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Molecular Characterization of a Novel Relapsing Fever *Borrelia* Species from the Desert Cottontail (*Sylvilagus audubonii*) in New Mexico, USA

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ABSTRACT: The *Borrelia* genus comprises vector-borne, spirochete bacteria infecting vertebrates worldwide. We characterized a novel relapsing fever *Borrelia* species from a desert cottontail (*Sylvilagus audubonii*) from New Mexico, US, using an established multilocus sequence analysis approach. Phylogenetic analysis of the flagellin gene (*flaB*) and four other protein-coding loci (*clpX*, *pepX*, *recG*, *rplB*) grouped the novel *Borrelia* species with hard tick relapsing fever borreliae *Borrelia lonestari*, *Borrelia theileri*, and *Borrelia miyamotoi*. The identity of the vectors and other vertebrate hosts, geographic distribution, and zoonotic potential of this novel *Borrelia* species deserve further investigation.

Key words: *Borrelia*, MLSA, multilocus sequence analysis, rabbit, relapsing fever, vector-borne bacteria.

Relapsing fever (RF) borreliae comprise several species infecting vertebrates worldwide (Cutler 2015). Most RF borrelioses remain poorly investigated with scant information on their ecology (Talagrand-Reboul et al. 2018). *Borrelia hermsii*, *Borrelia parkeri*, and *Borrelia turicatae* are the main RF borreliae in North America (Talagrand-Reboul et al. 2018). Reservoirs of RF spirochetes in the US include rodents and birds (*B. hermsii*, *B. parkeri*, *B. turicatae*, *Borrelia miyamotoi*), deer (*Borrelia lonestari*), and livestock (*Borrelia theileri*); dogs and humans are probably dead-end hosts (Talagrand-Reboul et al. 2018). Given the diversity of potential vertebrate hosts and tick species in North America, additional RF *Borrelia* species are probably yet to be characterized.

A recent survey of vector-borne bacteria in small mammals of New Mexico, US, detected a RF *Borrelia* species in a desert cottontail (*Sylvilagus audubonii*; Goodrich et al. 2020),

prompting our study to characterize the *Borrelia* sp. with multilocus sequence analysis (MLSA). This method more accurately delineates species and reflects deep evolutionary relationships among bacterial taxa than do single-locus analyses (Margos et al. 2009).

Cottontails were captured in the Eldorado subdivision of Santa Fe County, New Mexico, as part of a mark-recapture plague surveillance study between November 2002 and July 2004 (Morway et al. 2008). Animals were captured using Tomahawk traps (10×10×25 cm; Tomahawk Live Trap Company, Hazelhurst, Wisconsin, USA) baited with oats and peanut butter, and then the animals were anesthetized with isoflurane in oxygen. Blood (100–200 µL) was collected from the retro-orbital sinus and kept on dry ice until stored at –80 C in the laboratory. Genomic DNA was extracted from 100 µL of blood using the KingFisher Flex Purification System and MagMAX Pathogen RNA/DNA Kit (both Thermo Fisher Scientific, Waltham, Massachusetts, USA).

We detected *Borrelia* DNA in rabbit blood via multiplex quantitative real-time PCR targeting the flagellar filament cap gene (*fljD*) and the 16S ribosomal RNA gene (Goodrich et al. 2020). Additional primers were targeted with conventional PCR to further characterize the detected *Borrelia* DNA. We targeted the flagellin gene (*flaB*) and four housekeeping genes used for *Borrelia* MLSA: *clpX*, *pepX*, *recG*, and *rplB* (Margos et al. 2009). Primers are provided in Supplementary Material Table 1; detailed PCR protocols have been published (Schwan et al. 2005; Margos et al. 2015) or are available at PubMLST (Jolley et al. 2018). We separated PCR amplicons in 1.5%

agarose gels stained with Biotium GelGreen (Biotium, Hayward, California, USA) and purified them using the QIAquick PCR Purification Kit (QIAGEN, Valencia, California, USA). Products were sequenced in both directions on an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) and assembled using Lasergene (DNASTAR, Madison, Wisconsin, USA). Extraction of DNA, PCR, and sequencing were performed in separate laboratories to minimize contamination.

To produce a comprehensive *Borrelia* genus phylogeny, housekeeping gene sequences were compared with named *Borrelia* species available in GenBank (NCBI Resource Coordinators 2016) and PubMLST (Jolley et al. 2018; Supplementary Table 2). We included *Borrelia turcica*, *Candidatus B. mahuryensis*, and *Candidatus B. tachyglossi*, which constitute a third group of *Borrelia* distinct from the relapsing fever and Lyme disease clades (Binetruy et al. 2020). Flagellin gene sequences were compared to closely matching RF *Borrelia* sequences using BLAST (National Center for Biotechnology Information 2018). Sequences were aligned with MAFFT (Katoh and Standley 2013) using the L-INS-i method and trimmed. Phylogenetic model selection and maximum likelihood tree estimation were performed using IQ-Tree for concatenated housekeeping genes and *flaB* sequences separately (Minh et al. 2020).

Nine adult desert cottontails of unknown sex and one juvenile female were captured in April–October 2003; one cottontail, sampled September 2003, was positive for *Borrelia* sp. DNA (Goodrich et al. 2020). None of the 13 rodent species sampled in that study were positive. Sequences obtained from targeted loci were submitted to GenBank with accession numbers MZ408281–MZ408285 and were uploaded to PubMLST as isolate ID 3197. Our BLAST searches for each gene indicated that the novel *Borrelia* species from *S. audubonii* was most closely related to RF spirochetes *B. miyamotoi*, *B. theileri*, *B. lonestari*, and other *Borrelia* species identified in hard ticks or large mammal hosts (Supple-

mentary Table 3). The closest match (>94% sequence identity) for all genes was *Borrelia* sp. tHM16w from *Haemaphysalis megapinna* ticks collected from wild boar (*Sus scrofa*) in Japan (Furuno et al. 2017). The closest matching *flaB* sequences (>95% identity) were from Sika deer (*Cervus nippon*) and in *Haemaphysalis* spp. ticks from wild boar and Sika deer in Japan and in *Rhipicephalus sanguineus* ticks collected from vegetation or from sheep in Portugal and Iran (Supplementary Table 3). Phylogenetic analysis confirmed that the cottontail *Borrelia* belonged to a RF clade including *B. miyamotoi*, *B. theileri*, and *B. lonestari*, species associated with hard ticks (family Ixodidae) rather than the soft ticks (family Argasidae) typically associated with RF *Borrelia* species (Margos et al. 2020; Nakao et al. 2021). Although *clpX*, *pepX*, *recG*, and *rplB* sequences were not available for *B. theileri* and *B. lonestari*, the tree produced from concatenated sequences confirmed that the cottontail *Borrelia* species clustered with *Borrelia* sp. tHM16w and *B. miyamotoi* with 100% bootstrap support (Fig. 1). The *flaB* phylogeny showed that the cottontail *Borrelia* species clustered with the hard tick RF species *B. miyamotoi*, *B. theileri*, and *B. lonestari* with 100% bootstrap support, though it clustered more closely to *Borrelia* sp. tHM16w and other sequences from Eurasian hard ticks and large mammals with 93% bootstrap support (Fig. 2).

Borrelia spp. in the RF clade have not been reported in lagomorphs previously. Previous studies have detected *Borrelia burgdorferi* sensu lato (s.l.) in *Sylvilagus* rabbits and their associated ticks in North America (Burgess and Windberg 1989; Hamer et al. 2012). We did not detect *B. burgdorferi* s.l. in our small sample of *S. audubonii*. Additional surveys of *Sylvilagus* spp. in North America will be necessary to determine if the novel cottontail *Borrelia* or other RF *Borrelia* spp. are widespread in this host group, and if *B. burgdorferi* s.l. species are truly absent in the New Mexico population of *S. audubonii* or its ticks.

Phylogenetic analysis indicated that the novel cottontail *Borrelia* sp. is a member of

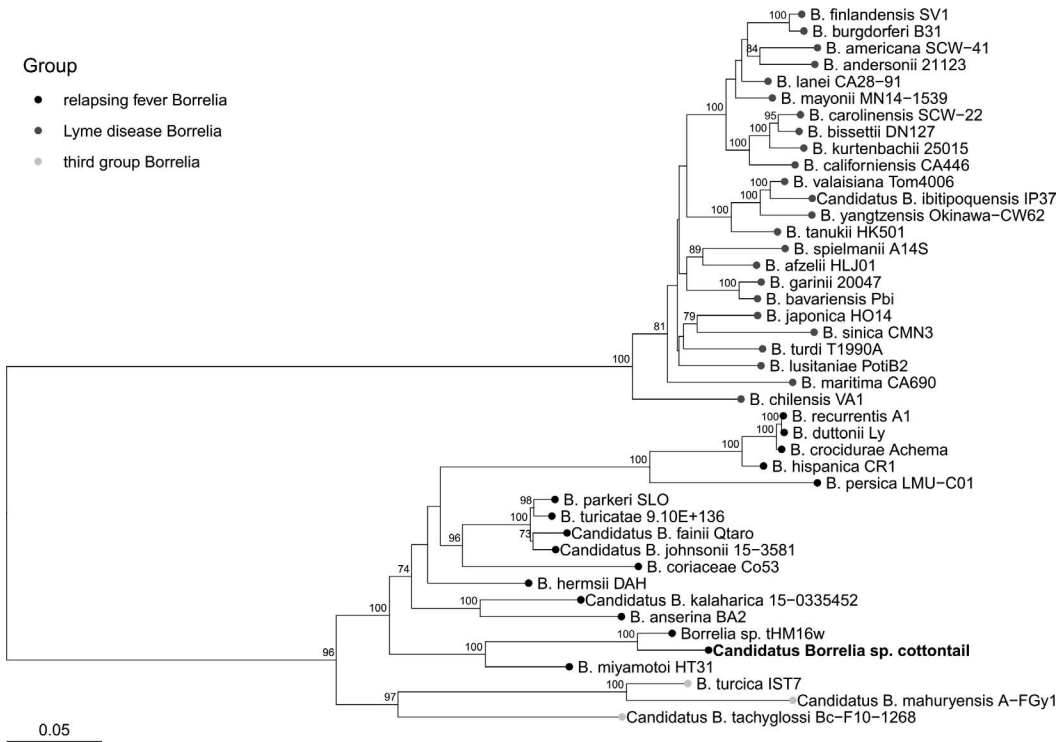


FIGURE 1. Maximum likelihood phylogenetic tree of concatenated *Borrelia* sequences produced from a 2468 base pair (bp) alignment of housekeeping genes (623 bp *clpX*, 570 bp *pepX*, 651 bp *recG*, 624 bp *rplB*). The best model of sequence evolution was GTR+F+I+G4 based on the Bayesian information criterion. The tree was rooted at the midpoint and bootstrap branch support values greater than 70% are shown next to branches. Strain names are labeled next to each *Borrelia* species name. GenBank accession numbers for genes from each strain are provided in Supplementary Material Table 2.

the hard tick RF clade with high bootstrap support (Figs. 1, 2), but the identity of its potential hosts beyond *S. audubonii* is difficult to infer. Close relatives of the novel species have been reported from hard ticks collected from deer, sheep, and wild boar (Supplementary Table 3). *Borreliae* of the hard tick RF clade have been detected in ungulates, rodents, birds, carnivores, and lemurs (Moore et al. 2003; Kang et al. 2018; Qurollo et al. 2018; Talagrand-Reboul et al. 2018). Detection of a novel *Borrelia* species in the desert cottontail widens the range of potential hosts of hard tick RF borreliae. The vectors of the novel *Borrelia* are unknown. New Mexico has multiple hard tick species, including nine *Ixodes* spp., four *Dermacentor* spp., *Haemaphysalis leporispalustris*, and *R. sanguineus* recorded (Bishopp and Trembley 1945; New

Mexico Department of Health 2021). Both *Dermacentor parumapertus* and *H. leporispalustris* are associated with lagomorphs (Bishopp and Trembley 1945). Joint surveys of *Borrelia* spp. in ticks and associated vertebrate hosts in New Mexico are needed to assess the full host and vector range of this novel cottontail *Borrelia* species.

Placement of the novel *Borrelia* species in the hard tick RF group raises questions about its zoonotic potential, though few *Borrelia* species are known to cause human illness (Cutler et al. 2017). Humans could be exposed to spirochetes in ticks parasitizing lagomorphs in peridomestic areas. At least two hard ticks parasitizing lagomorphs (*H. leporispalustris*, *Haemaphysalis longicornis*) have been reported to bite humans (Brown 1945; Wormser et al. 2020). The potential for

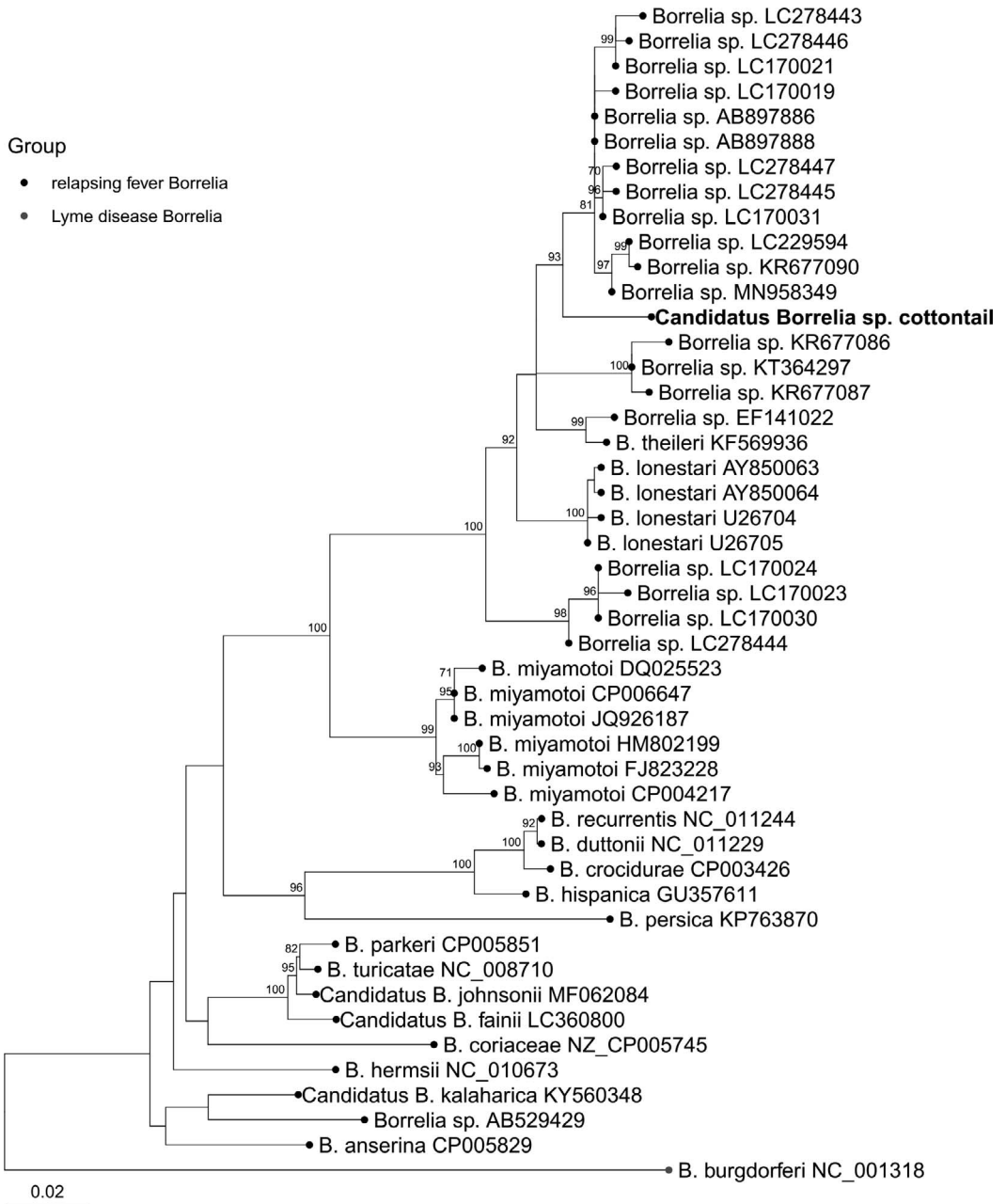


FIGURE 2. Maximum likelihood phylogenetic tree of hard tick relapsing fever *Borrelia flaB* sequences (1017 base pair, gaps included). The best model of sequence evolution was TIM3+F+G4 on the Bayesian information criterion. The tree was rooted at the midpoint and bootstrap branch support values greater than 70% are shown next to branches. GenBank accession numbers are given next to each *Borrelia* species name.

this novel *Borrelia* sp. to cause disease in humans is unknown.

We did not attempt to culture spirochetes from rabbit blood in this study due to the age

and limited volume of samples, thereby precluding more extensive genetic analysis (e.g., full genome sequencing). Attribution of the novel *Borrelia* sp. to the hard tick RF

clade may be strengthened by testing rabbit-associated ticks from the region. Experimental infection of *S. audubonii* and potential tick vectors with isolates of the novel *Borrelia* sp. could then establish host and vector competence (Eisen 2020). We recommend increased surveillance in lagomorphs and their ticks, together with expanded sampling from other geographic locations, to determine the prevalence and distribution of this novel cottontail *Borrelia* species.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at <http://dx.doi.org/10.7589/JWD-D-21-00148>.

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