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要約

The role of sika deer in the transmission of *Borrelia* spp. in Hokkaido, Japan

北海道における *Borrelia* spp.の伝播に対する鹿の役割

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Dedicated to my family and friends.

CONTENTS

PREFACE

The genus *Borrelia*, a group of vector-borne spirochetes, is known to consist of three phylogenetic clusters: Lyme disease (LD) borreliae, relapsing fever (RF) borreliae and reptile associated (REP) borreliae (Barbour, 2001; Takano et al., 2011a). The bond between each *Borrelia* sp. and its vector tick species looks quite solid and also connects with specific reservoir hosts. The strategies of propagation of the micro-organisms are different; for example, some RF borreliae can be inherited from female to eggs but not others (Barbour and Hayes, 1986). The best known species in these genera is *Borrelia burgdorferi* sensu lato (LD borreliae), especially *B. burgdorferi* in its life cycle, or transmission strategy, etc. (Steere et al., 2005).

In Hokkaido, Lyme disease and relapsing fever group borreliae have been reported; *B. garinii*, *B. afzelii*, *B. japonica* and *B. miyamotoi*. All of them use hard-bodied tick vectors, and only hard-bodied ticks have been reported in Hokkaido (Yamaguti et al., 1971; Ito and Takahashi, 2006). *Ixodes persulcatus* and *I. ovatus* have been considered as tick vectors for *B.garinii*, *B. afzelii* and *B. miyamotoi* and *B. japonica*, respectively reported in Hokkaido (Kawabata et al., 1993; Fukunaga et al., 1995; Eisen and Lane, 2002)(Table A). *I. pavlovsky* is regarded as another vector of *B. garinii* (Franke et al., 2012) and *B. miyamotoi* (unpublished). There are also several *Haemaphysalis* spp. ticks found in Hokkaido (Yamaguti et al., 1971), but reports of *Borrelia* spp. from *Haemaphysalis* ticks are limited .

Deer populations in Hokkaido have steadily increased since the late 19th century and are now in a state of overpopulation, accompanied by damage to forests and conflicts with humans. Local governments have tried to control deer populations by encouraging hunting and nuisance control schemes (Hokkaido Government, 2014). In addition to the direct effects of overpopulation on the environment and humans, indirect consequences, such as overpopulation-related emerging zoonoses are also significant issues. Deer are one of the main large-mammal hosts for ticks and all of the above mentioned vector ticks of *Borrelia* spp. are found on sika deer with regional and seasonal variations (Ito and Takahashi, 2006). The incompatibility of *Cervid* spp. as a reservoir host of LD borrelia was

proved by Telford 3rd et al. (1988) and reported in Hokkaido, as well (Taylor, 2013). Instead, Richter and Matuschka (2010) reported the elimination effect of domestic ruminants on the Lyme disease spirochetes from feeding ticks and there are several other studies reporting similar cases involving birds and hoofed animals (Matuschka and Spielman, 1992; Matuschka et al., 1993). Thus, one can wonder if it is possible for deer to decrease the prevalence of the pathogens as a dead-end host. Separate from LD borrelia, *B. lonestari* uses white-tailed deer as the reservoir host in the USA (Moore IV et al., 2003; Moyer et al., 2006) and Taylor (2013) has found very similar *Borrelia* sp. with *B. lonestari* from sika deer samples. Hard-bodied tick vector RF borrelia group, including *B. lonestari*, have not been studied much compared to LD borreliae.

The basic mode of transmission of *Borrelia* spp. is between vector ticks and animals during blood feeding. However, depending on the sensitivity of animals to each organism, the results will be different. Deer have many opportunities to come into contact with diverse species of borreliae via variety species of ticks. Studying the infection status of deer with respect to different species of borreliae will improve the understanding of spirochete dynamics in nature.

This study aims to define the role of sika deer related to LD borrelia, RF borrelia, their vectors and their different strategies of propagation in Hokkaido, Japan.

CHAPTER 1. ROLE OF DEER IN THE ECOLOGY OF RELAPSING FEVER BORRELIA

: A Relapsing fever group *Borrelia* sp. similar to *Borrelia lonestari* found among wild sika deer and *Haemaphysalis* spp. ticks in Hokkaido, Japan.

- The original paper of this part appeared in *Ticks Tick-borne Dis*. in press (2014).

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 of RF borreliae are transmitted by argasid (soft-bodied) ticks, except for *Borrelia recurrentis*, which is transmitted by lice. However, Some RF borreliae such as *Borrelia theileri*, *Borrelia miyamotoi*, and *Borrelia lonestari*, use hard-bodied ticks as a transmission vector: *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) (Table A). *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 hampered 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., 2007). Takano et al. (2012) reported a RF *Borrelia* sp. from the *Amblyomma geoemydae* collected in Okinawa prefecture, the most southern part of Japan, whose sequences clustered with *B. lonestari* and *B. miyamotoi* by phylogenetic analysis. These findings suggested the possibility that unknown *Borrelia* spp. are present worldwide.

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

Materials and Methods

Sampling from field

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

From May through September of 2012 and 2013, questing ticks on vegetation were collected by flagging with an approximately 1 m^2 white flannel sheet in Shiretoko and Shibetsu. Sampling was implemented in several locations over nature trails and pasture. Species and stages of collected ticks were identified and were kept at -20°C until DNA extraction.

DNA extraction

DNA from deer blood was extracted using the Wizard® genomic DNA purification kit (Promega, Madison, WI) by the recommended protocol using 3 ml of whole blood or unspun plasma or buffy coat prepared from 3 ml of blood sample. Tick DNA was extracted by using ammonium hydroxide (NH_4OH) as described in Barbour et al. (2009) with minor modifications. Tick was cut in half longitudinally with No.22 blade. Then it was incubated for two hours in 50ul of 140mmol/L NH_4OH at room temperature. 50ul of distilled H_2O was added to this before a second incubation at 95°C for

30 min. Each sample was centrifuged and supernatant was harvested into clean tube with 1ul of 100mmol/L EDTA and stored at -20°C until analysis.

Conventional PCR and sequencing

All deer blood DNA samples were examined using nested PCR to detect the *Borrelia* spp. flagellin gene (*flaB*) with the primer set of BflaPAD and BflaPDU for first PCR and BflaPBU and BflaPCR for nested PCR as previously described (Takano et al., 2010) (Table B) with the GeneAmp®+ PCRSystem9700 (Applied Biosystems, Foster City, CA). PCR was done with Takara Ex *Taq* (Takara Bio, Otsu, Japan) and the first PCR condition was 25 cycles of 20 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C, and nested PCR was performed at 30 cycles with the same conditions. Contamination and amplicon carryover were carefully checked by using distilled water as blank control in each experiment. After gel electrophoresis, the PCR product (323 bp) was purified with the NucleSpin®Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The forward primer of the nested PCR (BflaPBU) was used for direct sequencing of amplicon DNA using 27 cycles of 15 sec at 96°C, 5 sec at 50°C and 4-min at 60°C with the BigDye® Terminator v1.1 Cycling Sequencing Kit. The sequenced results were analysed on an ABI PRISM 310 Genetic Analyser (Applied Biosystem), and were compared in GenBank for the identification of species.

Real-time PCR and Quantification of borrelial DNA copy number

To confirm the positive cases of deer with *Borrelia* sp. (preliminarily designated *B. lonestari*-like), and to quantify the copy number of the borrelial genome in the blood, 16S rRNA gene detection by real-time PCR was performed on all deer blood samples for which *flaB* nested PCR was performed, with the exception of dried out samples.

(i) Construction of real-time PCR

To construct the real-time PCR, a part of the 16S rRNA gene (1363bp) of *B. lonestari*-like was amplified by sets of primers 16S-F1 and 16S-R4 (Takano et al., 2010) for the first PCR and 16SMF and 16SMR for nested PCR using 3 borrelial *flaB* PCR positive samples (Table B). The PCR

condition was 30 cycles of 10 sec at 95°C, 30 sec at 55°C (first PCR) or 58°C (nested PCR) and 90 sec at 72°C, using Takara Ex *Taq* (Takara Bio.). After sequencing of the 16S rRNA gene as the sequencing method described above for *flaB* nested PCR, the sequences were compared with the 16S rRNA gene of *B. miyamotoi* and *B. lonestari*-like using Sequencher 5.1 (Gene Codes Corporation, MI, USA). In this study, a real-time PCR protocol previously reported by Barbour et al. (2009) was used with minor modification to the probe. Forward and reverse primers were 16S RT-F and 16S RT-R, respectively and the dye-labeled probe to detect *B. lonestari*-like was modified by 1 bp from the VIC probe to detect *B. miyamotoi* (BM-16S) with the 3' end modified with a minor groove binding protein (Applied Biosystems) described by Barbour et al. (2009) as noted in Table B. 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 for 20 sec at 95°C, and second stage for 1 sec at 95°C and for 20 sec 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 the 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, 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 10^1 to 10^8 plasmid copies with 10-fold dilutions. As a result, the limit of detection consistently observed was a minimum of 10^1 plasmid copies, and quantification was confirmed between a range of 10^1 copies to 10^7 copies (data not shown).

Detection of borrelial DNA in ticks

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

Phylogenetic analysis

To define the genetic character of *B. lonestari*-like in Hokkaido, 2 positive female tick samples were examined by PCR and sequencing using sets of primers, BflaPAD and BflaPDU for *flaB* (429bp), 16S-F1 and 16S-R4 for 16S rRNA gene (1,537 bp) and glpQ-F and glpQ-R for the glycerophosphodiesterphosphodiesterase gene (*glpQ*) (appro. 1.5kbp) as previously described (Takano et al., 2011a). The sequences of all 3 genes of positive ticks (130707_13_HJF) were deposited to GenBank (Acc. No. AB897888, AB897889, and AB897891). Another tick (130708_80_HJF) was only positive for *flaB* and *glpQ*, and these sequences were 100% identical to that of 130707_13_HJF. In addition to these sequences, the sequences from deer blood samples described above (Acc. No. AB897886, AB897887; *flaB*, and AB897890; 16S rRNA gene) were also analysed using MEGA 5.2 software (http://www.megasoftware.net) (Tamura et al., 2011). Sequences were aligned using the Clustal-W and the phylogenetic inferences were analysed for *flaB* and *glpQ* by

Neighbor-Joining with the Kimura 2-parameter correction model and for 16S rRNA gene by Maximum likelihood with the Hasegawa-Kishino-Yano model with Gamma distribution. Internal node supports were calculated using a bootstrap with 1,000 replies. Pairwise alignments were performed with an open-gap penalty of 15 and a gap extension penalty of 6.66. Multiple alignments were also performed using the same values. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion) with the Neighbor-Joining method.

Culture for isolation of B. lonestari-like

Deer whole blood was inoculated into BSK media (modified BSK-H (BSK-M) medium or modified 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. For the preparation of BSK-M media, Bovine serum albumin Fr. V (Probumin Universal grade, Millipore, MA) and Rabbit serum (Sterile Non-hemolyzed grade, Pelfreeze Biologicals, AR) were used and the Rabbit serum was heat-inactivated at 56°C for 30 min before use. 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/mosqSurv-Soft.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 1-1.). 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 for 16s rRNA gene. The prevalence of *B. lonestari*-like in sika deer was 10.6% (25 of 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 1-1). When separated by age group, the prevalence in fawns (7 of 32, 21.9%) was twice as high as that of adults (18 of 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, it was 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 five heads in each pool. As a result, two *H. japonica* females and four 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 1-2). 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

samples were collected from Shiretoko (2 of 272 adults and 4 of 76 nymph pools, including 380 ticks). None of the ticks collected in Shibetsu (0 of 18 adults) were positive. Twenty four adults and 83 nymphs of host seeking *I. persulcatus* were tested to detect *Borrelia* spp., but none of them possessed *B. lonestari*-like.

Genome copy number in the deer and tick

The copy number of genome was determined by *B. lonestari*-like real-time PCR. The copy numbers were 233,352 and 788,251 (in Log_{10}, 5.4 and 5.9) in the heads of adult ticks, with a range 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 and 0.3 of SD) in a pool of 5 nymphs (Table 1-2). The number of genomes in 1 ml of deer blood ranged 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 of SD) (Table 1-2).

Phylogenetic analysis

The *flaB* sequencing of *B. lonestari*-like in this study (Acc. No. AB897886, AB897887, and AB897888) was most similar to *Borrelia* sp. BR (Acc. No. EF141022) at 97% similarity using BLAST in GenBank. The 16S rRNA gene (Acc. No. AB897890 and AB897891) and *glpQ* (Acc. No. AB897889) were most similar to those of *B. miyamotoi* LB-2001 (Acc. No. CP006647, at 99%) and *B. lonestari* MO2002-V1 (Acc. No. AY682922, at 92%), respectively. Phylogenetic trees were created for *flaB* (Fig. 1-2), 16S rRNA gene (Fig. 1-3), and *glpQ* (Fig. 1-4). *B. lonestari*-like in this study consistently clustered with *B. lonestari* and *Borrelia* sp. BR (Acc. No. EF141022).

Identification of feeding ticks on deer

Adult ticks were collected from the ears of 137 deer (69 from Shiretoko and 68 from Shibetsu) and their species, stage, and engorged states were identified morphologically. From Shiretoko, the mean number of ticks on deer ears was 59 per deer (from 0 to 553 adults) and 7 per deer (from 0 to 35 adults) for *Haemaphysalis* spp. and *Ixodes* spp., respectively. On the other hand, from Shibetsu, only *Ixodes* spp. were found, and the mean tick number on deer was 33 per deer (from 0 to 381 adults). Thus, *Haemaphysalis* spp. ticks infesting deer were more abundant in Shiretoko than in Shibetsu

(P<0.01, chi-square test). In this study, *H. japonica* and *I. ovatus* were the most common tick species found on deer.

Culture for isolation of B. lonestari-like

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

Discussion

In this study, *B. lonestari*-like was detected from blood of sika deer and from *Haemaphysalis* spp. ticks in Hokkaido. *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 this study, *B. lonestari*-like was detected from the blood of sika deer at a total prevalence of 10.6% (Table 1-1), 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 vertebrate hosts for LD 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, however, it was unable to isolate *B. lonestari*-like organisms from blood samples of sika deer. Up to now, it is still unknown why some *Borrelia* spp. are uncultivable *in vitro*. The culture condition of these RF *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 and 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 *B. lonestari*-like in future 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)

reported latent infections of *B. duttonii* in the brains of mice, and Taylor et al. (2013) stated that *B. miyamotoi* did not show age dependent infection rates in rodents, and inferred that *B. miyamotoi* may not cause persistent infections. In the case of *B. lonestari* of white-tailed deer in the United States, only serological studies have been reported on age variation, and the seroprevalences were not different between age groups (Murdock 2009). The reason that fawns have a higher prevalence of *B.lonestari*-like DNA than adults remains unclear. It may be related with immune reaction of deer, for example, fawn are more susceptible to the first exposure to this *Borrelia* sp. and, further investigation (e.g. examining seroprevalence among sika deer) may resolve this issue.

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

(Table 1-2), and the number of *Haemaphysalis* spp. ticks both seeking hosts and infesting deer in Shiretoko was greater than in Shibetsu, the regional variation in *B. lonestari*-like prevalence among sika deer may be correlated with the population of *Haemaphysalis* spp.

In this study, the prevalence of *B. lonestari*-like among sika deer, adult *Haemaphysalis* spp. ticks, and nymphs were 17%, 0.7%, and 1.1%, respectively. The prevalence of LD borreliae in a previous study in Hokkaido were 30% and 15.7%, and those of *B. miyamotoi* were 6.9% and 1.8% among mammalian hosts and vector ticks in Hokkaido, respectively (Taylor et al., 2013). The gap between the prevalence in host and tick in this study is wider than expected. However, unlike other *Borrelia* spp., since there is no study or report on the transmission strategy or efficiency of *B. lonestari*, it is difficult to evaluate whether this gap is appropriate or not. The prevalence of borreliae in ticks may be affected by transovarial, transstadial, or horizontal transmission. Furthermore, a larger sample size is necessary to determine a more accurate prevalence among ticks.

In the real-time PCR results, it was possible to know the copy number of DNA in the positive samples of deer and ticks. The copy number in tick samples (mean $Log_{10}4.7$) were larger than those of deer samples (mean $Log_{10}3.5$) and these results were higher than the *B. miyamotoi* spirochete number in *I. scapularis* nymphs and the mice blood, respectively. However, the original DNA amount of samples were not regulated in this study, the direct comparison of copy number was not possible. In addition, the original sample amount of deer blood were not equal as 3 ml of whole blood or 0.5 ml of buffy coat, it was not appropriate to compare the copy number between the group analysis. In future study, these problems should be corrected from the sampling stage.

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

This study has presented the status of a potentially novel *Borrelia* sp. genetically similar to *B. lonestari* in wild sika deer and *Haemaphysalis* spp. ticks of Hokkaido, Japan. This is the first report

on the presence of *B. lonestari*-like organisms in *Haemaphysalis* spp. ticks, and the first phylogenetic analysis of this *B. lonestari*-like in Asia. Through this study, it was suggest that *B. lonestari*-like is endemic in an area of Hokkaido, and the main mammalian reservoir is the sika deer and the vector candidate is *Haemaphysalis* spp. ticks. The pathogenecity of *B. lonestari* or *B. lonestari*-like to human or animal are still unknown. Further investigation of this *Borrelia* sp. will be beneficial in understanding the survival strategy of a cluster of RF borreliae transmitted by hard-bodied ticks, and in contributing to the clarification of the dynamics of vector borne diseases in general.

Tables and Figures 1

Table 1-1. 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)

a and b: Infection rates marked with the same letter were significantly different ($P<0.01$) using the chi-square test.

*Age unidentified deer were included in the Subtotal number: 6 and 5 in Shiretoko and Shibetsu, respectively. These contained no positive samples.

Table 1-2. 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)

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

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

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

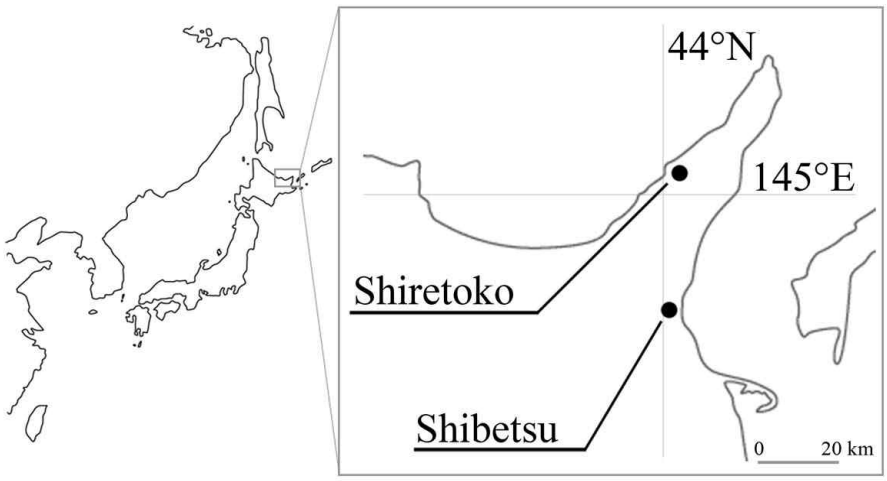

Figure 1-1. Map of sampling locations.

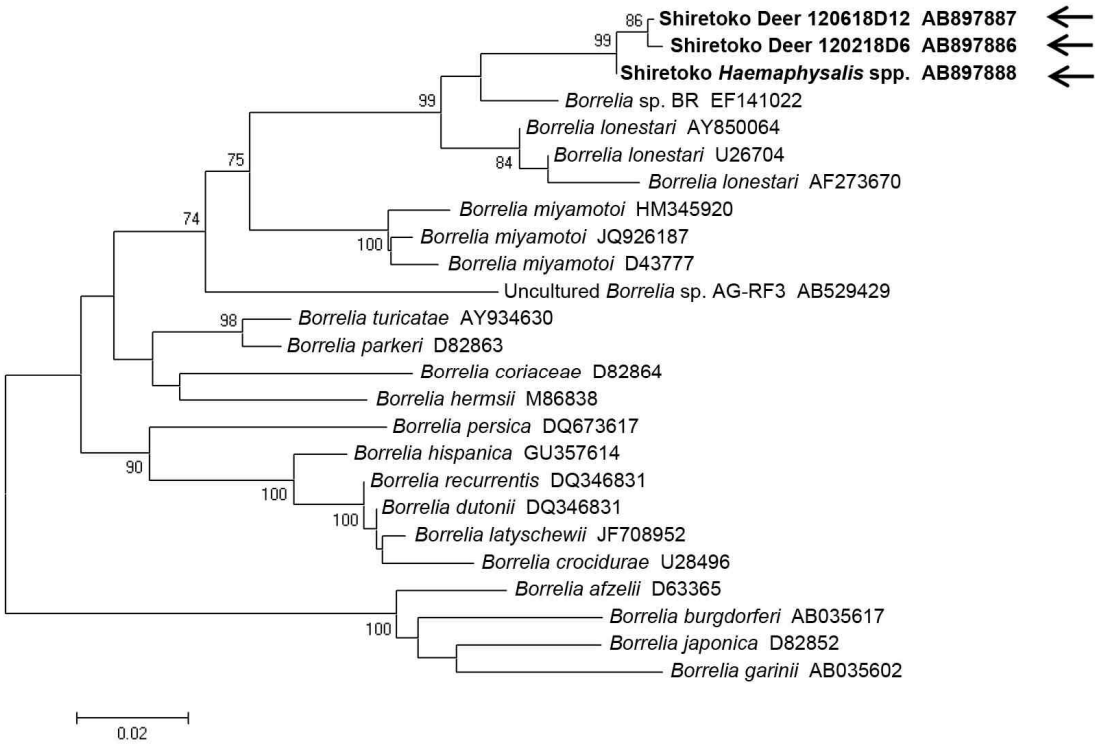

Figure 1-2. Phylogenetic analysis of *flaB* of *B. lonestari*-like in Hokkaido.

Arrows pointing to bold type indicate the results of this study. Constructed based on the Neighbor joining method with Kimura-2 parameter under pair-wise deletion option. There were a total of 1,762 positions in the final dataset.

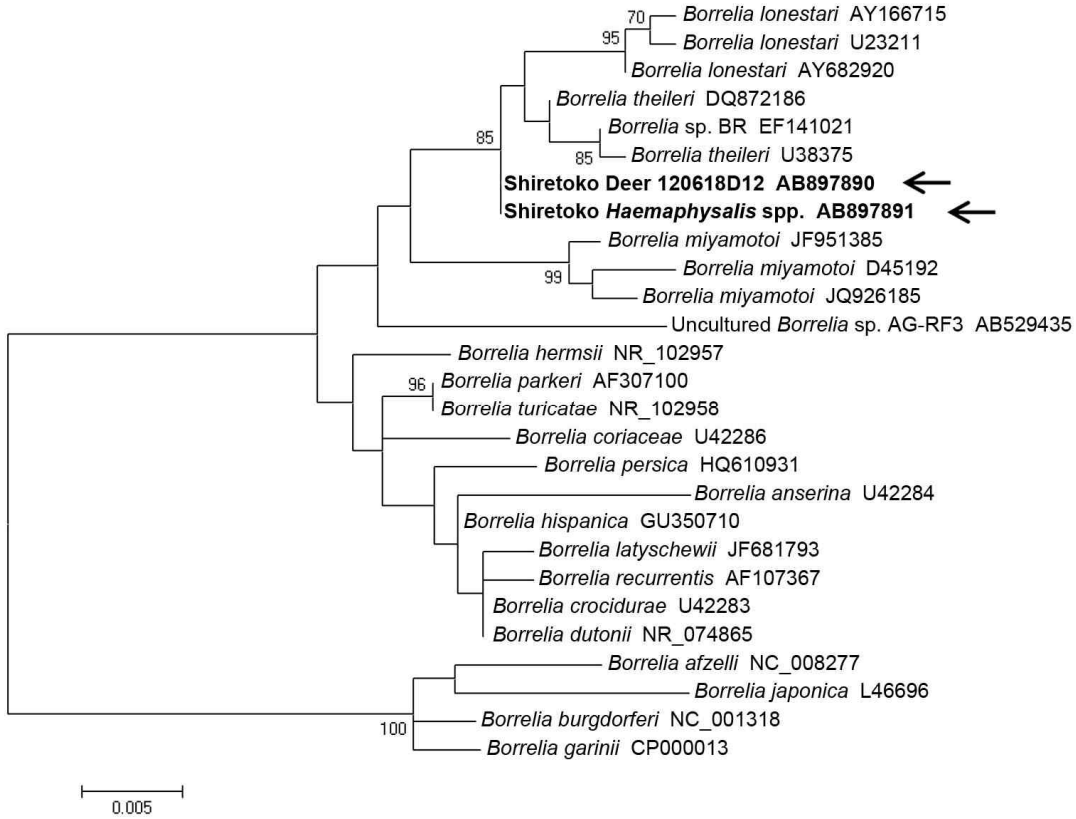

Figure 1-3. Phylogenetic analysis of 16S rRNA gene of *B. lonestari*-like in Hokkaido.

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

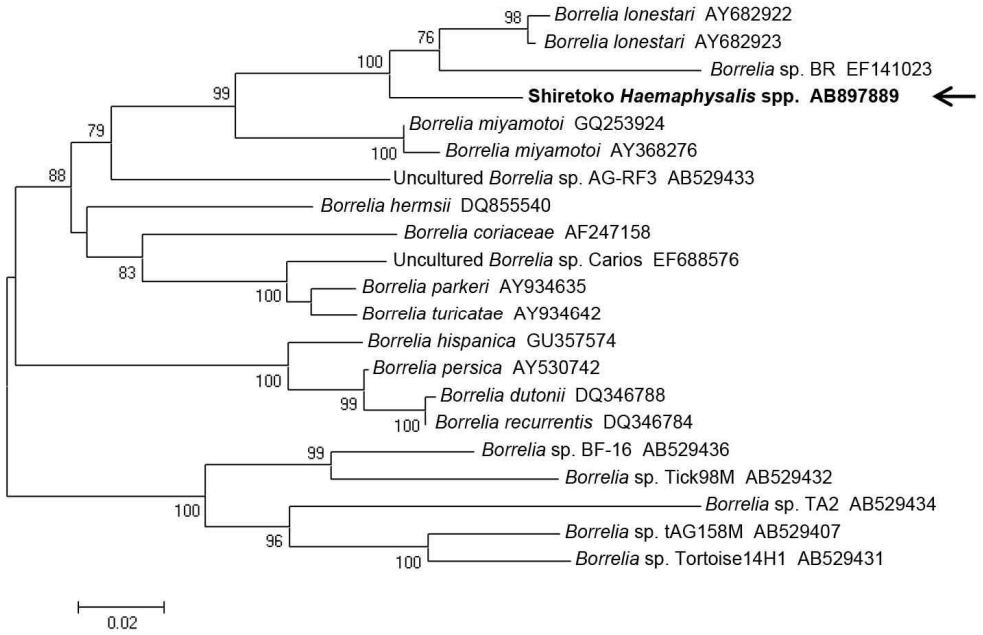

Figure 1-4. Phylogenetic analysis of *glpQ* of *B. lonestari*-like in Hokkaido.

Arrow pointing to bold type indicates the result of this study. Constructed based on the Neighbor joining method with Kimura-2 parameter under pair-wise deletion option. There were a total of 1,056 positions in the final dataset.

CHAPTER 2. ROLE OF DEER IN THE ECOLOGY OF LYME DISEASE BORRELIA

: Lower recovery rate of Lyme disease borreliae DNA in deer feeding *Ixodes persulcatus* than in host questing ticks in the vegetation.

- The original paper of this chapter will be submitted for publication, and thus cannot be shown at the time the thesis has been submitted to Hokkaido University.

Introduction

Deer are one of the main mammal hosts that provide blood meal to ticks infected with various pathogens and are involved directly and indirectly with the tick-borne disease ecology. For ecology of Lyme disease (LD) spirochetes, due to the infestation of vector ticks, deer species had been suspected as the reservoir host (Magnarelli et al., 1984; Isogai et al., 1991; Kimura et al., 1995). However, the incompatibility of deer as a reservoir of LD spirochetes was reported (Telford 3rd et al., 1988). And Nelson (2000) proposed that this was due to the serum complement-mediated killing effect of *Borrelia burgdorferi* found in deer. Similarly, in the study of the ecology of *Borrelia* spp. in Hokkaido, a comparable results were reported to show the incompatibility of deer as the reservoir of LD spirochetes (Taylor, 2013).

Such incompatibility as a reservoir of infectious pathogens may be understood through the investigation of other ecology. Richter and Matuschka (2010) reported that domestic ruminants eliminated LD borreliae from ticks which fed on them and the possibility of a zooprophylactic effect. Such low recovery rate of LD spirochetes from feeding ticks on cervid species have been reported (Telford 3rd et al., 1988; Jaenson and Tälleklint, 1992; Matuschka et al., 1993), but the status in Hokkaido, having different vector tick species and host dynamics compared with those found in Europe or North America, have yet to be investigated.

This study aims to present the possibility of sika deer producing a zooprophylactic effect in the transmission of LD borreliae by comparing the infection rates between *Ixodes persulcatus* feeding on deer and questing in the field.

SUPPLEMENTAL INFORMATION

Table A. *Borrelia* spp. its vector and reservoir animals.

Group	Species	Vectors/carriers	Reservoir (suggested)	References
Lyme Disease	*Borrelia burgdorferi*	*Ixodes scapularis,* *I. ricinus,* *I. pacificus.,* ***I. persulcatus***	Small mammals, birds	Baranton et al. (1992); Bacon et al. (2008)
	B. garinii	*I. ricinus,* ***I. persulcatus****,* *I. nipponensis,* ***I. pavlovski****,*	Small mammals, birds	Baranton et al. (1992); Miyamoto and Masuzawa (2002); Takano et al. (2011b)
	B. afzelii	*I. ricinus,* ***I. persulcatus,*** ***I. pavlovski*** ***Haemaphysalis spp.***	Small mammals, birds	Miyamoto and Masuzawa (2002); Chu et al. (2008); Franke et al. (2010)
	B. japonica	***I. ovatus*** ***Hae. longicornis***	Small mammals	Kawabata et al. (1993); Murase et al. (2013)
Relapsing fever	*B. dutonii*	*Ornithodoros. moubata*	Rodents	Dupont et al. (1997)
	B. hermsii	*O. hermsi*	Small mammals	Boyer et al. (1977); Dworkin et al. (1998)
	B. miyamotoi	***I. persulcatus****,* *I. scapularis*	Small mammals	Fukunaga et al. (1995); Scoles et al. (2001)
	B. theileri	*Boophilus.* Spp.	Cattle	Smith et al. (1978); Smith et al. (1985)
	B. lonestari	*Amblyomma americanum*	White-tailed deer	Moore IV et al. (2003); Moyer et al. (2006)
	Borrelia sp. BR	*Rhicephalus microplus*	Horse (?)	Yparraguirre et al. (2007)
Reptile-associated	*Borrelia* sp. BF-16	*A. trimaculatum*	Reptile	Takano et al. (2010)

Reported species and vectors in Hokkaido are written in bold face.

Table B. PCR Primer and probe sets used in this study.

Gene	Primer	Sequences	References
Borrelia flaB	BflaPAD	GATCA(G/A)GC(T/A)CAA(C/T)ATAACCA(A/T)ATGCA	Sato et al. (1997)
	BflaPDU	AGATTCAAGTCTGTTTTGGAAAGC	
	BflaPBU*	GCTGAAGAGCTTGGAATGCAACC	
	BflaPCR*	TGATCAGTTATCATTCTAATAGCA	
Borrelia glpQ	glpQ-F*	GGTATGCTTATTGGTCTTC	Bacon et al. (2004)
	glpQ-R*	TTGTATCCTCTTGTAATTG	
Borrelia 16SrRNA	16s F1	ATAACGAAGAGTTTGATCCTGGCT	Takano et al. (2010)
	16s R4r	AAAGGAGGTGATCCAGCC(A/G)CACT	
	rrs-F2 *	GGTGTAAGGGTGGAATCTGTTG	
	16s RT R*	GGCGGCACACTTAACACGTTAG	Barbour et al. (2009)
Tick-rrs[#]	Mtrrs-1	CTGCTCAATGATTTTTTAAATTGCTGTGG	Ushijima et al. (2003)
	Mtrrs-2	CCGGTCTGAACTCAGATCAAGTA	
Borrelia 16SrRNA[¶]	16s RT-F	GCTGTAAACGATGCACACTTGGT	Barbour et al. (2009)
	16s RT-R	GGCGGCACACTTAACACGTTAG	
	Probe		
Borrelia 16SrRNA	BM-VIC	5'CGGTACTAACCTTTCGATTA 3'MGB	Barbour et al. (2009)
	BB-FAM	5'TTCGGTACTAACTTTTAGTTAA 3'MGB	
	BS-FAM	5'CGGTACTAATCTTTCGATTA 3'MGB	In this study[§]

*: used for sequencing.

#: used to confirm the species identification of tick nymph samples.

¶: used in real-time PCR.

§: This primer was developed based on Barbour et al. (2009).

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SYNOPSIS

The role of sika deer (*Cervus nippon yesoensis*) in the transmission of *Borrelia* spp. in Hokkaido, Japan.

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The intent of this research was to describe the role of deer in the transmission of different species of *Borrelia* spp. Sika deer (*Cervus nippon yesoensis*) are the hosts of ticks of various species and stages in Hokkaido. Deer overpopulation has caused problems with forestry and agricultural production. The potential of deer involvement in zoonoses is also being watched. In this study, deer caught in the nuisance control scheme and ticks collected from deer and the field were tested to detect *Borrelia* spp.. These results were analyzed for the following two subjects.

Firstly, a survey was held related to a relapsing fever *Borrelia* sp. recently found in Hokkaido. A relapsing fever *Borrelia* sp. similar to *Borrelia lonestari* was detected from wild sika deer 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 methods. The prevalence was significantly higher in deer fawns compared to adults (21.9% and 9.4%, respectively). Additionally, there was a significant regional difference between two sampling areas, Shiretoko and Shibetsu with 17% and 2.8% prevalence, respectively. Regional differences were also found in tick species collected from the field and on deer. In the Shiretoko region, *Haemaphysalis* spp. were more abundant than *Ixodes* spp., while in Shibetsu, it was the opposite. 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 this result, *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 distribution of these ticks.

The second survey was on Lyme disease borreliae in deer with an aspect different from the common vector-reservoir relationship. In conclusion, it could be said that sika deer may be the zooprophylactic host for ticks harboring Lyme disease borreliae in Hokkaido.