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Determination of Novel *Borrelia* Genospecies in Swedish *Ixodes ricinus* Ticks

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A total of 301 adult questing *Ixodes ricinus* ticks were collected at 15 different locations along the south and east coasts of Sweden to determine the *Borrelia* genospecies diversity. Thirty-two ticks (11%) were found to be positive by nested PCR with *Borrelia burgdorferi* sensu lato-specific primers. Species determination was based on partial sequencing of the 16S rRNA gene and the flagellin gene. Five different *Borrelia* species were found. The nucleotide sequence of the *Borrelia* DNA found in two ticks differed extensively from the nucleotide sequences of the *Borrelia* DNA found in the other ticks, and analysis revealed that they were closely related to the relapsing fever borrelia species *Borrelia miyamotoi*. This is the first report of a *B. miyamotoi*-like borrelia in *I. ricinus* and in Europe. Moreover, the *Borrelia* DNA of two ticks (6%) clustered within the *B. valaisiana* complex. *B. valaisiana* has not previously been reported in Sweden. *B. afzelii* DNA was found in 14 ticks (44%), and *B. garinii* DNA was found in 10 ticks (31%). *B. burgdorferi* sensu stricto DNA was found in four ticks (13%). We conclude that all of the known human-pathogenic species (*B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto) and *B. valaisiana* found elsewhere in Europe are also present in the Swedish host-seeking tick population and that a *B. miyamotoi*-like *Borrelia* species seems to be present in *I. ricinus* ticks in Europe.

Ticks transmit many clinically important pathogens of the genus *Borrelia*. These spirochetes are responsible for two groups of human disease: Lyme borreliosis (LB) and relapsing fever (RF). LB is the most prevalent tick-borne zoonosis in Europe and North America and affects the skin, joints, heart, and nervous system (31). *Borreliae* that cause LB are transmitted by hard ticks of the genus *Ixodes*. In Europe the principal vector is *Ixodes ricinus*. The disease is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, which consists of 10 different named genospecies. Three species, all found in Europe, are known to be pathogenic for humans: *B. burgdorferi* sensu strico, *B. garinii* (4), and *B. afzelii* (4, 9). Another two species, *B. valaisiana* (34) and *B. lusitaniae* (17), have been isolated from European ticks. The pathogenic capabilities of the last two species are still uncertain, although *B. valaisiana* DNA has been amplified by PCR from patients with LB (29). Two additional *Borrelia* species have been found in European patients with LB; *B. bissettii* sp. nov. (26) has been isolated from patients in Slovakia (25), and a novel *Borrelia* species has been isolated from a patient in The Netherlands (35). There has been an increasing interest in the clinical and diagnostic implications of the different *Borrelia* species, since an association between the clinical manifestations of LB and the infective species has been suggested (3, 9, 22, 33). The infective *Borrelia* species also influences the immune response (8, 30).

Tick-borne RF, with periodic febrile episodes as the main symptom, is caused by a genetically and ecologically different group of *Borrelia* species. RF is rarely seen in Europe but is reported in the most southern parts of Europe (1). *Borrelia* species that cause tick-borne RF are usually considered vector species specific and are mostly transmitted by soft ticks (family Argasidae) of the genus *Ornithodoros* (24). Two RF-associated *Borrelia* species are exceptions and are found in hard ticks: *B. lonestari*, which is transmitted by *Amblyomma americanum* in North America (5), and *B. miyamotoi*, which is isolated from *Ixodes persulcatus* in Japan (10).

Different methods are used for *Borrelia* species determination. PCR detection and subsequent sequencing of the 16S rRNA gene is considered a sensitive and reliable method (36). Sequencing of the flagellin gene gives additional taxonomic data (11).

Since information on the *Borrelia* genospecies present in the tick population is essential to our understanding of the epidemiology, clinical spectrum, diagnosis, and prevention of LB, we conducted this study to determine the diversity of *B. burgdorferi* sensu lato among ground host-seeking ticks in Sweden.

MATERIALS AND METHODS

Study area and tick collection. During the summer of 1999 questing adult ticks were collected by flagging at 15 different locations with mixed vegetation along the south and east coasts of Sweden. A total of 301 adult unfed *I. ricinus* ticks were collected. Twenty-one ticks were collected at two locations in the province of Skåne, 233 ticks were collected at nine different locations in the province of Blekinge (108 ticks were collected from a single location), 24 ticks were collected at two locations in the province of Kalmar, 16 ticks were collected in the proximity of Stockholm, and 7 ticks were collected farther north at a location in the proximity of Gävle (Fig. 1). Of the ticks collected, 165 (55%) were male and 136 (45%) were female. The ticks were placed into coded tubes and stored at −20°C until September 2000.

DNA extraction. The ticks were processed individually. Each tick was washed in 70% ethanol and cut in half sagittally on a glass slide with a drop of phosphate-buffered saline. One half was saved for future use, and the other half was crushed and transferred to a test tube (Eppendorf; 1.5 ml) for DNA extraction. A QIAamp tissue kit (Qiagen) was used for DNA extraction according to the protocol of the manufacturer, with a few modifications. Samples were incubated

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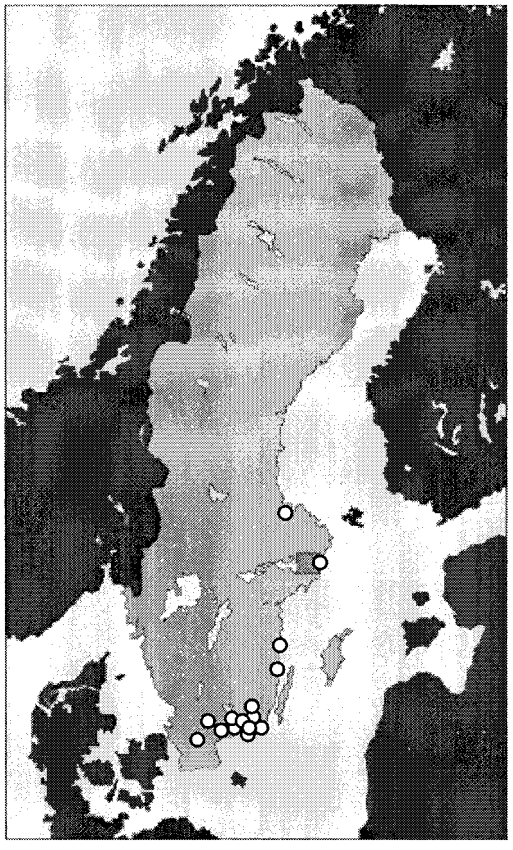

FIG. 1. Map of Sweden showing the locations of tick collection.

overnight with proteinase K solution and eluted twice with 100 μl of AE buffer in order to increase the DNA yield. Purified DNA was stored at −20°C.

PCR amplification. For detection of *Borrelia*-infected ticks, the 16S rRNA sequence was amplified by a nested PCR. Primers16S-F and 16S-R were used in the first amplification (19). The master mixture contained 0.5 μM each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 0.2 μM each deoxynucleoside triphosphate, and 1.25 U of *Taq* DNA polymerase. The reaction volume was set to 50 μl containing 5 μl of sample, and the amplification was conducted by using a program of 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 55°C for 60 s, 72°C for 90 s and finally 72°C for 7 min in a Perkin-Elmer 9600 thermocycler. Primers LD-1 and LD-2 (18) were used in the second reaction. The master mixture described above was used, except that 0.2 μM each primer was added and 2 μl of the PCR product from the first PCR was used as the template in the reaction. The volume was set to 50 μl, and amplification was conducted by using a program of 94°C for 2 min, followed by 40 cycles of 94°C for 45 s, 56°C for 60 s, 72°C for 60 s and finally 72°C for 7 min. The products were visualized by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. A negative control and a positive control were included in all PCR runs. Contamination was minimized by performing the different steps in separate rooms. Gloves and filter tips were always used.

Nucleotide sequence analysis. Partial sequencing of the 16S rRNA gene was performed for all positive samples. The products were extracted with a QIAquick gel extraction kit (Qiagen) according to the protocol of the manufacturer and sequenced by the BIG DYE method (Applied Biosystems) by direct sequencing by PCR. Each strand was analyzed with an ABI 3100 instrument (Applied Biosystems) by the Biomolecular Resource Facility at Lund University. Primers 16S-F and 569r (5′-TACGCCCAATAATCCCGAACAAC-3′) were used. The flagellin gene in four of the samples was partially sequenced by use of primer flaC (5′-ATTGAAAT AGAGCAACTTACAGA-3′) and primer flaL4 (5′-TTATCT AAGCAATGACAAAACATAT-3′). The DNA sequences were compared with the *Borrelia* gene sequences registered in the GenBank database. BioEdit software (T. A. Hall, Nucleic Acids. Symp. Ser. **41:**95-98, 1999) was used for analysis of the results.

Nucleotide sequence accession numbers. The 16S rRNA sequences of the borreliae from the following *Borrelia* strains determined in this study have been deposited in GenBank and given the indicated accession numbers: Ri11, AY083470; St1, AY083471; To60, AY083472; To72, AY083473; Ku10, AY083474; To76, AY083475; Al10, AY083476; As15, AY083477; Ga2, AY083478; Os8, AY083479; Ri13, AY083480; St12, AY083481; St13, AY083482; St8, AY083483; Tr2, AY083484; Ri9, AY083485; Al12, AY083486; Al16, AY083487; As18, AY083488; As7, AY083489; Ha3, AY083490; Ha5, AY083491; Os10, AY083492; Os2, AY083493; Osk2, AY083494; Osk3, AY083495; Sa5, AY083496; To106, AY083497; To16, AY083498; To89, AY083499; Na34, AY083500; and St4, AY083501. The flagellin gene sequences of the borreliae from the following *Borrelia* strains determined in this study have been deposited in GenBank and given the indicated accession numbers: St4, AY083502; Na34, AY083503; To76, AY083504; and Ku10, AY083505.

RESULTS

***Borrelia* PCR.** Of the 301 ticks, 32 (11%) were positive by use of the *B. burgdorferi* sensu lato-specific primers in the nested PCR. The PCR products of two of the tick samples yielded atypical bands by gel electrophoresis, but the two samples were

TABLE 1. Analysis of signature nucleotide positions of the partial 16S rRNA in the *Borrelia* strains studied

Borrelia DNA source(s)[a]	Nucleotide at the following 16S rRNA position[b]:								
	77	126	170	175	178	181	252	273	471
B. burgdorferi sensu strico B31 (U03396), Ri11, St1, To60, To72	C	T	G	G	A	T	A	A	T
B. afzelii DK1 (X85190), As18, Osk2, Osk3, To16, To89, To106, As7, Ha3, Os2, Al12, Al16, Sa5	C	T	G	A	A	T	G	G	C
B. garinii DK27 (X85202), Os8, St8, St12, St13, Ga2, As15, Ri13, Tr2, Al10	C	C	A	A	A	T	A	G	T
Ri9	C	T	A	A	A	T	A	G	T
B. valaisiana VS116 (X98232), To76, Ku10	T	T	G	A	A	T	G	G	T
B. miyamotoi HT31 (D45192)	T	C	A	A	A	A	G	T	C
Na34, St4	C	C	G	A	G	G	G	T	T
B. parkeri (AF307100)	C	T	G	A	G	A	A	T	T
B. recurrentis (AF107367)	C	T	G	G	G	A	A	T	T
B. turicatae (U42299)	C	T	G	A	G	A	A	T	T
B. lonestari (U23211)	T	C	A	A	G	T	A	T	T
B. hermsii (U42292)	C	T	G	A	G	A	A	T	T

[a] GenBank accession numbers are shown in parentheses. The nucleotides of *B. burgdorferi* sensu stricto B31, *B. afzelii* DK1, *B. garinii* DK27, *B. valaisiana* VS116, *B. miyamotoi* HT31, *B. parkeri, B. recurrentis, B. turicatae, B. lonestari*, and *B. hermsii* and their GenBank accession numbers are included for comparison purposes.
[b] *B. burgdorferi* B31 numbering; insertion of a C residue in position 213 has been ignored.

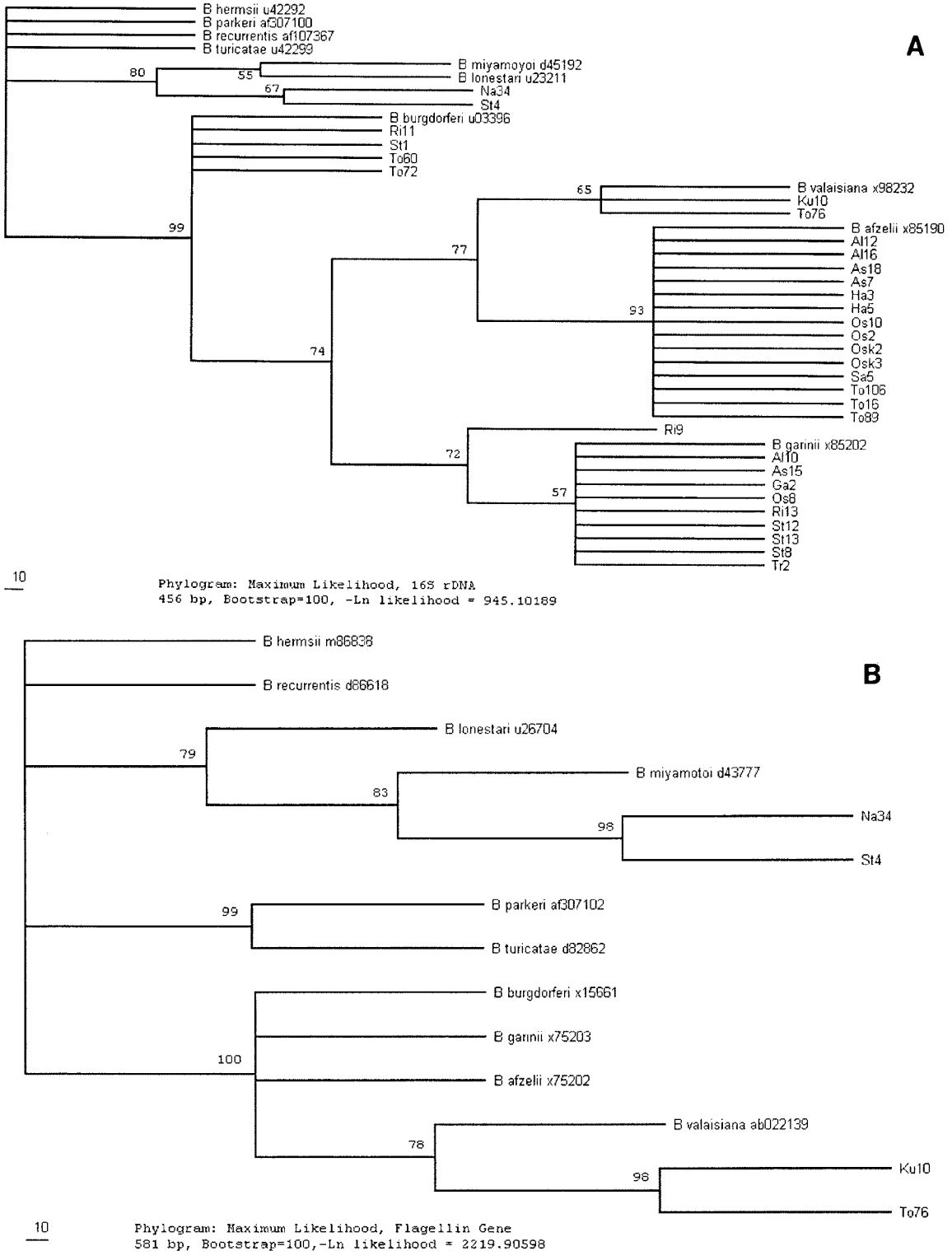

FIG. 2. (A) Phylogenetic tree, based on a comparison of the 16S rRNA sequences (456 bp) of *Borrelia* species, obtained by the maximum-likelihood method. *B. hermsii*, *B. parkeri*, *B. recurrentis*, *B. turicatiae*, *B. miyamotoi*, *B. lonestari*, *B. burgdorferi*, *B. valaisiana*, *B. afzelii*, and *B. garinii* and their GenBank accession numbers are included for comparison purposes. (B) Phylogenetic tree, based on a comparison of the flagellin gene sequences (581 bp) of *Borrelia* species, obtained by the maximum-likelihood method.

still considered positive. There were no differences in the prevalences of *Borrelia* spp. between male and female ticks.

***Borrelia* nucleotide sequence analysis.** Species determination was made by DNA nucleotide sequence analysis, and the sequences were compared with those for previously reported strains available in the GenBank database. Partial sequencing of the 16S rRNA gene, with a minimum of 496 bp analyzed, was performed for all 32 *Borrelia* 16S rRNA gene-positive ticks, and the results are summarized in Table 1. The flagellin gene was partially sequenced (a minimum of 608 bp was analyzed) in order to confirm and specify the findings for four of the *Borrelia*-positive ticks (Na34, St4, To76, and Ku10). Five different *Borrelia* species were identified in the ticks examined. DNA from two of the ticks (Na34 and St4, collected in Blekinge and Stockholm, respectively) yielded atypical PCR bands by gel electrophoresis (the strongest band was about 1,500 bp, and no LD primer-specific band was seen). The sequences of the 16S rRNA and flagellin genes obtained from the two ticks were identical but differed extensively from the sequences of the genes from the other ticks. Analysis indicated that they are closely related, but not identical, to the previously reported sequences of the genes from *B. miyamotoi* strains (Table 1). *B. afzelii* DNA was found in 14 ticks (44%), *B. garinii* DNA was found in 10 ticks (31%), and both species were widely distributed geographically. *B. burgdorferi* sensu stricto DNA was found in four ticks (13%) collected in Blekinge and Stockholm. The DNA of the 16S rRNA and flagellin gene sequenced in two ticks (6%), collected in Blekinge (from two different locations), belonged to the *B. valaisiana* genomic group. A phylogenetic tree was constructed from the DNA sequence data obtained (Fig. 2A and B).

DISCUSSION

The genetic diversity of *Borrelia* species in the tick population has not previously been studied in Sweden. In this study five different *Borrelia* species were identified. We obtained *Borrelia* DNA from two *I. ricinus* ticks that were not closely related to any of the *B. burgdorferi* sensu lato genospecies. Phylogenetic analysis of the flagellin and 16S rRNA sequences indicated that they were closely related to the *B. miyamotoi* genospecies, previously isolated only in Japan from *I. persulcatus* ticks (10). *B. miyamotoi* has not yet been shown to cause disease in humans and is phylogenetically most closely related to *B. lonestari* among the relapsing fever borreliae (11, 27). *B. lonestari* is transmitted by the hard *A. americanum* tick (classified within the Metastriata subfamily) (5) and can cause an erythema migrans-like rash (15). *B. lonestari* is also closely related to the agent of bovine borreliosis, *Borrelia theileri*, which is transmitted by hard ticks from the Metastriata subfamily (28). Phylogenetic analysis distinguishes two separate clusters within the group of RF borreliae that are transmitted by ixodid ticks: the Metastriata tick-transmitted species *B. lonestari* and *B. theileri* cluster together, and the *B. miyamotoi*-like borreliae transmitted by *Ixodes* spp. form a separate cluster. The present study is the first report of *B. miyamotoi*-like *Borrelia* species in Europe and in *I. ricinus* ticks. We found *B. miyamotoi*-like DNA in two ticks collected 400 km apart, which indicates that the findings were not isolated but, rather, that the species is geographically widespread. The monophyletic cluster of *B. miyamotoi*-like borreliae thus seems to be distributed on two continents and transmitted by two subspecies of *Ixodes* ticks. Although genetically classified within the RF borreliae, *B. miyamotoi* seems to have ecological characteristics of a borrelia of the *B. burgdorferi* sensu lato complex. Further studies are needed to characterize the biological properties of this *Borrelia* species. Isolation and more records are needed to confirm its presence in the European tick population.

The primers used in this study were not designed to find any borreliae other than those from the *B. burgdorferi* sensu lato complex. The tick templates in which the *B. miyamotoi*-like DNA was found reacted with the primers, but the PCR products were of a different size. The published data do not enable analysis of how the 16S rRNA-specific primers correspond to the 16S rRNA sequence of *B. miyamotoi*. The 3′ ends of the LD-1 and LD-2 primers differ at 3 and 2 bp, respectively, from the sequence of the *B. miyamotoi* type strain (strain HT31).

Of the *B. burgdorferi* sensu lato species found, *B. afzelii* and *B. garinii* dominated, with 44 and 31% of the *Borrelia* isolates being of these two species, respectively, which is consistent with clinical findings in Sweden (22) and with reports from Europe (13). *B. burgdorferi* sensu stricto has just recently been found in Swedish LB patients by PCR (23) and has previously been reported from *Ixodes* ticks collected from migrating birds arriving in Sweden (21). *B. valaisiana* has been reported from a number of European countries (13), but it has never before been reported in Sweden.

We found a *Borrelia* prevalence of 11% in the Swedish host-seeking tick population by use of the PCR technique. Previous studies have used immunofluorescence analysis (6, 12), phase-contrast microscopy (7, 14, 20, 32), or dark-field microscopy (2); and prevalences ranging between 7 and 29% have been recorded in adult ticks from locations in southern and central Sweden. Significant spatial and seasonal variations in the prevalence of *Borrelia*-infected ticks have been observed previously (16, 20); and the year, the location of sampling, and the method used for analysis may account for the differences between the reported prevalences.

We conclude that all of the known pathogenic species (*B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu strico) and *B. valaisiana* found elsewhere in Europe are also present in the Swedish host-seeking tick population. Of particular interest is our finding of *B. miyamotoi*-like *Borrelia* in Europe.

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