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Original article

New records of tick-associated spotted fever group *Rickettsia* in an Amazon-Savannah ecotone, Brazil

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ABSTRACT

Human rickettsiosis has been recorded in the Amazon Biome. However, the epidemiological cycle of causative rickettsiae has not been fully accounted for in the Amazon region. This study investigates the presence of spotted fever group (SFG) *Rickettsia* spp. in free-living unfed ticks of the *Amblyomma* genus. The study was conducted in seven municipalities in Rondonia State, Brazil, where the main biomes are Amazon forest, Brazilian Savannah and their ecotones (areas of ecological tension between open ombrophilous forest and savannah). The following tick species were collected: *Amblyomma cajennense* (sensu lato) s.l., *A. cajennense* (sensu stricto) s.s., *A. coelebs*, *A. naponense*, *A. oblongoguttatum*, *A. romitii*, *A. scalpturatum* and *A. sculptum*. A total of 167 adults, 248 nymphs and 1004 larvae were subjected to DNA extraction and polymerase chain reaction (PCR) to determine the presence of SFG *Rickettsia* spp. PCR-positive samples included: one *A. cajennense* s.s. female and one *A. cajennense* s.l. male from a rural area in Vilhena Municipality; 10 nymphs and a sample of larvae of *A. cajennense* s.l. from a peri-urban area in Cacoal Municipality; and an *A. oblongoguttatum* adult male from a rural area of Pimenta Bueno Municipality. All sequences obtained exhibited 100% identity with *Rickettsia amblyommatis* sequences. This is the first confirmation of SFG *Rickettsia* in an *A. oblongoguttatum* tick. Furthermore, this is the first record of SFG *Rickettsia* in the municipalities targeted by this study. These results warn that SFG *Rickettsia* circulation poses a threat in Rondonia State (among Amazon-Savannah ecotones), and that this threat is increased by the fact that SFG *Rickettsia* infect a human-biting tick species hitherto unconfirmed as a vector.

1. Introduction

Rickettsia is a genus of bacteria of the order Rickettsiales, α-Proteobacteria subgroup. These bacteria are obligate intracellular Gram-negative Coccobacilli (Parola et al., 2005). This genus includes: typhus group (TG), comprised of *Rickettsia typhi* and *R. prowazekii*; and spotted fever group (SFG), which includes several species, *R. rickettsii* being the most lethal (Raoult and Roux, 1997; Gillespie et al., 2007; Weinert et al., 2009; Tarragona et al., 2015).

In Brazil, SFG *Rickettsia* spp. infection is transmitted to humans by ticks. The primary transmission vectors are: *Amblyomma sculptum*, in the Brazilian Savannah biome; and *A. aureolatum*, in the Atlantic rainforest biome (Labruna, 2009). SFG *Rickettsia* spp. have been detected in several species of the *Amblyomma* genus; some of these species parasitize dogs and humans, and some have already been recorded in Rondonia State (Labruna et al., 2004a,b, 2005a,b, 2007; Barbieri et al., 2008; Szabó et al., 2013; Martins et al., 2014; Oliveira et al., 2016a,b).

The *Amblyomma* spp. fauna is more diversified in the western Amazon than in other regions of Brazil (Martins et al., 2014). Although many studies of the tick fauna and SFG *Rickettsia* have been conducted in this region, little is known about the dynamic dispersion of these pathogens and their relation to human illness is poorly understood

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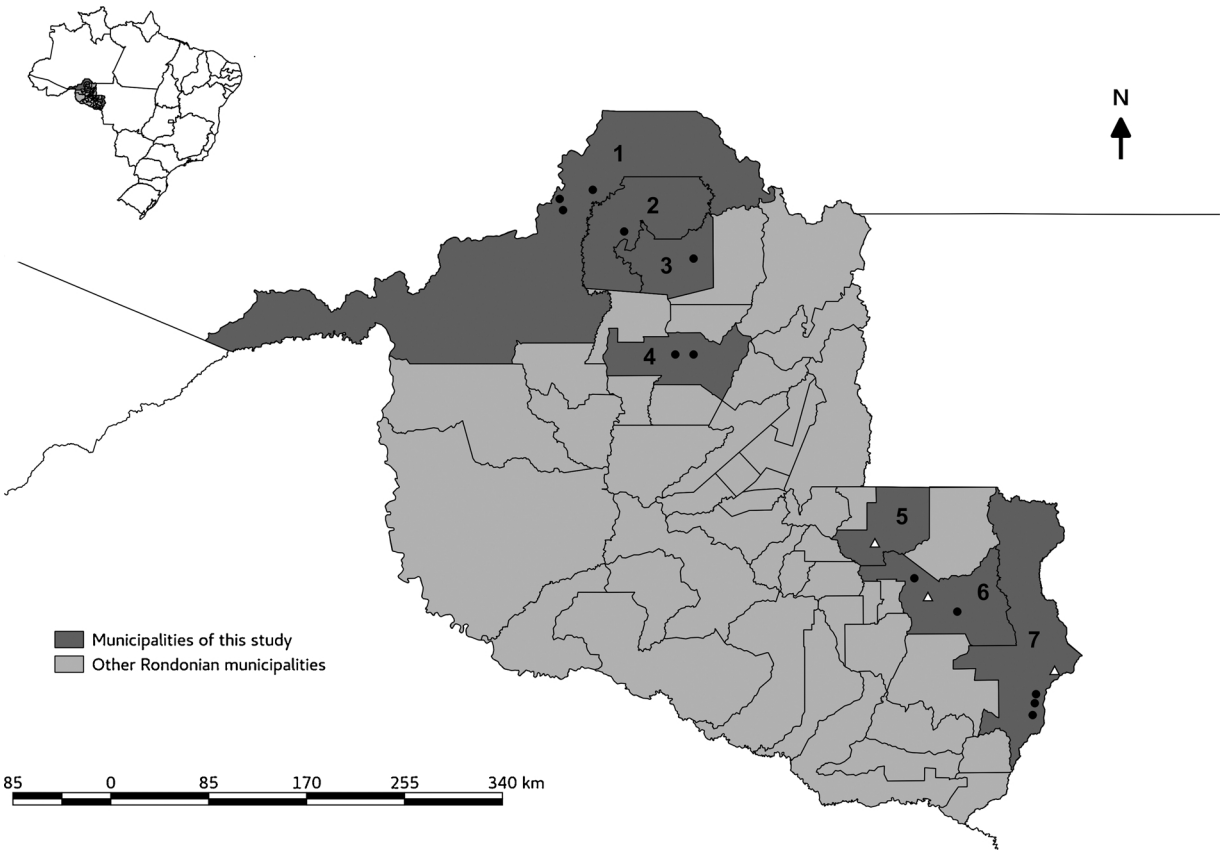

Fig. 1. Map representing Rondonia State, Brazil. Numbered areas in dark gray represent the municipalities in this study: Amazon biome (a) – (1) Porto Velho, (2) Candeias do Jamari, (3) Itapuã do Oeste, (4) Ariquemes; Amazon-Savannah ecotone region (b) – (5) Cacoal, (6) Pimenta Bueno, (7) Vilhena. Areas in light gray represent other Rondonian municipalities. Small black circles represent tick collection sites where samples were negative, and white triangles represent sites where samples were positive and confirmed for *Rickettsia amblyommatis*.

(Labruna et al., 2004a,b, 2005a,b, 2007; Martins et al., 2014; Oliveira et al., 2016a,b).

Ticks and dogs carrying SFG *Rickettsia* have been recorded in Rondonia State; this suggests that human infection may be occurring unreported. Brazilian spotted fever (BSF) presents symptoms that are similar to highly prevalent regional illnesses, such as dengue, malaria and leptospirosis, and BSF may therefore be subject to misdiagnosis (Labruna et al., 2004a,b, 2005a,b, 2007). The first confirmed case of BSF in Rondonia State was reported in 2016, by Oliveira et al. (2016a). All these concerns highlight the need for further studies.

This study addresses the potential for the dissemination of tick-borne diseases in Rondonia State, as evidenced by the first confirmed case of BSF (Oliveira et al., 2016a), and by several reports of SFG *Rickettsia* circulation in animals and ixodid vectors (Labruna et al., 2004a,b, 2005a, 2007). The aim of this study was to assess the presence of SFG *Rickettsia* in ixodid ticks via molecular analysis; samples were collected along animal trails where the likelihood of contact between humans and *Amblyomma* ticks was high, and collections were performed in two distinct biomes.

2. Material and methods

2.1. Study site

Tick collections (described below) were performed between April 2015 and December 2016, along animals' trails in forest fragments that provided favorable conditions for the transmission of BSF. Conditions were considered favorable if target locations were close to human shelters (peridomiciles and rural areas), near water sources (streams, brooks, ponds), and likely to be frequented by domestic animals (dogs and horses) and by wild animals (capybaras, tapirs, armadillos and small rodents). Collections were performed in two distinct biomes split between seven municipalities in Rondonia State, Brazil: (a) Amazon rainforest – including the municipalities of Porto Velho (08° 45′ 43″ S, 63° 54′ 14″ W), Candeias do Jamari (08° 48′ 35″ S, 63° 41′ 44″ W), Itapuã do Oeste (9° 11′ 51″ S, 63° 9′ 56″ W) and Ariquemes (9° 54′ 50″ S, 63° 2′ 38″ W); and (b) Amazon-Savannah ecotone – areas bordering BR-364 road, including the municipalities of Cacoal (11° 25′ 53″ S, 61° 26′ 52″ W), Pimenta Bueno (11° 40′ 29″ S, 61° 11′ 28″ W) and Vilhena (12° 44′ 3″ S, 60° 8′ 41″ W) (IBGE, 2012) (Fig. 1).

2.2. Tick collections and identification

Tick collections were performed using the following methods: CO_2 (dry ice) traps; drag flagging; and visual inspection of vegetation up to one meter beyond the edge of animal trails (Oliveira et al., 2000; Castro and Clover, 2010). Collected unfed ticks were taken to the laboratory alive. Adults and nymphs were identified taxonomically under a stereoscopic microscope (Barros-Battesti et al., 2006; Martins et al., 2010). Identification was verified for *A. cajennense* (sensu stricto) (s.s.) and *A. sculptum* females (both from the *A. cajennense* complex) according to Nava et al. (2014); each species exhibited clear differences on the genital aperture (Soares et al., 2015). Males and nymphs from the *A. cajennense* complex were identified as *A. cajennense* (sensu lato) s.l. The genus of larvae collected in bunches was identified morphologically (Barros-Battesti et al., 2006), and a portion of the larvae was allocated to feed on rabbits (Bechara et al., 1995) with the aim of producing nymphs for species identification (Martins et al., 2010). Following identification, ticks were preserved in absolute ethanol at −18 °C until DNA extraction and polymerase chain reaction (PCR). All procedures

with animals were carried out according to ARRIVE guidelines and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the Ethics Commission on the Use of Animals/CEUA of Fiocruz Rondônia, under protocol number 2015/18, approved this study.

2.3. DNA extraction, PCR and sequencing analysis

DNA was extracted from all samples and molecular analysis was performed using PCR. The presence of SFG *Rickettsia* was tested via amplification of the *ompA* gene fragment. Extractions were performed individually for adults, in pools of up to 10 specimens for nymphs, and in pools of bunches for larvae, following the guanidine isothiocyanate-phenol technique, as previously described by Sangioni et al. (2005). The obtained DNA was amplified using the primers Rr190.70p and Rr190.602n to amplify a 532-pb fragment of the *ompA* gene, present exclusively in only SFG *Rickettsia* (Regnery et al., 1991).

DNA from positive samples was purified using Purelink™ PCR Purification Kit (Invitrogen, CA, USA). Further sequencing was performed with the Sequencing and Genotyping DNA Platform in René Rachou Research Center, Oswaldo Cruz Foundation, Belo Horizonte, Brazil. The Sanger method (Sanger et al., 1977) was used in an ABI 3730 (Life Technologies) sequencer, and samples were sequenced in duplicate. Sequences were then aligned with CulstalW algorithm using the MEGA 7.0 program (Kumar et al., 2016). Sequences were deposited in GenBank, with major identity criteria determined by the BLASTn search tool.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) for phylogenetic tree analysis. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,6801)). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least one unambiguous base is present in at least one sequence for each descendant clade is shown next to each internal node in the tree. The analysis involved 38 nucleotide sequences. Codon positions included were 1° + 2° + 3° + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 program (Kumar et al., 2016).

3. Results

A total of 1420 unfed *Amblyomma* ticks was collected in this study. Of these, 178 were obtained from (a) region (Amazon biome) (4 larvae, 132 nymphs and 42 adults), and 1242 were obtained from (b) region (Amazon-Savannah ecotone) (1000 larvae, 117 nymphs and 125 adults). Table 1 shows the total number of ticks, life stages and species, and the results of SFG *Rickettsia* detection with respect to the relevant biomes and municipalities. Nine species were identified: *A. cajennense* s.l. (1173, including a bunch of 1000 larvae), *A. oblongoguttatum* (98), *A. naponense* (59), *A. cajennense* s.s. (43), *A. coelebs* (13), *A. scalpturatum* (13), *A. sculptum* (7), *A. ovale* (5), *A. romitii* (4), *A. calcaratum* (1), and *Amblyomma* spp. larvae (4). Additionally, four *A. oblongoguttatum* and four *A. ovale* adults (two males and two females each) were collected from a domestic dog in a rural area in Candeias do Jamari Municipality (data not shown).

Nine samples were PCR-positive, all from free-living ticks: two *A. oblongoguttatum* females from Candeias do Jamari Municipality; one *A. oblongoguttatum* male from Pimenta Bueno Municipality; one *A. cajennense* s.l. male and one *A. cajennense* s.s. female from Vilhena Municipality; three nymph pools containing 10 specimens each, and a bunch of 1000 larvae of *A. cajennense* s.l. from Cacoal Municipality. Nevertheless, from the nine samples PCR-positive, four were inadequate for sequencing due to low quality of DNA, and only five samples shared sequence homology with *ompA* sequences on GenBank. All five of these samples exhibited 100% identity with a *Rickettsia amblyommatis* sequence, An 13 isolate (GenBank: CP015012), as determined by BLASTn program analysis. Furthermore, all five samples exhibited 100% identity with more than 10 sequences from several "*Candidatus* Rickettsia amblyommii" records, and 99% identity with the "ARANHA" strain (GenBank AY360213), which has been recorded near the regions targeted by this study. The identity of *R. amblyommatis* was determined by phylogenetic analysis of *ompA* sequences (access numbers GenBank MF188911, MF188912, MF188913, MF188914) (Fig. 2).

4. Discussion

This is the first study to report the presence of SFG *Rickettsia* in a specimen of *A. oblongoguttatum* tick in South America. The specimen was collected in an Amazon-Savannah ecotone (IBGE, 2012), in Pimenta Bueno Municipality, Rondonia, Brazil. This is the first record of *A. oblongoguttatum* carrying SFG *Rickettsia ompA* already confirmed by 100% sequence identity. An earlier detection of SFG *Rickettsia* in *A. oblongoguttatum* was reported in Panama (Central America), but the authors of that study emphasized that the speciation analysis could not be completed due to a low number of DNA copies (Bermúdez et al., 2009). In addition, two specimens of *A. oblongoguttatum* from Candeias do Jamari Municipality tested positive for SFG *Rickettsia*; however, the species could not be confirmed by sequencing due most likely to low DNA integrity.

One larval and one nymphal DNA sample of *A. cajennense* s.l. from Cacoal Municipality, and an *A. cajennense* s.l. male and an *A. cajennense* s.s. female from Vilhena Municipality exhibited 100% sequence identity with *R. amblyommatis ompA* (along with other "*Candidatus* R. amblyommii" strains). Thus, this study is also the first confirmed record of *R. amblyommatis* in *A. cajennense* s.s., in Rondonia State (an adult female from Vilhena Municipality).

It is worth mentioning that *A. cajennense* s.s. belongs to a namesake complex which encompasses six species (including *A. sculptum*) (Nava et al., 2014), and that the last SFG *Rickettsia* report of this complex in Rondonia was made a decade before the complex was first described (Labruna et al., 2004b). Since there have also been reports in Rondonia State of *A. sculptum* (in Pimenta Bueno Municipality), records made prior to the complex description tend to be treated as *A. cajennense* s.l.

In this study, *A. cajennense* s.s. was identified in the municipalities of Cacoal, Pimenta Bueno, and Vilhena, which accords with previous reports (Martins et al., 2016). This indicates that the enzootic cycle and circulation of *R. amblyommatis* is complex, and may involve several tick vectors and potentially utilize several animal species as natural reservoirs. Aside from the records cited, the presence of *R. amblyommatis* is unprecedented in the municipalities of Rondonia that were the focus of this study.

Recently, "*Candidatus* R. amblyommii" was formally named *R. amblyommatis* sp. nov., according to the International Code of Nomenclature of Prokaryotes (Karpathy et al., 2016). The tick species most commonly infected by this pathogen is *A. americanum*, which has a rate of infection above 40% in North America (Burgdorfer et al., 1981; Karpathy et al., 2016). However, this pathogen has also been found in several Central and South American countries, where it infects many other *Amblyomma* species (Sánchez-Montes et al., 2016). In Brazil, this bacteria has already been detected in *A. auricularium*, *A. cajennense* s.s., *A. coelebs*, *A. geayi*, *A. humerale*, *A. longirostre*, *A. parkeri* and *A.*

Table 1
Total number of ticks collected and PCR analysis for spotted fever group *Rickettsia* spp. identification, categorized by biome and municipality, in Rondonia State, Brazil, 2015–2016.

			Ticks					
				Collected (n)				
Biomes	Municipalities	Geographic coordinates	Species	Larvae	Nymphs	Adults	Infected (n)	*Rickettsia* species
(a) Amazon	Ariquemes	9° 54′ 50” S, 63° 2′ 38” W	*Amblyomma naponense*		52		0	
			Amblyomma oblongoguttatum		57	4	0	
			Amblyomma ovale		1		0	
			Amblyomma scalpturatum			1	0	
	Candeias do Jamari	08° 48′ 35” S, 63° 41′ 44” W	*Amblyomma coelebs*		1	2	0	
			A. naponense			6	0	
			A. oblongoguttatum		5	12	0	
			A. ovale			4	0	
			A. scalpturatum		1	2	0	
			A. coelebs		6	1	0	
			Amblyomma spp.	4			0	
	Itapuã do Oeste	9° 11′ 51” S, 63° 9′ 56” W	*A. oblongoguttatum*			1	0	
	Porto Velho	08° 45′ 43” S, 63° 54′ 14” W	*A. oblongoguttatum*		5	8	0	
			A. scalpturatum		4	1	0	
			Total	**4**	**132**	**42**	**0**	
(b) Amazon-Savannah ecotone	Cacoal	11° 25′ 53” S, 61° 26′ 52” W	*Amblyomma cajennense* (s.l)	1000	105	28	1 larvae pool and 1 nymph pool	*Rickettsia amblyommatis*
			Amblyomma cajennense (s.s.)			18	0	
			Amblyomma romittii		4		0	
	Pimenta Bueno	11° 40′ 29” S, 61° 11′ 28” W	*A. cajennense* (s.l.)			20	0	
			A. cajennense (s.s.)			13	0	
			Amblyommma calcaratum		1		0	
			A. coelebs		2		0	
			A. naponense			1	0	
			A. oblongoguttatum		2	4	1 male	*R. amblyommatis*
			A. scalpturatum		2	1	0	
			Amblyomma sculptum			7	0	
	Vilhena	12° 44′ 3” S, 60° 8′ 41” W	*A. cajennense* (s.l.)			20	1 male	*R. amblyommatis*
			A. cajennense (s.s.)			12	1 female	*R. amblyommatis*
			A. coelebs			1	0	
			A. scalpturatum		1		0	
			Total	**1000**	**117**	**125**	**5**	
			TOTAL	**1004**	**249**	**167**	**5**	

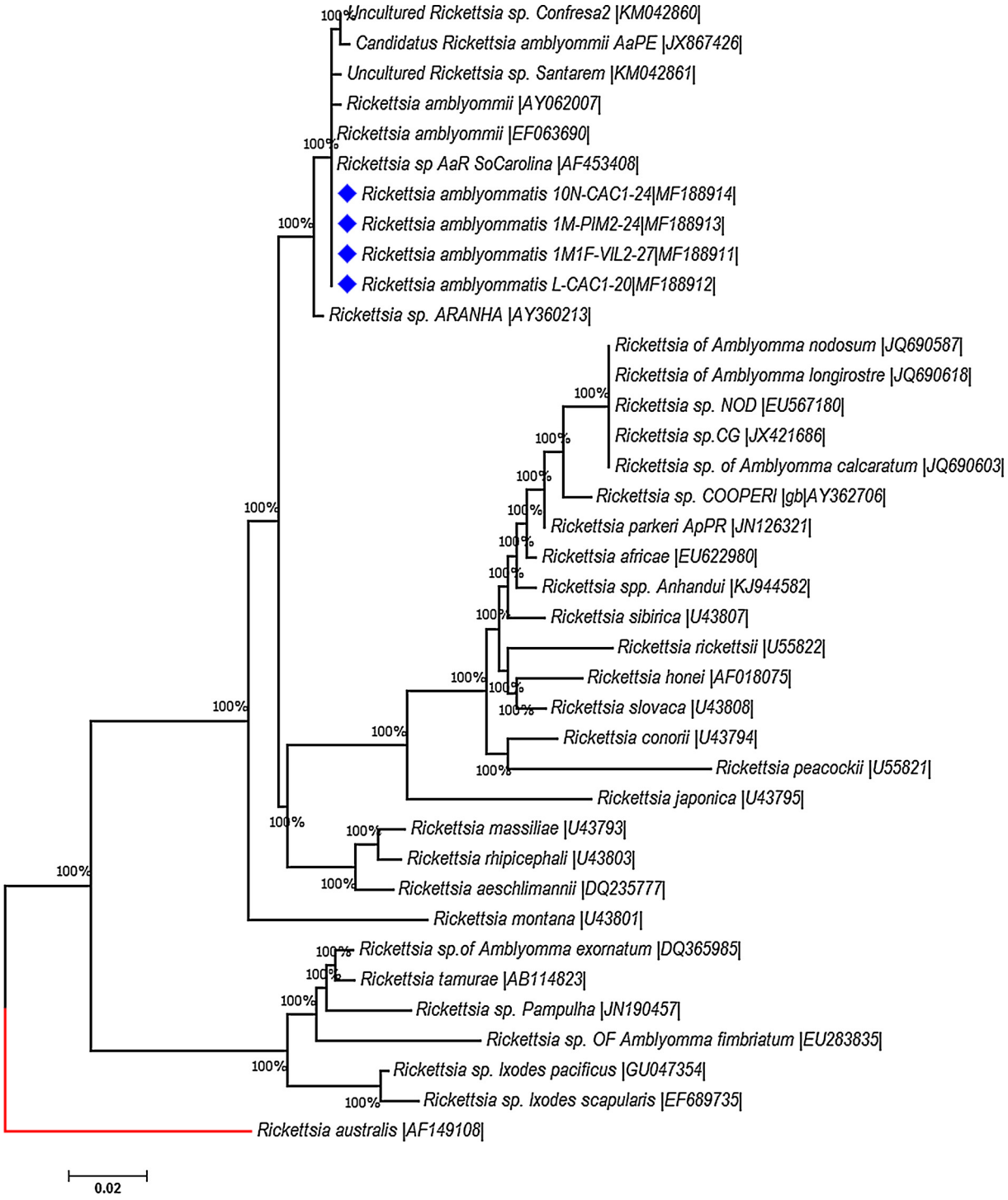

Fig. 2. Phylogenetic tree of *Rickettsia amblyommatis*. The evolutionary history was inferred by the Maximum likelihood method for the *ompA* gene. Sample sequences obtained in this study are indicated by blue rhombuses with species name followed by identification code for the sample from which the DNA was isolated (Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sculptum, and has been recorded in the states of Bahia, Mato Grosso, Pará, Paraná, Pernambuco, Rondônia and São Paulo (Labruna et al., 2004a,b; Ogrzewalska et al., 2008, 2010, 2011; Pacheco et al., 2012; Saraiva et al., 2013; Alves et al., 2014; Lopes et al., 2014; Soares et al., 2015).

BLAST analysis of all positive samples exhibited 100% identity with: the An13 isolate of *R. amblyommatis*; "*Candidatus* R. amblyommii" isolates 11-TC-1-1, GP4A, AcCR and TX116, and other rickettsiae isolates from North and Central America, including isolates that have been related to human pathogens by Billeter et al. (2007), (GenBank: EF063690); and with the AcaIII isolate of "*Candidatus* R. amblyommii" from the municipalities of Governador Jorge Teixeira and Monte Negro, Rondonia, an isolate that was not deposited on GenBank because it exhibited 100% identity with ArR/SoCarolina (GenBank: AF453408), according to Labruna et al. (2004b). The sequences reported here exhibited 99% identity with sequences from other studies conducted in Brazil, including: Ariquemes (RO), "ARANHA" strain (GenBank: AY360213), Confresa (MT), Confresa2 clone (GenBank: KM042860),

Ibimirim (PE), AaPE strain (GenBank: JX867426) and Santarém (PA), Santarem clone (GenBank: KM042861) (Labruna et al., 2004a; Saraiva et al., 2013; Soares et al., 2015).

Since the discovery of *R. amblyommatis*, its pathogenesis in humans has remained unclear. Laboratory assays with experimentally infected animals did not elicit evident clinical signs. In the same study, the authors evaluated humans in a region with high rates of tick-borne infection and constant exposure to *A. americanum* parasitism, yet no relevant clinical signs were detected (Burgdorfer et al., 1981). Nevertheless, recent reports suggest that some correlation exists between mild illness and rickettsiosis in people that test seropositive for *R. amblyommatis* and have had previous contact with *A. americanum* (Apperson et al., 2008; Delisle et al., 2016).

In Rondonia State, *Rickettsia* genera were described for the first time in Ariquemes Municipality, where *A. longirostre* was infected by the "ARANHA" strain (AY360213) (Labruna et al., 2004a). Shortly thereafter, a strain 100% identical to ArR/SoCarolina (AF453408) was detected infecting 26.8% of *A. cajennense* s.l. (collected from Governador Jorge Teixeira Municipality) and 14.3% of *A. coelebs* (collected from Monte Negro Municipality) (Labruna et al., 2004b). In Monte Negro, 11.6% of dogs from rural areas and 3.9% of dogs from urban areas tested seropositive for at least one of three *Rickettsia* species: *R. parkeri*; *R. amblyommatis* and *R. rhipicephali* (Labruna et al., 2007). After these studies were conducted, *Rickettsia* was not reported in Rondonia State until the first confirmed case of BSF in Ariquemes Municipality (Oliveira et al., 2016a,b). These authors had already confirmed *Rickettsia* spp. in *Dermacentor nitens*, *Rhipicephalus sanguineus* sensu lato and *R. microplus*, in Rondonia State. However, these authors did not identify the species of *Rickettsia* in the human case of BSF, nor did they identify the species of *Rickettsia* infecting the three types of tick. It is worth noting that up to now there has been no record of *A. cajennense* complex in Ariquemes Municipality, which suggests that other tick species are involved in the transmission cycle.

While performing collections, the authors of this study found *A. oblongoguttatum* and *A. ovale* parasitizing a dog in Candeias do Jamari Municipality. According to the literature, dogs may be accidental hosts for these ticks even though adult ticks normally parasitize wild carnivores (Labruna et al., 2000). Furthermore, dogs play an important role in the zoonotic cycle of rickettsiosis: dogs can serve as an amplifier host to several SFG *Rickettsia* spp. by transmitting ticks between wild animals and also by transmitting ticks from wild animals to humans (Labruna et al., 2007; Melo et al., 2011).

A. oblongoguttatum has been reported parasitizing humans in Brazil, mainly in Amazon forest areas (including in Rondonia State), and there have been additional reports in the South Region of Brazil, as well as in neighboring countries (Labruna et al., 2000, 2005b; Arzua et al., 2005; Guglielmone et al., 2006). *A. oblongoguttatum* is very common in the region studied. Now that we have confirmed *A. oblongoguttatum* as a potential vector of SFG *Rickettsia*, local public health authorities should be altered and systems for the control and surveillance of zoonotic diseases should include this tick as a species of concern.

Dogs are viable hosts for *A. oblongoguttatum* and humans are frequently bitten by this tick species (Labruna et al., 2000; Guglielmone et al., 2006; Martins et al., 2017), and SFG *Rickettsia* infection has been reported in both dogs and humans in the region studied. Given these facts, we would like to reiterate the strong possibility that the *A. oblongoguttatum* is involved in the zoonotic transmission cycle of BSF, and that this vector may pose a threat especially in areas where the *A. cajennense* complex has not been reported. Knowledge of this possible threat and the new records of SFG *Rickettsia* in Rondonia add information of great value to our limited understanding of the circulation dynamics of rickettsiosis in animals, ixodid vectors and humans, especially with respect to the Amazon region.

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