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DETECTION OF *BORRELIA* GENOMOSPECIES 2 IN *IXODES SPINIPALPIS* TICKS COLLECTED FROM A RABBIT IN CANADA

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ABSTRACT: Lyme disease is a serious health problem, with many patients requiring in-depth clinical assessment and extended treatment. In the present study, we provide the first records of the western blacklegged tick, *Ixodes pacificus*, and *Ixodes spinipalpis* parasitizing eastern cottontails, *Sylvilagus floridanus*. We also documented a triple co-infestation of 3 tick species (*Ixodes angustus*, *I. pacificus*, *I. spinipalpis*) feeding on an eastern cottontail. Notably, we discovered a unique member of the Lyme disease bacterium, *Borrelia burgdorferi* sensu lato (s.l.) in Canada. *Ixodes spinipalpis* ticks, which were collected from an eastern cottontail on Vancouver Island, British Columbia (BC), were positive for *B. burgdorferi* s.l. With the use of polymerase chain reaction amplification on the tick extracts and DNA sequencing on the borrelial amplicons, we detected *Borrelia* genomospecies 2, a novel subgroup of the *B. burgdorferi* s.l. complex. Based on 416 nucleotides of the flagellin B (*flaB*) gene, our amplicons are identical to the *Borrelia* genomospecies 2 type strain CA28. *Borrelia* genomospecies 2 is closely related genetically to other *B. burgdorferi* s.l. genospecies, namely *Borrelia americana*, *Borrelia andersonii*, and *B. burgdorferi* sensu stricto (s.s.) that cause Lyme disease. Like some other borrelial strains, *Borrelia* genomospecies 2 can be missed by current Lyme disease serology. Health-care providers must be aware that *Borrelia* genomospecies 2 is present in *I. pacificus* and *I. spinipalpis* ticks in far-western North America, and patients with clinical symptoms of Lyme disease need to be assessed for potential infection with this pathogen.

Lyme disease (Lyme borreliosis) is recognized as a major medical problem in many countries. This pernicious disease is caused by members of *Borrelia burgdorferi* sensu lato (s.l.) complex, and this spirochetal bacterium is typically transmitted by ixodid (hard-bodied) ticks (Acari: Ixodidae) (Burgdorfer et al., 1982). Economically, this debilitating multisystem disease costs society billions of dollars in loss of schooling, employment, and health, especially for medical travel, doctor visits, diagnosis, testing, and treatment. In the United States, the cost is calculated to be $1.3 billion (Adrion et al., 2015), with extrapolated costs for Canada estimated to be $130,000,000 per year.

Ixodes spinipalpis is a blood-sucking ectoparasite indigenous to western North America including California to British Columbia (BC) and Alberta (Durden and Keirans, 1996). All stages of this tick species parasitize rodents and lagomorphs and, additionally, larvae and nymphs will feed on birds (Keirans and Clifford, 1978; Durden and Keirans, 1996). Epidemiologically, *I. spinipalpis* is a competent vector of *B. burgdorferi* s.l., and maintains Lyme disease spirochetes in enzootic transmission cycles (Brown and Lane, 1992; Dolan et al., 1997; Burkot et al., 2000). In semiarid regions, *I. spinipalpis* has an ecological pattern of being a nidicolous tick (Maupin et al., 1994).

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Ixodes angustus is another nidicolous tick that commonly parasitizes small rodents (Keirans and Clifford, 1978), and occasionally feeds on shrews, voles, rabbits, cats, dogs, and humans. Biogeographically, *I. angustus* has wide distribution across the Holarctic region (Gregson, 1956; Durden and Keirans, 1996). Importantly, *I. angustus* is a competent vector of *B. burgdorferi* s.l., and contributes to the enzootic cycle of Lyme disease spirochetes in this region (Banerjee et al., 1994a; Peavey et al., 2000).

The western blacklegged tick, *Ixodes pacificus*, is indigenous from Baja California to BC (Durden and Keirans, 1996). In California, this tick species has been collected from a wide array of vertebrate species, including lagomorphs (Castro and Wright, 2007). All host-feeding life stages (larvae, nymphs, adults) of *I. pacificus* have been reported on black-tailed jackrabbits, *Lepus californicus*, and, similarly, adults have been noted on Audubon's cottontail, *Silvilagus audubonii*, and larvae and nymphs have been collected from the brush rabbit, *Sylvilagus bachmani* (Castro and Wright, 2007). Importantly, *I. pacificus* is a competent vector of *B. burgdorferi* s.l. in far-western North America (Burgdorfer et al., 1985; Lane et al., 1994; Peavey and Lane, 1995).

Worldwide, the *B. burgdorferi* s.l. complex consists of at least 23 genospecies (sometimes described as genomospecies). In North America, *Borrelia americana*, *Borrelia andersonii, Borrelia bissettii*, *B. burgdorferi* sensu stricto (s.s.), *Borrelia californiensis*, *Borrelia carolinensis*, *Borrelia garinii*, *Borrelia* genomospecies 2, *Borrelia kurtenbachii*, and *Borrelia mayonii* have been detected in ixodid ticks, and some have been found in mammalian hosts, including humans (Baranton et al., 1992; Marconi et al., 1995; Postic et al., 1998; Smith et al., 2006; Rudenko et al., 2009a, 2009b; Margos et al., 2010; Pritt et al., 2016). Of these genospecies, *B. americana*, *B. andersonii*, *B. bissettii*, *B. burgdorferi* s.s., and *B. mayonii* have been detected in Lyme disease patients in North America (Girard et al., 2011; Clark et al., 2013; Golovchenko et al., 2016; Pritt et al., 2016; Rudenko et al., 2016). Previously, *B. americana* (Scott and Foley, 2016), *B. bissettii*

(culture 1340) (Banerjee et al., 1994a), and *B. burgdorferi* s.s. (Scott et al., 2010) were discovered in ixodid ticks in BC.

The archetype isolates of *Borrelia* genomospecies 2 were obtained from ticks collected in California. Strain CA2 was cultured from an *I. spinipalpis* tick (listed as *Ixodes neotomae*, which has since been relegated to a junior synonym of *I. spinipalpis*) (Postic et al., 1994), and type strain CA28 was isolated from an *I. pacificus* tick (Schwan et al., 1993). With the use of multilocus sequence analysis (MLSA), Postic et al. (2007) grouped these 2 novel borrelial strains into *Borrelia* genomospecies 2.

In far-western North America, *I. spinipalpis* is involved in the enzootic transmission cycle of *B. burgdorferi* s.l. featuring rodents (i.e., deer mice, *Peromyscus maniculatus*; dusky-footed woodrats, *Neotoma fuscipes*), hares (i.e., white-tailed jackrabbits, *Lepus townsendii townsendii*) (Brown and Lane, 1992), and now rabbits (i.e., eastern cottontails, *Sylvilagus floridanus*). Deer mice and dusky-footed woodrats are reservoir-competent hosts of *B. burgdorferi* s.l. (Eisen et al., 2003). Also, prairie voles, *Microtus ochrogaster*, which are native to east-central Alberta, are competent reservoirs of *B. burgdorferi* s.l. when infected *I. spinipalpis* feed on them (Burkot et al., 2000; Zeidner et al., 2000). Along the U.S. East Coast, Anderson et al. (1989) isolated *B. burgdorferi* s.l. from eastern cottontails and from larvae and nymphs of *Ixodes dentatus* parasitizing them. In addition, larval *I. dentatus* acquired borrelial spirochetes from feral eastern cottontails, indicating that these lagomorphs are reservoirs of *B. burgdorferi* s.l. (Anderson et al., 1989; Telford and Spielman, 1989). These borreliae were later named *B. andersonii* (Marconi et al., 1995).

The main objective of this study was to determine tick vectors and potential reservoir-competent hosts of *B. burgdorferi* s.l. in far-western Canada and to assess the diversity of these Lyme disease spirochetes genetically. This study was part of ongoing investigation of tick–host associations in far-western Canada.

MATERIALS AND METHODS

Tick collection

All of the lagomorphs were collected in southwestern British Columbia, and were taken to wildlife rehabilitation centers because they were injured. Wildlife rehabilitators removed ticks during physical examination of the admitted animals. In one particular case, an adult male eastern cottontail was found in Metchosin, Vancouver Island, BC, Canada, and brought to BC SPCA Wild ARC, an animal rehabilitation center for wildlife, located near Metchosin, BC. Because this eastern cottontail had severe injuries, including a fractured leg, it was euthanized. Upon further examination, 3 engorged ticks were found, and removed with fine-point, stainless-steel tweezers. The ticks were put in a round-bottom, 8.5-ml polypropylene tube (15.7 mm × 75 mm) (Sarstedt, Montreal, Québec, Canada) with attached labels specifying date collected, host, geographic location, and collector's name. A 7-mm hole in the polyethylene push cap (15.7 mm) allowed ventilation for the ticks and, to prevent the ticks from escaping, fine tulle netting was stretched over the mouth of the vial before the push cap was inserted. The tube, which contained the ticks from a single host, was placed in a self-sealing, double-zipper plastic bag with a slightly moistened paper towel to maintain high humidity. The live ticks were sent to the laboratory (JDS), and identified with the use of taxonomic keys (Keirans and Clifford, 1978; Durden and Keirans, 1996).

The methodology to allow replete ticks to molt is as follows: fully engorged ticks were inserted in separate 8.5-ml polypropylene tubes with a slightly moistened piece (5 cm × 5 cm) of paper towel. The vented push caps allowed the ticks to have consistent humidity at ~95% moisture. Tubes were placed in a self-sealed plastic bags with slightly moistened paper. A full spectrum LifeLite A21/E26, 12-watt LED, light bulb (LifeEnergy Systems, Richmond Hill, Ontario, Canada), on a timer, was set for the summertime photoperiod of 16:8/L:D. Each tube was checked every 3–5 days to assess the progress of the molt and to ensure that adequate humidity was maintained. A log sheet was kept to record the dates checked, progress of molt, and the number of days to complete the molt.

Spirochete detection

After identification, the *I. spinipalpis* ticks were put in a 2-ml microtube containing 94% ethyl alcohol, and sent via courier to a separate molecular biology laboratory (KLC) for *B. burgdorferi* s.l. testing and molecular analysis. DNA extraction, polymerase chain reaction (PCR) amplification, and DNA sequencing of amplified products were performed as previously described (Scott et al., 2016a).

PCR testing methods for this study were as follows: Tick extracts were initially screened for *B. burgdorferi* s.l. with the use of a nested PCR assay that amplifies a portion of the 41-kDa chromosomal flagellin B (*flaB*) gene. Primary/outer reaction primers were 271F (5′-AAG-GAA-TTG-GCA-GTT-CAA-TCA-GG-3′) and 767R (5′-GCA-TTT-TCT-ATT-TTA-GCA-AGT-GAT-G-3′), which amplify a 497–base-pair (bp) fragment; inner reaction primers were 301F (5′-ACA-TAT-TCA-GAT-GCA-GAC-AGA-GG-3′) and 737R (5′-GCA-TCA-ACT-GTA-GTT-GTA-ACA-TTA-ACA-GG-3′), which amplify a 437-bp product. First-round PCR amplifications contained 2.5 µl of tick DNA extract in a total reaction volume of 50 µl. Each inner/nested reaction used 1 µl of outer reaction product as template. First-round amplifications utilized a hot start PCR master mix (HotMasterMix, 5 Prime, Gaithersburg, Maryland) resulting in a final concentration of 1.0 U of *Taq* DNA polymerase, 45 mM KCl, 2.5 mM Mg2+, 200 µM of each deoxynucleoside triphosphate, and 0.5 µM of each primer. Second-round amplifications used GoTaqGreen® PCR Master Mix (Promega, Madison, Wisconsin), which allowed samples to be directly loaded into agarose gels without the addition of a gel loading buffer. All PCRs were carried out in an Applied Biosystems AB2720 thermal cycler (Life Technologies, ThermoFisher Scientific, Waltham, Massachusetts). Each primary PCR consisted of initial denaturation at 94 C for 2 min, followed by 35 cycles at 94 C for 30 sec, primer annealing at 52 C for 30 sec, and extension at 65 C for 1 min, with a final extension at 65 C for 5 min. Nested reactions included initial denaturation at 94 C for 1 min, followed by 35 cycles of amplification with an annealing temperature of 55 C for 30 sec, and extension temperature of 72 C for 1 min. Positive results with the *flaB* PCR were confirmed by PCR and DNA sequencing of a portion of the 16S-23S rRNA intergenic spacer using primers and parameters described by Bunikis et al. (2004).

TABLE I. Presence of *Borrelia burgdorferi* s.l. in ticks collected from lagomorphs in southwestern British Columbia, 2012–2016. Abbreviations: neg, negative; pos, positive; L, larva(e); N, nymph(s); M, male(s); F, female(s).

Tick no.	Location	Date collected	Host	Tick species	Life stage	Infection results
12-5A53	Highlands	29 August 2012	Eastern cottontail	*Ixodes spinipalpis*	F	neg
12-5A105	Metchosin	5 October 2012	Eastern cottontail	*I. spinipalpis*	1N, 4F	neg, 4 neg
13-5A87A	Maple Ridge	13 June 2013	Snowshoe hare	*Ixodes pacificus*	M, F	pos*, pos*
13-5A87B	Maple Ridge	13 June 2013	Same host	*I. spinipalpis*	F, F	pos*, neg
14-5A124	Cobble Hill	12 June 2014	Eastern cottontail	*I. spinipalpis*	2F, M	neg, neg, pos*
15-5A22A	Metchosin	3 May 2015	Eastern cottontail	*I. spinipalpis*	3F	neg, neg, pos†
15-5A22B	Metchosin	3 May 2015	Same host	*I. spinipalpis*	3M	neg, pos†, neg
16-5A6A	Saanich	12 April 2016	Eastern cottontail	*I. spinipalpis*	N	neg
16-5A6B	Saanich	12 April 2016	Same host	*Ixodes angustus*	F	pos‡
16-5A34A	Victoria	27 May 2016	Eastern cottontail	*I. pacificus*	L→N	neg
16-5A34B	Victoria	27 May 2016	Same host	*I. spinipalpis*	L→N	neg
16-5A84§	Saanichton	6 September 2016	Eastern cottontail	*I. angustus*,	M, F	neg, neg
				I. pacificus,	N	pos
				I. spinipalpis	N→F	neg

* DNA sequencing was not conducted.
† *Borrelia* genomospecies 2.
‡ Not able to sequence for genomospecies.
§ Triple co-infestation.

PCRs were set up in an area separate from DNA extractions, and within a PCR clean cabinet (CleanSpot Workstation, Coy Laboratory Products, Grass Lake, Michigan) equipped with a germicidal UV lamp. Other precautions were employed to prevent carryover contamination of amplified DNA, including different sets of pipettes dedicated for DNA extraction, PCR setup, and postamplification activities. As an additional precaution, aerosol barrier filter pipette tips were used for handling DNA samples, and pipettes were soaked in 10% bleach solution after setting up each PCR. Each PCR test included negative control samples with nuclease-free TE buffer as a template. As a further measure to minimize DNA artifact contamination of PCR testing, no positive control samples were used. PCR products were electrophoresed in 2% agarose gels, which were stained with ethidium bromide, and visualized and recorded with a digital gel documentation unit.

Nucleotide sequences

The DNA nucleotide sequences for amplicons of the *B. burgdorferi* s.l. *flaB* gene were deposited in the GenBank: KX644891 for tick 15-5A22A3 (*I. spinipalpis* female) and KX644892 for tick 15-5A22B2 (*I. spinipalpis* male). Likewise, the sequences for the amplicons of the 16S-23S rRNA intergenic spacer gene are KX644893 for tick 15-5A22A3 and KX644894 for tick 15-5A22B2.

RESULTS

In all, 28 ticks were collected from 8 lagomorphs in southwestern British Columbia (Table I); 3 lagomorphs were co-infested with 2 tick species. Three tick species, namely *I. angustus* (male, female), *I. pacificus* (nymph), *I. spinipalpis* (nymph) were collected from an eastern cottontail, which was recovered on the outskirts of Saanichton, Vancouver Island, BC on 6 September 2016; this triple co-infestation constitutes a new multitick parasitism for eastern cottontail. An *I. spinipalpis* nymph, which was in the species triad, molted to a female in 58 days. In a separate parasitism, rehabilitation staff found 3 ticks on the ventral surface of a rabbit's neck in juxtaposition to the jugular veins (Fig. 1A, B). When the engorged ticks were sent to the laboratory (JDS), they were identified as *I. spinipalpis* females. In addition, 3 *I. spinipalpis* males were found mating on the underside of the females. In total, 6 *I. spinipalpis* adults (females 15-5A22A1-3, males 15-5A22B1-3) were collected from the eastern cottontail (Table I).

With the use of nested PCR amplification with primers of the *flaB* gene and the 16S-23S rRNA intergenic spacer gene, 2 *I. spinipalpis* adults (1 female, 1 male) were positive for *B. burgdorferi* s.l. After end-trimming of the 437-bp product of the *flaB* gene, we found a conserved *flaB* segment of 416 nucleotides from the 2 positive *I. spinipalpis* ticks to be 100% identical to the *Borrelia* genomospecies 2 type strain CA28 located in the GenBank database. Based on these molecular findings, we conclude that the 2 *I. spinipalpis* adults were infected with *Borrelia* genomospecies 2.

In addition, a fully engorged *I. pacificus* larva (16-5A34A) and a fully engorged *I. spinipalpis* larva (16-5A34B), which were co-feeding on another eastern cottontail, molted to nymphs in 33 and 36 days, respectively (Table I).

DISCUSSION

We document the first account of *Borrelia* genomospecies 2 in Canada. Our discovery follows earlier reports of this *B. burgdorferi* s.l. genomospecies in the state of California. Previously, *Borrelia* genomospecies 2 was isolated from *I. pacificus* and *I. spinipalpis* in California, which indicates that this *Borrelia* group is implicated in enzootic maintenance cycles along the West Coast of North America. Because of the lack of molecular, serological, and pathological information, we do not know if *Borrelia* genomospecies 2 is pathogenic to humans.

Eastern cottontails are an invasive rabbit species in BC, and were introduced to Metchosin, BC, on the southern shore of Vancouver Island, in 1964 and, subsequently, have been gradually expanding northward on the island (Nagorsen, 2005). Depending

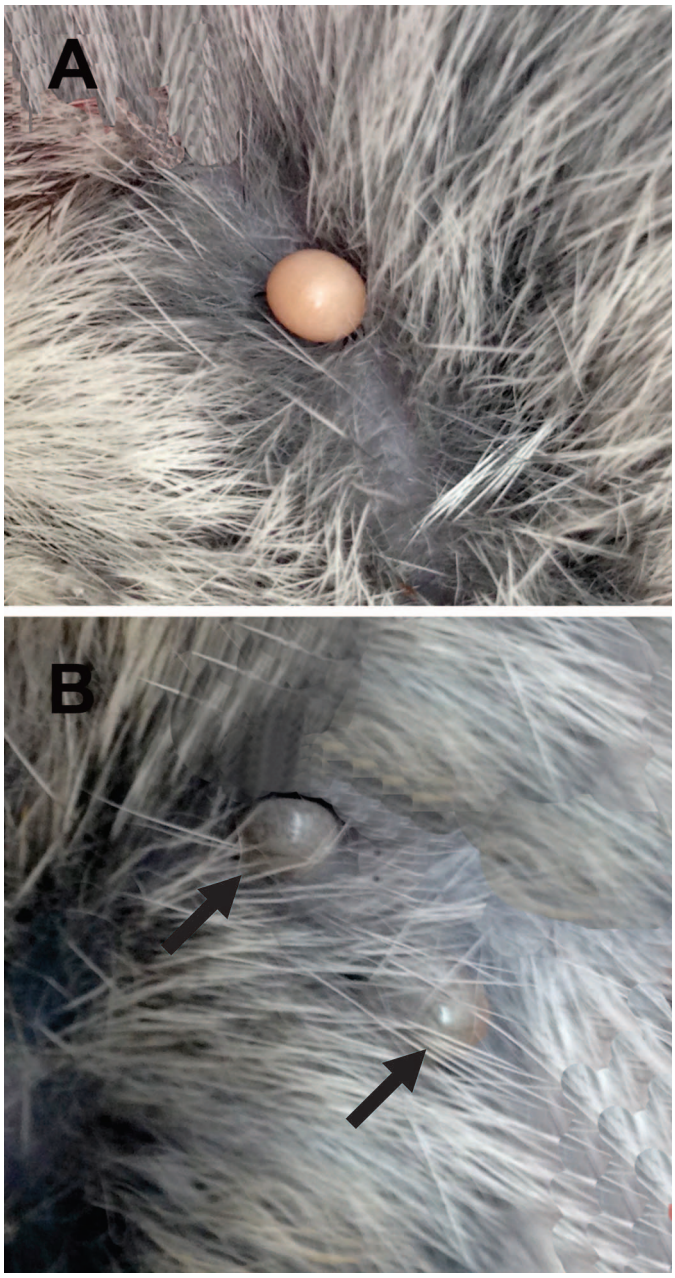

FIGURE 1. *Ixodes spinipalpis*, engorged females, parasitizing eastern cottontail: (**A**) 1 female feeding on the ventral surface of the neck, (**B**) these 2 females are well camouflaged and hidden in the long, thick hair of the rabbit's neck. The 3 mating males are not visible in either photos A or B. Photo credits: Vanessa Williams.

on the season and habitat, this terrestrial mammal has a home range of 0.5–16 ha (Trent and Rongstad, 1974). Ecologically, several vertebrates living in ecosystems of Garry oak, *Quercus garryana*, play a significant role in an enzootic cycle of *B. burgdorferi* s.l. along the West Coast (Banerjee et al., 1994a; Costanzo et al., 2011). Garry oak woodlands in southwestern BC provide an ideal habitat for a wide range of mammals, including eastern cottontails and snowshoe hares, *Lepus americanus*. Lagomorphs frequently inhabit Garry oak ecosystems, and are hosts of all host-feeding stages of *I. spinipalpis* and *I. pacificus* (Durden and Keirans, 1996; Castro and Wright, 2007). Ecologically, Garry oak habitats sustain birds, rodents, lagomorphs, and Columbian black-tailed deer, *Odocoileus hemonionus columbianus*, which act as hosts for Lyme disease vector ticks. Acorns from Garry oaks provide high-energy food for a diversity of vertebrates, such as deer mice and Columbian black-tailed deer. Because mice, chipmunks, and deer eat acorns, Garry oak ecosystems act as communal hubs for Lyme disease vector ticks and their hosts.

In California, lizards are the most common host of *I. pacificus* larvae and nymphs (Eisen et al., 2004), whereas, in BC, cricetid rodents (i.e., deer mice) and sciurid rodents (i.e., Townsend's chipmunk, *Neotamias townsendii*) are the most common hosts of *I. pacificus* immatures (Banerjee et al., 1994a, 1994b). Columbian black-tailed deer are the most important hosts for adult *I. pacificus* (Durden and Keirans, 1996). Although deer are not competent reservoirs of *B. burgdorferi* s.l. (Telford et al., 1988), they support and amplify the reproduction of *I. pacificus*. Of note, transovarial transmission of *B. burgdorferi* s.l. is not evident in *I. pacificus* (Schoeler and Lane, 1993).

First records of *Ixodes* ticks on eastern cottontails

We provide the first records of *I. pacificus* and *I. spinipalpis* parasitizing eastern cottontails, and are unaware of any previous records of these 2 tick species from eastern cottontails. An *I. pacificus* larva was collected from an eastern cottontail on 27 May 2016 at Victoria, BC (Table I); this collection represents the first record of an *I. pacificus* parasitizing an eastern cottontail. An *I. spinipalpis* female was collected from an eastern cottontail on 29 August 2012 at Highlands, BC; this parasitism signifies the first record of *I. spinipalpis* on an eastern cottontail (Table I). Enzootically, an *I. spinipalpis* male, which was infected with *B. burgdorferi* s.l., was collected from an eastern cottontail on 12 June 2014 at Cobble Hill, BC; this tick collection constitutes the first *B. burgdorferi* s.l.-infected *I. spinipalpis* parasitizing an eastern cottontail. Based on our findings, all host-feeding stages (larvae, nymphs, adults) of *I. spinipalpis* parasitize eastern cottontails. In addition, a fully engorged *I. angustus* female was collected from an eastern cottontail on 12 April 2016 at Saanich, BC (Table I); this parasitism stands for the first record of *I. angustus* on an eastern cottontail in Canada. Moreover, we provide the first account of 3 tick species (*I. angustus*, *I. pacificus*, *I. spinipalpis*) simultaneously feeding on an eastern cottontail (Table I). The neck is a preferred attachment site for lagomorph-feeding ticks because these ectoparasites are well protected in this cutaneous area, and out of the reach of biting teeth and grooming appendages, namely front and hind paws (Fig. 1A, B).

Eastern cottontails as *Borrelia* reservoirs

In the eastern United States, all host-feeding life stages of blacklegged ticks, *Ixodes scapularis*, have been collected from feral cottontail rabbits (Anderson and Magnarelli, 1999). Eastern cottontails act as reservoirs of certain Lyme disease spirochetes. Anderson et al. (1989) cultured *Borrelia* from eastern cottontails captured in eastern New York state and, likewise, from attached *I. dentatus*. These borrelial spirochetes were later named *B. andersonii* (Marconi et al., 1995).

In southwestern BC, we have collected *B. burgdorferi* s.l.-infected ticks (*I. angustus*, *I. pacificus*, *I. spinipalpis*) from invasive eastern cottontails and indigenous snowshoe hares (Table I). All

of these tick species are competent vectors of *B. burgdorferi* s.l. (Eisen and Lane, 2002). Scott et al. (2014) reported *I. pacificus* adults on a snowshoe hare in southwestern BC, and 1 of these ticks was infected with *B. burgdorferi* s.l.; this heavily infested lagomorph died of tick paralysis. Furthermore, co-infestations of 2 species of ticks have been found on lagomorphs in this coastal habitat, namely *I. angustus* with *I. spinipalpis* and, similarly, *I. pacificus* with *I. spinipalpis* (Table I). Such co-infestations provide ample opportunity for transmission of *B. burgdorferi* s.l. from 1 tick species to another. Furthermore, the triple co-infestation of *I. angustus*, *I. pacificus*, and *I. spinipalpis* on an eastern cottontail shows the potential to transmit *B. burgdorferi* s.l. simultaneously to 3 attached tick species.

Genetic association of *Borrelia* genomospecies 2

As a member of the *B. burgdorferi* s.l. complex, *Borrelia* genomospecies 2 is part of the group of microorganisms that cause Lyme disease. The type strain, CA28 was initially isolated from an *I. pacificus* tick in California and, likewise, the isolate CA2 was obtained from an *I. spinipalpis* tick in the same state (Schwan et al., 1993; Postic et al., 1994). In the present study, a 416-bp segment of the *flaB* gene was 100% homologous to *Borrelia* genospecies 2 type strain CA28. With the use of MLSA concatenated sequences of 7 loci, Postic et al. (2007) found that *Borrelia* genomospecies 2 is genetically most similar to *B. americana* strains (differing by 5 nucleotides in the *flaB* fragment analyzed in the present study) and, similarly, closely related to *B. burgdorferi* s.s. type strain B31 (6 nucleotide differences in the *flaB* fragment). The close genetic relationship of *Borrelia* genomospecies 2, which was detected in the *flaB* amplicons from *I. spinipalpis* ticks (15-5A22A3, 15-5A22B2) collected in BC, indicates there has been north–south movement of *Ixodes* ticks infected with *Borrelia* genomospecies 2 along the Pacific Coast.

Epidemiology of *Borrelia* genomospecies 2 in Canada

Prior to the present study, the closest known location for this *Borrelia* genomospecies 2 was California. The presence of *Borrelia* genomospecies 2 in *I. spinipalpis* ticks collected from an eastern cottontail in BC suggests that this zoonotic bacterium is widely distributed by bird-transported ticks. As a competent vector, *I. spinipalpis* transmits *B. burgdorferi* s.l. from infected hosts to noninfected hosts, including rodents, lagomorphs, and birds. Although an uncommon occurrence, *I. spinipalpis* is known to parasitize humans (Cooley and Kohls, 1945; Gregson, 1956; Maupin et al., 1994; Dolan et al., 1997; Merten and Durden, 2000; Eisen et al., 2006; Zeidner et al., 2000). In nature, *I. spinipalpis* is an enzootic vector of Lyme disease spirochetes, and *I. pacificus* is a bridge vector to humans (Brown and Lane, 1992; Clover and Lane, 1995). In California and Oregon, *I. spinipalpis* functions as an enzootic vector of *B. burgdorferi* s.l. among dusky-footed woodrats and, when *I. pacificus* feeds on *B. burgdorferi* s.l.-infected woodrats, this tick species can then bite and transmit Lyme disease spirochetes to humans (Clover and Lane, 1995). Epidemiologically, the discovery of *Borrelia* genomospecies 2 in *I. spinipalpis* adults collected from eastern cottontails represents another potential vertebrate reservoir for Lyme disease spirochetes in Canada.

This is the first record of *Borrelia* genomospecies 2 in Canada and, moreover, the first account of this *Borrelia* group in *I. spinipalpis* ticks in Canada. In addition, this is the first documentation of ticks infected with *Borrelia* genomospecies 2 parasitizing a lagomorph in North America. Furthermore, this parasitism represents the northernmost record of *Borrelia* genomospecies 2 in North America, and constitutes a new distribution record.

Vector competence of *I. spinipalpis*

Recent tick-host studies reveal that *I. pacificus* and *I. spinipalpis* co-infest birds (Scott et al., 2012), and also co-feed on mammals in southwestern BC. In the present study, we recorded 3 individual hosts co-infested with *I. spinipalpis* and other tick species (Table I). Of note, *I. pacificus* and *I. spinipalpis* were cofeeding on a snowshoe hare and, likewise, co-feeding on eastern cottontails. Because *I. pacificus* and *I. spinipalpis* parasitize rodents, lagomorphs, and birds, each of these vertebrates could be reservoir hosts of *Borrelia* genomospecies 2. Because eastern cottontails have a localized home range, and are parasitized by all 3 host-feeding life stages of *I. pacificus* and *I. spinipalpis*, it is likely that these lagomorphs are temporary or long-term reservoirs of *Borrelia* genomospecies 2. During co-infestation by Lyme disease vector ticks, *Borrelia* genomospecies 2 could be transmitted from *I. spinipalpis* to *I. pacificus*, and vice versa. In tick-conducive habitats in far-western Canada, *I. spinipalpis* could transmit *Borrelia* genomospecies 2 to avian and mammalian vertebrates, and may subsequently pass Lyme disease spirochetes to bridge vector ticks, such as *I. pacificus*, and then onward to humans and domestic animals (Brown and Lane, 1992).

Birds involved in the enzootic cycle of *B. burgdorferi* s.l.

Wild birds provide an interconnecting link between BC and California where *Borrelia* genomospecies 2 was initially discovered. During bidirectional migration in spring and fall, migratory songbirds play a key role in the wide dispersal of *I. pacificus* and *I. spinipalpis* ticks (Morshed et al., 2005; Scott et al., 2010, 2012, 2015; Scott and Foley, 2016). Along BC's coast, the avian coastal tick, *Ixodes auritulus*, which is exclusively an ectoparasite of birds, had a 31% infection prevalence of *B. burgdorferi* s.l. (Scott et al., 2015). Notably, *I. auritulus*, *I. pacificus*, and *I. spinipalpis* parasitize birds in this bioregion, and help to maintain the enzootic transmission cycle of Lyme disease spirochetes (Scott et al., 2013, 2015, 2016a; Scott and Foley, 2016). Of enzootic significance, Scott et al. (2012) reported 3 different tick species (i.e., *I. auritulus*, *I. pacificus*, *I. spinipalpis*) co-feeding on a song sparrow. When any 1 of these 3 tick species is infected with *Borrelia* genomospecies 2, it can transmit spirochetes to other bird-feeding ticks by co-feeding or by sequential feeding on reservoir-competent birds.

Some birds are reservoir hosts of *B. burgdorferi* s.l. (Richter et al., 2000). Lyme disease spirochetes have been cultured from birds and skin biopsies (Anderson and Magnarelli, 1984; Anderson et al., 1986, 1990; McLean et al., 1993; Durden et al., 2001). Recently, Newman et al. (2015) reported *B. bissettii* and *B. burgdorferi* s.s. in the blood of songbirds collected in northwestern California. Throughout the western hemisphere, Neotropical songbirds have the capacity to transport ticks thousands of kilometers, and widely disperse them across continental United States and Canada during northward spring migration (Morshed et al., 2005; Ogden et al., 2008; Scott et al., 2001, 2010, 2012, 2016b; Scott and Durden 2015a, 2015b, 2015c, 2015d). Scott et al.

(2015) reported *I. pacificus* and *I. spinipalpis* on songbirds (Passeriformes) in western Canadian provinces and, moreover, on gallinaceous birds (Scott et al., 2016a). These bird-feeding ticks can be infected with a wide array of *B. burgdorferi* s.l. genospecies/genomospecies, including *Borrelia* genomospecies 2. Because Vancouver Island is surrounded by water, migratory birds provide a natural mode of transport for bird-feeding ticks to and from neighboring islands and the mainland. Furthermore, passerine migrants furnish a natural geographic link for *Borrelia* genomospecies 2 between California and Vancouver Island, and beyond.

Pathogenicity of *Borrelia* genomospecies 2

The pathogenicity of *Borrelia* genomospecies 2 to humans has yet to be determined. Based on phylogenetic analysis of 5 loci of *B. burgdorferi* s.l. type strains, Rudenko et al. (2009a) found that *Borrelia* genomospecies 2 is closely related to *B. americana*, *B. andersonii*, and *B. burgdorferi* s.s.; all of the latter strains are pathogenic to humans (Clark et al., 2013). As with some other borrelial strains, *Borrelia* genomospecies 2 may be missed by current Lyme disease serology. In far-western Canada, Lyme disease patients have been diagnosed for decades (Banerjee et al., 1994b), but the different *Borrelia* genomospecies have not been determined in these human cases. Because medical professionals in western Canadian provinces have not been actively screening patients for specific borrelial genotypes, we do not know if *Borrelia* genomospecies 2 is causing Lyme disease in patients. Because *Borrelia* genomospecies 2 is closely associated genetically with pathogenic strains of *B. burgdorferi* s.l., it has the formidable potential to be pathogenic to humans.

In conclusion, we document the northernmost location of *Borrelia* genomospecies 2 in the western hemisphere. With this novel discovery, we reveal the presence of at least 4 genomospecies of *B. burgdorferi* s.l. in British Columbia. *Borrelia* genomospecies 2 is harbored by *I. pacificus* and *I. spinipalpis* ticks and, ultimately, may be transmitted to people. Because *Borrelia* genomospecies 2 is closely related genetically to other members of the *B. burgdorferi* s.l. complex that cause pernicious Lyme disease, we imply that *Borrelia* genomospecies 2 may be a member of the *Borrelia* group that is pathogenic to humans. In order to determine the pathogenicity of *Borrelia* genomospecies 2, patients exhibiting clinical signs and symptoms of Lyme disease need to be studied and tested with laboratory methods capable of identifying *Borrelia* genomospecies 2.

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