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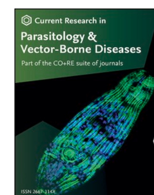

Isolation and molecular characterization of a novel relapsing fever group *Borrelia* from the white-eared opossum *Didelphis albiventris* in Brazil

Barbara C. Weck [a,**], Adriana Santodomingo [b], Maria Carolina A. Serpa [a], Glauber M.B. de Oliveira [a], Felipe R. Jorge [a], Sebastián Muñoz-Leal [b], Marcelo B. Labruna [a,*]

[a] *Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva 87, São Paulo, SP, 05508-270, Brazil*
[b] *Department of Animal Science, Faculty of Veterinary Sciences, University of Concepción, Chillán, Ñuble, Chile*

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ABSTRACT

This study aimed to detect, isolate and to characterize by molecular methods a relapsing fever group (RFG) *Borrelia* in white-eared opossums (*Didelphis albiventris*) from Brazil. During 2015–2018, when opossums (*Didelphis* spp.) were captured in six municipalities of the state of São Paulo, Brazil, molecular analyses revealed the presence of a novel RFG *Borrelia* sp. in the blood of seven opossums (*Didelphis albiventris*), out of 142 sampled opossums (4.9% infection rate). All seven infected opossums were from a single location (Ribeirão Preto municipality). In a subsequent field study in Ribeirão Preto during 2021, two new opossums (*D. albiventris*) were captured, of which one contained borrelial DNA in its blood. Macerated tissues from this infected opossum were inoculated into laboratory animals (rodents and rabbits) and two big-eared opossums (*Didelphis aurita*), which had blood samples examined daily *via* dark-field microscopy. No spirochetes were visualized in the blood of the laboratory animals. Contrastingly, spirochetes were visualized in the blood of the two *D. aurita* opossums between 12 and 25 days after inoculation. Blood samples from these opossums were used for a multi-locus sequencing typing (MLST) based on six borrelial loci. Phylogenies inferred from MLST genes positioned the sequenced *Borrelia* genotype into the RFG borreliae clade basally to borreliae of the Asian-African group, forming a monophyletic group with another Brazilian isolate, "*Candidatus* B. caatinga". Based on this concatenated phylogenetic analysis, which supports that the new borrelial isolate corresponds to a putative new species, we propose the name "*Candidatus* Borrelia mimona".

1. Introduction

Tick-borne spirochetes of the relapsing fever group (RFG) occur in sylvatic transmission cycles in tropical and subtropical regions of the world, infecting wild vertebrates and mainly ticks of the genus *Ornithodoros* (Margos et al., 2008). Ticks get infected while feeding on spirochetemic vertebrates (e.g. mammals and birds) and maintain the infection for several weeks to years (Barbour et al., 2005). Humans may be exposed to these pathogens in environments where *Ornithodoros* spp. occur (Barbour et al., 2005). When bitten by infected ticks, humans can experience episodes of recurrent fever, which is a typical symptom of tick-borne relapsing fever, accompanied by non-specific symptoms (Madison-Antenucci et al., 2020). In Brazil, nine species of *Ornithodoros* have already been reported parasitizing humans: *Ornithodoros brasiliensis*, *Ornithodoros rostratus*, *Ornithodoros fonsecai*, *Ornithodoros marinkellei*, *Ornithodoros mimon*, *Ornithodoros rietcorreai*, *Ornithodoros rudis*, *Ornithodoros tabajara* and *Ornithodoros hasei* (Aragão, 1923; Cançado et al., 2008; Labruna and Venzal, 2009; Labruna et al., 2011, 2014; De Oliveira et al., 2018; Muñoz-Leal et al., 2018, 2021a, 2021b). Although six genotypes of RFG *Borrelia* have been detected in some of those human-biting *Ornithodoros* ticks (Muñoz-Leal et al., 2021a), only two species have been isolated in the country: *Borrelia venezuelensis* from *O. rudis* (Muñoz-Leal et al., 2018), and "*Candidatus* Borrelia caatinga" from an *Ornithodoros* sp. phylogenetically related to *Ornithodoros tabajara* (Oliveira et al., 2023a). All these findings have been obtained from ticks, while vertebrate hosts or reservoirs in nature remain almost unknown.

South American small mammals were implicated as hosts of *Borrelia*

* Corresponding author.
** Corresponding author.
E-mail addresses: barbara.weck@nih.gov (B.C. Weck), labruna@usp.br (M.B. Labruna).

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spirochetes at the beginning of the 20th century (Dunn and Clark, 1933; Pifano, 1941). More recently, molecular detection of RFG borreliae were made in rodents from Chile (Thomas et al., 2020) and in opossums (*Didelphis marsupialis*) from Colombia (López et al., 2023). As there are no studies of RFG *Borrelia* infecting small mammals in Brazil, the present study aimed to detect, isolate and to characterize by molecular methods a RFG *Borrelia* detected in white-eared opossums (*Didelphis albiventris*) from Brazil.

2. Materials and methods

During 2015–2018, small wild mammals were captured in six areas of the state of São Paulo for an extensive study on ticks and tick-borne rickettsiae, as reported elsewhere (Serpa et al., 2021). These areas were located in six municipalities (Americana, Araras, Piracicaba, Pirassununga, Ribeirão Preto, and São Paulo), and were composed by degraded/regenerated forest fragments of the Cerrado or Atlantic Forest biomes. Descriptions and illustrations of the six areas have been provided elsewhere (Luz et al., 2019). Small mammals were captured with Tomahawk and Sherman-like traps and anesthetized with an intramuscular injection of ketamine (100 mg/kg)-xylazine (10 mg/kg), as previously described (Serpa et al., 2021). Blood samples were collected in 1.5-ml tubes from all trapped animals (Serpa et al., 2021); however, for the present study, we evaluated only the samples collected from opossums (*Didelphis* spp.).

2.1. Spirochete detection

DNA was extracted from opossum blood by using the DNeasy Blood and Tissue and Blood Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. To verify the success of extraction, an initial PCR targeting the mammalian mitochondrial cytochrome *b* gene (*cytb*) was performed (Steuber et al., 2005). Positive samples were then screened for *Borrelia* DNA with real-time PCR using genus-specific primers and a probe targeting a 148-base pair (bp) fragment of the *Borrelia 16S* rRNA gene (Parola et al., 2011). Real-time PCR-positive samples were submitted to different conventional PCR protocols to obtain fragments of three borrelial genes, *16S* rRNA, *flaB*, and *rrs-rrlA* intergenic spacer (IGS) (Table 1). PCR assays were performed in a total volume of 25 µl, using DreamTaq Green PCR Master Mix (Thermo Scientific, Carlsbad, CA, USA). DNA of *B. venezuelensis* RMA01 (Muñoz-Leal et al., 2018) was employed as a positive control and negative controls consisted of ultrapure water.

PCR products were resolved in 1.5% agarose gels; amplicons with expected size were purified and prepared for sequencing with the BigDye kit (Applied Biosystems, Foster City, CA, USA). An ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was employed for sequencing purposes using the PCR primers. The obtained sequences were subjected to BLASTn analyses to check their highest identities with the congeneric organisms available in GenBank (Altschul et al., 1990).

2.2. Spirochete isolation

Because *Borrelia* DNA was detected by PCR in opossums from one specific area during the 2015–2018 field study, in May 2021 we attempted to capture opossums by using 60 Tomahawk traps during four nights in that same area, with the purpose to obtain samples to isolate viable spirochetes. Captured animals were blood-sampled, euthanized by increasing anesthetic doses, and necropsied to collect fragments of spleen, liver, and lungs. Blood and organs were collected in 2-ml cryotubes, immediately frozen in a box containing dry ice, which was transported to the laboratory where the cryotubes were stored in liquid nitrogen for further tentative isolation of spirochetes. Moreover, duplicated samples were also collected in 1.5-ml tubes and stored at −20 °C for DNA extraction and molecular analyses.

Blood and spleen samples from the 2021-captured opossums were submitted to DNA extraction with the DNeasy Blood and Tissue Kit. A PCR targeting the mammalian mitochondrial cytochrome *b* (*cytb*) gene (Steuber et al., 2005) was performed; then the DNA samples were screened for *Borrelia* DNA with the same above-mentioned real-time PCR assay (Parola et al., 2011). Attempts to isolate spirochetes were performed with cryopreserved duplicates of blood and spleen of one white-eared opossum (*D. albiventris*) that yielded amplicons by real-time PCR screening. For this purpose, the samples were thawed at room temperature and both the blood and spleen were macerated together in 5 ml of sterile phosphate-buffered saline (PBS). The opossum tissue homogenate was inoculated intraperitoneally (0.5 ml per animal) into two guinea pigs (*Cavia porcellus*), two vesper mice (*Calomys callosus*), two golden hamsters (*Mesocricetus auratus*), two New Zealand white rabbits (*Oryctolagus cuniculus*), and two big-eared opossums (*Didelphis aurita*). All these animals were obtained from laboratory animal rooms, except for the *D. aurita* opossums, which were captured three weeks before in a small forest fragment of the University of São Paulo campus in São Paulo City. At the inoculation day (Day 0), blood samples (≈1 ml) were collected from the tail vein of the two opossums immediately prior to inoculation and were tested for *Borrelia* DNA using the PCR method described above, to certify that they did not contain borreliae in their

Table 1
Primer pairs used in the present study for amplification of three *Borrelia* genes by conventional PCR assays.

Gene/Primer		Primer sequence (5′-3′)	Amplicon size (bp)	Reference
16S rRNA	FD3	F: AGAGTTTGATCCTGGCTTAG	1540	Ras et al. (1996)
	T50	R: GTTACGACTTCACCCTCCT		
	FD3	F: AGAGTTTGATCCTGGCTTAG	729[a]	Schwan et al. (2005)
	16S-1	R: TAGAAGTTCGCCTTCGCCTCTG		
	16S-2	F: TACAGGTGCTGCATGGTTGTCG	513[a]	Schwan et al. (2005)
	T50	R: GTTACGACTTCACCCTCCT		
	Rec-4	F: ATGCTAGAAACTGCATGA	520[a]	Ras et al. (1996)
	Rec-9	R: TCGTCTGAGTCCCCATCT		
flaB	FlaLL	F: ACATATTCAGATGCAGACAGAGGT	665	Stromdahl et al. (2003)
	FlaRL	R: GCAATCATAGCCATTGCAGATTGT		
	FlaLL	F: ACATATTCAGATGCAGACAGAGGT	485[a]	
	FlaRS	R: CTTTGATCACTTATCATTCTAATAGC		
	FlaLS	F: AACAGCTGAAGAGCTTGGAATG	522[a]	
	FlaRL	R: GCAATCATAGCCATTGCAGATTGT		
rrs-rrlA	IGS-F	F: GTATGTTTAGTGAGGGGGGTG	987	Bunikis et al. (2004)
	IGS-R	R: GGATCATAGCTCAGGTGGTTAG		
	IGS-Fn	F: AGGGGGGTGAAGTCGTAACAAG	945[a]	
	IGS-Fr	R: GTCTGATAAACCTGAGGTCGGA		

Abbreviations: F, forward; R, reverse.
[a] Amplified by a nested or heminested reaction.

blood prior to inoculation).

One day before inoculation, a drop of blood (≈2.5 μl) was obtained from each animal (guinea pigs, mice, hamsters, rabbits, opossums), by ear or tail vein-puncture, expressed onto glass slides, and visually monitored by dark-field to detect the presence of motile spirochetes. This procedure was repeated daily from Day 0 (inoculation day) to 14 days after inoculation. The animals that did not show any motile spirochetes during this 14-day period were considered negative and were not bled anymore. When showing spirochetes, daily examinations by dark-field microscopy were extended to Day 30 post-inoculation. The mean number of spirochetes per field was calculated by counting the total number of motile spirochetes in 50 microscope fields at 200× magnification, dividing it by 50; results as decimal numbers were always rounded up, as previously described (Oliveira et al., 2023a). Within this period, blood was collected into 2-ml cryotubes and stored at −80 °C for future studies, and in 1.5-ml tubes to perform DNA extraction and genetic characterization of the detected spirochetes (using the same above-described PCR protocols). After sequencing those three loci (*16S* rRNA, *flaB*, and IGS) and identifying by BLASTn analyses (Altschul et al., 1990) that the detected *Borrelia* sp. belonged to the RFG, we attempted to perform a multi-locus sequence typing (MLST) by amplifying portions of the *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *nifS*, *rlpB*, and *uvrA* genes with degenerate primers available in the *Borrelia* MLST database (http://pubmlst.org/borrelia).

2.3. Phylogenetic analyses

Orthologous sequences recovered from GenBank (Sayers et al., 2020) and PubMLST database (https://pubmlst.org/organisms/borrelia-spp) coupled with sequences obtained in this study were aligned with MAFFT using default parameters (Katoh and Standley, 2013). Subsequently, the alignments were curated with Block Mapping and Gathering with Entropy (BMGE) using default parameters to map informative regions for phylogenetics inferences (Criscuolo and Gribaldo, 2010).

Phylogenetic analyses were conducted using Maximum likelihood (ML) (Felsenstein, 1981) and Bayesian inference (BI) (Rannala and Yang, 1996; Yang and Rannala, 1997) methods in IQ-TREE v. 1.6.12 (Nguyen et al., 2015) and MrBayes v. 3.2.6 (Ronquist et al., 2012), respectively. As protein-coding genes present different nucleotide exchange rates (heterogeneity) at the first, second and third codon position (Yang, 1996; Ronquist et al., 2012); datasets were partitioned into the three codon positions (position-1, position-2 and position-3) (Yang, 1996; Lanfear et al., 2012; Ronquist et al., 2012; Kainer and Lanfear, 2015).

ML best-fit evolutionary models and best-partition scheme for protein-encoding datasets were calculated using the ModelFinder command “TESTNEWONLYMERGE -mrate G” (Kalyaanamoorthy et al., 2017). To assess the robustness of the inferred tree, we employed a combination of hill-climbing approaches and a stochastic disturbance method, complemented by an ultrafast bootstrap approach (UFBoot) with 1000 iterations (Minh et al., 2013; Nguyen et al., 2015). UFBoot values $< 70\%$, between 70 and 94%, and $\geq 95\%$ indicated non-significant, moderate, and high statistical support, respectively (Minh et al., 2013).

BI phylogenies were constructed based on nucleotide substitution models selected with the MrBayes command “lset nst = mixed rates = invgamma” (Huelsenbeck et al., 2004; Ronquist et al., 2012; Lanfear et al., 2012), with partition schemes determined by ModelFinder. The robustness of the inferred BI tree was evaluated by sampling trees every 1000 generations, with the first 25% removed as “burn-in”, implementing four Markov chain Monte Carlo (MCMC) chains through two independent tests of 20×10^6 generations. The correlation and effective sample size (ESS) of the MCMCs were confirmed using Tracer v1.7.1 (Rambaut et al., 2018). Nodes with Bayesian posterior probabilities (BPP) > 0.70 were considered high statistical support (Huelsenbeck and Rannala, 2004). All best-fit models and partitions schemes were selected based on Bayesian Information Criteria (BIC) (Schwarz, 1978). Trees were visualized and edited with FigTree v 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v. 1.1 (https://inkscape.org/es/). Consensus trees for both ML and BI were generated following the approach outlined by Santodomingo et al. (2022).

3. Results

Overall, blood samples were collected from 142 opossums (131 *D. albiventris* and 11 *D. aurita*) during the 2015–2018 field study in six areas of the state of São Paulo (Table 2). PCR assays yielded fragments of expected size for the borrelial *16S* rRNA, *flaB* and *rrs-rrlA* (IGS) genes of seven blood samples (opossums RP001, RP014, RP018, RP069, RP22, RP34 and RP58) all yielding identical sequences for each locus. After BLASTn comparisons, the *16S* rRNA (1346 bp) and *flaB* (504 bp) sequences were 100% identical with *Borrelia* sequences retrieved from the tick *Ornithodoros mimon* (MT013211and MT076262, respectively) collected from a human household in the urban area of the State of Mato Grosso, Brazil (Muñoz-Leal et al., 2021a). The IGS partial sequence was 88% identical to sequences of a *Borrelia* sp. (MN598782, MN598783) obtained from the blood of yellow-rumped leaf-eared mice (*Phyllotis xanthopygus*) from Chile (Thomas et al., 2020). The seven opossums that yielded borrelial DNA were captured in the same area, Ribeirão Preto, at different years, as follows: opossum RP001 during 2015; opossums RP014, RP018, RP022 and RP034 during 2016; and opossums RP58 and RP069 during 2017. No borrelial DNA was detected in the opossums from the remaining five sampled areas (Table 2).

In 2021, we performed a four-night field work in the same forest fragment of Ribeirão Preto (Fig. 1), when only two opossums (*D. albiventris*) were captured. No ticks were recovered on the captured animals. One of these animals (BW2) had spleen and blood samples that produced amplicons of expected size for *16S* rRNA, *flaB* and *rrs-rrlA* (IGS) genes. These sequences were identical to those described previously from opossums RP001, RP014, RP018, RP022, RP034, RP058 and RP069).

In the attempts to isolate the detected borrelial agent through animal inoculations, only the two *D. aurita* opossums (#1 and #2) developed detectable spirochetemia, as shown by motile spirochetes in their blood. Some of these spirochetes were also shown by Giemsa-stained blood smears (Fig. 2). The other inoculated animals (guinea pigs, vesper mice, hamsters, and rabbits) did not show spirochetes in the dark-field microscopy during the experiment. In opossum #1, a mean of ≤ 1 spirochete/microscope field was visualized from Day 12 to Day 15 post-inoculation, then increased to 20 spirochetes/field from Day 16 to Day 18, decreasing gradually until ≤ 1 spirochete/field on Day 25, the last day when spirochetes were observed (Fig. 3). In opossum #2, spirochetes were observed from Day 12 to Day 21 after inoculation, with maximal numbers of 4 spirochetes/field on Days 16 and 17.

We collected blood samples from opossums #1 and #2 at the inoculation day (Day 0) and on Days 3, 9, 15, 16, and 30 post-inoculation, and performed a DNA extraction and real-time PCR to evaluate the

Table 2
Results of molecular tests for the detection of *Borrelia* DNA in the blood of opossums (*Didelphis* spp.) captured in forest fragments in six municipalities of the state of São Paulo, Brazil, during 2015–2018.

Municipality	No. of opossums with *Borrelia*/No. of tested opossums (% positivity)		
	Didelphis albiventris	*Didelphis aurita*	Total
Americana	0/38 (0)		0/38 (0)
Araras	0/10 (0)		0/10 (0)
Piracicaba	0/18 (0)	0/1 (0)	0/19 (0)
Pirassununga	0/24 (0)		0/24 (0)
Ribeirão Preto	7/41 (17.1)		7/41 (17.1)
São Paulo		0/10 (0)	0/10 (0)
Total	7/131 (5.3)	0/11 (0)	7/142 (4.9)

Fig. 1. Location where *Borrelia*-infected opossums (*Didelphis albiventris*) were trapped in Ribeirão Preto municipality, Brazil. Note the bamboo grove in the background, where the traps were placed in and around for four consecutive nights in 2021.

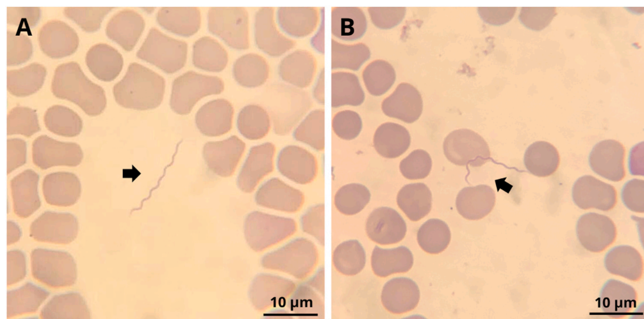

Fig. 2. Giemsa-stained blood smear of opossum #1 (**A**) and opossum #2 (**B**) showing spirochetes (*black arrows*). Original magnification: 1000× .

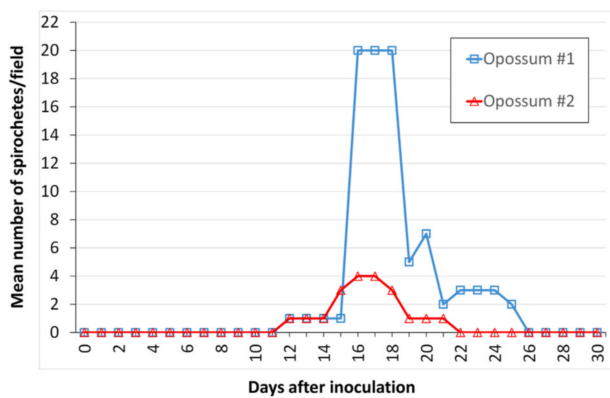

Fig. 3. Results of dark-field examination of blood samples of opossums #1 and #2 (*Didelphis aurita*) according to the number of days after inoculation with blood- and spleen samples that were collected from a *Borrelia*-infected opossum (*Didelphis albiventris*) from Ribeirão Preto, Brazil. Values presented as the mean number of motile spirochetes per microscope field at 200× magnification in each sampling day.

presence of spirochetes in blood. On Days 0, 3 and 30 the real-time was negative, on Day 9 it was positive even without spirochetes in dark field microscopy observation. Samples from days 15 and 16 were also PCR-positive. The sample from Day 16 (spirochetemia peak) from opossum #1 was submitted to molecular characterization (MLST) of the borrelial isolate.

As the *16S* rRNA (1346 bp) and *flaB* (504 bp) sequences showed 100% of identity with *Borrelia* sp. from *O. mimon* (MT013211 and MT076262, respectively), we did not construct phylogenetic trees with these genes because these sequences have been represented in phylogenetic trees reported by Muñoz-Leal et al. (2021a). Here, phylogenies were inferred for IGS and MLST sequences. The IGS tree depicts the detected *Borrelia* sp. into a monophyletic clade with other two *Borrelia* spp. that matched closer after BLASTn comparisons (*Borrelia* sp. BoA10 and *Borrelia* sp. Bo44) (Fig. 4). In the MLST, expected amplicons were obtained for six genes (*clpX*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*). Phylogenies inferred from MLST genes positioned the sequenced *Borrelia* genotype into the clade of the RFG borreliae basally to borreliae of the Asian-African group (Fig. 5). Notably, "*Candidatus* B. caatinga" formed a monophyletic group with the *Borrelia* sp. detected in this study. This clade also exhibited a close relationship with "*Candidatus* Borrelia octodonta", recently reported infecting *Ornithodoros octodontus* in Chile (Santodomingo et al., 2024). Based on this concatenated phylogenetic analysis, which supports that the new borrelial isolate corresponds to a putative new species, we propose the name "*Candidatus* Borrelia mimona", in allusion to the tick species (*O. mimon*) in which it was firstly reported by Muñoz-Leal et al. (2021a).

4. Discussion

In this study, we detected a novel RFG borrelial agent, "*Candidatus* B. mimona" infecting opossums (*D. albiventris*) in the state of São Paulo, Brazil. Besides a detection rate of 17.1% of infected opossums in our first field campaign during 2015–2018 in Ribeirão Preto (Table 2), we further isolated the agent from an opossum that was captured during the 2021 field-study. Our repeated findings of the agent in different opossums in the same area during different years from 2015 to 2021 indicates that "*Candidatus* B. mimona" is an agent probably established in

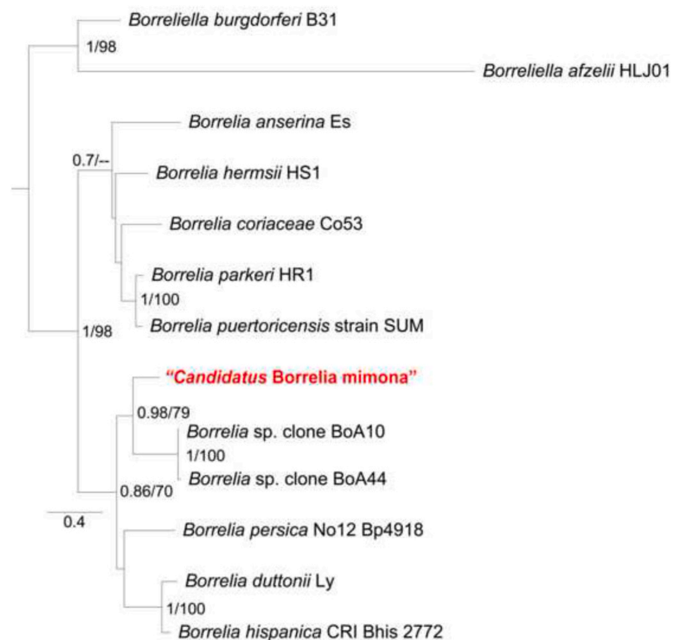

Fig. 4. Phylogenies of *Borrelia* spp. inferred for *rrs-rrlA* intergenic spacer (IGS). The IGS tree is based on 13 sequences and an alignment of 501 bp. Best-fit evolutionary models calculated for Maximum likelihood and Bayesian inference methods were HKY+F+I+G4, and M_{50}, M_{85}, M_{15}, M_{122}, M_{147}, M_{177}, M_{152}, M_{157}, respectively. The position of "*Candidatus* Borrelia mimona" is highlighted in bold red font. Ultrafast bootstrap values and Bayesian posterior probabilities (BPP) are indicated above or below each branch. The scale-bar indicates the number of nucleotide substitutions per site. GenBank accession numbers of the selected sequences are shown in Supplementary Table S1.

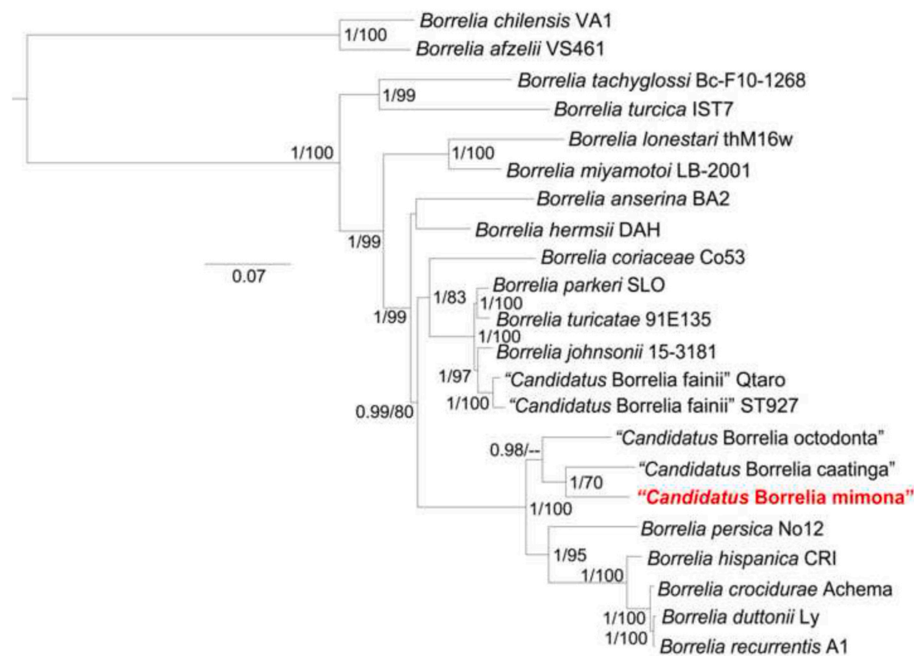

Fig. 5. Phylogenies of *Borrelia* spp. from the relapsing fever group (RFG) inferred for concatenated *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *nifS*, *rplB*, and *uvrA* genes (MLST scheme). The MLST tree is based on 22 sequences and an alignment of 4776 bp. Gaps were treated as missing data. Best-fit evolutionary models calculated for Maximum Likelihood and Bayesian inference methods were GTR+F+G4 (position-1); GTR+F+G4 (position-2); TVM+F+G4 (position-3), and M_{202}, M_{175}, M_{203}, M_{193} (position-1); M_{200}, M_{203}, M_{134}, M_{198}, M_{189}, M_{190} (position-2); M_{195}, M_{157}, M_{147} (position-3), respectively. The position of the "*Candidatus* Borrelia mimona" characterized in the present study is highlighted in red font. Ultrafast bootstrap values and Bayesian posterior probabilities (BPP) are indicated above or below branches. The scale-bar indicates the number of nucleotide substitutions per site. Sequence Type (ST) numbers of the sequences used for the MLST phylogeny are shown in Supplementary Table S2.

the opossum population of the study area of Ribeirão Preto.

Marsupials of the genus *Didelphis* are mammals geographically restricted to the American continent (Gardner, 2008), and their implication as a reservoir for borreliae has already been addressed in earlier studies in Panama and Venezuela, where spirochetes were visualized in blood smears of *Didelphis marsupialis* (Dunn and Clark, 1933; Pifano, 1941). Although the opossum species mentioned by Pifano (1941) was *D. aurita*, it is currently accepted that this species does not occur in Venezuela, where the only *Didelphis* species with black ears is *D. marsupialis* (Emmons and Feer, 1997). More recently, López et al. (2023) reported the molecular detection of *Borrelia puertoricensis* in the blood of opossums (*D. marsupialis*) from Colombia.

Partial sequences of the *16S* rRNA and *flaB* genes of "*Candidatus* B. mimona" were 100% identical to *16S* rRNA and *flaB* haplotypes of *Borrelia* sp. O mimon 2 MT, recently detected in *O. mimon* from a household in the urban area of Cuiabá City, Mato Grosso State, central-western Brazil (Muñoz-Leal et al., 2021a). This result indicates that they represent the same agent; therefore, *O. mimon* could represent a possible vector of "*Candidatus* B. mimona". Interestingly, opossums of the genus *Didelphis* are among the most common hosts of *O. mimon* in Brazil (Labruna et al., 2014; Sponchiado et al., 2015). In fact, at least one of the captured opossums of our 2015–2018 field study was infested by *O. mimon*, as reported by Serpa et al. (2021). Indeed, the role of *O. mimon* as vector of "*Candidatus* B. mimona" must be investigated in further studies, especially because *O. mimon* is a common human-biting tick in Brazil (Nogueira et al., 2022), where it colonizes human households, most of the times associated with synanthropic opossums (Labruna et al., 2014; Dantas-Torres et al., 2022; Oliveira et al., 2023b).

Phylogenies inferred for alignments of MLST scheme sequences showed that "*Candidatus* B. mimona" grouped within a clade containing two other South American borreliae detected in soft ticks ("*Candidatus* B. caatinga") and rodents ("*Candidatus* B. octodonta"), and several Asian-African *Borrelia* spp. of the RFG. These results add additional evidence that Old and New World RFG spirochetes do not necessarily have defined geographical distributions. Unfortunately, we were not able to amplify two genes (*clpA* and *nifS*) from de MLST scheme, similarly to the results of the MLST analysis of "*Ca.* B. caatinga" (Oliveira et al., 2023a) and "*Ca.* B. octodonta" (Santodomingo et al., 2024). It is likely that this South American group of RFG *Borrelia* species have polymorphic loci or diverge genetically, since the primers designed using allochthonous species do not anneal. Genome-wide analyses of South American RFG borreliae will solve this gap in the future.

5. Conclusions

This study reports the detection and isolation of a novel RFG spirochete, "*Candidatus* B. mimona", from opossums *D. albiventris* in southeastern Brazil. Experimental infections demonstrated that another opossum species, *D. aurita*, is susceptible to this novel agent, as demonstrated by detectable spirochetemia for several consecutive days. Phylogenetic analysis indicated that "*Candidatus* B. mimona" is more closely related to Old World than North American RFG *Borrelia* species. The formal description and validation of the taxon "*Candidatus* B. mimona" remains dependent on its establishment in axenic media and determination of its entire genome.

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Ethical approval

This study has been approved by the Brazilian Government - IBAMA/ICMBio (SISBIO 43259-3, 77618-2), the São Paulo Forestry Institute (Cotec permit 260108–000.409/2015), and the Institutional Animal

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CRediT authorship contribution statement

Barbara C. Weck: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Adriana Santodomingo:** Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. **Maria Carolina A. Serpa:** Data curation, Investigation, Methodology, Writing – review & editing. **Glauber M.B. de Oliveira:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Felipe R. Jorge:** Investigation, Methodology, Writing – review & editing. **Sebastián Muñoz-Leal:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Marcelo B. Labruna:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data collected and analyzed during the study are provided in the article and its supplementary files. The newly generated DNA sequences of "*Candidiatus* B. mimona" are deposited in the GenBank database under the accession numbers PP729468 (*16S* rRNA), PP735920 (IGS), PP736195 (*flaB*), PP736196 (*rplB*), PP736197 (*recG*), PP736198 (*pepX*), PP736199 (*clpX*), PP736200 (*uvrA*), PP736201 (*pyrG*).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2024.100193.

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