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Molecular detection and phylogenetic analysis of *Borrelia miyamotoi* strains from ticks collected in the capital region of Finland

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ABSTRACT

Borrelia miyamotoi is an emerging pathogen that shares high similarity with relapsing fever *Borrelia,* but has an atypical clinical presentation. Within the framework of tick-borne disease surveillance in Finland, human serum samples suspected for tick-borne encephalitis (n=974) and questing ticks (n=739) were collected from the capital region in Finland to determine the prevalence of *B. miyamotoi.* All tested human samples were negative and 5 (0.68 %) *Ixodes ricinus* ticks were positive for *B. miyamotoi*. Partial sequencing of the flagellin (*flaB*) gene of 3 positive samples and 27 *B. miyamotoi*-positive tick samples obtained from previous studies across Finland were amplified, sequenced, and included in the phylogenetic analysis.

The phylogenetic tree revealed that most *B. miyamotoi* strains isolated from ticks in Finland share high similarity with other European strains, including strains related to human infection. Possible disease transmission may occur during exposure to tick bites. A single strain collected from an *I. persulcatus* tick in Pajujärvi grouped with an outlier of *B. miyamotoi* strains isolated from Russia and Far East Asian countries. Further studies should investigate the pathogen's role in human infection in Finland.

Another important finding is the occurrence of *I. persulcatus* ticks (8%) collected by crowdsourcing from the coastal southern part of Finland. This suggests a regular introduction and a possible wide expansion of this tick species in the country. This could be associated with transmission of new pathogens.

1. Introduction

Borrelia miyamotoi is a Gram-negative spirochete that shares a high nucleotide-level similarity with relapsing fever *Borrelia* and other *Borrelia* species such as *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis. *Borrelia miyamotoi* was first isolated in *Ixodes persulcatus* ticks from Hokkaido, Japan in 1995. Circulation of *B. miyamotoi* in *Ixodes* ticks was also confirmed in the United States, Sweden, and Russia before its role as a human pathogen was revealed (Fraenkel et al., 2002; Korotkov et al., 2008; Scoles et al., 2001).

Species of the genus *Ixodes* (hard ticks) are considered vectors of *B. miyamotoi*. The main vectors are species of the *I. ricinus* complex (*I. scapularis, I. pacificus, I. ricinus*, and *I. persulcatus*), which are common in Europe, parts of Asia, and North America. Some rodents and passerine birds may serve as animal reservoirs of *B. miyamotoi* (Franck et al., 2020; Krause et al., 2015; Salkeld et al., 2018).The medical importance of *B. miyamotoi* was not recognized until 2011, when the first human infection of *B. miyamotoi* was reported in Russia (Platonov et al., 2011). Furthermore, a retrospective study on sera collected between 1990–2010 confirmed human infection with *B. miyamotoi* in the United States (Krause et al., 2013). In 2012, the first reported meningoencephalitis case in Europe (Netherlands) drew attention to the possible dissemination of this infectious agent in other parts of the European continent (Hovius et al., 2013). The presence of the bacterium was also

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serologically confirmed in the serum of two patients from Hokkaido in Japan (Sato et al., 2014).

Several studies from different European countries confirmed the circulation of *B. miyamotoi* in hard ticks (Cutler et al., 2019). In 2018, the first reported human cases in Scandinavia were reported in Sweden in two elderly immunocompromised female patients; one of these patients was suffering from bacterial meningitis due to *B. miyamotoi* (Henningsson et al., 2019).

While *B. miyamotoi* is phylogenetically closely related to *Borrelia* species that cause relapsing fever, *B. miyamotoi* does not induce a typical relapsing fever, although the infection can persist with repeated febrile episodes (Telford et al., 2015). Clinical manifestations range from non-specific febrile flu-like symptoms to serious neurologic damage and meningoencephalitis, especially in immunocompromised patients. More recently, the presence of *B. miyamotoi* DNA was confirmed in 43 patients with persistent polymorphic syndrome after tick bites in France. The clinical symptoms included fatigue and neurocognitive manifestations and some patients showed primary and late stages of dermatological symptoms associated with Lyme borreliosis infection (Franck et al., 2020).

Borrelia miyamotoi can be cultivated in modified Kelly-Pettenkofer medium and can be identified serologically with anti-GLPQ antibodies for antibody detection in patient serum (Wagemakers et al., 2014). However, current routine diagnosis is mainly based on detection of *B. miyamotoi* nucleic acid in clinical samples by targeting specific genes. The flagellin gene (*flaB*) of *B. miyamotoi*, which encodes the 38-kDa flagellin protein, is a useful marker for phylogenic studies and has adequate diversity within closely related *Borrelia* species that enables accurate classification among *Borrelia* (Fukunaga and Koreki, 1995). The *flaB* gene has also been widely used for the detection of *B. miyamotoi* in ticks and human samples.

The presence of *B. miyamotoi* was confirmed for the first time in Finnish ticks collected by blanket dragging between 2013–2014 from different localities in the southern part of country (Sormunen et al., 2016).

Borrelia miyamotoi was also detected by qPCR in *I. ricinus* and *I. persulcatus* ticks collected by a crowdsourcing nationwide study in Finland. The prevalence of *B. miyamotoi* in these tick species was 0.2 % and 0.4 %, respectively (Laaksonen et al., 2017). No human cases due to *B. miyamotoi* have been reported in Finland. Therefore, the main aims of this study were i) to investigate the presence of *B. miyamotoi* DNA in human samples, ii) to study the prevalence of this bacterium within ticks in an urban capital region in Finland, and iii) to study the phylogeny of the Finnish strains using the *flaB* gene.

2. Materials and methods

2.1. Collection of blood samples and DNA extraction

Blood samples (n = 974) sent to Helsinki University Hospital laboratory (HUSLAB, Helsinki, Finland) were retrieved from 916 individual patients suspected to have tick-borne encephalitis (TBE) from May to November 2018. The same set of samples was used for investigations on Alongshan virus (Kuivanen et al., 2019). Most of the samples were taken between July to September 2018 (n = 595), which is the peak TBE season in Finland. The patients were living in different hospital districts throughout Finland. Most samples came from the Helsinki and Uusimaa hospital district (n = 529), Nordlab (n = 207), Northern Savo (n = 97), and Åland (n = 86), located in south, north, mid, and southwest of Finland, respectively. In addition, single or multiple samples came from Northern Karelia, Vaasa, Päijänne-Tavastia, Kymenlaakso, and Eastern and Southern Savo. Patient age ranged from 2 to 89 years and median age was 48 years. Serum samples were archived and DNA was extracted using a MagNA Pure LC instrument (Roche) and Total Nucleic Acid kit (Roche) with elution of 50 µl.

2.2. Collection of ticks and DNA extraction

Questing ticks were collected from 3 localities in the capital region including: Espoo/ Kauniainen (n = 652) and Lauttasaari (n = 87) for the purpose of screening of different pathogens. Some ticks were found by blanket flagging and dragging (n = 33) during the year 2016: thereafter the majority of samples (n = 706) were collected mainly from local residents and their family pets by the end of 2017. Local residents were instructed to bring ticks to the vaccination busses, along with an accompanying information form for each tick. The collected information included dates, areas of collection/ localities, life stage, source of collection (i.e. 1. the environment, 2. on the fur of a pet, 3. attached to a pet, 4. on the skin or clothes of a person, 5. attached to a person). The ticks collected in crowdsourcing were also used in a previous study on tick-borne encephalitis virus (TBEV) in ticks from Finland (Smura et al., 2019).

A total of 739 ticks were collected separately in Eppendorf tubes and transferred to the University of Helsinki for DNA analysis. Ticks were frozen at -80 °C and then processed for total DNA extraction. Each tick was mechanically homogenized with Tissuelyser for 30 cycles/s with 1 ml of TRIzol LS reagent and two 5 mm stainless steel beads. Total DNA was extracted and eluted to 100 µl from the homogenate according to the manufacturer's instructions. DNAs samples were preserved at −20 °C until use.

2.3. Molecular identification of ticks

Macroscopically, all the ticks belonged to *Ixodes* spp, and identification was performed by a duplex qPCR and amplification of the ITS2 gene as described by (Sormunen et al., 2016) with a slight modification. Briefly, primers and probes were used as follows: forward primer IXO-I2-F4 ITS2 TCTCGTGGCGTTGATTTGC, reverse primer: IXO-I2-R4 ITS2 CTGACGGAAGGCTACGACG and two species-specific probes: Ipe-I2-P4 probe (*I. persulcatus*) ITS2 [FAM]-TGCGTGGAAAGAAAACGAG-[BHQ1] and Iri-I2-P4 (*I. ricinus*) ITS2 [HEX]-TGCTCGAAGGAGAGAACGA-[BHQ1]. We used 2x Maxima Probe qPCR Master Mix or 5x HOT FIREPol Probe Universal qPCR Mix according to kit availability. Reactions were performed according to the manufacturer's instructions.

2.4. Mapping

A map of tick species occurrence was created with ESRI ArcGIS (version 10.3.1) by using open source GIS data (HSY, 2019; SYKE, 2018a,b) (HSY, 2019; SYKE, 2018a). The water area dataset was created from CORINE land cover 2018 (SYKE, 2018a,b) in ArcMap (SYKE, 2018b).

2.5. qPCR detection of B. miyamotoi

For the purpose of qPCR for the detection of *B. miyamotoi*, the primers Bm-fla-F:AGAAGGTGCTCAAGCAG, Bm-fla-R: TCGATCTTTGAAAGTGACATAT and the probe Bm-fla-P: [6FAM]-AGCACAACAGGAGGGAGTTCAAGC-[BHQ] were used as previously described (Laaksonen et al., 2017). A mixture of 4 µl 5x HOT FIREPol Probe Universal qPCR Mix, 600 nM of each primer and 100 nM of probe, 11.4 µl of water and 2 µl of template DNA were used. The thermal cycler was programmed as follows: a polymerase activation step at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and annealing at 60 °C for 60 s. For each reaction, positive and negative controls were used to confirm the results.

2.6. Nested PCR

A more specific nested PCR was performed for the *flaB* gene on all positive samples as previously described (Kim et al., 2019).

Furthermore, 27 positive *B. miyamotoi* samples obtained from ticks collected across Finland in a nationwide crowdsourcing study were included. Two sets of primers were utilized for the nested amplification as described by (Kim et al., 2019). Phusion Flash High-Fidelity PCR Master Mix was used and the thermal cycler was programmed as follows: 10 s at 98 °C, 30 cycles of 1 s at 98 °C, annealing at 55 °C and 56 °C for 5 s for the external and internal sets of primers, respectively, 15 s at 72 °C, and final extension of 1 min at 72 °C. The PCR products were visualized by gel electrophoresis and all positive samples proceeded to sequencing.

2.7. DNA sequencing and sequence analysis

A total of 12 µl of nested PCR product for each positive sample was used for library preparation and sequencing. The PCR products were first purified using GeneJET PCR Purification Kit (Thermo Fisher). Libraries were prepared using Nextera XT (Illumina, San Diego, USA). Library fragment sizes were measured using agarose gel and library concentrations were measured by Qubit. Sequencing was performed with MiSeq sequencer (Illumina) using MiSeq Reagent Kit V2 with 150-bp reads. The raw sequences were trimmed and low quality (quality score <30) and short (<50 nt) sequences were removed using Trimmomatic (Bolger et al., 2014) followed by assembly against the *flaB* gene of the *B. miyamotoi* reference strain LB-2001 (CP006647.2) using BWA-MEM algorithm implemented in SAMTools version 1.8 (Li et al., 2009; Li, 2013).

Strains were identified using Blastn program available at NCBI. For phylogenetic analysis, sequences of the following *B. miyamotoi* strains deposited at GenBank were used for construction of the phylogenetic tree: *B. miyamotoi* DB15F6-04, *B. miyamotoi* SW249-12, *B. miyamotoi* 6T04-2, *B. miyamotoi* CM1132-12 (Poland), *B. miyamotoi* CZ-F1E (Czechia), *B. miyamotoi* HoHe (Germany), *B. miyamotoi* Yekat-18, *B. miyamotoi* Vologda (Russia), *B. miyamotoi* Chosun T5-30 (South Korea), *B. miyamotoi* FR64b (Japan), *B. miyamotoi* 14T114 (China), *B. miyamotoi* CB1 (USA).

Sequences were aligned using the ClustalW algorithm implemented in MEGA-X program (Kumar et al., 2018) followed by manual trimming. The phylogenetic tree was constructed and visualized using Neighbor Joining method with 1000 bootstrap replicates using Tamura-Nei substitution model implemented in MEGA-X program.

3. Results

3.1. Identification of ticks

A total of 739 ticks were collected from Espoo/Kauniainen and Lauttasaari. Duplex qPCR enabled differentiation between two different *Ixodes* species. Most of collected ticks were *I. ricinus* (680, 92.0 %) and the remaining ticks were *I. persulcatus* (59, 8.0 %). Furthermore, the additional 27 ticks from other studies were identified as *I. ricinus* (26 samples) and only a single male adult tick was identified as *I. persulcatus*. Espoo, Kauniainen, and Lauttasaari were mainly dominated by *I. ricinus*.

Ixodes persulcatus was found in Espoo, primarily at the districts of the Suvisaaristo archipelago and other small areas near the coast (Fig. 1). Table S1 (supplementary file) summarizes all data related to ticks harboring *B. miyamotoi* genetic material (life stage, species, number of collected ticks, collection area and localities, and year of collection).

3.2. Real-time and nested PCRs

All tested human samples were negative for *B. miyamotoi*. Five out

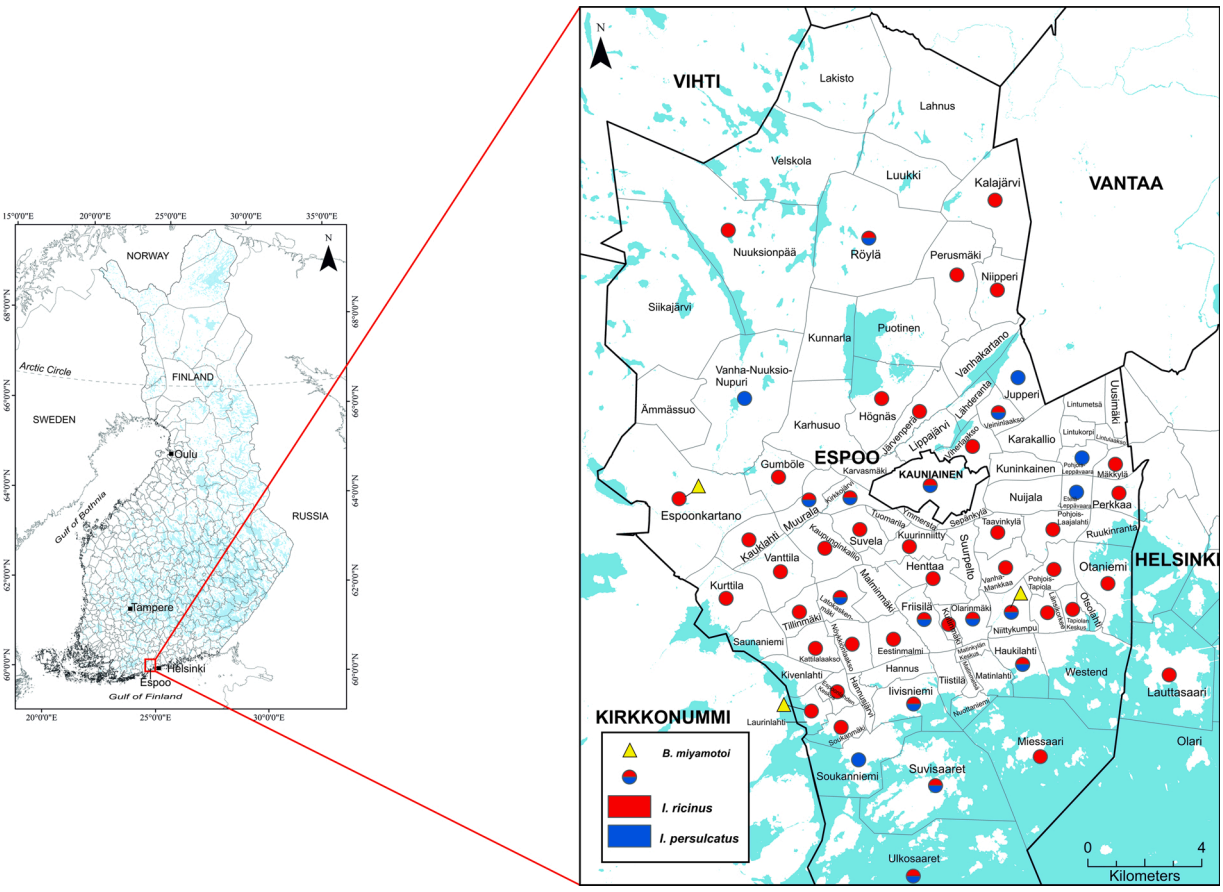

Fig. 1. Map of tick species occurrence in the capital region of Finland in small areas. Points represent the geographical mean centers of each small area. Colors (red/blue) inside the points corresponds to tick species (*I. ricinus* and *I. persulcatus* ticks). Yellow triangles indicate *B. miyamotoi* positive samples.

739 ticks (0.68 %) from the Helsinki region were qPCR positive. All *B. miyamotoi* positive tick samples in this study were detected in *I. ricinus* ticks collected from Espoo, which is an urban region. These samples and the additional 27 *B. miyamotoi* positive tick samples collected in other studies were amplified by nested PCR targeting the *flaB* gene followed by sequencing. Two samples were positive with very low DNA copy numbers in qPCR and did not show amplification in nested PCR.

3.3. NGS and phylogenetic analysis

The final purified nested PCR products were sequenced (n = 30) and compared with other available sequences from international databases. Blastn results showed that most *B. miyamotoi* strains isolated from ticks in Finland share high similarity (99–100 %) with other European strains isolated in other European countries and the United States. A phylogenetic tree was constructed and two distinct clusters were observed. Significantly, most *B. miyamotoi* strains obtained from Finland clustered with other European strains *(B. miyamotoi* DB15F6-04, *B. miyamotoi* SW249-12, *B. miyamotoi* 6T04-2, *B. miyamotoi* CM1132-12, *B. miyamotoi* CZ-F1E, *B. miyamotoi* HoHe), including both tick- and human-associated strains. Only a single strain (*B. miyamotoi*-Pajujarvi-1753) was clearly and separately grouped with an outlier composed of *B. miyamotoi* strains isolated from Russia, South Korea, Japan, and China (*B. miyamotoi* Yekat-18, *B. miyamotoi* Vologda, *B miyamotoi* Chosun T5-30, *B. miyamotoi* FR64b, *B. miyamotoi* 14T114). Notably, strain Pajujarvi-1753 was the only strain detected in *I. persulcatus* in this study. The American strain *B. miyamotoi* CB1 (USA) was closer to the European strains, including Finnish strains (Fig. 2).

4. Discussion

The most frequently encountered human *Borrelia* pathogens in Finland are *B. burgdorferi* S.l species, which cause Lyme borreliosis. The estimated seroprevalence reached 20 % in the last century as shown in a recent retrospective study performed on sera samples collected between 1960–1970. The authors attributed the current reduction of prevalence to changes in living style (a third of study subjects worked in agriculture) and improvements in health quality in recent decades in Finland (Cuellar et al., 2020). Nevertheless, reports collected from 1995 to 2014 showed an increased incidence from 7 to 31/100,000 in disseminated Lyme borreliosis (Sajanti et al., 2017).

After confirming the circulation of *B. miyamotoi* in ticks from Sweden and Estonia (Fraenkel et al., 2002; Geller et al., 2012), several molecular studies also confirmed *B. miyamotoi* in ticks from Finland without any reported cases of human infection (Laaksonen et al., 2017; Sormunen et al., 2016). The continuous circulation of *B. miyamotoi* in ticks mandates regular investigation and surveillance of this pathogen in human samples.

To investigate the presence of *B. miyamotoi* DNA in humans and ticks, we performed a retrospective study on 974 human serum samples collected from samples received for tick-borne encephalitis virus antibody determination, therefore representing patients with suspicion of tick-borne encephalitis typically with neurological/febrile manifestations (Kuivanen et al., 2019). Ticks were also collected from the most populated region in Finland (Espoo/Kauniainen and Lauttasaari, Helsinki). Espoo is the second largest city in Finland and a part of the urban capital region in Finland. With more than 270,000 inhabitants, Espoo also surrounds the small municipality of Kauniainen. Lauttasaari is an island in the southern part of Helsinki and is considered the second biggest island in population with active daily connections to the center of Helsinki.

The absence of bacterial nucleic acid does not exclude exposure to the bacterium during a certain period. In a recent study performed on samples from Alsace in France, the authors did not confirm the presence of *B. miyamotoi* DNA in patients. However, some patients showed positive serological evidence of a previous exposure to *B. miyamotoi* (Boyer et al., 2020). In addition, a recent study confirmed the presence of *B. miyamotoi* DNA in 43 patients from France (Franck et al., 2020).

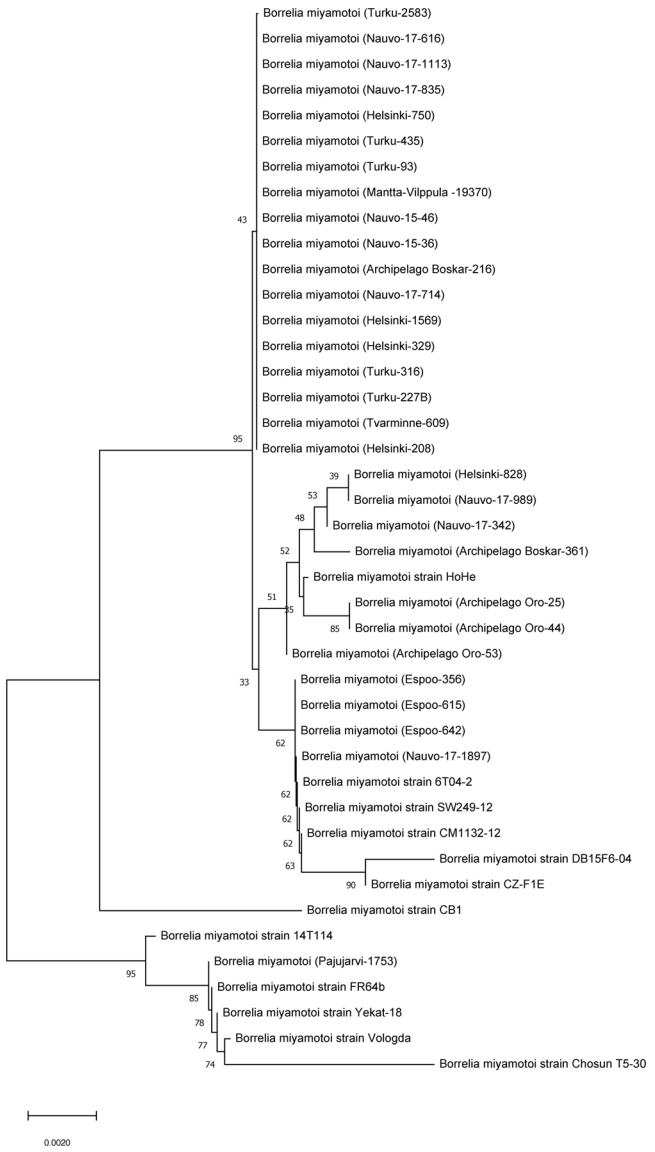

Fig. 2. Phylogenetic analysis of 30 *B. miyamotoi* strains in ticks from Finland.

Serological assays for the detection of *B. miyamotoi* antigens are more sensitive for sera collected in the convalescent phase and not in the acute phase (Harris et al., 2019). In addition, possible cross-reactivity with the C6 antigen of *B. burgdorferi* could also represent a second challenge for appropriate diagnosis of relapsing fever borreliae (Molloy et al., 2018). However, a recent immune-proteomic and a comprehensive antigen profiling study revealed promising data on the use of recombinant putative lipoprotein for the serological detection of *B. miyamotoi* in sera from infected patients without cross-reactivity to Lyme borreliosis antigens and could be used in future studies (Harris et al., 2019).

Phylogenetic analysis revealed that most of our isolates are closely related to other European strains and were closer to an American strain, showing a possible common ancestor. Additionally, one strain from Pajujärvi (located in eastern Finland) was detected in an *I. persulcatus* male adult tick and clustered with strains from Russia and Far East Asian countries (South Korea, Japan, and China).

At the local level, we report for the first time the occurrence of *I. persulcatus* ticks in the capital region (coastal southern part of Finland), mainly in the districts of the Suvisaaristo archipelago and other closed areas along the coast, showing a regular introduction of

I. persulcatus in other parts of Finland. Most ticks were collected by crowdsourcing and the citizens provided data regarding the tick's source. Most ticks were found on the fur of animals, were attached to animals or were found on humans or their clothing.

Ixodes persulcatus ticks were found in Finland at the Western coast in 2002 (Jääskeläinen et al., 2006). Recent studies revealed that while *I. persulcatus* ticks are distributed in the northern, central, western, and eastern parts of Finland, the southern-western part is dominated by *I. ricinus* ticks (Laaksonen et al., 2018; Sormunen et al., 2020). The expansion of *I. persulcatus* distribution could lead to the transmission of novel pathogens to new areas. For instance, (Laaksonen et al., 2018) observed that the prevalence of new pathogens such as *Candidatus* Rickettsia tarasevichiae and *Rickettsia monacensis* is higher in *I. persulcatus* than *I. ricinus* (35.8 % and 3.8 % versus 0.5 % and 0.5 %, respectively) and most pathogens were found in adult ticks collected by crowdsourcing. The introduction of new strains of pathogens could be associated with *I. persulcatus*, as the Pajujärvi/1753 strain that was observed to cluster with other Asian and Russian strains as similarly demonstrated for *I. persulcatus* versus. *I. ricinus* for the detected *B. miyamotoi* strains in Estonia (Geller et al., 2012).

5. Conclusion

To the best of our knowledge, this is the first phylogenetic study of *B. miyamotoi* strains in Finland, which may be indicative of future and possible transmission to humans. Therefore, we recommend a further seroprevalence study for investigation about relapsing fever *Borrelia* in Finland, considering all possible vectors and related *Borrelia* species.

Author contribution

FZ performed lab experiments, analyzed the results, and wrote the first draft of the manuscript. AJ collected human samples and provided data related to patients. GVT and JC collected ticks from Espoo and Lauttasaari. RU designed the map and analyzed geographical data. TS performed NGS and participated in analysis of results. TS and OV conceived, re-drafted, and revised the manuscript. All authors reviewed drafts and approved the final draft.

Data availability

The sequences of *B. miyamotoi* are deposited in GenBank under accession numbers MT991107-MT991136.

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CRediT authorship contribution statement

Fathiah Zakham: Methodology, Data curation, Validation, Writing - original draft, Writing - review & editing. **Anne J. Jääskeläinen:** Methodology, Writing - review & editing. **Janne Castrén:** Methodology, Writing - review & editing. **Jani J. Sormunen:** Methodology, Writing - review & editing. **Ruut Uusitalo:** Methodology, Data curation, Validation, Writing - review & editing. **Teemu Smura:** Methodology, Data curation, Validation, Writing - review & editing. **Gabriel Von Troil:** Methodology, Writing - review & editing. **Suvi Kuivanen:** . **Tarja Sironen:** Conceptualization, Writing - review & editing, Supervision, Project administration. **Olli Vapalahti:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101608.

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