Molecular detection of *Borrelia burgdorferi* (*sensu lato*) and *Rickettsia* spp. in hard ticks distributed in Tokachi District, eastern Hokkaido, Japan

Kiyoshi Okada, Paul Franck Adjou Moumounda, Seung-Hun Lee, Thillaiampalam Sivakumar, Naoki Yokoyama, Kozo Fujisaki, Hiroshi Suzuki, Xuanan Xuan, Rika Umemiya-Shirafuji

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**ABSTRACT**

Ticks transmit various pathogens, including parasites, bacteria and viruses to humans and animals. To investigate the ticks and the potentially zoonotic pathogens that they may carry, questing ticks were collected in 2017 from 7 sites in Tokachi District, eastern Hokkaido, Japan. A total of 1563 ticks including adults (male and female), nymphs and larvae were collected. Four species of ticks were identified: *Ixodes ovatus*, *Ixodes persulcatus*, *Haemaphysalis japonica* and *Haemaphysalis megaspinosa*. Of the 1563 ticks, 1155 were used for DNA extraction. In total, 527 individual tick DNA samples prepared from adults (*n* = 484), nymphs (*n* = 41) and larvae (*n* = 2); and 67 pooled tick DNA samples prepared from larval stages (*n* = 628) were examined using PCR methods and sequencing to detect *Borrelia burgdorferi* (*sensu lato*) and *Rickettsia* spp. The phylogenetic analysis of *Borrelia* spp. flaB gene sequences showed the presence of the human pathogenic *B. burgdorferi* (*s.l.*) species (*Borrelia garinii*, *Borrelia bavariensis* and *Borrelia afzelii*) in *I. persulcatus*, whereas the non-pathogenic species *Borrelia japonica* was found only in *I. ovatus*. In *I. persulcatus*, *B. garinii* and/or its closely related species *B. bavariensis* was detected in both adults and nymphs at a prevalence of 21.9% whereas *B. afzelii* was found only in adults (1.8%). The prevalence of *B. japonica* in adult *I. ovatus* was 21.8%. *Rickettsia* spp. were identified through phylogenetic analysis based on 16S rRNA, ompB and sacA genes. Four genotypes were detected in the samples which were classified into three species. The prevalence of human pathogenic *Rickettsia helvetica* was 26.0% in *I. persulcatus* adults and nymphs, 55.6% in *I. persulcatus* larval pools, and 1.7% in *H. megaspinosa* larval pools. The prevalence of “*Candidatus R. tarasevichiae*” was 15.4% in *I. persulcatus* adults and nymphs and 33.3% in *I. persulcatus* larval pools. The prevalence of “*Candidatus R. priscigi*” in *H. megaspinosa* adults and nymphs was 11.1% whereas it was detected in 3.4% of the *H. megaspinosa* larval pools. These results indicate that most of the risks of Lyme borreliosis and spotted fever group rickettsiosis infection in eastern Hokkaido, Japan, are restricted to *I. persulcatus*.

1. Introduction

In Japan located in East Asia, the dynamics of tick-borne pathogens is changing as the incidence of related human disease cases is increasing (Yamaji et al., 2018). Mapping of the distribution of ticks and the zoonotic pathogens they carry, in each locality of the country is one of the constant key efforts to assess the risk of the occurrence of infectious diseases. Tokachi District in eastern Hokkaido, Japan, is famous for its agriculture and dairy farming that utilize the area’s vast plains. Because dairy cows live in meadows shared with wild animals such as sika deer, they frequently suffer tick infestations while grazing, are at great risk of pathogenic infections (Ota et al., 2009; Shibata et al., 2018) and may serve as a source of infected ticks. Likewise, not only dairy farmers but also farmers and hunters are at high risk for tick bites which potentially transmit various tick-borne zoonotic diseases (Kubo et al., 1992; Chaligiannis et al., 2018). Moreover, outdoor nature-based recreational activities such as hiking, fishing and barbecuing are popular in the natural environment of Tokachi District surrounded by mountains and might also be associated with risks of tick bites.

Lyme borreliosis and rickettsioses are important tick-borne zoonotic diseases. Despite substantial efforts to improve surveillance and control of Lyme borreliosis, it remains prevalent in the northern hemisphere.
DNA extraction, PCR and sequencing to detect ticks from seven sites of the district in 2017. The tick samples were ticks in Tokachi District, eastern Hokkaido, Japan, we collected questing 2.1. Tick collection and identi
2. Materials and methods spp. (Eisen 1985; Yamaguti et al., 1971). Identification was processed individually or in pools for larvae, whereas nymph and adult ticks were processed individually. Identified larvae were pooled according to collection month, collection site, and species (2–10 individuals per pool). For Ixodes ovatus, one larva was tested. The ethanol-preserved ticks were rinsed twice with 70% ethanol and then immersed in distilled water (Yu et al., 2016). Ticks were homogenized in 75 μl of PBS using a PowerMasher II homogenizer (Nippi, Tokyo, Japan) and sterilized homogenization tube BioMaster II (Nippi). DNA was extracted using NucleoSpin Tissue (Macherey, Düren, Germany) according to the manufacturer’s instructions. DNA was eluted from columns with 50 μl of elution buffer. To confirm the purity of the eluates, the concentration of each DNA extract was assessed by Nanodrop spectrophotometers (Thermo Fisher Scientific, MA, USA). The extracted samples were stored at −30 °C until use.

2.3. PCR detection of B. burgdorferi (s.l.) A nested PCR reaction was run on all DNA samples to detect B. burgdorferi (s.l.). Specific primers for B. burgdorferi (s.l.) (Table 1) were used to amplify the flaB gene encoding the flagellin protein (Yu et al., 2016). A 0.75 μl of DNA sample was added to 9.25 μl of reaction mixture that contained 1 μl of 10× PCR buffer, 1 μl of dNTPs, 0.2 μl of each primer (primers flaB outer primer F and flaB outer primer R, Table 1), 0.1 μl of Taq DNA polymerase (Blend Taq-Plus; Toyobo, Osaka, Japan) and 6.75 μl of sterile Milli-Q water. The reaction conditions for the first PCR involved 4 min of pre-denaturation at 94 °C followed by 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The second PCR was performed in the same buffer using 0.5 μl of the first PCR products as a template and primers flaB nested primer F and flaB nested primer R (Table 1). The protocol for the second PCR was initial denaturation (94 °C, 4 min); followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, with the final extension step at 72 °C for 10 min. Amplicons of the second PCR were electrophoresed and stained with ethidium bromide as described previously (Yu et al., 2016). Detection of a band close to 411 bp under UV was considered positive.

The species of the B. burgdorferi (s.l.) complex detected in the samples were later identified through sequencing of the amplicons of the second PCR. When the flaB sequence did not allow a precise identification, the rrf (5S)-rrl (23S) ribosomal RNA (5S–23S) gene was amplified and sequenced for confirmation of the B. burgdorferi (s.l.) species. The primer sets used are shown in Table 1. PCR conditions were the same as previously reported (Choi et al., 2007).
**Table 1**

| Organism       | Target gene | Oligonucleotide primer | Primer sequence (5'-3') | Amplicon size (bp) | Reference          |
|----------------|-------------|------------------------|-------------------------|--------------------|--------------------|
| *B. burgdorferi* (s.l.) | flaB        | Outer primer F         | CTCGGCCAGACGGGATTTCT    | 725                | Yu et al. (2018)    |
|                |             | Outer primer R         | TCAATTCGATCACTGACTCT    |                    |                    |
|                |             | Nested primer F        | GGCGGTACGTCGCTAAGG     | 411                | Yu et al. (2018)    |
|                |             | Nested primer R        | AGAAGGTCTGCAGGAGGAGGG  |                    |                    |
| *Rickettsia* spp. | gltA        | CS-78                  | ACATACTCTCTGTTTGAGAC   | 373                | Choi et al. (2007)  |
|                |             | CS-323                 | TAAGGTGATGTAATAATTACAC |                    |                    |
|                |             | 5SCB                   | ACCATGAGCTTCTTTGAGGACA  | 227                | Choi et al. (2007)  |
|                |             | CS-322                 | GAGAAGGTATGTTTGCCAGG   | 401                | Labruna et al. (2004) |
|                |             | 16S rRNA               | ATCGAAGGAAATACCTTTA    | 1328               | Anstead & Chilton (2013) |
|                |             | Rick_16S_F3            | TGGCTCTGGTGGATGCTCA    |                    |                    |
|                |             | Rick_16S_F4            | AGGCTTTCTTATGCTGCA     |                    |                    |
|                |             | rs2_seq1               | CGCCGCTATCTCCTCCTC     |                    |                    |
|                |             | rs2_seq2               | CTCAGGGCTGACTGAAAGC    |                    |                    |
|                |             | R16S_Fw1               | AGAAGGGCGGCCGCTTACCC   |                    | This study          |
|                |             | R16S_Rv1               | CCATGCAAACCTGTTGTTG    |                    |                    |
|                |             | ompB                   | AAACAAATCAAGGATCTGT    | 816                | Roux & Raoult (2000) |
|                |             | 120_2788               | TACTCGCGTGTCAGAAGAGT   |                    |                    |
|                |             | 120_3599               | ATGAGGAAGAAGGATACCT    |                    |                    |
|                |             | sca4                   | AAGCTATGAGTGGCAATGCG   | 928                | Sekeyova et al. (2001) |

* Primers that were used only for the sequencing of amplicons.

2.4. **PCR detection of *Rickettsia* spp.**

PCR preparation was carried out in sterile conditions as described above. All of the tick DNA samples were subjected to PCR targeting *gltA* as a screening test for *Rickettsia* spp. DNA. Primers and PCR conditions have been described previously (de Sousa et al., 2018, Table 1). One microliter of DNA sample was added to a 9-μl reaction mixture that contained 1 μl of 10× PCR buffer, 1 μl of dNTPs, 0.2 μl of each primer set (primers CS-78 and CS-323, Table 1), 0.1 μl of Taq DNA polymerase (Blenda Taq-Plus; Toyobo) and 6.5 μl of sterile Milli-Q water. Detection of a band around 401 bp under UV was considered positive. Additional PCR assays were performed based on three genes: 16S ribosomal RNA gene (*ompB*), outer membrane protein B gene (*ompB*), and surface cell antigen-4 (*sca4*) gene. The primer sets used for each reaction are shown in Table 1. PCR conditions were the same as previously reported by Thu et al. (2019) except for the annealing temperatures for *ompB* and *sca4* PCR (54 °C for *ompB* PCR and 56 °C for *sca4* PCR).

The *Rickettsia* species detected in the samples were identified in two steps. First, *gltA* genotyping was performed through sequencing of the amplicons of *gltA* PCR. Then, selected-sample representatives of the *gltA* genotype were submitted to further characterization of *Rickettsia* spp. through sequencing of 16S rRNA, *ompB* and *sca4* genes.

2.5. **Sequencing**

The amplified PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Each PCR product was subjected to DNA sequencing (Fasmac, Kanagawa, Japan), and ClustalW was used to align the sequences. The newly generated DNA sequences were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under the accession numbers: LC496815-LC496832 (*Borellia* flaB); LC496811-LC496814 (*Rickettsia* gltA); MT378425-MT378437 (*Rickettsia* 16S rRNA); LC544128-LC544135 (*Rickettsia* ompB); and LC544136-LC544138 (*Rickettsia* sca4). Inter-species comparison of sequences based on BLASTn was performed using NCBI software Megablast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide collection (nr/nt) database, which links GenBank, EMBL, DDBJ, PDB and RefSeq sequences was used.

2.6. **Phylogenetic analysis**

DNA sequences obtained were aligned with sequences of representative *Borellia* spp. or *Rickettsia* spp. using ClustalW 1.6 as implemented in MEGA 7 (Kumar et al., 2016). After manual confirmation, phylogenetic trees were constructed using the maximum likelihood method according to the Tamura 3-parameter (Tamura, 1992) model with bootstrap tests of 1000 replicates using MEGA 7.

2.7. **Statistical tests**

Microorganism prevalence in the individual tick DNA samples and their 95% confidence intervals (95% CI) were calculated using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Microorganism prevalence in larvae was estimated by taking into account the number of pools and the number of larvae per pool. We assumed a maximum positive rate if all larvae of a positive pool were infected and a minimum positive rate if only one larva in a positive pool was infected.

3. **Results**

3.1. **Tick collection**

In total, 1563 ticks (599 adults, 191 nymphs and 773 larvae) were collected between May and November 2017 from 7 sites in Tokachi District. The number of ticks, tick species, and developmental stages collected varied based on sampling month and site. Four species of ticks were identified based on morphological criteria: *I. ovatus* (n = 364), *Ixodes persulcatus* (n = 296), *Haemaphysalis japonica* (n = 143) and *Haemaphysalis megaspinosa* (n = 760). *Ixodes persulcatus* was found in all sites examined across Tokachi District. The detailed features of the tick samples (sampling month, sampling sites, species and developmental stage) are presented in Supplementary Table S1. The 1563 live ticks that were collected by flagging, was divided into two sets. One set was allocated to laboratory rearing in other experiments (Umemiya-Shirafuji et al., unpublished data) and the remaining samples were submitted to DNA extraction: a total of 1155 ticks were used for DNA extraction and microorganism detection in the present study. Overall, 594 DNA samples including 527 individual tick DNA samples and 67 pooled larval tick DNA samples were prepared. The individual tick DNA samples were prepared from 295 *I. ovatus* (123 males; 171 females; and 1 larva), 169 *I. persulcatus* (72 males; 77 females; and 20 nymphs), 18 *H. japonica* (6 males; 10 nymphs; and 1 larva), and 45 *H. megaspinosa* (26 males; 8 females; and 11 nymphs). The pooled tick DNA samples were prepared from larval stages of *I. persulcatus* (61; 9 pools) and *H. megaspinosa* (567; 58 pools) (Supplementary Table S1).
3.2. Detection of B. burgdorferi (s.l.) in ticks

*Borrelia burgdorferi* (s.l.)-positive ticks were detected in 6 (Memuro, Shikaoi, Urahoro, Shimizu, Taiki and Ashoro) of the 7 sites surveyed (Supplementary Table S1). DNA fragments of *Borrelia* spp. were detected in *I. ovatus* and *I. persulcatus* in the present study. The overall prevalence of *B. burgdorferi* (s.l.) was 21.8% (64/294; 95% CI: 17.2–26.9%) in *I. ovatus* (8/123 males; 56/171 females). One larva of *I. ovatus* tested was negative. The prevalence in *I. persulcatus* was 23.7% (40/169; 95% CI: 17.5–30.8%) and positive ticks included adults (12/72 males; 26/77 females,) and nymphs (2/20). All larval pools were negative (0/9 pools).

3.3. flaB genotyping and species classification of *B. burgdorferi* (s.l.)

Of the 104 samples that tested positive for *B. burgdorferi* (s.l.), 83 flaB PCR amplicons were successfully sequenced. The species identity of the remaining 21 samples was confirmed by sequencing the SS–23S gene. From analyzing the 83 flaB sequences, 17 different flaB genotypes (referred to as flaB to BG17) were identified. Fifteen genotypes were detected in *I. persulcatus* (BG1, BG2, BG3, BG4, BG5, BG8, BG9, BG10, BG11, BG12, BG13, BG14, BG15, BG16 and BG17) whereas the remaining two (BG6 and BG7) were only found in *I. ovatus*. The flaB sequences obtained in this study shared 99 or 100% identity with the closest *Borrelia* species sequences available in the GenBank database (Table 2). In the phylogenetic tree inferred from flaB sequences, 14 genotypes (BG1, BG2, BG3, BG4, BG5, BG8, BG9, BG10, BG11, BG12, BG13, BG14, BG15 and BG16) were found in the same clade with *B. garinii* and the closely related *Borrelia bavariensis*. The BLASTn analysis of the SS–23S gene sequences obtained from the corresponding samples (data not shown) identified some of these genotypes as *B. garinii* and others as *B. bavariensis*. The 14 genotypes were therefore classified as *B. garinii/B. bavariensis* (Fig. 2). BG6 and BG7 formed a distinct cluster with *Borrelia japonica* and were identified as *B. japonica*. The SS–23S gene sequences (data not shown) also confirmed the presence of *B. japonica* in the samples from which BG6 and BG7 were amplified. BG17 clustered with *B. afzelii* (Fig. 2).

3.4. Detection of *Rickettsia* spp. in ticks

Ticks positive for *Rickettsia* spp. were detected in 6 (Memuro, Shikaoi, Urahoro, Hiroo, Taiki and Ashoro) out of the 7 provinces (Supplementary Table S1). Tick samples that showed positivity for *Rickettsia* spp. flaB included adults, nymphs and larvae of *I. persulcatus* and *H. megaspinosa*. All of the *I. ovatus* and *I. japonica* samples were negative. The overall prevalence of *Rickettsia* spp. was 41.4% (70/169; 95% CI: 34.5–49.8%) in *I. persulcatus* adults and nymphs (21/72 males; 44/77 females; 5/20 nymphs). Most of the *I. persulcatus* larval pools were positive (8/9), and the maximum and minimum positive rates among the larvae were estimated at 90.2% (55/61) and 13% (8/61), respectively. Among *H. megaspinosa* ticks the prevalence was 11.1% (5/45; 95% CI: 3.7–24.1%) for adults and nymphs (3/26 males; 2/8 females; 0/11 nymphs). Three out of the 58 larval pools were positive, suggesting 5.29% (30/567) and 5.3% (3/567) for the maximum and minimum positive rates among the *H. megaspinosa* larvae.

3.5. gltA genotyping and species classification of *Rickettsia* spp.

PCR amplicons of 86 samples found positive for *Rickettsia* spp. were sequenced and differentiated into 4 gltA genotypes (RG1, RG2, RG3 and RG4). RG1 was obtained from *I. persulcatus* (adult, nymph and larva) and a larval pool of *H. megaspinosa*. RG2 and RG4 were recovered from *I. persulcatus* (adult, nymph and larva) whereas RG3 was detected in *H. megaspinosa* (adult and larva). In the multiple gene sequencing of samples representing the gltA genotypes, the 16S rRNA gene was successfully amplified for all genotypes. The ompB gene was amplified in all samples of 3 genotypes (RG1, RG3 and RG4) except for *H. megaspinosa* (Hmegen2017 Hiroo L007; RG1). The sca4 gene, however, was successfully amplified only for RG3. The gltA sequences obtained in this study shared 98–100% identity with the closest published *Rickettsia* species sequence (Table 2). Phylogenetic trees inferred from gltA (Fig. 3), 16S rRNA (Fig. 4), ompB (Fig. 5) and sca4 (Fig. 6) sequences were constructed using the data from our study and public sequences of validated *Rickettsia* species. Four out of the five representatives of the RG1 genotype are close to *R. helvetica* and formed a distinct cluster with *R. helvetica* in the gltA and ompB phylogenetic trees. One RG1 obtained from a larval pool of *H. megaspinosa* was located in the *R. helvetica* gltA cluster but formed a divergent branch in the 16S rRNA phylogenetic tree (ompB and sca4 could not be amplified for that sample; Table 3). RG2 species identity was assessed using gltA and 16S rRNA phylogenetic trees, both of which located the genotype in a cluster with “Ca. R. tarasevichiae”. The RG3 genotype was classified as “Ca. R. principis” in the gltA phylogenetic tree. However, in the 16S rRNA, ompB and sca4 phylogenetic trees, RG3 sequences formed a cluster distinct from other validated *Rickettsia* species. RG4 was identified in the “Ca. R. tarasevichiae” cluster of the gltA phylogenetic tree, whereas in the 16S rRNA and ompB trees, it was located within the cluster of *R. helvetica*.

Altogether, the BLAST and phylogenetic analyses based on the gltA, 16S rRNA, ompB and sca4 sequences, showed that the *Rickettsia* spp.
detected in the samples include species related to *R. helvetica*, “Ca. R. principis”, and “Ca. R. tarasevichiae”.

3.6. Distribution of *B. burgdorferi* (s.l.) and Rickettsia species

The presence of *Borrelia* spp. and *Rickettsia* spp. in tick samples collected from Tokachi District in 2017 is summarized in Table 4. *Borrelia japonica* was only detected in adult *I. ovatus* samples with a prevalence of 21.8% (64/294; 95% CI: 17.2–26.9%). *Borrelia garinii/B. bavariensis* was detected in both adult and nymphal *I. persulcatus* at a prevalence of 21.9% (37/169; 95% CI: 15.9–28.9%). *Borrelia afzelii* was found only in adult *I. persulcatus* at a prevalence of 1.8% (3/169; 95% CI: 0.4–5.1%). Concerning *Rickettsia* species, *R. helvetica* was detected at a prevalence of 26.0% (44/169; 95% CI: 19.6–33.3%) in adult and nymphal *I. persulcatus* and in 55.6% (5/9) of the larval pools. Among the *H. megaspinosa* larval pools, one (1.7%; 1/58) was positive for *R. helvetica*. The prevalence of “Ca. R. tarasevichiae” was 15.4% (26/169; 95% CI: 10.3–21.7%) in *I. persulcatus* adults and nymphs and 33.3% (3/9) in the larval pools. “*Candidatus R. principis*” was related to *H. megaspinosa* with a prevalence of 11.1% (5/45; 95% CI: 3.7–24.1%) and 3.4% (2/58) for adults and nymphs, and larval pools, respectively. The prevalence of these microorganisms in female ticks appeared to be higher than the values recorded in male ticks (Table 4). However, larger sample sizes will be needed to compare statistically the prevalence of the microorganisms between female and male ticks. The distribution of *Borrelia* and *Rickettsia* species varied across the sampling sites (Supplementary Table S1). *Rickettsia helvetica* and “Ca. R. tarasevichiae” found in 6 sites (Memuro, Shikaoi, Urahoro, Hiroo, Taiki and Ashoro) were the most widely distributed, followed by *B. japonica* (Memuro, Urahoro, Shimizu, Taiki and Ashoro) and *B. garinii/B. bavariensis* (Memuro, Shikaoi and Urahoro). “*Candidatus R. principis*” and *B. afzelii* were found in 2 (Hiroo, Taiki) and 1 (Memuro) site, respectively.

Fig. 2. Phylogenetic tree based on the sequences of the *flaB* gene (321 bp) for *Borrelia* spp. Seventeen different *flaB* genotypes (BG1 to BG17) were identified in the present study (red-colored letters). Numbers in parentheses represent GenBank accession numbers. The unit of branch length is nucleotide substitutions per site.
### Table 3

Results of the BLAST analysis of the *Rickettsia* spp. *gltA*, 16S rRNA, *ompB*, and *sca4* sequences obtained in this study

| RG No. | Tick ID          | Tick species and developmental stage | % Identity with the closest *Rickettsia* species (GenBank ID) | *gltA*          | 16S rRNA          | *ompB*          | *sca4*          |
|--------|------------------|--------------------------------------|--------------------------------------------------------------|-----------------|------------------|-----------------|-----------------|
| RG1 (n = 50) | Ipers20170019     | *I. persulcatus* (male)               | 100% with *R. helvetica* (KU310588)                          | 100% with *R. helvetica* (MH618376) | 99% with *R. helvetica* (MF163037) | na              | na              |
|          | Ipers20170021     | *I. persulcatus* (female)             | 100% with *R. helvetica* (KU310588)                          | 100% with *R. helvetica* (MH618376) | 99% with *R. helvetica* (MF163037) | na              | na              |
|          | Ipers20170135     | *I. persulcatus* (nymph)              | 100% with *R. helvetica* (KU310588)                          | 100% with *R. helvetica* (MH618376) | 99% with *R. helvetica* (MF163037) | na              | na              |
|          | Ipers2017 Shikaoi L001 | *I. persulcatus* (larva)           | 100% with *R. helvetica* (KU310588)                          | 100% with *R. helvetica* (MH618376) | 99% with *R. helvetica* (MF163037) | na              | na              |
|          | Hmega2017 Hiroo L007 | *H. megapisiosa* (larva)          | 100% with *R. helvetica* (KU310588)                          | 98% with *R. rauludi* (MK304546) | 98% with *R. rauludi* (MG544987) | 98% with *R. raoultii* (MK304546) | 98% with *R. conorii* (NR_074480) | 98% with *R. heilongiangensis* (AP019865) |
| RG2 (n = 28) | Ipers20170103     | *I. persulcatus* (male)               | 100% with “Ca. R. tarasevichiae” (MN450397)                   | 100% with “Ca. R. tarasevichiae” (MN446745) | na              | na              | na              |
|          | Ipers20170025     | *I. persulcatus* (female)             | 100% with “Ca. R. tarasevichiae” (MN450397)                   | 100% with “Ca. R. tarasevichiae” (MN446745) | na              | na              | na              |
|          | Ipers2017184      | *I. persulcatus* (nymph)             | 100% with “Ca. R. tarasevichiae” (MN450397)                   | 100% with “Ca. R. tarasevichiae” (MN446745) | na              | na              | na              |
|          | Ipers2017 Taiki L001 | *I. persulcatus* (larva)          | 100% with “Ca. R. tarasevichiae” (MN450397)                   | 100% with “Ca. R. tarasevichiae” (MN446745) | na              | na              | na              |
| RG3 (n = 7) | Hmega20170010     | *H. megapisiosa* (male)              | 100% with “Ca. R. principis” (AY578115)                      | 99% with *R. rauludi* (MK304546) | 99% with “Ca. R. principis” (MG544987) | 99% with *R. rauludi* (MK304546) | 99% with “Ca. R. rauludi” (NR_074480) | 98% with *R. helongiangensis* (AP019865) |
|          | Hmega20170004     | *H. megapisiosa* (female)            | 100% with “Ca. R. principis” (AY578115)                      | 99% with *R. rauludi* (MK304546) | 99% with “Ca. R. principis” (MG544987) | 99% with *R. rauludi* (MK304546) | 99% with “Ca. R. rauludi” (NR_074480) | 98% with *R. helongiangensis* (AP019865) |
|          | Hmega2017 Hiroo L009 | *H. megapisiosa* (larva)          | 100% with “Ca. R. principis” (AY578115)                      | 99% with *R. rauludi* (MK304546) | 99% with “Ca. R. principis” (MG544987) | 99% with *R. rauludi* (MK304546) | 99% with “Ca. R. rauludi” (NR_074480) | 98% with *R. helongiangensis* (AP019865) |
| RG4 (n = 1) | Ipers20170166     | *I. persulcatus* (male)               | 99% with “Ca. R. tarasevichiae” (MN450397)                     | 99% with “Ca. R. tarasevichiae” (KU310588) | 100% with *R. helvetica* (MH618376) | 99% with *R. helvetica* (MF163037) | na              | na              |

**Abbreviations:** Ca., *Candidatus*; na, not amplified; RG, *gltA* genotype of *Rickettsia* spp.

* Identities with “Ca. R. principis” 16S rRNA and *sca4* are not shown due to absence of reference sequences in the database.

b Query coverage was 95%.
In this study, four hard tick species namely *I. ovatus*, *I. persulcatus*, *H. japonica* and *H. megaspinosa* were collected in 2017 from Tokachi District, Japan. The identified tick species are consistent with previous surveys on tick species in Tokachi (Inokuma et al., 2007), suggesting that these species are still dominant hard ticks in this area. *Ixodes ovatus* and *I. persulcatus* are the main causative species of human tick bites in Hokkaido to Honshu, northern to central Japan (Natsuaki, 2021). Some human cases with *H. japonica* bites were found in Hokkaido and Honshu (Yamauchi et al., 2010; Yamauchi & Nakatani, 2016; Sasaki et al., 2021). Although the primary hosts of *H. megaspinosa* are large mammals (e.g. deer, boar and Japanese serow) (Yamaguti et al., 1971), human cases of tick bites by *H. megaspinosa* were reported in Honshu (Seishima et al., 2000; Tsunoda, 2004; Hasegawa et al., 2016). The present PCR assays using DNA samples of questing ticks revealed that *I. persulcatus* carried *B. garinii*, *B. bavariensis*, *B. afzelii*, *R. helvetica*, and “Ca. R. Helvetica” and “Ca. R. principis”, while none of the *H. japonica* ticks collected in the present study was positive for the microorganisms that were targeted.

Currently, the *B. burgdorferi* (s.l.) species complex consists of more than 20 pathogenic, potentially pathogenic, non-pathogenic and unknown pathogenic bacteria that utilize *Ixodes* ticks as vectors (Stanek & Sirle, 2018; Margos et al., 2019). *Borrelia garinii*, *B. bavariensis* and *B. afzelii* are human pathogens and cause Lyme borreliosis, whereas the pathogenicity of *B. japonica* is unknown. *Borrelia garinii* and *B. bavariensis*, previously referred to as *B. garinii* genospecies, are closely related species sharing the same geographical distribution and tick vectors (Margos et al., 2019). Discrimination of these *Borrelia* species requires a multilocus genotyping approach including eight genes. In our study, a two-gene approach confirming the occurrence of both species was selected and the samples of *B. burgdorferi* (s.l.) showing sequence identity with any of these two species were classified as *B. garinii*/*B. bavariensis*. *Ixodes persulcatus* is a suspected vector for *B. garinii*, *B. bavariensis* and *B. afzelii* (Masuzawa et al., 2005; Margos et al., 2019; Eisen, 2018). Furthermore, *I. persulcatus* was considered as a primary tick vector for Lyme borreliosis in Hokkaido (Fukunaga et al., 1993; Murase et al., 2012). The presence of *B. garinii*/*B. bavariensis* and *B. afzelii* in *I. persulcatus* from Tokachi area relates therefore to the vector role of this tick and its importance in Lyme borreliosis transmission.

Despite sharing the same geographical range and vector, *B. garinii*, *B. bavariensis* and *B. afzelii* seem to have different relative frequency. Fukunaga & Hamase (1995) indicated that *B. garinii* was predominant among isolates obtained from Lyme borreliosis patients and ticks in Japan. Meanwhile, Murase et al. (2012) showed that *B. garinii* infection rate was higher than that of *B. afzelii* in *I. persulcatus* from Hokkaido. In accordance, in our study, 92.5% (37/40) of the pathogens found in *I. persulcatus* was identical to *B. afzelii*. However, the infection rate (21.9%; 95% CI: 15.9–28.9%) of *B. garinii*/*B. bavariensis* in *I. persulcatus* was lower than the *B. garinii* infection rate (33.8%) obtained by Murase et al. (2012) in the same tick species but in another site in Hokkaido Prefecture. The infection rate of *B. afzelii* (1.8%; 95% CI: 0.4–5.1%) showed in our study was similar to the value (4.9%) obtained by Murase et al. (2012). These authors investigated a ranching farm where a confirmed case of Lyme borreliosis has been reported, whereas we did not collect ticks in such farm in our study. It might suggest that the positive rates of pathogens vary depending on the characteristics of the study areas. While *B. garinii*, *B. bavariensis* and *B. afzelii* are found in Europe and Asia,
B. japonica seems to be restricted to Japan (Kawabata et al., 1993; Li et al., 1998). Previous studies indicated that I. ovatus carries only B. japonica (Kawabata et al., 1993; Masuzawa, 2004; Murase et al., 2012) and Nakao et al. (1994) indicated that the tick and Borrelia species are not related to Lyme borreliosis. The presence of B. japonica and not the other B. burgdorferi (s.l.) genospecies in I. ovatus collected in this study, therefore relates to this tick species not being a vector of Lyme borreliosis.

Borrelia japonica prevalence in I. ovatus (21.8%; 64/294, 95% CI: 17.2–26.9%) was also similar to the values (19.5%; 8/41) obtained by Murase et al. (2012). We did not find I. persulcatus larvae carrying B. garinii/B. bavariensis or B. afzelii. In the absence of transovarial transmission of B. burgdorferi (s.l.) in I. persulcatus (Nefedova et al., 2004), acquisition of B. garinii, B. bavariensis and B. afzelii by its vector obviously results from feeding on the blood of host animals. The principal reservoir host for the species of Borrelia transmitted by I. persulcatus immatures in Hokkaido is the wood mouse, Apodemus speciosus ainu (Nakao & Miyamoto, 1993a). The Borrelia isolated from A. speciosus ainu was formerly identified as B. garinii (currently probably B. bavariensis) (Kawabata et al., 1993; Margos et al., 2012). In contrast, the long-tailed shrew Sorex unguiculatus is a reservoir for B. japonica and also a host animal for I. ovatus immatures in Hokkaido (Nakao & Miyamoto, 1993b). Tokachi area is a sympatric region for I. ovatus and I. persulcatus. Although the host ranges of I. ovatus and I. persulcatus partially overlap for their immature stages (Nakao & Miyamoto, 1993b), our results and previous studies suggest that Borrelia species detected from questing adults of both tick species are clearly distinguishable (Table 4). Further experimental investigations such as xenodiagnosis are needed to test the vector competence of the two tick species for different Borrelia species and observe the effect of these Borrelia infections on tick physiology.

In the present study, Rickettsia species were detected not only in adults and nymphs, but also in larvae. Of the three Rickettsia spp. detected, R. helvetica and “Ca. R. tarasevichiae” are pathogenic to humans, whereas “Ca. R. principis”, pathogenicity remains unknown. R. helvetica was first reported in Europe (Parola et al., 1998). Its prevalence in Europe has been evidenced in France (Fournier et al., 2000) and then in Japan (Fournier et al., 2002; Noji et al., 2005). It is known that vectors of R. helvetica in Japan are I. persulcatus, I. ovatus and Ixodes monospinosus (Fournier et al., 2002; Ishiguro et al., 2008). In the present study, we detected R. helvetica gltA genotype in I. persulcatus, corresponding to the finding of Inokuma et al. (2007) and suggesting that I. persulcatus could be a vector of R. helvetica in Tokachi District. Furthermore, the detection of R. helvetica in questing I. persulcatus larvae suggests transovarial transmission, although contaminations or partial feeding could not be excluded. Rickettsia helvetica gltA genotype (RG1) was also found in one pool of H. megaspinosa larvae (Hmega2017 Hiroo L007) that included 10 individual larvae; however, the sequence of the
Fig. 5. Phylogenetic tree based on the *Rickettsia ompB* gene (815 bp). The tree was constructed using the maximum likelihood method with the Tamura 3-parameter model. The analysis was performed with bootstrap tests of 1000 replicates. DNA sequences obtained in the present study are indicated in red-highlighted-tick ID. Numbers in parentheses represent GenBank accession numbers. The units of branch length are nucleotide substitutions per site. The long branches are shortened and presented as interrupted branches (see full-size image in Supplementary Fig. S2).

Fig. 6. Phylogenetic tree based on the *Rickettsia sca4* (847 bp) gene. The tree was constructed using the maximum likelihood method with the Time reversible model. The analysis was performed with bootstrap tests of 1000 replicates. DNA sequences obtained in the present study are indicated in red-highlighted-tick ID. Numbers in parentheses are GenBank accession numbers. The units of branch length are nucleotide substitutions per site.
16S rRNA gene from this pool formed a different clade with *R. helvetica* (Fig. 4; HmeGa2017 Hiroo L007 RG1 (MT378429)). Although such finding indicates that *H. megaspinosa* might play a role as a vector for *R. helvetica*, further research is needed to fully characterize the *Rickettsia* species/strain carried by these larvae.

“*Candidatus* R. tarasevichiae” was regarded as non-pathogenic until 2012, when five patients with recent tick bites sought treatment in a hospital of northeastern China and were found to be infected with this pathogen (Jia et al., 2013). More recently, a retrospective investigation in eastern central China found that 56 patients who showed severe fever with thrombocytopenia syndrome-like illnesses were infected with “*Ca. R. tarasevichiae*” (Liu et al., 2016). The identification of “*Ca. R. tarasevichiae*” in *I. persulcatus* in our study is in agreement with a previous study (Inokuma et al., 2007) in the same district. “*Candidatus* R. tarasevichiae” was detected not only in adults and nymphs but also in larvae, suggesting that transovarial transmission occurred under natural conditions. Among the samples in which “*Ca. R. tarasevichiae*” *gltA* was identified, one (RG4 (n = 1); Ipers20170166) showed 16S rRNA and *ompB* sequences that clustered with *R. helvetica* sequences (Figs. 4 and 5). This may be explained by the tick being infected with both “*Ca. R. tarasevichiae*” and *R. helvetica*. On the other hand, the life-cycle and pathogenicity for humans of “*Ca. R. principis*” remain unknown (Mediannikov et al., 2006). This rickettsial agent has previously been detected in the ticks *Haemaphysalis flava* and *H. japonica* in Japan (Gaowa et al., 2013) and in *Haemaphysalis danieli* in China (Chahan et al., 2007). A recent survey of SFG rickettsiae in questing ticks in Japan identified a *Rickettsia* genotype that was obtained from adult *H. megaspinosa* and clustered in the same clade with “*Ca. R. principis*” (Thu et al., 2019). In our study, *Rickettsia gltA* genotype RG3 was detected in *H. megaspinosa* adults and larvae and located in “*Ca. R. principis*” clade. In the BLASTn search, the *ompB* sequences of RG3 showed 100% identity with previously published “*Ca. R. principis*” sequences, although the query coverage was 95%. Because published “*Ca. R. principis*” sequences were shorter (*ompB*) or not available (16S rRNA and *sca4*), RG3 sequences formed a cluster distinct from other validated *Rickettsia* species in the *ompB*, 16S rRNA and *sca4* phylogenetic trees. Based on BLASTn search (*gltA* and *ompB*) and *gltA* phylogenetic tree, RG3 was identified as genetically related to “*Ca. R. principis*”. These results support previous reports and also provide, to our knowledge, the first evidence of the occurrence of “*Ca. R. principis*” in larvae of *H. megaspinosa*. The detection of “*Ca. R. principis*” in questing *H. megaspinosa* larvae indicates transovarial transmission of “*Ca. R. principis*” in the tick species.

5. Conclusions

Our findings suggest that major risks of Lyme borreliosis and SFG rickettsiosis agents in the Tokachi District are currently restricted to *I. persulcatus*. It would be informative for farmworkers, hunters, local residents as well as tourists to make aware of infection risks associated with tick bites. Furthermore, the sampling design (sampling season and frequency) as well as tick species life-cycle patterns might explain the differences in tick species and tick life stage between sampling sites. In the future, annual or yearly surveys of ticks and their carrying microorganisms will be needed to reveal their seasonal occurrence in the region to constantly avoid the risk for tick-borne diseases.

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**Ethical approval**

Not applicable.

**CRediT author statement**

Kiyoshi Okado: methodology, validation, formal analysis, investigation, writing - original draft, visualization. Paul Franck Adjou Moumouni: methodology, validation, formal analysis, investigation, resources, writing - review and editing. Seung-Hun Lee: methodology, validation, formal analysis, investigation, writing - review and editing. Chih-Liang Chang: conceptualization, investigation, writing - review and editing. Hiroshi Suzuki: conceptualization, writing - review and editing. Hiroshi Fujisaki: conceptualization, writing - review and editing. Seung-Hun Lee: conceptualization, investigation, writing - review and editing. Kozo Fujiwara: conceptualization, writing - review and editing. Thilakampalam Sivakumar: formal analysis, writing - review and editing. Naoaki Yokoyama: conceptualization, investigation, writing - review and editing. Koko Fujisaki: conceptualization, writing - review and editing. Hiroshi Suzuki: conceptualization, investigation, writing - review and editing. Xuanan Xuan: conceptualization, investigation, resources, project administration, funding acquisition. Rika Umemiya-Shirafuji: conceptualization, investigation, resources, writing - review and editing, visualization, supervision, funding acquisition. All authors read and approved the final manuscript.

**Data availability**

The newly generated DNA sequences were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under the accession number DDBJ.
Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crpvbd.2021.100059.

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