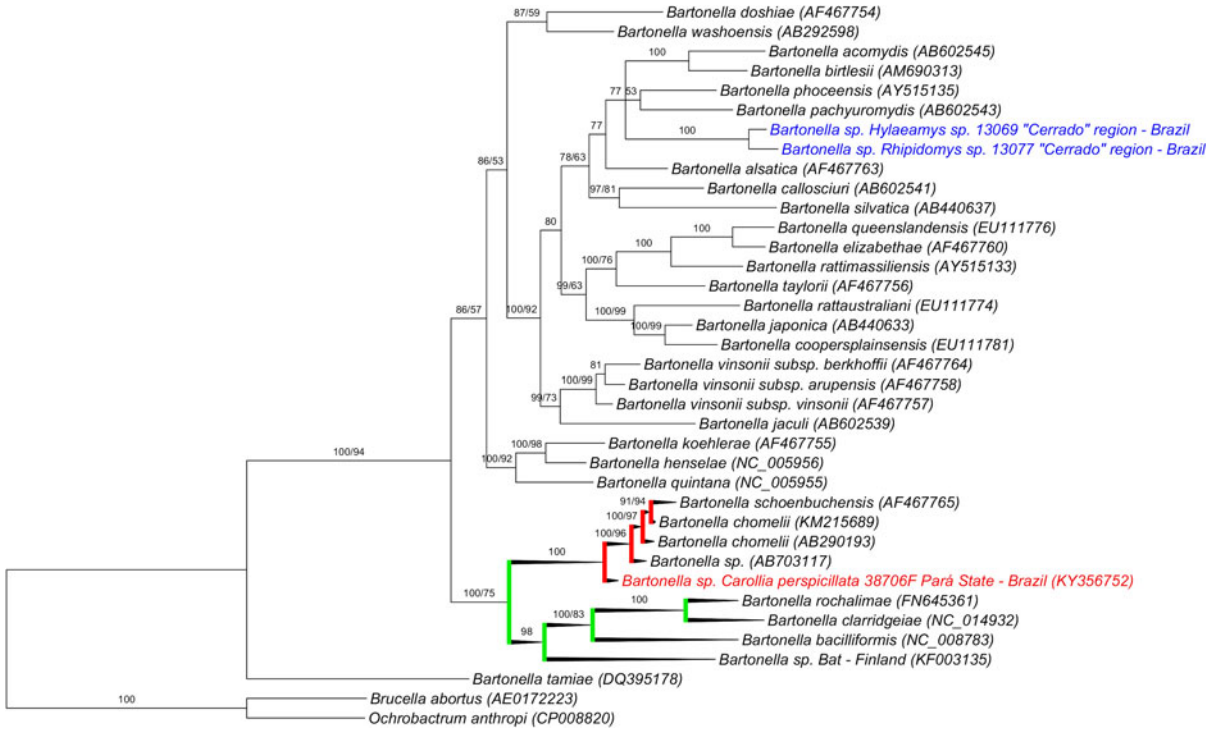

Fig. 4. Phylogenetic analysis of *Bartonella ftsZ* sequences (790 bp after alignment) based on the topology generate on the BI method. The values of support of posterior probability/bootstrap higher than 50% are shown in each branch. The *Bartonella* sequence of the present work was highlighted in red. The *Bartonella* sequences highlighted in blue were previously detected in rodents from the same research group. The *Bartonella* sequence detected in a specimen of *Carollia perspicillata* was positioned alone in one branch and clustered with *Bartonella* sp. detected in a deer from Asia and other *Bartonella* spp. with 100% of probability (red branches). A *Bartonella* genotype detected in a bat sampled in Finland was closely positioned with the sequence of the present study, with 100/75% of probability (green branches). *Brucella abortus* and *Ochrobactrum anthropi* were used as outgroups.

overall prevalence ranged from 24·1% to 33·3% [29, 34, 35].

Although a low occurrence of *Bartonella* in *S. lilium* and *G. soricina* bat specimens is reported [35], these animals together with *C. perspicillata* were the main Chiroptera species that showed positivity for *Bartonella* in the present study. Despite the high number of *Molossus* spp. individuals (29·01% (47/162)) sampled in the present study, none of them was positive for *Bartonella* sp., as previously reported [29] as well. *Bartonella* genotypes have already been described previously in the bat species sampled in the present work [29, 35], except for *N. espiritosantensis*.

Even though it is suggested that the spleen is the sample of choice to detect *Bartonella* spp. [69], no difference was observed among the tissues collected in the present study, since seven were from blood, six from splenic tissue, and four from liver tissue.

Bartonella host transitions are supposed to occur with bats within the same family, but transitions between bats belonging to different families, superfamilies and suborders seem to occur infrequently [70]. It had already been hypothesized that a different pressure on the coevolutionary relationships between Old and New World bats host species, since Old World species seem to be more rustic and show a longer evolutionary time for the establishment of this sort of relationship [71]. This hypothesis could explain the different positioning of *Bartonella* genotypes detected in neotropical bats, including those sampled in the present study, when compared with those from Africa (Megachiroptera/Yangpteroptera). However, it is noteworthy to observe that *Bartonella* genotypes detected in bats from the present study were closely related to those detected in wild mammals, such as rodents and deer from Asia, based on *ftsZ* and *rpoB* phylogenetic analyses. Regarding to these findings, intra- and inter-specific *Bartonella* transmission has been already suggested, since different *Bartonella* species/genotypes can be found in only one individual [35].

Interestingly, *Bartonella* genotypes detected in Brazilian rodents were positioned in the same clade

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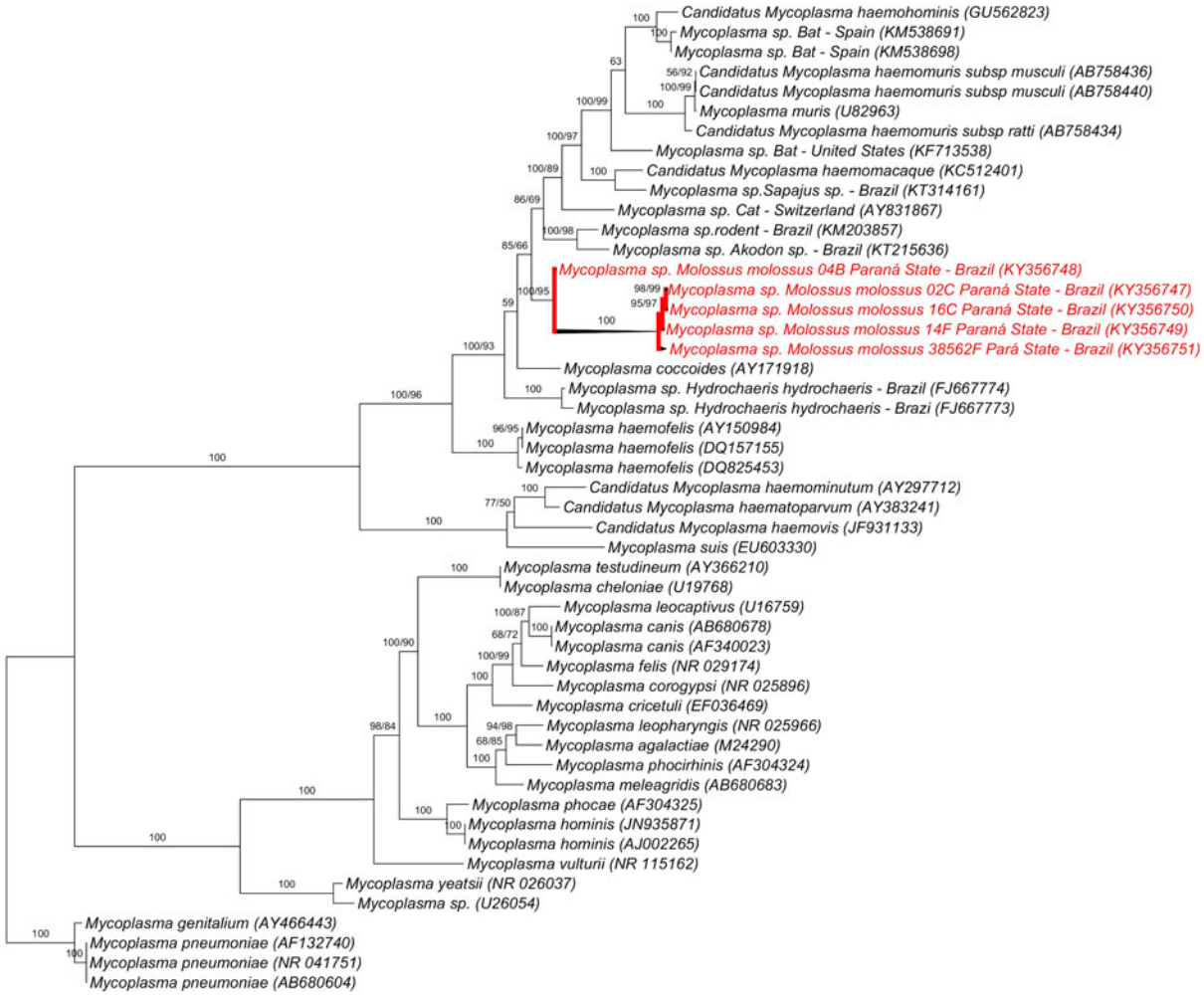

Fig. 5. Phylogenetic analysis of hemoplasmas 16SrRNA sequences (1770 bp after alignment) based on the topology generate on the BI method. The values of support of posterior probability/bootstrap higher than 50% are shown in each branch. The sequences of the present work were highlighted in red and placed together in a same branch with 100/95% of probability (red branches). *Mycoplasma pneumoniae* was used as outgroup.

with the sequences obtained from bats sampled in the present study with a 59/71% of node support based on the concatenated *nuoG* and *gltA* analysis. However, a *Bartonella* genotype detected in a specimen of *G. soricina* sampled in the state of Tocantins was positioned distant from the other *Bartonella* genotype detected in the state of Paraná, clustering with a *Bartonella* genotype detected in specimens of *C. sowelli* and its Streblidae dipteran, namely *S. guajiro from* Costa Rica, based in *gltA*/*nuoG* concatenated analysis. These findings suggest the occurrence of different *Bartonella* genotypes among bats in Brazil. On the other hand, *Bartonella* genotypes amplified from different tissue samples (spleen and liver) belonging to a specimen of *S. lilium* sampled in Paraná state clustered together, probably representing the same *Bartonella* genotype.

Regarding the occurrence of hemoplasmas among bats, *Mycoplasma* spp. has been poorly reported in this group of mammals. Despite the *Molossus* spp. was the main group of bats positive for hemoplasmas in the present study, to the best authors' knowledge, this is the first molecular detection of hemoplasmas in eight bat species, namely *A. planirostris*, *Eptesicus* sp., *Eumops auripendulus*, *G. soricina*, *M. molossus*, *M. rufus*, *M. nigricans* and *S. lilium.*

High prevalence rates for hemoplasmas have been found in small populations of bats sampled in the USA (47% (32/68)) [12] and Spain (41·93% (13/31)) [13]. Interestingly, hemoplasmas genotypes detected in bats from both studies were similar to each other, but different for *Mycoplasma* genotypes detected in bats sampled in Brazil. The hemoplasmas sequences

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detected in bats sampled in the USA and Spain clustered with '*Candidatus* Mycoplasma haemohominis' that was detected in a human patient in England [72], and with *Mycoplasma haemomuris*, which has been detected in domestic, laboratory, and wild rodents [8]. Based on the phylogenetic positioning, hemoplasmas genotypes detected in bats in the present study seem to be different from those previously detected in bats sampled in the USA and Spain, since comprised a monophyletic group with high node support and closely related to *M. coccoides*.

Despite the higher positivity to hemoplasmas than to *Bartonella* spp., only one animal was co-positive for both studied pathogens. The pathogenic potential of both group of pathogens in bats have not been investigated.

Finally, it is important to highlight that *Bartonella* and *Mycoplasma* genotypes detected in bats from the present study were closely related to previously reported genotypes detected in rodents. From an evolutionary point of view, bats and rodents are large groups belonging to ancient orders of mammals [71], which have shown a high adaptation as hosts for different groups of pathogens.

To the best of authors' knowledge, the present work presented the first molecular evidence of circulation of *Bartonella* and hemoplasmas among bats in Brazil. Future studies aiming at assessing the role of bats as reservoirs for *Bartonella* and *Mycoplasma* species showing zoonotic potential are much needed.

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DECLARATION OF INTEREST

None.

ETHICAL STANDARDS

The authors assert that all procedures contributing to this work comply with the ethical standards according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and to the 2000 Report of the AVMA Panel on Euthanasia (American Veterinary Medical Association, 2001).

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