Research Square

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Surveillance of Borrelia Miyamotoi-Carrying Ticks and Genomic Analysis of Isolates in Inner Mongolia, China

H Gaowa (melody_gaowa@163.com)
Hetao College
W Wulantuya
Hetao College
Kozue Sato
National Institute of Infectious Diseases
Dan Liu
Hetao College
Yunhong Cui
Hetao College
Xuhong Yin
Hetao College
Lihua Zhang
Hetao College
Hong Li
Hetao College
Tingfu Wang
Hetao College
Rongxin Liu
Hetao College
Lijing Wu
Hetao College
Saixia Lu
Hetao College
Ting Gao
Hetao College
Zitong Zhang
Hetao College
Minzhi Cao
Bayan Nur Centers for Disease and Prevention
Guodong Wang
Hulunbuir Centers for Disease Control and Prevention
Chunpu Li
Hulunbuir Centers for Disease Control and Prevention
Dacheng Yan
Hulunbuir Centers for Disease Control and Prevention
Norio Ohashi
University of Shizuoka
Shuji Ando
National Institute of Infectious Diseases

Hiroki Kawabata
National Institute of Infectious Diseases: Kokuritsu Kansensho Kenkyujo

Research

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Abstract

Background: *Borrelia miyamotoi* is a newly described relapsing fever spirochete transmitted by Ixodid tick species. Little is known about the prevalence of *B. miyamotoi* infections in humans and ticks in Inner Mongolia, China. Therefore, we investigated the prevalence of *B. miyamotoi* in *Ixodes persulcatus*, and we aimed to isolate *B. miyamotoi* from *I. persulcatus* ticks from four regions of Greater Hinggan, Inner Mongolia, China.

Methods: During May to June of 2016-2019, host-seeking adult ticks of *I. persulcatus* were collected from vegetation. Genomic DNA was prepared from half of each tick body for PCR template, and the remaining half was used to cultivate *B. miyamotoi* in BSK-M medium. We employed quantitative real-time PCR (qPCR) to detect *Borrelia* DNA in the ticks and to calculate the prevalence of *B. miyamotoi* and other borreliae infections. For the characterization of isolated *B. miyamotoi*, we performed draft genome sequencing and multi-loci sequencing analysis (MLSA).

Results: A total of 2,656 *I. persulcatus* adult ticks were collected. The over-all prevalence of relapsing fever (RF) borreliae in ticks was 5.0% (134/2,656) and that of Lyme disease (LD) borreliae was 43.8% (1,164/2,656). Co-infection by RF and LD borreliae was observed in 63 ticks (2.4%). Ticks that were positive for RF borreliae on qPCR were subjected to glycerophosphodiester diester phosphodiesterase gene (*glpQ*) PCR amplification and sequencing, through which we identified the RF borreliae specimens as *B. miyamotoi*. In this study, we successfully isolated the *B. miyamotoi* strain Hetao-1 from *I. persulcatus*, and a draft genome sequence was obtained for the isolate. Genomic sequencing revealed the strain Hetao-1 genome to be approximately 906.1 kbp in length (28.9% average GC content), and MLSA analysis identified the strain as ST633, which was previously reported in Japan and Mongolia.

Conclusion: We detected *B. miyamotoi* from *I. persulcatus* ticks collected in Inner Mongolia, and successfully isolated a *B. miyamotoi* strain. To our knowledge, this is the first study to culture a *B. miyamotoi* isolate in China. The data on the prevalence of *B. miyamotoi* and other borreliae in *I. persulcatus* ticks will be fundamental for future epidemiological studies of *B. miyamotoi* disease in Inner Mongolia.

Background

Borrelia miyamotoi and the other genetically related relapsing fever (RF) borreliae are transmitted by Ixodid tick species. *Ixodes* ticks are also vectors of Lyme disease agents [1, 2]. *B. miyamotoi* was first discovered from *I. persulcatus* tick and *Apodemus argenteus* in Japan [3] and is considered an emerging pathogen of humans [4]. Recently, the spirochete *B. miyamotoi* was shown to cause infectious disease in humans, designated as *B. miyamotoi* disease (BMD), which has been described in patients in Russia, the United States, several European countries, Japan, and China [4–12]. BMD manifests as a high fever (up to 40℃), fatigue, headaches, myalgia, chills, nausea, and arthralgia, and meningoencephalitis has been reported in immunocompromised patients [8, 13]. To date, *B. miyamotoi* has been found in *I. scapularis* and *I. pacificus* ticks in North America [14, 15], *I. ricinus* in Europe [16], and *I. persulcatus, I. ovatus*, and *I. pavlovskyi* in Asia [17, 18]. *Ixodes* ticks are found across the northern hemisphere and are the most common reservoir and vector of many pathogenic agents, including viruses, bacteria, and protozoa of medical importance.

In China, cases of Lyme disease (LD) have been reported in Greater Hinggan and Lesser Hinggan in the north east, where the principal LD vector, *I. persulcatus*, is abundant [19]. *I. persulcatus* is also a very common vector of *B. miyamotoi*. Human infection with *B. miyamotoi* was also reported in northeastern China in 2018 and has been detected in *I. persulcatus* ticks [12]. However, there has been a lack of wide-area surveys of *B. miyamotoi* infection in tick populations, including *I. persulcatus* and other *Ixodes* species. Not only do the epidemiological data in China remain unclear but also the genetic characteristics of *B. miyamotoi* because of the difficulty with cultivating the bacteria. This basic information on the prevalence of *B. miyamotoi* infection in ticks and the genetic characterization of the pathogen are urgently required for risk assessments of BMD in northeastern China.

The Greater Hinggan region in northeastern China offers favorable environmental conditions for the survival and proliferation of *I. persulcatus*. In this area, tick ectoparasitic are highly prevalent on humans, and human ixodid tick-borne infections, including those caused by LD borreliae and tick-borne encephalitis virus (genus *Flavivirus*), are endemic and transmitted by the same tick species [20]. However, some febrile patients have presented with a recent tick bite that could not have been infected with the tick-borne pathogen diagnosed. In this study, large-scale surveillance for *B. miyamotoi* was conducted in Greater Hinggan to estimate the infection rate of host-seeking adult *Ixodes* ticks. The tick-derived isolates of *B. miyamotoi* discovered in this study were subjected to molecular analyzes to characterize their genetic profile. The resultant field and laboratory data will serve as a baseline for research aiming to understand the epidemiology of *B. miyamotoi* in Inner Mongolia, China.

Methods

Study area

The tick samples in this study were collected in different forested areas throughout Greater Hinggan in Hulun Buir City of Inner Mongolia, northeastern China (Fig. 1). The Greater Hinggan forest region of Inner Mongolia is in the northernmost area of the Greater Hinggan Mountains, accounting for 46% of the total area, with geographical coordinates ranging from 119°36'30" to 125°24'00" E and 47°03'40" to 53°20'00" N. The main habitat is primeval forest at an altitude of 250-1,745 meters, an average annual temperature of − 3.5℃, and an annual precipitation of 300–450 mm. In these areas, no specific permissions were required for the collection of ticks, and this study did not involve endangered or protected species.

Tick collection, DNA extraction, and borrelial cultivation

During 2016–2019, from May to June each year, host-seeking adult ticks were collected by flagging from vegetation. The collected tick samples were placed in a collection tube, which were classified and numbered according to the sampling time and place. *I. persulcatus* ticks were identified on the basis of the morphological characteristics. Ticks were washed with 0.1% sodium hypochlorite and 75% ethanol containing povidone iodine for 5 min, washed again with 3% hydrogen peroxide for 5 min, and then rinsed with sterile water. Genomic DNA PCR template was prepared from half of each tick body according to Yamazaki-Matsune et al. [21]. The remaining half was used to cultivate *B. miyamotoi* in modified Barbour-Stoenner-Kelly medium (BSK-M: using minimal essential medium alpha [Bio West, Germany] as a substitute for CMRL-1066) under microaerophilic conditions [17, 22]. The tick samples that were positive for RF borreliae and negative for LD borreliae on qPCR were cultivated at 30℃ for 4 weeks, and the growth of spirochetes was examined by dark-field microscopy every 2 weeks.

Detection of borrelial DNA from ticks

Tick lysates were subjected to qPCR assay to detect borrelial infection. The assay was designed to specifically detect RF borreliae, including *B. miyamotoi*, and LD-related spirochetes. The system was originally established by Barbour et al. [23], and the borrelial DNA in the tick lysates was detected by multiplex qPCR targeting the 16S rRNA gene (*16S rDNA*). To allow detection of most *Borrelia* spp., common primers were designed to conserved sequences, and specific DNA probes conjugated to non-fluorescent quencher (NFQ) and minor groove-binder architectural protein (MGB) were designed. The two probes were labeled with either the fluorescence reporter group FAM or VIC, and the multi-qPCR reaction system was able to simultaneously detect RF and LD borreliae. The forward and reverse primers were 5'-GCTGTAAACGATGCACACTTGGT-3' and 5'-GGCGGCACACTTAACACGTTAG-3', respectively. The corresponding dye-labeled probes, FAM-TTCGGTACTAACTTTTAGTTAA-NFQ-MGB and VIC-CGGTACTAACCTTTCGATTA-NFQ-MGB, were purchased from Applied Biosystems (Foster City, CA). The qPCR was performed using Premix Ex *Taq* (Probe qPCR, Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions and run on a Bio-Rad CFX96 system with 42 PCR cycles.

Conventional PCR and phylogeny reconstruction using glpQ sequences

To confirm the qPCR results, we performed conventional PCR on the tick-derived isolates. Ticks that were found to be RF-DNA-positive by qPCR were subjected to glycerophosphodiester diester phosphodiesterase gene (*glpQ*) analysis with PCR-based DNA sequencing [24] using primers purchased from Nanjing GenScript Biological Technology Company: forward primer (*glpQ*-

F), 5'-CATACGCTTATGCYTTRGGMGCTGA-3', and reverse primer (*glpQ*-R), 5'-GCAACCTCTGYCATACCTTCTTSTG-3'. The amplicon was approximately 610 bp in length. The reaction conditions of the first PCR were 3 min 94℃, then 30 cycles of 30 s at 94℃, 30 s annealing at 53℃, 30 s at 72℃, and finishing with 5 min at 72℃. In the second PCR, the annealing temperature was changed to 55℃. We employed the Blend Tag-Plus enzyme (TOYOBO, Osaka, Japan) in the PCR reactions, and the operation was conducted in accordance with the instructions. A negative control was used in each PCR amplification. After amplification, 5 μL of PCR product was separated on 1% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR products containing the target fragment were sent to the Nanjing GenScript Biological Technology Company for bidirectional sequencing. We conducted phylogenetic analyses based on the nucleotide sequences of *glpQ* (555 bp) using the maximum likelihood method [25] in MEGA 6.0 [26]. Homologous sequences were searched for with BLAST in NCBI and downloaded. ClustalW software was used for sequence alignment analysis, and its reliability was tested with bootstrap analysis with 1000 replicates.

De novo sequencing and multi-loci sequencing analysis based on draft genome data of cultured isolate

Genomic DNA was extracted from the *B. miyamotoi* strain Hetao-1 according to Lim et al. [27]. For genomic library construction, 1 μg of DNA was used for DNA sample preparation, and sequencing libraries were generated using the Next Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's instructions. Briefly, the DNA sample was fragmented by sonication to approximately 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. The PCR products were purified (AMPure XP system), and libraries were analyzed for size distribution on the Agilent 2100 Bioanalyzer and quantified using real-time PCR. The whole genome of *B. miyamotoi* strain Hetao-1 was sequenced using Illumina NovaSeq PE150. For genome assembly, the raw data were independently assembled using SOAP denovo v.1.0 [28], SPAdes [29], and ABySS v.2.0 [30]. The assembly results for the three software packages were integrated with CISA software [31], and the assembly result with the fewest scaffolds was selected. *De novo* sequencing and assembling were performed at the Beijing Novogene Bioinformatics Technology.

Multi-loci sequencing analysis (MLSA) was performed using the MLSA dataset proposed by Margos et al. extracted from the draft genome sequence of strain Hetao-1. The loci of eight genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) were concatenated and used in this analysis according to Margos et al. [32].

Results

Ticks infected with borreliae in Inner Mongolia

A total of 2,656 adult *I. persulcatus* ticks were collected from the Daxingan mountains in Hulun Buir City of Inner Mongolia, China (Fig. 1). All the collected ticks were screened for borreliae DNA by qPCR targeting of the 16S rDNA. As shown in Table 1, ticks harboring *B. miyamotoi* were found from four districts: Genhe, Yakeshi, Arong Banner, and Arxan. The overall prevalence of RF borreliae, including *B. miyamotoi*, in ticks of Hulun Buir was 5.0% (134/2656). In more detail, the percentages of ticks positive for RF borreliae were 8.6% in Genhe, 5.1% in Yakeshi, 2.6% in Arong Banner, and 14.0% in Arxan (Table 1). The overall prevalence of LD borreliae was 43.8% (1164/2656), and the percentages of ticks positive for LD borreliae were 59.5% in Genhe, 45.0% in Yakeshi, 31.3% in Arong Banner, and 55.8% in Arxan (Table 1). Co-infection by RF and LB borreliae was observed in 46 ticks (1.7%) in Hulun Buir.

Identification of B. miyamotoi in ticks

To identify the RF borrelia in ticks from Hulun Buir, we performed sequence analysis followed by *glpQ* qPCR of RF-borrelia-positive samples (134 samples). Of these 134 tick samples, we successfully sequenced partial *glpQ* from 105, and these sequences were 100% identical to each another and to that of the *B. miyamotoi* strain FR64b (accession number: CP004217) (Fig. 2). Nucleotide sequences of the representative *B. miyamotoi* isolate from Hulun Buir were deposited in the DDBJ/GenBank DNA database with the following accession numbers: LC570864-LC570882. In 29 of the tick specimens, weak or no amplification of *glpQ* was seen. The reason for this is unknown; however, the negative control qPCR showed no amplification.

Thus, we believe it is possible that this absence of a signal in qPCR may be due to the cross-contamination of *B. miyamotoi* in this study.

Genetic characterization of B. miyamotoi **DNA from cultured isolates and ticks using** glpQ **genes**

We successfully cultured one *B. miyamotoi* isolate from *I. persulcatus* tick using BSK-M medium. This isolate was used in the initial qPCR confirmation of the pathogen and for analyzing the *glpQ* sequences. Based on the amplified region of the *glpQ* gene, the Hetao-1 isolated in this study (accession number: LC557152) clustered together with Siberian *B. miyamotoi* strains isolated in Japan and Russia (Fig. 2).

MLSA by draft genome sequence

A draft genomic sequence of the *B. miyamotoi* isolated from ticks sampled in Inner Mongolia was obtained to characterize the Hetao-1 strain. The chromosome of the strain was estimated to be approximately 906.1 kb in length with a GC content of 28.9%. The chromosome sequence showed 46 single nucleotide polymorphisms (SNPs) without Ins/Del compared with the *B. miyamotoi* strain Izh-4 (accession number: CP024390) [33]. Using the genome assembly data, MLST was carried out using eight genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) isolated from the draft genome sequence. Analysis of the eight concatenated housekeeping gene sequences (4,776 nucleotides) identified the Chinese Hetao-1 isolate from *I. persulcatus* as ST633 and to be identical to *B. miyamotoi* Japanese isolate HT31 (Japan) and M12C4 (Mongolia) (Fig. 3).

DNA Accession number

Accession numbers obtained in this study is as follows; *B. miyamotoi* strain Hetao-1 CTP synthase gene (*pyrG*), DNA helicase gene (*recG*), ATP-dependent endopeptidase clp ATP-binding subunit gene (*clpX*), Aspartyl aminopeptidase gene (*pepX*), Excinuclease ABC subunit A gene (*uvrA*), Cysteine desulfurase gene (*nifS*), ATP-dependent Clp protease subunit A gene (*clpA*), LSU ribosomal protein L2P gene (*rplB*), *16SrRNA*, and flagellin gene (*flaB*) are LC557142, LC557143, LC557144, LC557145, LC557146, LC557147, LC557148, LC557149, LC557150, and LC557151, respectively.

Discussion

The *B. miyamotoi* bacterium is a newly described emerging pathogen. Before this study, no isolates of this bacterium from China had been cultured, and there was little information on *B. miyamotoi* infections in human and ticks [12, 34, 35]. In this study, we detected *B. miyamotoi* in *I. persulcatus* ticks and successfully isolated a *B. miyamotoi* strain from *I. persulcatus* collected in Greater Hinggan, Inner Mongolia, China. Our findings demonstrated that *B. miyamotoi* infection of *I. persulcatus* is widespread across the regions examined. Similar to in Russia and Japan, *I. persulcatus* ticks in Greater Hinggan, China, are mainly abundant in forested regions. To date, there have been no reports on the prevalence of *B. miyamotoi* infections in humans and ticks in Greater Hinggan; however, human tick bites and tick-borne LD or TBE are known to occur frequently in this region. We conducted large-scale tick surveillance for *B. miyamotoi* in Greater Hinggan, as human cases of *B. miyamotoi* infection were confirmed in Jilin and Heilongjiang, China, in 2018, and *B. miyamotoi* has been found previously in *I. persulcatus* ticks [12]. From our research, the frequency of *B. miyamotoi* infections in *I. persulcatus* was shown to be approximately 5%, which is similar to the prevalence shown by the most recent study in China (approximately 3% in *I. persulcatus*) [12, 35]. Most previous reports suggest that *Ixodes* ticks are transmission vectors of *B. miyamotoi* in North America, Europe, and other Asian countries. Our results support the hypothesis that *I. persulcatus* is an important *B. miyamotoi* transmission vector in Inner Mongolia, China.

Before this study, other tick species (*Haemaphysalis* and *Dermacentor* ticks) were found to carry *B. miyamotoi* in China [12, 34, 35]. The potential of *Haemaphysalis* and *Dermacentor* ticks to act as vectors of *B. miyamotoi* remains unclear; however, *Haemaphysalis* is suggested to be a vector of *Borrelia* species related to *B. thaileri* in Japan [36, 37]. The *Borrelia* species (i.e., *Borrelia* sp. HL) was classified as a hard-tick-borne RF borreliae; however, it is clearly distinguishable from *B. miyamotoi*. Thus, further study may be required on the vectorial capacity of these tick species to clarify the risk of BMD in China.

The data collated in this study provide information on the risk of *B. miyamotoi* infection (Table 1). Additionally, we detected LD borreliae from 43.8% of *I. persulcatus* ticks, which are thought to be the vectors of *B. miyamotoi* and LD borreliae in Inner Mongolia. Although the prevalence of *B. miyamotoi* is lower than that of LD borreliae in Greater Hinggan, *B. miyamotoi*, as a cause of fever and various other symptoms, is also a risk to public health.

Using MLSA, we revealed that *B. miyamotoi* ST633, which was previously found in Mongolia and Japan [38], is distributed in several regions of Inner Mongolia. Furthermore, the draft genome sequence revealed the Inner Mongolia isolate has only 46 chromosomal SNPs compared with the *B. miyamotoi* strain Izh-4, although no geographical relationship was observed between these strains. The reason for the similarity is unknown; however, clonal expansion of *B. miyamotoi* may have occurred with the migration of vectors/reservoirs throughout Asian countries, including Russia. To resolve this question, further epidemiological studies of *B. miyamotoi* infection are required.

Conclusion

In this study, we detected *B. miyamotoi* in *I. persulcatus* ticks from Inner Mongolia, China, and successfully isolated a strain of *B. miyamotoi*. To our knowledge, this is the first report of the isolation of *B. miyamotoi* from China. Further epidemiological studies investigating the prevalence of *B. miyamotoi* and other borreliae in *I. persulcatus* ticks will provide new insights into the epidemiological aspects of *B. miyamotoi* infection in Inner Mongolia, China.

Declarations

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and materials

The datasets used and/or analyzed in the current study are available from Gaowa on reasonable request.

- Competing interests

The authors declare that they have no competing interests.

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- Authors' contributions

Gaowa designed the study and wrote the manuscript. All authors contributed to tick collection. All PCR analyses were performed by Gaowa. K.S. and H.K. supported borrelial culture in Inner Mongolia. H.K. provided helpful advice and discussion. All the authors agreed to the final version of the manuscript.

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Tables

Table 1. Prevalence of Borreliae in Ixodes persulcatus tick												
Location in Hulun Buir	No. of ticks			RF borreliae (including B. miyamotoi) positive No. (%)			LD borreliae positive No. (%)			Co-infection No. (%)		
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
Genhe	56	60	116	6 (10.7)	4 (6.7)	10 (8.6)	36 (64.3)	33 (55.0)	69 (59.5)	3 (5.4)	0	3 (2.6)
Yakeshi	1023	1090	2113	58 (5.7)	50 (4.6)	108 (5.1)	439 (42.9)	512 (47.0)	951 (45.0)	11 (1.1)	43 (3.9)	54 (1.6)
Arong Banner	217	167	384	4 (1.8)	6 (3.6)	10 (2.6)	78 (35.9)	42 (25.1)	120 (31.3)	1 (0.5)	4 (2.4)	5 (2.1)
Arxan	22	21	43	4 (18.2)	2 (9.5)	6 (14.0)	9 (40.9)	15 (71.4)	24 (55.8)	1 (4.5)	0	1 (2.3)
Total	1315	1341	2656	72 (5.5)	62(4.6)	134 (5.0)	562 (42.6)	602 (45.0)	1164 (43.8)	16 (1.2)	47 (3.5)	63 (2.4)

Figures

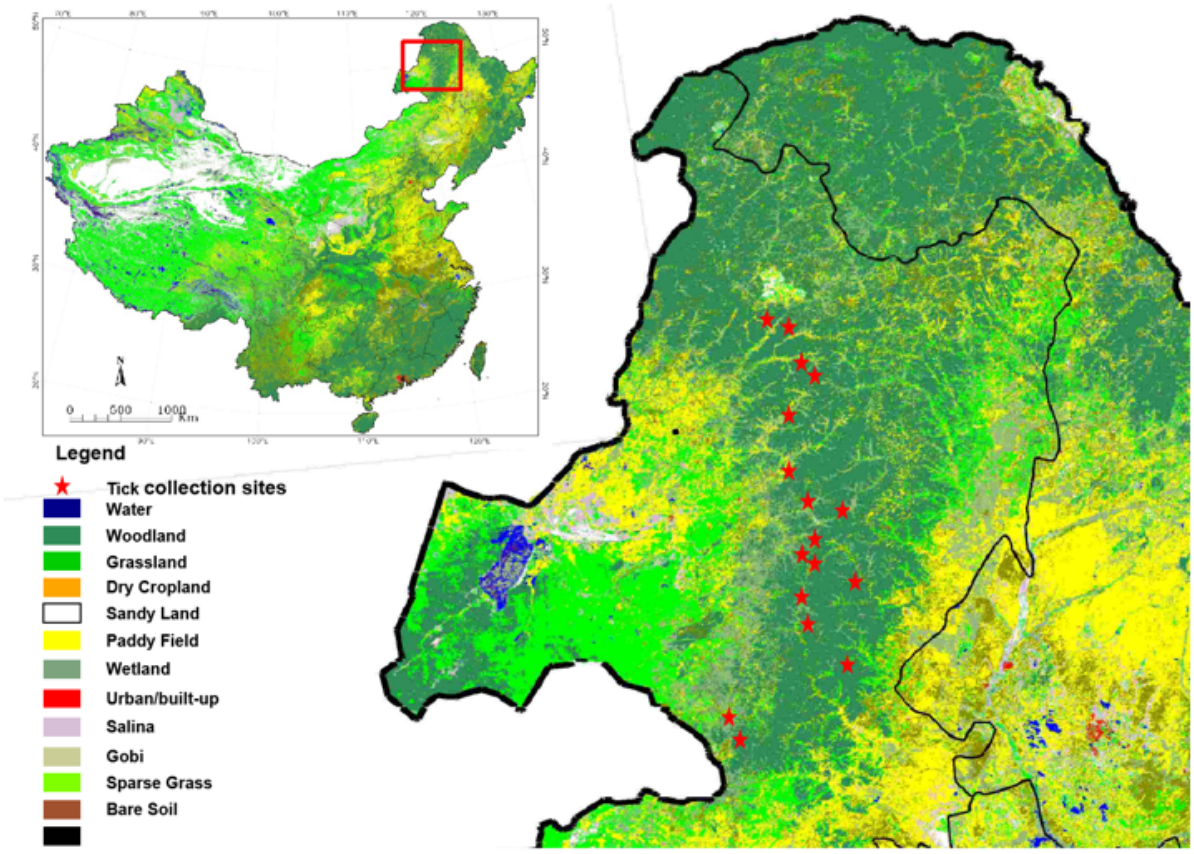

Figure 1

Tick collection areas in this study. Ticks were collected from the areas shown by red stars. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of

Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

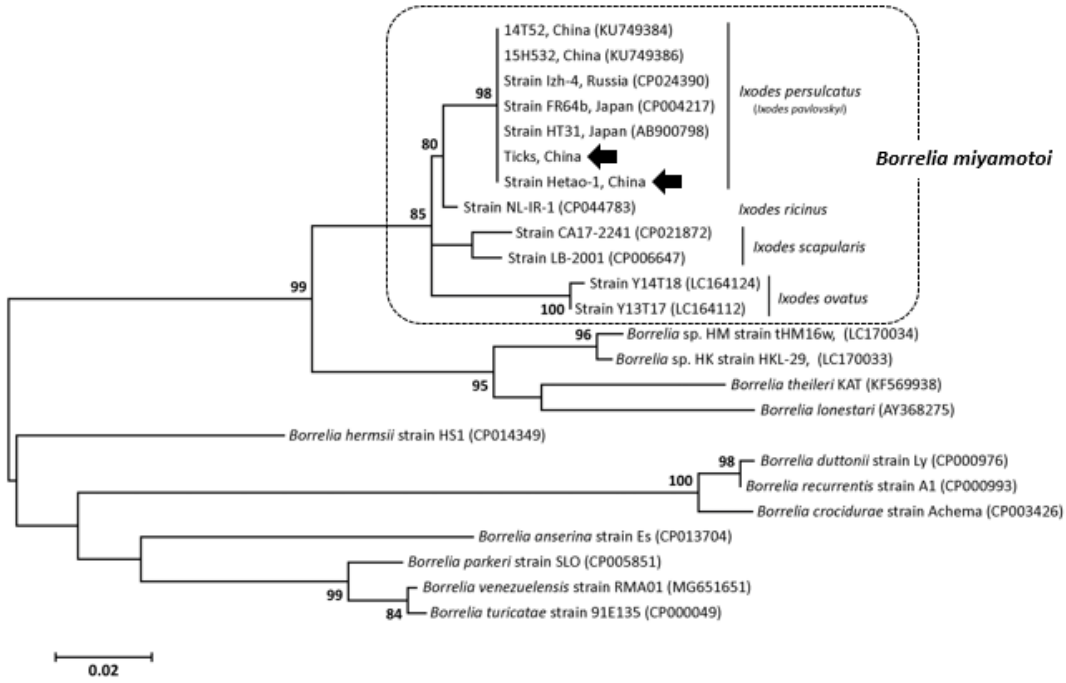

Figure 2

Phylogenetic analysis of RF borreliae based on glpQ. The tree was constructed based on glpQ sequences by the Maximun Likelihood method based on the Kimura 2-parameter model with 1,000 bootstraps resamplings. The bar indicates the percentage of sequence divergence. Borrelia miyamotoi found in this study, was pointed by black arrows. Putative tick vector of each B. miyamotoi group was listed after each lineages. The number in parentheses indicates Accession Number in GenBank. Accession number of B. miyamotoi “Ticks, China” was from LC557142 to LC557151. Accession number of glpQ gene sequence of B. miyamotoi Hetao-1 is LC557152.

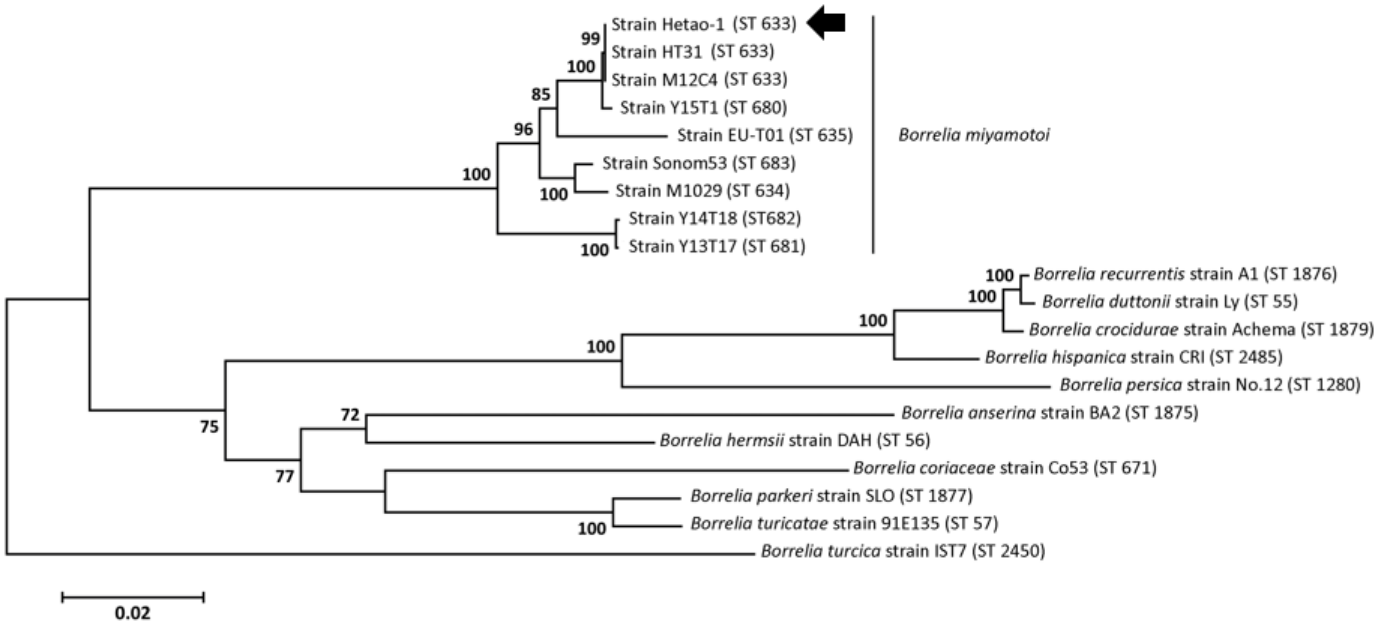

Figure 3

MLSA analysis of B. miyamotoi Hetao-1 and other Relapsing fever borreliae. A phylogenetic inference of the concatenated housekeeping gene sequences of the representative Relapsing fever borreliae is shown. The arrow indicates the B. miyamotoi strain Hetao-1 isolated in this study. Consensus sequences for the eight housekeeping genes were isolated from the draft genome sequence of B. miyamotoi strains Hetao-1, trimmed to lengths and concatenated in the order: clpA, clpX, nifS, pepX, pyrG, recG, rplB, and uvrA according to the Borrelia PubMLST database. For phylogenetic reconstruction, the Maximum likelihood model based on the Kimura 2-parameter model with MEGA 6.0 was used with 1000 bootstrap replicates. Borrelia turcica IST7 was used for outgroup. The ST number designated in each strain indicates the “sequence type” number registered in Borrelia PubMLST database.

Supplementary Files

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