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Original article

# Molecular evidence of *Borrelia burgdorferi* sensu stricto and *Rickettsia massiliae* in ticks collected from a domestic-wild carnivore interface in Chihuahua, Mexico

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ABSTRACT

Sixty-five wild carnivores and twenty free-roaming dogs from the Janos Biosphere Reserve (JBR), northwestern Chihuahua, Mexico, were inspected for ticks which were tested by molecular assays to identify *Borrelia* and *Rickettsia* infections. Overall, 45 ticks belonging to five taxa, including *Dermacentor parumapertus, Ixodes hearlei*, *Ixodes kingi*, *Rhipicephalus sanguineus* s.l., and *Ornithodoros* sp. were collected from 9.2% of the wild carnivores and 60% of the free-roaming dogs. *Borrelia burgdorferi* s.s. DNA was detected in an *I. kingi* tick collected from a kit fox (*Vulpes macrotis*), while *Rickettsia massiliae* was detected in two (6.5%) of the 31 *Rh. sanguineus* s.l. collected from free-roaming dogs. Our results revealed host associations between free-roaming dogs and wild carnivore hosts and their ticks in the JBR. The presence of the etiological agents of Lyme disease and spotted fever rickettsiosis in ticks raises the potential risk of tick-borne diseases at the human-domestic-wildlife interface in northwestern Mexico.

## 1. Introduction

Vector-borne pathogens are associated with emerging and re-emerging diseases in the last decades. Some pathogens have expanded their geographical distribution or host range, increasing concern for human and veterinary medicine worldwide (Kilpatrick and Randolph, 2012). Anthropogenic land use changes are widely recognized as one of the most important drivers for emerging diseases (Keesing et al., 2010). Expansion of human settlements, agriculture encroachment, and habitat fragmentation increase the human-domestic-wildlife interfaces as well as the probability of exchange of ectoparasites and pathogens (Gottdenker et al., 2014). Janos Biosphere Reserve (JBR), located in northwestern Chihuahua, Mexico, is a priority area for biodiversity in North America, with several wild carnivore species (List et al., 2010). However, suburbanization and intensive agriculture are increasing in JBR (Ceballos et al., 2010), which may increase contact rates and the risk of pathogen transmission among humans, domestic animals, and wildlife.

Several studies have proposed that members of the order Carnivora are common elements in the transmission cycles of vector-borne pathogens, either as reservoirs, as incidental hosts, or as hosts for vectors. For example, raccoons (*Procyon lotor*) are reservoir-competent for *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis (HGA) (Stuen et al., 2013), while ticks of the genus *Ixodes* can become infected with the spirochete *Borrelia burgdorferi* when feeding upon infected raccoons and striped skunks (*Mephitis mephitis*) (Fish and Daniels, 1990). At the domestic-wildlife interface, both wild and domestic carnivores could play bidirectional roles as bridging or alternate hosts for ectoparasites (Dobler and Pfeffer, 2011), promoting translocation of ectoparasites, including those carrying pathogens,

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across the interface. For instance, the eastern blacklegged tick (*Ixodes scapularis*), the primary vector for HGA and Lyme borreliosis agents in eastern and central North America (Schotthoefer and Frost, 2015), feeds on a number of wild hosts and also is frequently found on dogs (Durden et al., 2016). The brown dog tick (*Rhipicephalus sanguineus* s.l.) is the vector of the rickettsial pathogens *Ehrlichia canis* around the world and *Rickettsia rickettsii* in southwestern U.S. and northwestern of Mexico (Dantas-Torres, 2008); the maintenance host for the brown dog tick is the domestic dog and yet the tick will occasionally parasitize other hosts including wild carnivores (Dantas-Torres, 2010).

To date, about 10 tick-borne bacterial pathogens have been described in Mexico, of which six are considered zoonotic. Three of them belong to the family Anaplasmataceae (*A. phagocytophilum*, *E. canis*, and *Ehrlichia chaffeensis*) (Rojero-Vázquez et al., 2017; Sosa-Gutierrez et al., 2016); two to the spotted fever group (SFG) of the family Rickettsiaceae, (*Rickettsia parkeri* and *R. rickettsii*) (Labruna et al., 2011; Sánchez-Montes et al., 2018); and one is the spirochete *B. burgdorferi* s.l. (Gordillo-Pérez et al., 2018). In Mexico, tick-borne pathogens have been studied mainly in ticks collected from dogs (Eremeeva et al., 2011; Peniche-Lara et al., 2015) and rodents (Solís-Hernández et al., 2016; Sosa-Gutierrez et al., 2016). However, little is known about the presence and genetic diversity of tick-borne pathogens in ticks on wild carnivores, particularly in the northern transboundary region of Mexico where outbreaks of spotted fever rickettsiosis are reported (Álvarez-Hernández et al., 2017). The aim of this study was to identify: 1) tick-host associations on free-roaming dogs and wild carnivores; and 2) the presence of the members of genera *Borrelia* and *Rickettsia* in ticks collected from free-roaming dogs and wild carnivores in northern Chihuahua, Mexico.

## 2. Material and methods

### 2.1. Study area, animal sampling, and tick collection

The study was performed in the Janos Biosphere Reserve (JBR) in northern Chihuahua, Mexico (Fig. 1). JBR is in a transition zone between the Sierra Madre Occidental and the Chihuahuan Desert, and comprises a mosaic of grasslands, mesquite shrubland, oak forest, and riparian vegetation. We collected ticks from wild carnivores during fall 2013 (October-November) and spring 2014 (April-May) in five localities (El Cuervo [30° 41' 19"N, 108° 15' 05"W], La Bascula [30° 53' 08"N, 108° 25' 57"W], Monte Verde [30° 58' 28"N, 108° 42' 40"W], Ojitos [30° 46' 52"N, 108° 32' 14"W] and Rancho San Pedro [30° 41' 12"N, 108° 32' 46"W), and from free-roaming dogs during late winter 2015 (early March) in two settlements (Ejido San Pedro [30° 51' 21"N, 108°23'22"W] and Ejido Casa de Janos [30° 42' 58"N, 108° 25' 21"W]) (Fig. 1).

Domestic dog sampling was conducted at households with prior authorization by the owners. Wild carnivores were captured at each of the five locations over nine consecutive days per season, with 16 trapping sets placed at intervals of 500–800 meters along a 10-km transect (Fig. 1). Each set contained one box trap (Tomahawk Live Trap Inc. 76 × 76 × 178 cm or 152 × 51 × 71 cm) and one leg-hold trap (#1.75 or #3 Victor Coil Soft Catch) with at least 30 m distance between them. The traps were baited with canned sardine, chicken and commercial lure (Kishel´s, East Aurora, NY) and checked at least once a day. Each wild carnivore was chemically immobilized with a mixture of ketamine hydrochloride (Pisa, Atitalaquia, Hidalgo, Mexico) and xylazine hydrochloride (Pisa®) according to doses reported by Kreeger and Arnemo (2012). Animals were weighed, sexed, and identified to species. Each free-roaming dog and wild carnivore was visually examined for ticks, which were collected into cryovials containing 96% ethanol. All wild carnivores were marked with uniquely numbered metal eartags and released at the site of capture. All procedures for trapping and handling carnivores followed the guidelines of the American Society of Mammalogists (Sikes and Gannon, 2011) and were approved by the Mexican Secretary of Environment and Natural Resources (Permit FAUT-0250), and by the Ethics and Research Committee of the Medical Faculty of the UNAM (Universidad Nacional Autónoma de Mexico) (FMED/CI/JMO/004/2012).

### 2.2. Laboratory methods

Ticks were identified using morphological keys (Cooley and Kohls, 1944; Cooley, 1946; Keirans and Clifford, 1978; Durden and Keirans, 1996; Yunker et al., 1986). DNA was extracted from each ectoparasite using the Chelex 100 Chelating Resin (Biorad, USA) protocol (Aguilar-Domínguez et al., 2019). To verify the presence of amplifiable DNA in each sample, and for molecular identification of ticks we used a PCR protocol targeting an approximately 400 bp fragment of the mitochondrial 16S rRNA gene (Norris et al., 1996). For *Rickettsia* sp. screening, we amplified a conserved fragment of 805 bp of the citrate synthase gene (*gltA*) (Sánchez-Montes et al., 2016) with the following conditions: initial denaturation (2 min at 94 °C) followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min, followed by a final extension (7 min at 72 °C). All PCR-positive samples were further characterized by PCR amplification of the sequences of the additional outer membrane protein B gene (*ompB*) using published primers (Roux and Raoult, 2000) with the following conditions: initial denaturation (3 min at 95 °C) followed by 40 cycles of 95 °C for 15 s, 48 °C for 30 s, and 72 °C for 30 s, followed by a final extension (7 min at 72 °C). PCR was performed in a 25 µL reaction mixture containing 6.5 µL nuclease-free water, the corresponding pair of primers (100 ng each), 12.5 µL of GoTaq® Green Master Mix, 2X of Promega Corporation (Madison, WI, USA), and 100 ng of DNA. In all reactions, we included a negative (reaction mix without DNA) and a positive control (*Rickettsia lusitaniae* isolate RLOYYUCMEX002 DNA).

For *Borrelia* sp. DNA detection, we amplified a fragment of 470 bp of the flagellin (*flaB*) gene using the primers in Rudenko et al. (2009), with the following conditions: initial denaturation (5 min at 94 °C) followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, followed by a final extension (10 min at 72 °C). Negative control consisted of the reaction mix with nuclease free water; reptile-associated, DNA sequence-confirmed *Borrelia* sp. DNA obtained from *Amblyomma dissimile* collected in Los Tuxtlas, Veracruz, Mexico was used as positive control (Colunga-Salas et al. unpublished data, GenBank accession number KY389373). To verify the presence of amplicons of the expected size, PCR products were first electrophoresed in 1.5% ethidium bromide-stained agarose gels and visualized by UV-transillumination. Amplicons of the proper size were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in a 24-capillary 3500xL Genetic Analyzer at the Laboratorio de La Biodiversidad y la Salud, Instituto de Biología, UNAM and at the UC Davis DNA Sequencing core facility.

### 2.3. Sequence analyses

Consensus sequences were generated in Geneious and compared with those of references in GenBank using the Basic Local Alignment Search Tool (BLAST). All sequences for each gene were aligned using Muscle in Mega 6.0 (Tamura et al., 2013). Phylogenetic trees were constructed using Maximum Likelihood with 1000 bootstrap replications. The nucleotide substitution model for each alignment was selected based on the lowest Akaike information criterion (AIC) value using JModelTest (Darriba et al., 2012).

## 3. Results

We sampled 20 free-roaming dogs and 65 wild carnivores belonging to eight species, including coyotes (*Canis latrans*, 6 females, 12 males), kit foxes (*Vulpes macrotis*, 8 females, 6 males), gray foxes (*Urocyon cinereoargenteus*, 4 females, 3 males), bobcats (*Lynx rufus*, 2 females, 3

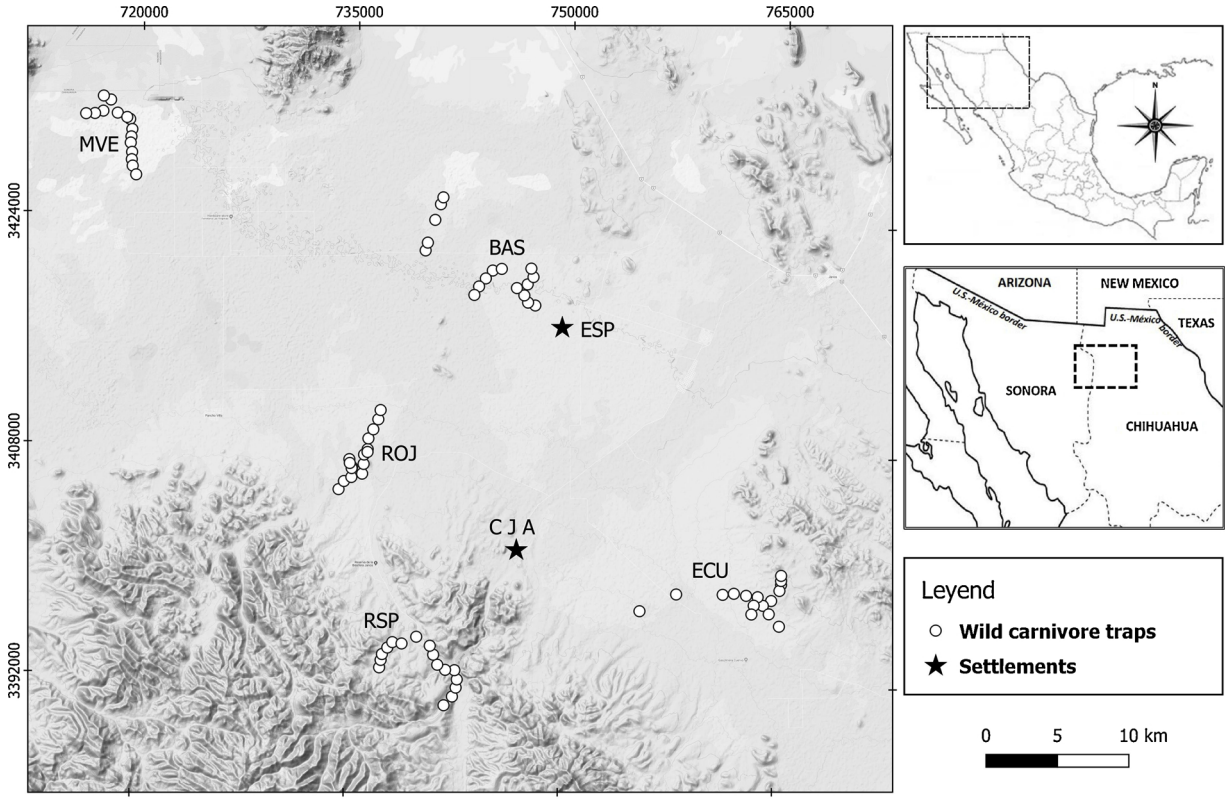


**Fig. 1.** Sampling sites along the Janos Biosphere Reserve, Chihuahua, Mexico. Capital letters refer to locations (MVE: Monte Verde; BAS: La Bascula; ESP: Ejido San Pedro; ROJ: Rancho Ojitos; CJA: Casa de Janos; RSP: Rancho San Pedro ECU: El Cuervo).

**Table 1**
Ticks collected from carnivores in five localities in Chihuahua, Mexico.

| Carnivore Host | Locations (Total host / infested host) | Tick species | Stages | *Rickettsia* sp. | *Borrelia* sp. |
|---|---|---|---|---|---|
| *Canis lupus familiaris* | Ejido San Pedro (2/4) | *Rhipicephalus sanguineus* s.l. | 5♀ | 0 | 0 |
| | Casa de Janos (10/16) | *Rhipicephalus sanguineus* s.l. | 7♀, 19♂ | 1♀, 1♂ | 0 |
| *Canis latrans* | Monte Verde (1/1) | *Dermacentor parumapertus* | 1♂ | 0 | 0 |
| *Urocyon cinereoargenteus* | Rancho San Pedro (1/7) | *Ornithodoros* sp. | 4 N | 0 | 0 |
| *Vulpes macrotis* | El Cuervo (1/4) | *Ornithodoros* sp. | 1L | 0 | 0 |
| | Monte Verde (1/5) | *Ixodes kingi* | 1♀ | 0 | 1♀ |
| *Mephitis macroura* | Rancho Ojitos (3/1) | *Ixodes hearlei* | 1♀ | 0 | 0 |
| *Mephitis mephitis* | Rancho San Pedro (2/6) | *Ixodes hearlei* | 4♀, 1N | 0 | 0 |
| | | *Ixodes* sp. | 1L | 0 | 0 |

♀ = Adult females; ♂ = Adult males; N = Nymphs; L = Larvae.

males), raccoons (2 females, 2 males), striped skunks (*Mephitis mephitis*, 3 females 5 males), hooded skunks (*Mephitis macroura*, 3 males), and badgers (*Taxidea taxus*, 2 females, 4 males) in northwestern Chihuahua, Mexico. None of these carnivores was recaptured. We collected 45 ticks belonging to two families (Ixodidae and Argasidae) from 12 (60%) free roaming dogs and six (9.2%) wild carnivores. Thirty-one ticks were *Rhipicephalus sanguineus* s.l., six were *Ixodes hearlei*, one larva was *Ixodes* sp., five were *Ornithodoros* sp., one was *Ixodes kingi*, and one was *Dermacentor parumapertus* (Table 1). *Ixodes kingi* and *Rh. sanguineus* s.l specimens were confirmed using DNA sequencing and the recovered sequences were deposited in GenBank (acc. no. MK370993, MK680295). We were unable to confirm the *Ornithodoros* sp. nymphs by sequencing. However, based on the presence of a dorso-ventral groove reaching only the middle part of the body and a micromammillated integument, those specimens were consistent with *Ornithodoros* sp. We deposited one female, one nymph, and one larva identified as *I. hearlei*, one nymph and one larva identified as *Ornithodoros* sp., and one female and one male identified as *Rh. sanguineus* in the Colección del Laboratorio de Acarología, Facultad de Ciencias, Universidad Nacional Autónoma de Mexico (UNAM) in Mexico City.

*Borrelia* DNA was detected by the amplification of the *fla*B gene in one *I. kingi* adult tick collected from a kit fox, while *Rickettsia* DNA were detected by amplification of *gltA* and *ompB* genes in two of the 31 *Rhipicephalus sanguineus* s.l. (6.5%) collected from free-roaming dogs. The *flaB* sequence obtained from the *Borrelia* in *I. kingi* was 100% identical to the *Borrelia burgdorferi* s.s. strain B31_NRZ (GenBank acc. no. CP019767) (Fig. 2). Based on *gltA* and *ompB* genes, the two rickettsial sequences obtained from *Rh. sanguineus* s.l. in JBR were identical and clustered with *Rickettsia massiliae*. They had an identity of 99.6% (795/798) and 99.3% (794/799) to AZT80, CABA and Bar29 strains previously described in *Rh. sanguineus* s.l. collected from dogs in Arizona (GenBank acc. no. DQ212705, DQ503428.1) (Eremeeva et al., 2006), Argentina (GenBank acc. no. KT032119, KT032122) (Cicuttin et al., 2015) and Spain (GenBank acc. no. U59720, AF123710) (Beati et al., 1996), respectively. Additionally, the phylogenetic reconstruction clustered all of them into a single group with a support value of 95

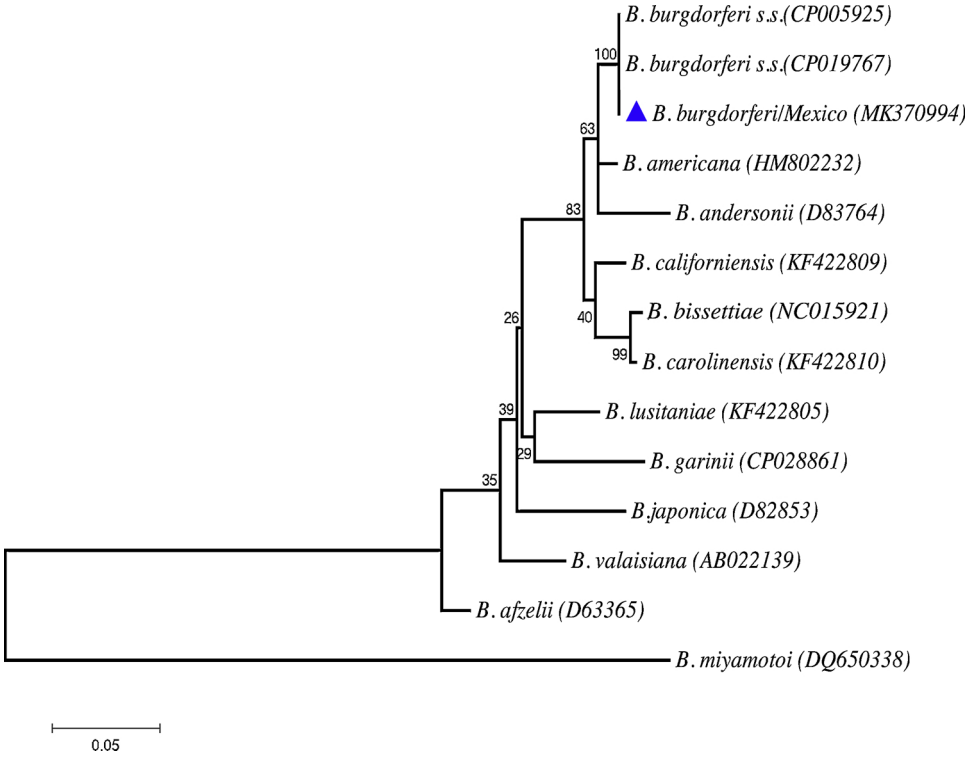


**Fig. 2.** Maximum likelihood (ML) phylogenetic tree generated with the General Time Reversible Model with invariant sites and Gamma distribution (GTR + I+G) using a total of 456 bp of the *flaB* gene from several members of the genus *Borrelia*. Each sequence is indicated by its GenBank accession number. The blue triangle refers the sequence obtained from an *Ixodes kingi* in northwestern, Mexico.

(Fig. 3). Sequences recovered in this study were deposited in GenBank with the following accession numbers: MK066334 (*Rickettsia gltA*), MK066335 (*Rickettsia ompB),* MK370994 (*Borrelia fla*B).

## 4. Discussion

Here, based on an eco-epidemiology approach, we provide the first record of *B. burgdorferi* s.s. in *I. kingi* and the first records for *I. kingi*, *I. hearlei*, and *R. massiliae* in Mexico. These results extend the southern known distribution of *I. kingi*, and *I. hearlei*, which so far have been described throughout western and central regions of Canada and the U.S. (Guglielmone et al., 2014).

Ticks on carnivores in the Janos Biosphere Reserve showed host-associations, with domestic dogs being infested with the brown dog tick

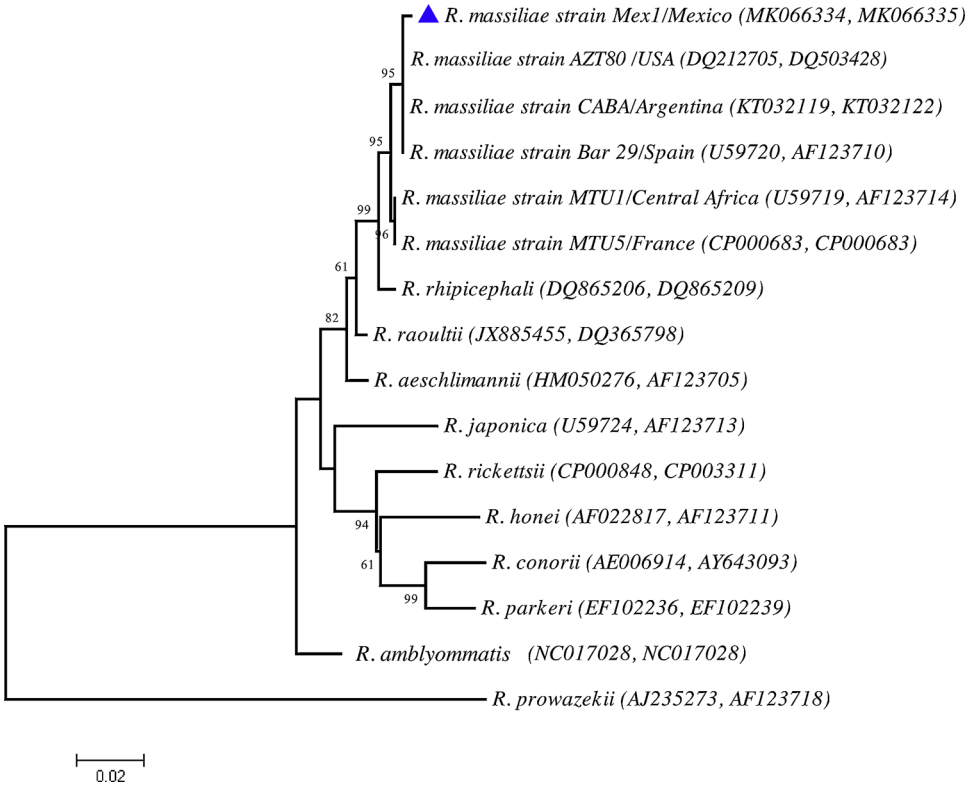


**Fig. 3.** Maximum likelihood (ML) phylogenetic tree generated with the General Time Reversible Model with Gamma distribution (GTR + G) using a total of 1,597 bp of the *gltA* and *ompB* genes concatenated from several members of the genus *Rickettsia*. Each sequence is indicated by its GenBank accession number. The blue triangle refers the sequences obtained from *Rh. sanguineus* s.l. in northwestern, Mexico.

while wild carnivores hosted an array of sylvatic ticks. Despite the premise that domestic and wild carnivores could play bidirectional roles as alternate hosts for ectoparasites (Dobler and Pfeffer, 2011) and high densities and free-roaming activity of domestic dogs at JBR (Almuna, 2016), we did not find brown dog ticks on wild carnivores or sylvatic ticks on dogs. The presence of some of these tick species on wild carnivores from the JBR might be explained by predator-prey relationships or use of burrows. For instance, we collected three tick species from wild carnivores that are commonly found on sciurids (*I. hearlei*), lagomorphs, and rodents (*D. parumapertus*, *I. kingi*) (Guglielmone et al., 2014). Some of these primary mammal hosts are found in JBR (Rubio et al., 2014; Sánchez-Montes et al., 2018).

Lyme disease, caused by the spirochete *Borrelia burgdorferi* s.s., is the most common vector-borne disease in the United States (Rosenberg et al., 2018). Serological and molecular evidence of this spirochete have been reported in humans (Gordillo-Pérez et al., 2018) and ticks from Mexico (Gordillo-Pérez et al., 2009), respectively; however this is the first work that provides sequences for the *fla*B gene in the country. Several ticks of the genus *Ixodes* are recognized as enzootic or bridging vectors of the Lyme disease agents (Eisen and Lane, 2002), but our results increase the range of potential vector species adding *I. kingi* to the list and raise questions about the possible role of this tick as vector for *B. burgdorferi*. *Ixodes kingi* rarely parasitizes humans (Guglielmone, 2018); thus their role in Lyme disease cycles is likely to be as an enzootic vector rather than bridging the pathogen to humans. Some authors report that *I. kingi* is not a vector for *B. burgdorferi,* but as far as we know, this assumption was based on few studies in Alberta, Canada (Fitzgerald, 2012; Government of Alberta, 2018). Given that wild carnivores are not considered reservoir-competent (Brunner et al., 2008), it is plausible that the tick might have acquired the infection from a rodent and maintained the bacterium through its molt (transstadially) (Gern and Humair, 2002). However, future studies are warranted collecting and testing more *I. kingi* specimens for *Borrelia* infection in medium-sized and small mammals, including the whited-footed mice (*Peromyscus leucopus*) which occurs in the JBR (Rubio et al., 2014), may be a host of *I. kingi* nymphs (Salkeld et al., 2006), and is considered the main reservoir of Lyme disease agents (Brunner et al., 2008).

*Rickettsia massiliae* was first described in 1990 from a *Rhipicephalus turanicus* tick in Marseille, France (Beati and Raoult, 1993) and has since been associated with humans and *Rhipicephalus* ticks in several countries of southern Europe (Psaroulaki et al., 2003), Africa (Letaïef, 2006), and South (Cicuttin et al., 2015) and North America (Eremeeva et al., 2006). Our results continue extending the southern geographic distribution of *R. massilae* in North America, which has been reported in California (Beeler et al., 2011) and Arizona (Eremeeva et al., 2006) in the U.S. Phylogenetic analysis shows at least two genotypes (across its geographic range) of *R. massiliae* in North America. Our sequences are closer to those recovered from brown dog ticks collected from dogs in Arizona (Eremeeva et al., 2006). Rocky Mountain spotted fever (RMSF), caused by *Rickettsia rickettsii*, is the only spotted fever disease recognized to date in humans from northwestern Mexico (Álvarez-Hernández et al., 2017). However, the finding of *R. massiliae* could indicate potential risk for humans in this region. This bacterium has been implicated in eight human cases of mild rickettsiosis in France, Italy, Romania, Greece, Tunisia, and Argentina (Eldin et al., 2018). Nevertheless, because the SFG rickettsioses are difficult to differentiate clinically with similar signs and symptoms, as well as in the laboratory (due to high antigenic cross-reaction among them) (Biggs et al., 2016), it is possible that some cases attributed to RMSF in North America could be caused by *Rickettsia* species other than *R. rickettsii*, especially in cases of milder disease.

In addition to the present results, recent studies in northwestern Chihuahua have reported other vector borne agents, such as *Bartonella* species (Rubio et al., 2014; López-Pérez et al., 2017) and *Rickettsia parkeri* (Sánchez-Montes et al., 2018), indicating a relatively complex eco-epidemiology of vector-borne diseases in this region. Moreover, northern Chihuahua is part of the transboundary region at the U.S.-Mexico border with vector-borne diseases that can affect wildlife, domestic animals, and people in both countries. Future international research can help understand ecological similarities and differences in different regions and countries at the border.

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