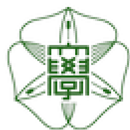

HOKKAIDO UNIVERSITY

Title	A relapsing fever group Borrelia sp similar to Borrelia lonestari found among wild sika deer (Cervus nippon yesoensis) and Haemaphysalis spp. ticks in Hokkaido, Japan
Author(s)	Lee, Kyunglee; Takano, Ai; Taylor, Kyle; Sashika, Mariko; Shimozuru, Michito; Konnai, Satoru; Kawabata, Hiroki; Tsubota, Toshio
Citation	Ticks and Tick-borne Diseases, 5(6), 841-847 https://doi.org/10.1016/j.ttbdis.2014.06.006
Issue Date	2014-10
Doc URL	http://hdl.handle.net/2115/57634
Type	article (author version)
File Information	TicksTickBorneDis v.5p.841.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

1 Journal: Ticks and Tick-Borne Diseases

2 Article types: Original Research Papers

3

4 Title: A Relapsing fever group *Borrelia* sp. similar to *Borrelia lonestari* found among wild sika deer

5 (*Cervus nippon yesoensis*) and *Haemaphysalis* spp. ticks in Hokkaido, Japan.

6 Kyunglee Lee[1], Ai Takano[2], Kyle Taylor[3], Mariko Sashika[1], Michito Shimozuru[1], Satoru Konnai[4],

7 Hiroki Kawabata[5], and Toshio Tsubota*[1]

8

9 Affiliations:

10 1. Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido

11 University, Sapporo, Japan

12 2. Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Yamaguchi University,

13 Yamaguchi, Japan

14 3. Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of

15 Florida, Florida, USA

16 4. Laboratory of Infectious Disease, Graduate School of Veterinary Medicine, Hokkaido University,

17 Sapporo, Japan

18 5. Department of Bacteriology-1, National Institute of Infectious Disease, Tokyo, Japan

19

20 * Corresponding author: Toshio Tsubota, Laboratory of Wildlife Biology and Medicine, Graduate

21 School of Veterinary Medicine, Hokkaido University, Kita18Nishi9, Kita-ku, Sapporo 060-0818,

22 Japan.

23 TEL: 81-11-706-5101. FAX: 81-11-706-5569. E-mail: tsubota@vetmed.hokudai.ac.jp

24

25

26

Abstract

A relapsing fever *Borrelia* sp. similar to *Borrelia lonestari* (herein referred to as *B. lonestari*-like) was detected from wild sika deer (*Cervus nippon yesoensis*) and *Haemaphysalis* ticks in the eastern part of Hokkaido, Japan. The total prevalence of this *Borrelia* sp. in tested deer blood samples was 10.6% using conventional PCR and real-time PCR. The prevalence was significantly higher in deer fawns compared to adults (21.9% and 9.4%, respectively). Additionally, there was significant regional difference between our two sampling areas, Shiretoko and Shibetsu with 17% and 2.8% prevalence, respectively. Regional differences were also found in tick species collected from field and on deer. In the Shiretoko region, *Haemaphysalis* spp. were more abundant than *Ixodes* spp., while in Shibetsu, *Ixodes* spp. were more abundant. Using real-time PCR analysis, *B. lonestari*-like was detected from 2 out of 290 adult *Haemaphysalis* spp. ticks and 4 out of 76 pools of nymphs. This is the first report of a *B. lonestari*-like organism in *Haemaphysalis* spp. ticks, and the first phylogenetic analysis of this *B. lonestari*-like organism in Asia. Based on our results, *Haemaphysalis* spp. are the most likely candidates to act as a vector for *B.lonestari*-like; furthermore, regional variation of *B.lonestari*-like prevalence in sika deer may be dependent on the population distribution of these ticks.

Keywords: *Borrelia lonestari*-like, *Haemaphysalis* spp., sika deer, ticks, Hokkaido

Introduction

The genus *Borrelia* is comprised of three phylogenetic groups: Lyme disease (LD) borreliae, which include the agents of Lyme diseases, Relapsing fever (RF) borreliae, and Reptile-associated (REP) borreliae (Takano et al., 2010; Franke et al., 2012). LD and REP borreliae are transmitted by ixodid (hard-bodied) ticks while most RF borreliae are transmitted by argasid (soft-bodied) ticks, except for *Borrelia recurrentis*, which is transmitted by lice. Some RF borreliae such as *Borrelia theileri*, *Borrelia miyamotoi*, and *Borrelia lonestari*, however, use hard-bodied ticks as vectors: *Rhipicephalus* spp., *Ixodes* spp., or *Amblyomma* spp., respectively (Smith et al., 1978; Fukunaga et al., 1995; Armstrong et al., 1996; Barbour et al., 1996; Scoles et al., 2001; Barbour, 2005). *B. theileri* is the causative agent of bovine theileriosis (Smith et al., 1985). *B. miyamotoi* was originally isolated in Japan (Fukunaga et al., 1995), and was considered a non-pathogenic species until recently, when Platonov et al. (2011) reported the first evidence of human infections in Russia. This was followed by human case reports from the United States and Holland, including two patients who developed menigoencephalitis (Chowdri et al., 2013; Gugliotta et al., 2013; Hovius et al., 2013). The pathogenicity of *B. lonestari* in humans is still unclear (Feder et al., 2011), although it was once suspected to be the agent of Southern Tick-Associated Rash Illness, a disease with Lyme disease-like symptoms associated with *Amblyomma americanum* (Burkot et al., 2001; James et al., 2001; Stromdahl et al., 2003). Understanding the biology of these RF borreliae, which are transmitted by hard-bodied ticks, has advanced slowly due to the difficulty of cultivation.

In the United States, there is strong evidence implicating the white-tailed deer (*Odocoileus virginianus*) as the main reservoir of *B. lonestari* (Moore IV et al., 2003; Moyer et al., 2006; Varela-Stokes, 2007). *B. lonestari* DNA prevalence in *A. americanum* from 29 sites in 4 states was 2.5%, in total (Mixson et al., 2006). The prevalence of *B. lonestari* antibody in deer was overall 15% throughout 20 eastern states, and there was regional difference, with higher prevalence in southern states (17.5%) than in northern states (9.2%) (Murdock et al., 2009). There have been few reports of *B. lonestari* outside of the United States. In Brazil, a RF *Borrelia* sp. closely related to *B. lonestari* and *B. theileri* was detected from a *Rhipicephalus microplus* feeding on a horse (Yparraguirre et al.,

1 2007). Takano et al. (2012) reported a RF *Borrelia* sp. from the *Amblyomma geoemydae* collected in
2 Okinawa prefecture, the most southern part of Japan, who's sequences clustered with *B. lonestari* and
3 *B. miyamotoi* by phylogenetic analysis. These findings suggested the possibility that unknown
4 *Borrelia* spp. exist worldwide.

5 In a previous survey of *Borrelia* spp. among wild animals in Hokkaido, a northern island of
6 Japan, borrelial DNA fragments which were similar to *B. lonestari* (Taylor, 2013) were found among
7 blood samples from sika deer (*Cervus nippon yesoensis*). However, *Amblyomma* spp., *Rhipicephalus*
8 spp., or soft ticks, which are the heretofore known vectors of RF borreliae, have never been reported
9 in Hokkaido (Yamaguti et al., 1971; Shimada et al., 2003; Taylor, 2013; Yamauchi et al., 2013). To
10 understand how this borrelial organism is maintained in the ecosystem, in this study, we conducted
11 surveillance of wild sika deer and ticks in Hokkaido, Japan.
12

1 **Materials and Methods**

2 *Sampling from field*

3 To examine the infection rate of *Borrelia* spp. among sika deer, we surveyed deer samples from
4 hunting and nuisance control culling held in the eastern part of Hokkaido from July 2011 to August
5 2013. Two regions were selected for sample collection: Shiretoko and Shibetsu, which are separated
6 by approximately 40 km and the Shiretoko mountain range (Fig. 1). Blood samples were collected
7 from veins or heart and dispensed into EDTA·Na tubes and plain tubes. EDTA blood was kept at 4°C
8 until DNA extraction, which was performed within 2 days. Buffy coat or unspun plasma were collect
9 on the day of sampling and kept in -20°C until DNA extraction, which was performed within a week.
10 Deer were identified to sex, and individuals were grouped into fawns (lesser than 1 year old) and
11 adults (1 year or older) based on a tooth formula (Koike and Ohtaishi, 1985). Sampling was divided
12 into two seasons based on snow covering from November through April (winter) and from May
13 through October (summer). When possible, a portion of an entire ear from the dead deer was collected
14 and kept at -20°C for tick counts and species and stage identification.

15 From May through September of 2012 and 2013, questing ticks on vegetation were collected by
16 flagging with a 1 m^2 white flannel sheet in Shiretoko and Shibetsu. Sampling was implemented in
17 several locations over nature trails and pasture. Collected ticks were identified to species and stage,
18 and were kept at -20°C until DNA extraction.

19 *DNA extraction*

20 DNA from deer blood was extracted using the Wizard® genomic DNA purification kit (Promega,
21 Madison, WI) by the recommended protocol using 3 ml of whole blood or unspun plasma or buffy
22 coat from 3 ml of blood sample. Tick DNA was extracted by using ammonium hydroxide (NH_4OH) as
23 described in Barbour et al. (2009) with minor modification. Harvested DNA samples were stored at -
24 20°C until analysis.

25 *Conventional PCR and sequencing*

26 All deer blood DNA samples were examined using nested PCR to detect the *Borrelia* spp.
27 flagellin gene (*flaB*) with the primer set of BflaPAD and BflaPDU for first PCR and BflaPBU and

1 BflaPCR for nested PCR as previously described (Takano et al., 2010) with the GeneAmp®+
2 PCRSystem9700 (Applied Biosystems, Foster City, CA). PCR was done with Takara Ex *Taq* (Takara
3 Bio, Otsu, Japan) and the first PCR condition was 25 cycles of 20 s at 94°C, 30 s at 55°C and 30 s at
4 72°C, and nested PCR was performed at 30 cycles with the same conditions. Contamination and
5 amplicon carryover were carefully checked by using distilled water as blank control in each
6 experiment. After gel electrophoresis, the PCR product (323 bp) was purified with the NucleSpin®Gel
7 and PCR clean-up kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's
8 instructions. The forward primer of the nested PCR was used for direct sequencing of amplicon DNA
9 using 27 cycles of 15 s at 96°C, 5 s at 50°C and 4-min at 60°C with the BigDye® Terminator v1.1
10 Cycling Sequencing Kit. The sequenced results were analysed on an ABI PRISM 310 Genetic
11 Analyser (Applied Biosystem), and were compared in GenBank for identification to species.
12 *Real-time PCR and Quantification of borrelial DNA copy number*
13 To confirm the positive cases of deer with *Borrelia* sp. (preliminarily designated *B. lonestari*-
14 like), and to quantify the copy number of the borrelial genome in the blood, 16S rRNA gene detection
15 by real-time PCR was performed on all deer blood samples for which *flaB* nested PCR was
16 performed, with the exception of dried out samples.
17 (i) Construction of real-time PCR
18 To construct the real-time PCR, a part of the 16S rRNA gene (1363bp) of *B. lonestari*-like was
19 amplified by sets of primers 16S-F1 and 16S-R4 (Takano et al., 2010) for the first PCR and 16SMF
20 (5'-GCGAACGGGTGAGTAACG-3') and 16SMR (5'-CCTCCCTTACGGGTTAGAA-3') for nested
21 PCR using 3 borrelial *flaB* PCR positive samples. The PCR condition was 30 cycles of 10 s at 95°C,
22 30 s at 55°C (first PCR) or 58°C (nested PCR) and 90 s at 72°C, using Takara Ex *Taq* (Takara Bio.).
23 After sequencing of the 16S rRNA gene as described above for *flaB* nested PCR, the sequences were
24 compared with the 16S rRNA gene of *B. miyamotoi* and *B. lonestari*-like using Sequencher 5.1 (Gene
25 Codes Corporation, MI, USA). In this study, we used a real-time PCR protocol previously reported by
26 Barbour et al. (2009) with minor modification to the probe. Forward and reverse primers were,
27 respectively, 16S RT-F (5'- GCTGTAAACGATGCACACTTGGT -3') and 16S RT-R (5'-

GGCGGCACACTTAACACGTTAG -3') and the dye-labelled probe was modified by 1 bp from the VIC probe described by Barbour et al. (2009) as follows: BS-16S (FAM 5'-CGGTACTAATCTTTCGATTA -3') with the 3' end modified with a minor groove binding protein (Applied Biosystems). The real-time PCR was performed using the *Premix Ex Taq*™ (Perfect Real Time) (Takara Bio Inc.) according to the manufacturer's instructions and run on ABI StepOne or StepOne Plus apparatuses (Life Technologies Corporation, Gaithersburg, MD). The thermal cycle protocol was performed as follows: first incubation stage, 20 s at 95°C; second stage, 1 s at 95°C and 20 s at 60°C. The second stage was repeated 45 times. For analysis of PCR results, the threshold line was fixed at 0.4 to avoid detection of nonspecific fluorescence.

(ii) Specificity and sensitivity of 16S rRNA gene based-real-time PCR

To evaluate specificity of the real-time PCR, 14 strains of *Borrelia* spp. were used: 2 REP borreliae (*Borrelia* sp. TA2 and *Borrelia* sp. tAG158M), 5 LD borreliae (*Borrelia burgdorferi* B31, *Borrelia garinii* HkIP1, *B. garinii* J-14, *Borrelia afzelii* HkIp7, and *Borrelia japonica* HO14), and 4 RF borreliae (*B. miyamotoi* HT31, *Borrelia duttonii* Ly, *Borrelia coriaceae* Co53, and *Borrelia hermsii*), respectively. Borrelial strains HkIP1 and HkIp7 and J-14 were isolated from *I. persulcatus* and human patient skin biopsies in Japan, respectively (Takano et al., 2011b). Plasmid DNA pBSrrs8 was established as a control DNA in this study. A part of the 16S rRNA gene of the *B. lonestari*-like positive deer blood samples was amplified by PCR using the primer set 16S RT-F and 16S RT-R. The amplicon (70bp) was cloned into the plasmid vector pGEM-T (Promega), and the plasmid DNA was subsequently propagated by *E. coli* JM109 strain (Nippon Gene, Tokyo, Japan), as previously described (Takano et al., 2011a). The sequences of inserted fragments were confirmed by direct sequencing of the plasmid. None of the REP borreliae or LD borreliae were detected using this assay. Moreover, among the RF borreliae, only the targeted *B. lonestari*-like showed signals of FAM fluorescence.

To determine the sensitivity of the real-time PCR, an external standard template was included in each run. For the standard DNA, the concentration of plasmid DNA pBSrrs8 was measured with a NanoDrop 2000c spectrophotometer (Thermo scientific, Wilmington, Delaware, USA) and adjusted to

1 10^1 to 10^8 plasmid copies with 10 fold dilutions. As a result, the limit of detection consistently
2 observed was a minimum of 10^1 plasmid copies, and quantification was confirmed between a range of
3 10^1 copies to 10^7 copies (data not shown).

4 *Detection of borrelial DNA in ticks*

5 The real-time PCR was performed using a portion of frozen tick DNA samples. The positive
6 samples were subsequently examined using *flaB* nested PCR and sequencing for confirmation and
7 characterization of borreliae.

8 *Phylogenetic analysis*

9 To define the genetic character of *B. lonestari*-like in Hokkaido, 2 positive female tick samples
10 were examined by PCR and sequencing using sets of primers, BflaPAD and BflaPDU for *flaB*
11 (429bp), 16S-F1 and 16S-R4 for 16S rRNA gene (1537 bp) and glpQ-F and glpQ-R for the
12 glycerophosphodiester phosphodiesterase gene (*glpQ*) (appro. 1.5kbp) as previously described
13 (Takano et al., 2011a). The sequences of all 3 genes of positive ticks (130707_13_HJF) were
14 deposited to GenBank (Acc. No. AB897888, AB897889, and AB897891). Another tick
15 (130708_80_HJF) was only positive for *flaB* and *glpQ*, and these sequences were 100% identical with
16 that of 130707_13_HJF. In addition to these sequences, the sequences from deer blood samples
17 described above (Acc. No. AB897886, AB897887; *flaB*, and AB897890; 16S rRNA gene) were also
18 analysed using MEGA 5.2 software (http://www.megasoftware.net) (Tamura et al., 2011). Sequences
19 were aligned using the Clustal-W and the phylogenetic inferences were analysed for *flaB* and *glpQ* by
20 Neighbor-Joining with the Kimura 2-parameter correction model and for 16S rRNA gene by
21 Maximum likelihood with the Hasegawa-Kishino-Yano model with Gamma distribution. Internal
22 node supports were calculated using a bootstrap with 1000 replies. Pairwise alignments were
23 performed with an open-gap penalty of 15 and a gap extension penalty of 6.66. Multiple alignments
24 were also performed using the same values. All positions containing alignment gaps and missing data
25 were eliminated in pairwise sequence comparisons (pairwise deletion) with the Neighbor-Joining
26 method.

27 *Culture*

Deer whole blood was inoculated into modified BSK medium (BSK-M or BSK-II medium: using minimal essential medium alpha [BioWest, Germany] as a substitute for CMRL-1066) (Barbour, 1984; Takano et al., 2011b) and incubated at 32°C for cultivation. Rabbit serum (Sterile Non-hemolyzed grade, Pelfreeze Biologicals, AR) was heat-inactivated at 56°C for 30 min before use. Bovine serum albumin Fr. V (Probumin Universal grade, Millipore, MA) was also used for BSK-M medium preparation. The inoculated media were examined under 200x dark field microscopy from 1 month of inoculation and checked every other week for another 2 months.

Statistics

Statistical differences were analysed using SPSS version 18 (SPSS, Chicago, IL) and Microsoft® Office Excel® 2007 for Windows. All the comparisons of prevalence between each group: region, season, age, and sex were made with the chi-square test and then all four factors were analysed with the logistic regression with a set confidence value of 95%. To estimate the prevalence of nymphs infected with *B. lonestari*-like, $\acute{P}$ value (the estimate of infection rate) (Chiang and Reeves, 1962), minimum infection rate (MIR), and maximum likelihood corrected for bias (MLE-C) were calculated with Mosquito Surveillance software Ver. 4 (http://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html).

Results

Prevalence in sika deer

In total, 235 blood samples were collected from sika deer in Shiretoko and Shibetsu, located in eastern Hokkaido (Fig 1.). A total of 25 sika deer blood samples were confirmed positive for DNA of an unknown *Borrelia* sp. using both nested PCR for *Borrelia* spp. *flaB* and the real-time PCR. The prevalence of *B. lonestari*-like in sika deer was 10.6% (25/235). There was a significant difference in the prevalence between the two sampling regions with 17.0% and 2.8% in Shiretoko and Shibetsu, respectively ($P<0.01$, chi-square test) (Table 1). When separated by age group, the prevalence in fawns (7/32, 21.9%) was greater than twice that of adults (18/192, 9.4%) ($P<0.01$, chi-square test). There was no significant statistical difference in the prevalence between sex or between winter and summer seasons in either region (data not shown). Using logistic regression, comparing four factors: age, season, sex, and region, we found that region and age were confirmed to be the main risk factors for *B. lonestari*-like infection ($P<0.01$, Odds ratio (OR) 13.06, 95% Confidence Interval (CI) 6.99-24.03 and $P<0.01$, OR 4.23, 95% CI 2.38-7.53, respectively).

Prevalence in host seeking ticks (*Haemaphysalis spp.)*

A total of 1,513 ticks were collected in Shiretoko and Shibetsu from May to September of 2012 and 2013 by the flagging method. *Haemaphysalis* spp. were more abundant in Shiretoko (736/940: number of *Haemaphysalis* spp. ticks over the total collected ticks) than in Shibetsu (32/573) ($P<0.01$, chi-square test). *Haemaphysalis japonica* was the main *Haemaphysalis* sp. collected, and *Ixodes ovatus* was the most common *Ixodes* sp. In these 768 host seeking *Haemaphysalis* ticks, 670 ticks including 290 adults and 380 nymphs were tested for *B. lonestari*-like using real-time PCR. Nymphs were pooled, with 5 heads in each pool. As a result, two *H. japonica* females and 4 nymph pools (two pools of *Haemaphysalis megaspinosa*, one pool of *H. japonica*, and one pool of unidentified *Haemaphysalis* spp.) were positive for *B. lonestari*-like DNA using real-time PCR, and were confirmed by *flaB* nested PCR (Table 2). The estimated prevalence was calculated: $\acute{P}$ value was 1.1, MIR (%) was 1.1 (95% CI [0.03, 2.08]), and MLE-C (%) was 1.1 (95% CI [0.35, 2.55]). All positive

1 samples were collected from Shiretoko (2/272 adults and 4/76 nymph pools, including 380 ticks).
2 None of the ticks collected in Shibetsu (0/18 adults) were positive.

3 *Genome copy number in the deer and tick*

4 The copy number of genome was determined by *B. lonestari*-like real-time PCR. The copy
5 numbers were 233,352 and 788,251 (in Log_{10}, 5.4 and 5.9) in the heads of adult ticks, with a range
6 from 15,634 to 87,913 (in Log_{10}, range: 4.2 - 4.9) and a mean of 51,894 (in Log_{10}, 4.7. 4.1~5.4 of CI
7 and 0.3 of SD) in a pool of 5 nymphs (Table 2). The number of genomes in 1 ml of deer blood ranged
8 from 14 to 608,213 (in Log_{10}, 1.2 to 5.8) with a mean of 47,054 (in Log_{10}, 3.5. 1.2~5.7 of CI and 1.2
9 of SD) (Table 2).

10 *Phylogenetic analysis*

11 The *flaB* sequencing of *B. lonestari*-like in this study (Acc. No. AB897886, AB897887, and
12 AB897888) was most similar to *Borrelia* sp. BR (Acc. No. EF141022) at 97% similarity using
13 BLAST in GenBank. The 16S rRNA gene (Acc. No. AB897890 and AB897891) and *glpQ* (Acc. No.
14 AB897889) were most similar to *B. miyamotoi* LB-2001 (Acc. No. CP006647, at 99%) and *B.*
15 *lonestari* MO2002-V1 (Acc. No. AY682922, at 92%), respectively. Phylogenetic trees were created
16 for *flaB* (Fig. 2), 16S rRNA gene (Fig. 3), and *glpQ* (Fig. 4). *B. lonestari*-like in this study consistently
17 clustered with *B. lonestari* and *Borrelia* sp. BR (Acc. No. EF141022).

18 *Identification of feeding ticks on deer*

19 Adult ticks were collected from the ears of 137 deer (69 from Shiretoko and 68 from Shibetsu)
20 and morphologically identified to species, stage, and engorged states. From Shiretoko, the mean
21 number of ticks on deer ears was 59 per deer (from 0 to 553 adults) and 7 per deer (from 0 to 35
22 adults) for *Haemaphysalis* spp. and *Ixodes* spp., respectively. On the other hand, from Shibetsu, only
23 *Ixodes* spp. were found, and the mean tick number on deer was 33 per deer (from 0 to 381 adults).
24 Thus, *Haemaphysalis* spp. ticks infesting deer were more abundant in Shiretoko than in Shibetsu
25 ($P<0.01$, chi-square test). In this study, *H. japonica* and *I. ovatus* were the most common tick species
26 found on deer.

27 *Culture*

1 Of the total 59 deer blood samples incubated in BSK-II or BSK-M media, including 17 PCR
2 positive cases, there was no growth in all culture tubes at 30 days post-inoculation and during 2
3 subsequent months of incubation.
4

Discussion

In this study, *B. lonestari*-like was detected from blood of sika deer and from *Haemaphysalis* spp. ticks in Hokkaido, Japan. *B. lonestari* was originally detected from the lone star tick, *A. americanum,* (Schulze et al., 1984; Luckhart et al., 1992). In the United States, *B. lonestari* has been detected from 8.7% of wild white-tailed deer (Moore IV et al., 2003), and in the experimental inoculation of 4 species of animals, including white-tailed deer, C3H mice, holstein cattle, and beagle dogs, only the white-tailed deer developed spirochetemia (Moyer et al., 2006). Based on those results, white-tailed deer were considered the natural vertebrate reservoirs of *B. lonestari* in the United States. In our study, *B. lonestari*-like was detected from the blood of sika deer at a total prevalence of 10.6% (Table 1), and bacteremia (average Log_{10}, 3.5) was observed in deer blood. In a previous study, *B. lonestari*-like was never found among 879 wild rodents, including 5 species: *Apodemus argenteus, Apodemus speciosus, Myodes rex, Myodes rufocanus,* and *Myodes rutilus*, in Hokkaido (Taylor et al., 2013), where *A. speciosus* and *M. rufocanus* are the most abundant rodent species, and are implicated as the main hosts for Lyme disease borreliae (Nakao and Miyamoto, 1993; Taylor et al., 2013). Given the above, sika deer likely play a greater role than rodents in maintaining *B. lonestari*-like in Hokkaido. In this study, we were unable to isolate *B. lonestari*-like organisms from blood samples of sika deer. To our knowledge, it is still unknown why some *Borrelia* spp. are uncultivable in vitro. The culture condition of these *Borrelia* spp. may be more fastidious than relatively well-known *B. burgdorferi* sensu lato. The borrelia found in this study is genetically similar to *Borrelia lonestari* found in the United States. Varela and colleagues (2004) reported *B. lonestari* was successfully isolated by co-cultivation with a tick cell line. Although it remains unclear how tick cells contribute to the borrelial growth in vitro (or ex vivo), this method may be beneficial in isolating the *Borrelia* sp. found in this study.

Secondly, the prevalence of *B. lonestari*-like was higher among fawns compared to older deer. In a previous study, LD borreliae showed age dependent infection rates, and this was interpreted as persistent infection in mice (Schwan et al., 1991). In the cases of RF borreliae, Larsson et al. (2006)

1 reported latent infections of *B. duttonii* in the brains of mice, and Taylor et al. (2013) stated that *B.*
2 *miyamotoi* did not show age dependent infection rates in rodents and inferred that *B. miyamotoi* may
3 not cause persistent infections. In the case of *B. lonestari* of white-tailed deer in the United States,
4 only serological studies have been reported on age variation, and the seroprevalences were not
5 different between age groups (Murdock 2009). The reason that fawns have a higher prevalence of
6 *B.lonestari*-like DNA than adults remains unclear; however, further investigation (e.g. examining
7 seroprevalence among sika deer) may resolve this issue.

8 Our data indicate that the prevalence of *B. lonestari*-like is different by region. The prevalence
9 among the deer caught in Shiretoko (17%) was much higher than in Shibetsu (2.8%) (Table 1). Such
10 regional variation of *Borrelia* spp. prevalence has been often reported, including in *B. lonestari*
11 studies, and it has mainly been correlated with vector distribution (Moore IV et al., 2003; Murdock et
12 al., 2009). This is true in the case of LD borreliae and other tick-borne diseases as well (Kirstein et al.,
13 1997; Mixson et al., 2006). The regional variation in prevalence in our study may also be due to
14 differences in vector population between the two different areas. Although *A. americanum* is the
15 vector of *B. lonestari* in the United States (Varela-Stokes, 2007), there has been no report of
16 *Amblyomma* spp. ticks in Hokkaido. Moreover, only *Ixodes* spp. and *Haemaphysalis* spp. have been
17 reported in our sampling areas (Yamaguti et al., 1971; Ito and Takahashi, 2006), and the ticks we
18 collected in this study were composed of these two genera. The host-seeking ticks and blood feeding
19 ticks on deer were significantly different between regions. In Shiretoko, where there was a higher
20 prevalence of *B. lonestari*-like among deer than in Shibetsu, *Haemaphysalis* spp. ticks were more
21 abundant. In a previous study, Ito and Takahashi reported that the primary host of *H. japonica* was
22 sika deer (2006). Additionally, although there have been several previous surveillance studies on the
23 prevalence of borrelial DNA among *Ixodes* spp. ticks, *B. lonestari*-like has never been reported in
24 these ticks (Hamer et al., 2011; Murase et al., 2012). Since we detected *B. lonestari*-like from host-
25 seeking *Haemaphysalis* spp., including *H. japonica* and *H. megaspinosa*, collected in Shiretoko
26 (Table 2), and the number of *Haemaphysalis* spp. ticks both seeking hosts and infesting deer in
27 Shiretoko was greater than in Shibetsu, the regional variation in *B. lonestari*-like prevalence among

1 sika deer may be correlated with the population of *Haemaphysalis* spp.

2 In this study, the prevalence of *B. lonestari*-like among sika deer, adult *Haemaphysalis* spp. 3 ticks, and nymphs were 17%, 0.7%, and 1.1%, respectively. The prevalence of LD borreliae in a 4 previous study in Hokkaido were 30% and 15.7%, and that of *B. miyamotoi* were 6.9% and 1.8% 5 among mammalian hosts and vector ticks in Hokkaido, respectively (Taylor et al., 2013). The gap 6 between the prevalence in host and tick in our study is wider than expected. However, unlike other 7 *Borrelia* spp., since there is no study or report on the transmission strategy or efficiency of *B.* 8 *lonestari*, it is difficult to evaluate whether this gap is appropriate. The prevalence of borreliae in ticks 9 may be affected by transovarial, transstadial, or horizontal transmission. Furthermore, a larger sample 10 size is necessary in order to determine a more accurate prevalence among ticks.

11 In the phylogenetic analyses, *B. lonestari*-like associated closely with *B. lonestari*, *Borrelia* sp. 12 BR, or *B. theileri* but is distinguishable by *flaB*, 16s rRNA and *glpQ* gene sequence alignments. To 13 identify this *Borrelia* sp. and to clarify the relationship in this cluster, further analysis (e.g. genome 14 sequencing) is necessary.

15 We have presented the status of a potentially novel *Borrelia* sp. genetically similar to *B.* 16 *lonestari* in wild sika deer and *Haemaphysalis* spp. ticks of Hokkaido, Japan. This is the first report 17 on the presence of *B. lonestari*-like organisms in *Haemaphysalis* spp. ticks, and the first phylogenetic 18 analysis of this *B. lonestari*-like in Asia. Through this study, we suggest that *B. lonestari*-like is 19 endemic in an area of Hokkaido, and the main mammalian reservoir is the sika deer and the vector 20 candidate is *Haemaphysalis* spp. ticks. Further investigation of this *Borrelia* sp. will be beneficial in 21 understanding the survival strategy of a cluster of RF borreliae transmitted by hard-bodied ticks, and 22 in contributing to the clarification of the dynamics of vector borne diseases in general.

23

1 **Acknowledgements**

2 The authors thank H. Nishizawa for his help in sampling and technical assistance. Additionally,
3 we are grateful to M. Osada (Shibetsu town office), M. Akaishi, Y. Fujimoto (NPO of South Shiretoko
4 Brown bear Information Center), S. Kasai (Shiretoko Nature Foundation), and K. Tomita (Shiretoko
5 Ezosika Farm), as well as Y. Yanagawa, R. Nakao, S. Hirano and N. Nagano (University of Hokkaido)
6 for deer and tick sampling and identification. We also thank H. Kim (Korean Cancer Center Hospital)
7 for her advice on statistical analysis.

8 This research was funded in part by a grant for research on emerging and re-emerging
9 infectious disease from the Japan Ministry of Health, Labor, and Welfare, and by the Global COE
10 program "Establishment of International Collaboration Centers for Zoonosis Control," MEXT, Japan.

11 **Author Disclosure Statement**

12 This paper was written to partially fulfil the requirements for a Ph. D. degree at the Hokkaido
13 University, Graduate School of Veterinary Medicine, for the lead author, Kyunglee Lee. No other
14 author has any interests that conflict with the publication of this paper.

15

References

Armstrong, P., Rich, S., Smith, R., Hartl, D., Spielman, A. and Telford III, S., 1996. A new *Borrelia* infecting Lone Star ticks. The Lancet. 347, 67-68.

Barbour, A.G., 1984. Isolation and cultivation of Lyme disease spirochetes. Yale. J. Biol. Med. 57, 521.

Barbour, A.G., 2005. Relapsing fever. In: Jesse, L., Goodman, D.T.D. and Sonenshine, D.E. (Eds.), Tick-borne diseases of humans. ASM Press, Washington, DC, pp. 268-291.

Barbour, A.G., Maupin, G.O., Teltow, G.J., Carter, C.J. and Piesman, J., 1996. Identification of an uncultivable *Borrelia* species in the hard tick *Amblyomma americanum*: possible agent of a Lyme disease-like illness. J. Infect. Dis. 173, 403-409.

Barbour, A.G., Bunikis, J., Travinsky, B., Hoen, A.G., Diuk-Wasser, M.A., Fish, D. and Tsao, J.I., 2009. Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am. J. Trop. Med. Hyg. 81, 1120-1131.

Biggerstaff, B.J., 2009. "PooledInfRate, Version 4.0 : a Microsoft® Office Excel© Add-In to compute prevalence estimates from pooled samples.". from http://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html.

Burkot, T.R., Mullen, G.R., Anderson, R., Schneider, B.S., Happ, C.M. and Zeidner, N.S., 2001. *Borrelia lonestari* DNA in adult *Amblyomma americanum* ticks, Alabama. Emerg. Infect. Dis. 7, 471-473.

Chiang, C.L. and Reeves, W.C., 1962. Statistical estimation of virus infection rates in mosquito vector populations. Am. J. Epidemiol. 75, 377-391.

Chowdri, H.R., Gugliotta, J.L., Berardi, V.P., Goethert, H.K., Molloy, P.J., Sterling, S.L. and Telford, S.R., 2013. *Borrelia miyamotoi* Infection Presenting as Human Granulocytic Anaplasmosis: A Case Report. Ann. Intern. Med. 159, 21-27.

Feder, H.M., Hoss, D.M., Zemel, L., Telford, S.R., Dias, F. and Wormser, G.P., 2011. Southern tick-associated rash illness (STARI) in the north: STARI following a tick bite in Long Island, New York. Clin. Infect. Dis. 53, e142-e146.

Franke, J., Hildebrandt, A. and Dorn, W., 2012. Exploring gaps in our knowledge on Lyme borreliosis

spirochaetes–updates on complex heterogeneity, ecology, and pathogenicity. Ticks. Tick. Borne. Dis. 4, 11-25.

Fukunaga, M., Takahashi, Y., Tsuruta, Y., Matsushita, O., Ralph, D., McClelland, M. and Nakao, M., 1995. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int. J. Syst. Bacteriol. 45, 804-810.

Gugliotta, J.L., Goethert, H.K., Berardi, V.P. and Telford III, S.R., 2013. Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. New. Engl. J. Med. 368, 240-245.

Hamer, S.A., Hickling, G.J., Sidge, J.L., Rosen, M.E., Walker, E.D. and Tsao, J.I., 2011. Diverse *Borrelia burgdorferi* strains in a bird-tick cryptic cycle. Appl. Environ. Microb. 77, 1999-2007.

Hovius, J.W., de Wever, B., Sohne, M., Brouwer, M.C., Coumou, J., Wagemakers, A., Oei, A., Knol, H., Narasimhan, S. and Hodiamont, C.J., 2013. A case of meningoencephalitis by the relapsing fever spirochaete *Borrelia miyamotoi* in Europe. The Lancet. 382, 658.

Ito, T. and Takahashi, K., 2006. The ecology of ticks infesting on Hokkaido sika deer. In: Kaji, K., Miyaki, M. and Uno, H. (Eds.), The conservation and management of Yezo sika deer. Hokkaido University Press, Sapporo, pp. 165-181 (in Japanese).

James, A.M., Liveris, D., Wormser, G.P., Schwartz, I., Montecalvo, M.A. and Johnson, B.J., 2001. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. J. Infect. Dis. 183, 1810-1814.

Kirstein, F., Rijpkema, S., Molkenboer, M. and Gray, J., 1997. The distribution and prevalence of *B. burgdorferi* genomospecies in *Ixodes ricinus* ticks in Ireland. Eur. J. Epidemiol. 13, 67-72.

Koike, H. and Ohtaishi, N., 1985. Prehistoric hunting pressure estimated by the age composition of excavated sika deer (*Cervus Nippon*) using the annual layer of tooth cement. J. Archaeol. Sci. 12, 443-456.

Larsson, C., Andersson, M., Pelkonen, J., Guo, B.P., Nordstrand, A. and Bergström, S., 2006. Persistent brain infection and disease reactivation in relapsing fever borreliosis. Microbes.

1 Infect. 8, 2213-2219.

2 Luckhart, S., Mullen, G.R., Durden, L.A. and Wright, J.C., 1992. *Borrelia* sp. in ticks recovered from
3 white-tailed deer in Alabama. J. Wildl. Dis. 28, 449-452.

4 Mixson, T.R., Campbell, S.R., Gill, J.S., Ginsberg, H.S., Reichard, M.V., Schulze, T.L. and Dasch,
5 G.A., 2006. Prevalence of *Ehrlichia, Borrelia*, and *Rickettsial* agents in *Amblyomma*
6 *americanum* (Acari: Ixodidae) collected from nine states. J. Med. Entomol. 43, 1261-1268.

7 Moore IV, V.A., Varela, A.S., Yabsley, M.J., Davidson, W.R. and Little, S.E., 2003. Detection of
8 *Borrelia lonestari*, putative agent of southern tick-associated rash illness, in white-tailed deer
9 (*Odocoileus virginianus*) from the southeastern United States. J. Clin. Microbiol. 41, 424-427.

10 Moyer, P., Varela, A., Luttrell, M., Moore IV, V., Stallknecht, D. and Little, S., 2006. White-tailed deer
11 (*Odocoileus virginianus*) develop spirochetemia following experimental infection with
12 *Borrelia lonestari*. Vet. Microbiol. 115, 229-236.

13 Murase, Y., Konnai, S., Githaka, N., Hidano, A., Taylor, K., Ito, T., Takano, A., Ando, S., Kawabata, H.
14 and Tsubota, T., 2012. Prevalence of Lyme *Borrelia* in *Ixodes persulcatus* Ticks from an Area
15 with a Confirmed Case of Lyme Disease. J. Vet. Med. Sci. 75, 215-218.

16 Murdock, J.H., Yabsley, M.J., Little, S.E., Chandrashekar, R., O'Connor, T.P., Caudell, J.N., Huffman,
17 J.E., Langenberg, J.A. and Hollamby, S., 2009. Distribution of antibodies reactive to *Borrelia*
18 *lonestari* and *Borrelia burgdorferi* in white-tailed deer (*Odocoileus virginianus*) populations
19 in the eastern United States. Vector. Borne. Zoonotic. Dis. 9, 729-736.

20 Nakao, M. and Miyamoto, K., 1993. Reservoir competence of the wood mouse, *Apodemus speciosus*
21 *ainu*, for the Lyme disease spirochete, *Borrelia burgdorferi*, in Hokkaido, Japan. Jpn. J. Sanit.
22 Zool. 44, 69-84.

23 Platonov, A.E., Karan, L.S., Kolyasnikova, N.M., Makhneva, N.A., Toporkova, M.G., Maleev, V.V.,
24 Fish, D. and Krause, P.J., 2011. Humans infected with relapsing fever spirochete *Borrelia*
25 *miyamotoi,* Russia. Emerg. Infect. Dis. 17, 1816-1823.

26 Schulze, T.L., Bowen, G.S., Bosler, E.M., Lakat, M.F., Parkin, W.E., Altman, R., Ormiston, B.G. and
27 Shisler, J.K., 1984. *Amblyomma americanum*: a potential vector of Lyme disease in New

1 Jersey. Science. 224, 601-603.
2 Schwan, T.G., Karstens, R.H., Schrumpf, M.E. and Simpson, W.J., 1991. Changes in antigenic
3 reactivity of *Borrelia burgdorferi*, the Lyme disease spirochete, during persistent infection in
4 mice. Can. J. Microbiol. 37, 450-454.
5 Scoles, G.A., Papero, M., Beati, L. and Fish, D., 2001. A relapsing fever group spirochete transmitted
6 by Ixodes scapularis ticks. Vector. Borne. Zoonotic. Dis. 1, 21-34.
7 Shimada, Y., Beppu, T., Inokuma, H., Okuda, M. and Onishi, T., 2003. Ixodid tick species recovered
8 from domestic dogs in Japan. Med. Vet. Entomol. 17, 38-45.
9 Smith, R., Brener, J., Osorno, M. and Ristic, M., 1978. Pathobiology of *Borrelia theileri* in the
10 tropical cattle tick, *Boophilus microplus*. J. Invertebr. Pathol. 32, 182-190.
11 Smith, R., Miranpuri, G., Adams, J. and Ahrens, E., 1985. *Borrelia theileri*: isolation from ticks
12 (*Boophilus microplus*) and tick-borne transmission between splenectomized calves. Am. J. Vet.
13 Res. 46, 1396-1398.
14 Stromdahl, E.Y., Williamson, P.C., Kollars, T.M., Evans, S.R., Barry, R.K., Vince, M.A. and Dobbs,
15 N.A., 2003. Evidence of *Borrelia lonestari* DNA in *Amblyomma americanum* (Acari:
16 Ixodidae) removed from humans. J. Clin. Microbiol. 41, 5557-5562.
17 Takano, A., Goka, K., Une, Y., Shimada, Y., Fujita, H., Shiino, T., Watanabe, H. and Kawabata, H.,
18 2010. Isolation and characterization of a novel *Borrelia* group of tick-borne borreliae from
19 imported reptiles and their associated ticks. Environ. Microbiol. 12, 134-146.
20 Takano, A., Fujita, H., Kadosaka, T., Konnai, S., Tajima, T., Watanabe, H., Ohnishi, M. and Kawabata,
21 H., 2011a. Characterization of reptile-associated *Borrelia* sp. in the vector tick, *Amblyomma*
22 *geoemydae*, and its association with Lyme disease and relapsing fever *Borrelia* spp. Environ.
23 Microbiol. Rep. 3, 632-637.
24 Takano, A., Nakao, M., Masuzawa, T., Takada, N., Yano, Y., Ishiguro, F., Fujita, H., Ito, T., Ma, X.
25 and Oikawa, Y., 2011b. Multilocus sequence typing implicates rodents as the main reservoir
26 host of human-pathogenic *Borrelia garinii* in Japan. J. Clin. Microbiol. 49, 2035-2039.
27 Takano, A., Sugimori, C., Fujita, H., Kadosaka, T., Taylor, K.R., Tsubota, T., Konnai, S., Tajima, T.,

Sato, K. and Watanabe, H., 2012. A novel relapsing fever *Borrelia* sp. infects the salivary glands of the molted hard tick, *Amblyomma geoemydae*. Ticks. Tick. Borne. Dis. 3, 259-261.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

Taylor, K.R. 2013. The Ecologies of *Borrelia* spp. in Hokkaido, Japan. PhD thesis, Hokkaido University, Hokkaido, Japan. Retrieved from http://hdl.handle.net/2115/52271

Taylor, K.R., Takano, A., Konnai, S., Shimozuru, M., Kawabata, H. and Tsubota, T., 2013. *Borrelia miyamotoi* Infections among Wild Rodents Show Age and Month Independence and Correlation with *Ixodes persulcatus* Larval Attachment in Hokkaido, Japan. Vector. Borne. Zoonotic. Dis. 13, 92-97.

Varela-Stokes, A.S., 2007. Transmission of bacterial agents from lone star ticks to white-tailed deer. J. Med. Entomol. 44, 478-483.

Varela, A.S., Luttrell, M.P., Howerth, E.W., Moore, V.A., Davidson, W.R., Stallknecht, D.E. and Little, S.E., 2004. First culture isolation of *Borrelia lonestari,* putative agent of southern tick-associated rash illness. J. Clin. Microbiol. 42, 1163-1169.

Yamaguti, N., Tipton, V., Keegan, H. and Toshioka, S. 1971. Ticks of Japan, Korea, and the Ryukyu islands. Vol. 15. Brigham Young University Science Bulletin, Provo, Utah.

Yamauchi, T., Satô, M., Ito, T., Fujita, H., Takada, N., Kawabata, H., Ando, S., Sakata, A. and Takano, A., 2013. Survey of tick fauna and tick-borne pathogenic bacteria on Rishiri Island, off north Hokkaido, Japan. Int. J. Acarol. 39, 3-6.

Yparraguirre, L.A., Machado-Ferreira, E., Ullmann, A.J., Piesman, J., Zeidner, N.S. and Soares, C.A., 2007. A hard tick relapsing fever group spirochete in a Brazilian *Rhipicephalus (Boophilus) microplus*. Vector. Borne. Zoonotic. Dis. 7, 717-722.

1 ## Legends to illustrations

2 **Figure 1. Map of sampling locations.**

3

4 **Figure 2. Phylogenetic analysis of *flaB* of *B. lonestari*-like in Hokkaido.**

5 Arrows pointing to bold type indicate the results of this study. Constructed based on the
6 Neighbor joining method with Kimura-2 parameter under pair-wise deletion option. There were a total
7 of 1762 positions in the final dataset.

8

9 **Figure 3. Phylogenetic analysis of 16S rRNA gene of *B. lonestari*-like in Hokkaido.**

10 Arrows pointing to bold type indicate the results of this study. Constructed based on the
11 Maximum likelihood method with HKY+G model. There were a total of 882 positions in the final
12 dataset.

13

14 **Figure 4. Phylogenetic analysis of *glpQ* of *B. lonestari*-like in Hokkaido.**

15 Arrow pointing to bold type indicates the results of this study. Constructed based on the
16 Neighbor joining method with Kimura-2 parameter under pair-wise deletion option. There were a total
17 of 1056 positions in the final dataset.

18

1 **Tables**

2 **Table 1. DNA detection of *B. lonestari*-like in deer blood samples with age and region.**

	Shiretoko			Shibetsu			Total
	Fawn	**Adult**	**subtotal***	**Fawn**	**Adult**	**subtotal***	
No. of deer	10	113	129	22	79	106	235
No. of positive	6	16	22	1	2	3	25
(% positive)	(60.0[b])	(14.2[b])	(17.0[a])	(4.5)	(2.5)	(2.8[a])	(10.6)

3 a and b: Infection rates marked with the same letter were significantly different (P<0.01) using the

4 chi-square test.

5 *Age unidentified deer were included in the Subtotal number: 6 and 5 in Shiretoko and Shibetsu, respectively.

6 These contained no positive samples.

1 **Table 2. Prevalence and loads of *B. lonestari*-like among sika deer and *Haemaphysalis* spp.**

Source		No. of sample	No. of positive	DNA copy number of genome/ml of blood or tick in Log_{10} (average / 95%CI / SD)
Deer-blood		235	25 (10.6%)	1.2 ~ 5.8 (3.5/1.2~5.7/1.2)
Tick	Adult	290	2 (0.7%)	5.4 and 5.9 #
	Nymph	380 (76*)	4 (1.1%§)	4.2~4.9 (4.7~5.4/0.3)

2 *: Nymphs were pooled, with 5 heads per pool.

3 §: Minimum Infection Rate (MIR) with 95% CI is 0.03-2.08. MLE was 1.1% with 95% CI (0.35-2.55).

4 # : There were only two values not enough to give the average, 95% CI and SD.

5

6

7

8