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Genetic and Phenotypic Analysis of *Borrelia miyamotoi* sp. nov., Isolated from the Ixodid Tick *Ixodes persulcatus*, the Vector for Lyme Disease in Japan

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The ixodid tick *Ixodes persulcatus* is the most important vector of Lyme disease in Japan. Most spirochete isolates obtained from *I. persulcatus* ticks have been classified as *Borrelia burgdorferi* sensu lato because of their genetic, biological, and immunological characteristics. However, we found that a small number of isolates obtained from *I. persulcatus* contained a smaller 38-kDa endoflagellar protein and single 23S-5S rRNA gene unit. Representative isolate HT31^T (T = type strain) had the same 23S rRNA gene physical map as *Borrelia turicatae*. The DNA base composition of strain HT31^T was 28.6 mol% G+C. DNA-DNA hybridization experiments revealed that strain HT31^T exhibited moderate levels of DNA relatedness (24 to 51%) with *Borrelia hermsii*, *B. turicatae*, *Borrelia parkeri*, and *Borrelia coriaceae*. However, the levels of DNA reassociation with the previously described Lyme disease borreliae (*B. burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii*) were only 8 to 13%. None of the previously described species examined exhibited a high level of DNA relatedness with strain HT31^T. In addition, the 16S rRNA gene sequence (length, 1,368 nucleotides) of strain HT31^T was determined and aligned with the 16S rRNA sequences of other *Borrelia* species. Distance matrix analyses were performed, and a phylogenetic tree was constructed. The results showed that isolate HT31^T is only distantly related to both previously described Lyme disease borreliae and relapsing fever borreliae. Thus, the spirochetes isolated from *I. persulcatus* and closely related isolates should be classified as members of a new *Borrelia* species. We propose the name *Borrelia miyamotoi* sp. nov. for this spirochete; strain HT31 is the type strain.

We previously demonstrated the usefulness of a restriction fragment length polymorphism (RFLP) ribotyping system based on the 23S-5S rRNA gene repetition in *Borrelia burgdorferi* sensu lato associated with Lyme disease (14, 32). Many spirochete isolates were examined with our RFLP ribotyping system by using rRNA gene probes. The strains isolated in the United States and Europe were placed into three distinct RFLP groups. The North American isolates clustered in ribotype group I (*B. burgdorferi* sensu stricto), and the European isolates were placed in ribotype groups I and II (*Borrelia garinii*) and ribotype group III (*Borrelia afzelii*). These groups are completely consistent with the three previously described Lyme disease agent species (2, 7, 18). Our findings also showed that there are no *B. burgdorferi* sensu stricto representative strains in Japan and that some Japanese isolates belong to ribotype groups II and III. In addition, most of the Japanese isolates produced RFLP patterns that were quite distinct from those of the North American and European isolates and were tentatively classified as ribotype group IV strains (14). *Borrelia japonica* is carried by *Ixodes ovatus* ticks, and it is thought that this microorganism is restricted to Japan. Moreover, some atypical spirochetes have been isolated in United States and Europe (38), and these spirochetes produced unique RFLP ribotype patterns.

Two medically important diseases, relapsing fever and Lyme borreliosis, are caused by *Borrelia* species (4). Both of these diseases are transmitted by hematophagous arthropods, and each pathogen is associated with particular vectors. Soft-bodied argasid ticks and the human body louse are the vectors of relapsing fever borreliae, while the vectors of Lyme disease borreliae are hard-bodied ixodid ticks, especially members of the *Ixodes ricinus* species complex (6, 17). The *Borrelia* pathogens differ in their clinical spectra. Periodic fever is the main symptom caused by relapsing fever borreliae (4); these organisms multiply in the bloodstream, and they have a high mutation rate in the genes which encode the outer membrane proteins and thus escape the host immune response. In contrast, Lyme disease borreliae cause various inflammatory reactions involving the skin, joints, heart, and central nervous system (22, 43); emergence of these borreliae into the bloodstream is rare, suggesting that they have a unique mechanism to escape the host immune response. Thus, the two related pathogens are quite different from one another in their adaptations to their arthropod and vertebrate hosts.

In our experimental survey, we found that a small number of the spirochetes examined contained a 38-kDa flagellin protein similar to that found in relapsing fever borreliae; in contrast, a 41-kDa flagellin protein is found in previously described Lyme disease borreliae. In this paper we describe the genetic and phenotypic characteristics of spirochetes isolated from *Ixodes persulcatus* ticks and a rodent. We also describe the results of a comparative 16S rRNA gene sequence analysis and a DNA-DNA relatedness analysis in which we identified strain HT31^T (T = type strain) and related isolates as members of a new *Borrelia* species. Our results indicated that these spirochete isolates obtained from *I. persulcatus* actually belong to a new *Borrelia* species. We propose the name *Borrelia miyamotoi* for this new species.

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TABLE 1. Organisms used in this study

Strain or species	Source	
	Location	Organism
Japanese isolates		
HT24	Hokkaido, Japan	*Ixodes persulcatus*
HT31^T	Hokkaido, Japan	*Ixodes persulcatus*
Hk004	Hokkaido, Japan	*Ixodes persulcatus*
NB103/1	Hokkaido, Japan	*Ixodes persulcatus*
FR64b	Hokkaido, Japan	*Apodemus argenteus*
B. japonica strains		
Am105	Aomori, Japan	*Ixodes ovatus*
HO14^T (= JCM 8951^T)	Hokkaido, Japan	*Ixodes ovatus*
IKA2	Shizuoka, Japan	*Ixodes ovatus*
B. burgdorferi sensu stricto strain B31^T (= ATCC 35210^T)	United States	*Ixodes scapularis*
B. garinii 20047^T (= CIP 103362^T)	France	*Ixodes ricinus*
B. afzelii VS461^T (= CIP 103469^T)	Switzerland	*Ixodes ricinus*
Unnamed genomospecies strain DN127	United States	*Ixodes pacificus*
B. hermsii	United States	*Ornithodoros hermsi*
B. turicatae	United States	*Ornithodoros turicata*
B. parkeri	United States	*Ornithodoros parkeri*
B. coriaceae Co53	United States	*Ornithodoros coriaceus*
B. anserina	Unknown	*Argas persicus*

MATERIALS AND METHODS

Bacterial strains and culture conditions. Spirochete isolates were obtained by culturing *I. persulcatus* midgut tissues or rodent blood in BSKII medium at 31°C. *B. japonica* Am105 isolated from *I. ovatus* midgut tissue (45), *B. japonica* IKA2 and HO14^T (25), and unnamed genomospecies DN127 (38) were also used in this study. All of the other *Borrelia* strains used were provided by R. C. Johnson (University of Minnesota) and G. Baranton (Institut Pasteur, Paris, France). All of the strains used in this study are shown in Table 1.

SDS-PAGE and Western blotting. Whole-cell lysates of spirochetes were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting) as described previously (13, 33, 34). Monoclonal antibody H9724 was used as the probe in the Western blot analysis. Monoclonal antibody H9724 reacts with a protein in borrelial periplasmic flagella (5).

DNA purification. Cells were cultivated in 100 ml of BSKII medium for 7 to 14 days at 31°C and harvested by centrifugation (13). Total DNA was extracted as described previously (11). Deproteinized DNA was dissolved in 200 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and mixed with 5 μl of RNase (1 mg/ml). After incubation for 30 min at 37°C, the DNA solution was mixed with 120 μl of a polyethylene glycol solution (20% polyethylene glycol [molecular weight, 8,000 to approximately 10,000], 2.5 M NaCl) and chilled in ice for 1 h. The DNA was precipitated by centrifugation and washed with 900 μl of 70% ethanol. The DNA was then dried and dissolved in 50 μl of TE buffer. A small aliquot of DNA was electrophoresed in an agarose gel and stained with ethidium bromide. The DNA concentration was determined by densitometry (ATTO Co., Ltd., Osaka, Japan).

Southern blot hybridization. Spirochete DNAs were digested with *Eco*RV or *Hin*cII, electrophoresed, and transferred to a membrane as described previously (12). The 23S rRNA gene probes of *B. burgdorferi* B31^T designated NP and Sty were labeled with [α-^{32}P]dCTP as described previously and then used for hybridization (14).

PFGE. Spirochetes were grown in 100 ml of BSKII medium at 31°C, collected by centrifugation, washed, and used immediately for pulsed-field gel electrophoresis (PFGE). The procedures used to prepare high-molecular-weight genomic DNAs have been described previously (45). Genomic DNAs in plugs were cleaved and electrophoresed in a 0.5× TBE (1× TBE is 89 mM Tris, 89 mM boric acid, and 2 mM EDTA [pH 8.3])-buffered contour-clamped homogeneous electric field model DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) as described previously (45). Molecular size markers consisting of lambda phage DNA concatemers or low-range PFG markers (New England Biolabs, Beverly, Mass.) were used to calculate the sizes of the fragments.

G+C content of DNA. The G+C content was determined by high-performance liquid chromatography (HPLC). A DNA preparation was dissolved in distilled water and boiled for 5 min. After the sample was chilled in ice, 10 μl (3 to ~5 μg) of denatured DNA was degraded with nuclease P1 (DNA-GC kit; Seikagaku Kogyo, Tokyo, Japan) according to the manufacturer's instructions. The hydrolysate and a standard solution (10 μl each) were subjected to HPLC in a Shimadzu model LC-10AS apparatus by using a YMC pack AQ-312 column (6 by 150 mm; YMC Co., Ltd., Kyoto, Japan). The mobile phase used was 10 mM phosphate buffer (pH 3.5), and the flow rate was 1.5 ml/min. The A_{270} of the effluent was monitored with a Shimadzu model SPD-10A UV-visible light detector.

DNA-DNA hybridization. Levels of DNA reassociation were determined by the membrane filter method (26). A 2-μg portion of DNA was loaded onto a nylon membrane (Zeta probe membrane; 15 by 15 mm; Bio-Rad Laboratories) and denatured twice with 0.5 M NaOH. The membrane was then neutralized twice with 1 M Tris-HCl (pH 7), dried at 65°C for 30 min, and used for hybridization experiments (11). The DNA was labeled with [1′,2′,5′-^{3}H]dCTP (2.37 TBq/mmol; Amersham Japan, Ltd., Tokyo, Japan) by using a random primer labeling kit (Takara Shuzo Co., Ltd., Kyoto, Japan) as described previously, and this DNA was used as a probe. The filter membrane containing bound DNA was incubated at 60°C for 5 h in hybridization buffer containing 10% dextran sulfate (Pharmacia, Uppsala, Sweden), 1% SDS, 1 M NaCl, and 100 μg each of salmon sperm DNA and yeast RNA per ml (10). The membrane was then hybridized with the radiolabeled probe at 60°C for 15 h. After hybridization, the membrane was washed twice with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) and three times with 2× SSC at 60°C for 30 min. The filter membrane was then dried and counted with a liquid scintillation counter by using 10 ml of scintillation cocktail. Three experiments were performed independently, and the results were normalized by homologous DNA hybridization.

Cloning the 16S rRNA gene and sequencing. The nucleotide primers which we used (5′-GCT GGC AGT GCG TCT TAA GCA TGC-3′ [positions 35 to 58, *Escherichia coli* numbering] and 5′-GTG ACG GGC GGT GTG TAC AAG GCC C-3′ [positions 1384 to 1408]) were synthesized and used for PCR amplification. PCR amplification was performed in a 100-μl reaction mixture for 30 cycles in a thermal controller (ATTO Zymoreactor) by using a denaturation step (94°C for 40 s), an annealing step (45°C for 30 s), and an extension step (72°C for 45 s). The other experimental conditions were the same as those described previously (14). A DNA fragment obtained by PCR amplification was purified as described above (polyethylene glycol precipitation), was ligated into vector plasmid pGEM5Zf by using a pGEM-T vector cloning kit (Promega Biotech, Madison, Wis.), and then introduced into competent *E. coli* JM109 cells by following the manufacturer's instructions. The nucleotide sequence was determined by the dideoxy chain termination method; the preparation was primed with a series of custom-synthesized primers by using an Autoread sequencing kit and an ALFred sequencer (Pharmacia). The sequences of both strands of two independent clones were determined by using custom primers. No differences in the sequences of the two clones were observed.

Different protocols were used to determine the sequences of strains DN127, IKA2, and HO14^T. PCR primers 16S-1507 (5′-CCA GAT CTG AGC TCA AGG AGG TGA TCC AGC-3′) and 16S-11 (5′-GGC TGC AGT CGA CGT TTG ATC CTG GCT CAG-3′) were used to amplify the 16S rRNA genes from 10 ng of *Borrelia* genomic DNA. These primers contained a *Bgl*II site and a *Sal*I site, respectively, and these sites were used for cloning. The PCR conditions were as follows: 30 cycles consisting of a denaturation step (94°C for 1 min), an annealing step (55°C for 1 min), and an extension step (72°C for 2 min). The PCR products were extracted with phenol-chloroform, precipitated, redissolved in water, and digested sequentially with *Bgl*II and *Sal*I. Ligation with vectors Bluescript SK and KS (Stratagene) and transformation into XL1-blue cells (Stratagene) were performed by following the instructions of the manufacturer. Templates for sequence determinations were prepared as phagemids rescued from insert-containing clones. Cloning into the two vectors permitted both orientations to be sequenced, and sequences were determined by using a Sequenase kit (U. S. Biochemicals, Cleveland, Ohio) and α-^{35}S-dATP. Sequencing reaction mixtures were primed either with vector-encoded universal or RV primers or with a series of custom-synthesized primers spaced uniformly across the entire gene. At least two clones (and up to four clones) were sequenced for each PCR product.

Multiple alignments of the sequences were prepared, and a neighbor-joining phylogenetic tree (39) was constructed by using the DNASTAR program (DNASTAR, Inc., Madison, Wis.) and the Clustal method (16).

Nucleotide sequence accession numbers. The 16S rRNA gene sequence of strain HT31^T has been deposited in the GSDB, DDBJ, EMBL, and NCBI data libraries under accession number D45192. The GenBank accession numbers for the 16S rRNA gene sequences of strain DN127 and *B. japonica* HO14^T and IKA2 are L40596, L40597, and L40598, respectively. The accession numbers for the reference sequences which we used are as follows: *B. burgdorferi* B31^T, M59293; *B. burgdorferi* 1352, M64309; *B. garinii* G1, M64311; *B. afzelii* Ip3, M75149; *Borrelia hermsii* HS1, M60968; *Borrelia coriaceae*, M60970; and *Borrelia anserina*, M64312.

RESULTS AND DISCUSSION

Unfed ticks were collected by flagging or dragging vegetation in various locations in Hokkaido, the northern island of Japan, where Lyme disease is endemic (27–32). Ticks were collected from birds at Nemuro, Hokkaido, Japan, with the

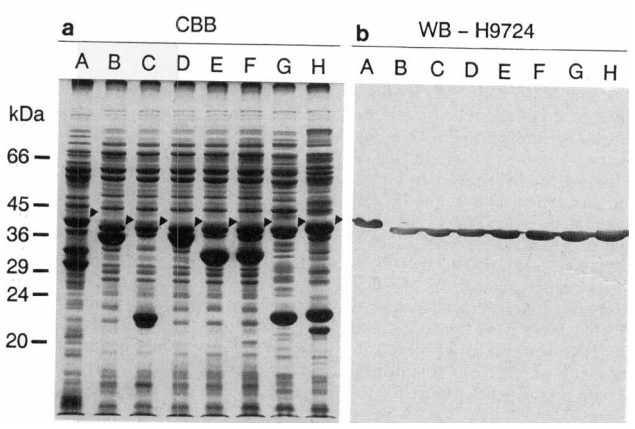

FIG. 1. Coomassie brilliant blue-stained proteins in whole-cell lysates of borrelial isolates and antigenic characteristics with monoclonal antibody H9724. An SDS-PAGE analysis (a) and a Western blot analysis (b) were performed as described previously (33, 34). Murine monoclonal antibody H9724 was obtained as a hybridoma supernatant. Lane A, *B. burgdorferi* B31^T; lane B, strain HT24; lane C, strain HT31^T; lane D, strain Hk004; lane E, strain NB103/1; lane F, strain FR64b; lane G, *B. hermsii*; lane H, *B. anserina*. The positions of molecular mass standards are indicated on the left. The arrowheads indicate the position of the protein that reacted with monoclonal antibody H9724. CBB, Coomassie brilliant blue; WB-H9724, Western blot with monoclonal antibody H9724.

collaboration of many bird banders. Mice were captured in Sherman box traps as described previously (31). Spirochetes were grown by culturing tick midgut tissues or rodent blood in BSKII medium (3) at 31°C. The origins of the five isolates which we studied are as follows: strain HT24, an unfed female tick collected at Shiretoko, Hokkaido, Japan; strain HT31^T, an unfed female tick collected at Shiretoko, Hokkaido, Japan; strain Hk004, an unfed tick nymph collected at Shibecha, Hokkaido, Japan; strain NB103/1, a tick nymph that fed on a bird (*Emberiza spodocephala*) captured at Nemuro, Hokkaido, Japan; and strain FR64b, blood from a rodent (*Apodemus argenteus*) trapped at Furano, Hokkaido, Japan. All culture sources were collected from 1990 through 1992.

The molecular weight of the endoflagellar protein was estimated from the results of SDS-PAGE and Western blotting (Fig. 1). Monoclonal antibody H9724, which is specific for *Borrelia* flagellar protein, was used as a probe for Western blotting. Although a 41-kDa flagellar protein is found in previously described Lyme disease borreliae, the five isolates obtained in Hokkaido contained a 38-kDa flagellar protein similar to that found in relapsing fever borreliae.

The rRNA genes of the *B. burgdorferi* sensu lato strains associated with Lyme disease have a unique organization. Two sets of 23S and 5S rRNA genes are repeated directly, are 3.2 kb long, and are separated from the single 16S rRNA gene (9, 15, 42). As shown in Fig. 2A and C, two hybridization signals were detected in the Lyme disease borreliae that produced the 3.2-kb band. In contrast, genomic Southern hybridization of the new spirochete isolates used in this study yielded a single radioactive band for each isolate. It has been shown that there is only one copy of the 23S and 5S rRNA genes in relapsing fever borreliae (42). We constructed physical maps of the rRNA genes and their flanking regions for our spirochetes, and the maps revealed that there is a single gene unit, indicating that the 23S and 5S rRNA genes are not repeated. We performed an RFLP analysis associated with the rRNA gene tandem cluster (12–15). The sizes of the restriction bands were determined, and the borrelial isolates were classified into several RFLP ribotype groups. This RFLP analysis in which tandem repetition was used was useful for identifying closely related *Borrelia* isolates, and all *B. burgdorferi* sensu lato isolates associated with Lyme disease exhibited this unique rRNA gene repetition (14, 42). However, the spirochete isolates obtained in Hokkaido and the relapsing fever borreliae produced a single band, indicating that the 23S-5S rRNA gene unit was not repeated (Fig. 2A).

Physical maps of the rRNA genes and flanking regions of representative strain HT31^T and *Borrelia turicatae* were constructed by using a previously described method (15). As shown in Fig. 2B, the restriction sites in the 23S rRNA gene were identical in these two borreliae, but the restriction sites in the flanking region were different. We also examined the rRNA gene organization of other borreliae, including *B. anserina*, *Borrelia parkeri*, *B. hermsii*, and *B. coriaceae*, and no repetition was observed in any of these organisms (data not shown). The organization of rRNA genes has also been described by other workers (9, 42). The organization of rRNA genes is highly conserved among prokaryotes, and the three rRNA genes are closely linked in the gene set in the following order: 16S-23S-5S (35). In contrast, in the genus *Borrelia*, the 16S rRNA gene is separated from the other two rRNA genes, and it is thought that tandem repetition of the 23S and 5S rRNA genes is limited to *Borrelia* species that infect ixodid ticks. However, an exception has been described by Liveris et al. (21), who found that gene duplication was absent in *B. japonica* IKA2 isolated from an *I. ovatus* tick in Japan. All five isolates obtained in Hokkaido had the same physical map (data not shown).

Spirochete genomic DNAs were digested with restriction enzymes *Mlu*I, *Apa*I, and *Sal*I and separated by PFGE. The fragment configurations obtained with *Mlu*I are shown in Fig. 3; when the other endonucleases were used, the five isolates obtained in Hokkaido produced the same PFGE profiles (data not shown). When *Mlu*I, *Apa*I, and *Sal*I were used, the PFGE profiles of the five isolates obtained in Hokkaido were different from the profiles of the previously described Lyme disease and relapsing fever borreliae. The PFGE gels were then blotted and hybridized with Lyme disease borrelia-specific gene probe P39 provided by T. G. Schwan (Rocky Mountain Laboratories) (40). This probe hybridized with previously described Lyme disease borreliae but not with the five isolates obtained in Hokkaido or with the relapsing fever borreliae (data not shown).

The relative binding ratios obtained in hybridization experiments performed by using the membrane filter method are shown in Table 2. The levels of hybridization between labeled strain HT31^T genomic DNA and DNAs from strains B31^T, 20047^T, VS461^T, and Am105 ranged from 8 to 13%. The genomic DNA of strain HT31^T, which was isolated from *I. persulcatus*, was quite similar to the DNA of strain FR64b, which was isolated from *A. argenteus* blood. Representatives of *B. hermsii*, *B. turicatae*, *B. parkeri*, *B. coriaceae*, and *B. anserina* exhibited levels of relatedness of 24 to 51% with strain HT31^T. The levels of DNA similarity between labeled strain B31^T DNA and DNAs from strains 20047^T, VS461^T, and Am105 were similar to values obtained in previous reports (2, 19), which supported the validity of the data which we obtained. It is generally assumed that the level of DNA similarity for two separate species should be less than 70% under optimal conditions (47). The results of our DNA-DNA hybridization experiments suggest that strain HT31^T isolated from *I. persulcatus* actually represents a new *Borrelia* species.

We cloned the 16S rRNA gene of strain HT31^T and the nucleotide sequence was determined in order to quantitatively assess the level of phylogenetic divergence of this strain from

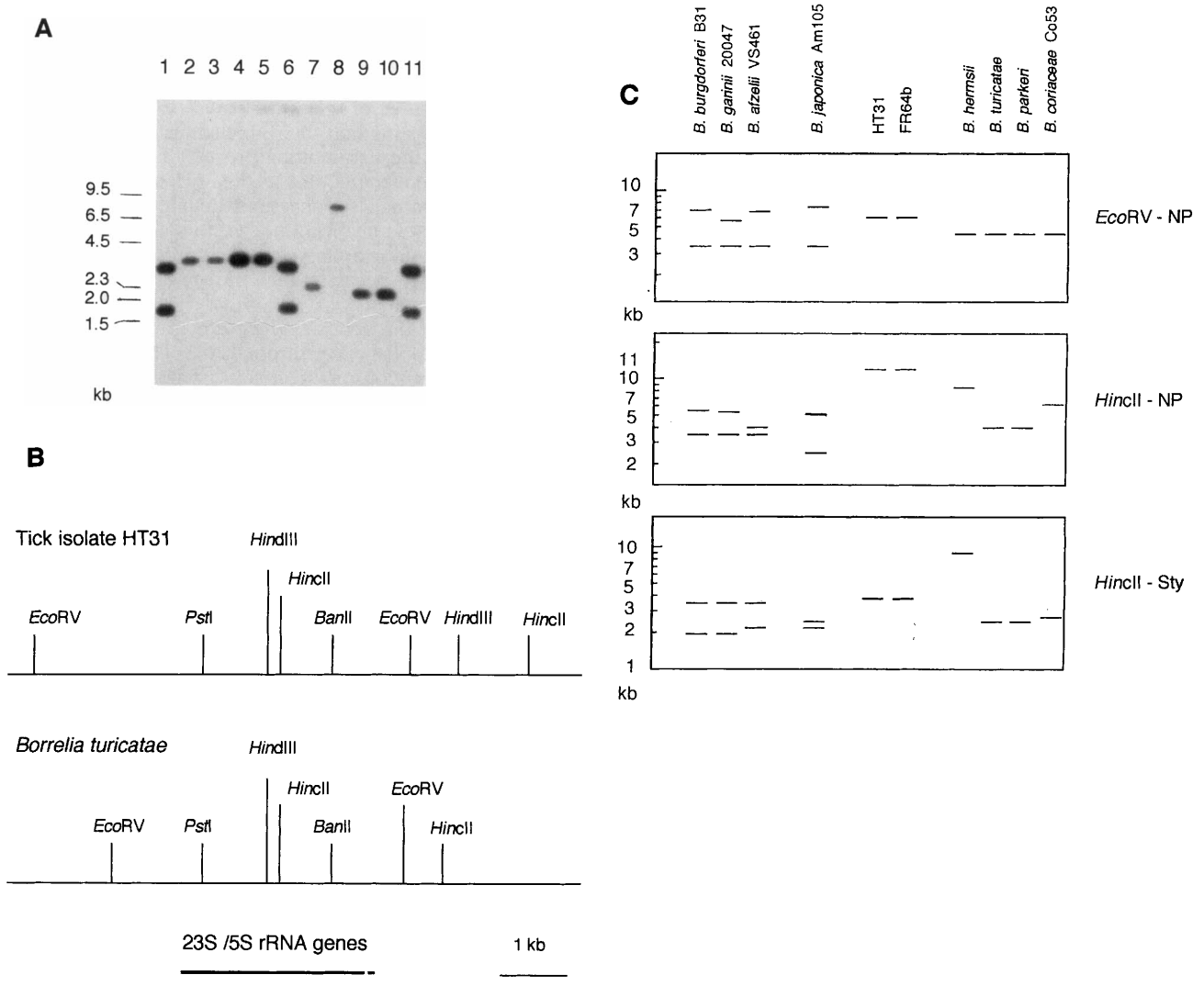

FIG. 2. Hybridization of the rRNA gene probe with restriction enzyme digests of *Borrelia* genomic DNAs, physical maps of the regions surrounding the rRNA genes, and schematic representation of genomic hybridization. Genomic DNAs were extracted, digested with enzymes, electrophoresed, blotted, and hybridized with probes as described previously (12, 14). (A) Each genomic DNA was digested with *Hin*cII, electrophoresed in a 1% agarose gel, and blotted onto a nylon membrane (Zeta probe membrane; Bio-Rad Laboratories). Hybridization probe Sty was radiolabeled with [α-^{32}P]dCTP by using a random primer labeling kit according to the instructions of the manufacturer (Takara Shuzo) and was used as a probe. Lanes 1, 6, and 11, *B. burgdorferi* B31^T; lane 2, strain NB103/1; lane 3, strain HT24; lane 4, strain HT31^T; lane 5, strain Hk004; lane 7, *B. coriaceae*; lane 8, *B. hermsii*; lane 9, *B. parkeri*; lane 10, *B. turicatae*. (B) Genomic DNAs of strain HT31^T and *B. turicatae* were digested with enzymes, and physical maps of the regions surrounding the 23S and 5S rRNA genes of these organisms were constructed by digestion, partial digestion, and double digestion with enzymes and by genomic Southern hybridization as described previously (12, 15). Probes corresponding to the 5′ part of the 23S rRNA gene (probe NP) and the 5S rRNA gene were also used, and the exact locations of the genes were determined as described previously (15). (C) Spirochete genomic DNAs were digested with *Eco*RV or *Hin*cII. The resulting DNA fragments were electrophoresed, transferred, and hybridized with the 23S rRNA gene fragments of *B. burgdorferi* B31^T designated NP and Sty. The hybridization results obtained with the probes are presented schematically.

representatives of other *Borrelia* species (23, 37). The sequence which we obtained was 1,368 bp long and included positions 35 to 1408 (*E. coli* numbering). This sequence was aligned and compared with the sequences of other *Borrelia* species by using the DNASTAR program. Nucleotide sequence pair similarity values for the sequences were calculated by using the Clustal method (16) (Table 3). A neighbor-joining phylogenetic tree constructed on the basis of the sequence similarity matrix data is shown in Fig. 4. According to this tree, the *Borrelia* strains which we compared could be divided into two major clusters. *B. miyamotoi* HT31^T belonged to a lineage distinct from the lineage that contained the previously described Lyme disease borreliae and to a cluster that included *B. hermsii*, *B. anserina*, and *B. coriaceae*. Our evolutionary analysis revealed that the members of this cluster are not closely related to each other. In contrast, North American strain DN127, which was isolated from *Ixodes pacificus*, and *B. japonica* HO14^T and IKA2 clustered in a distinct group along with the previously described Lyme disease borreliae. The tree clearly showed that the *B. japonica* strains are not closely related to all of the members of *B. burgdorferi* sensu lato which were compared. *B. japonica* seems to have diverged from *B. burgdorferi* sensu lato strains and to have adapted to *I. ovatus* ticks. *B. japonica* has been detected frequently in *I. ovatus* ticks collected in various locations in Japan (29, 34). Strain DN127 has been reported to be an unusual strain among the North American isolates (38, 48). Our phylogenetic analysis based on 16S rRNA gene sequences revealed that strain DN127 diverged at a level consistent with

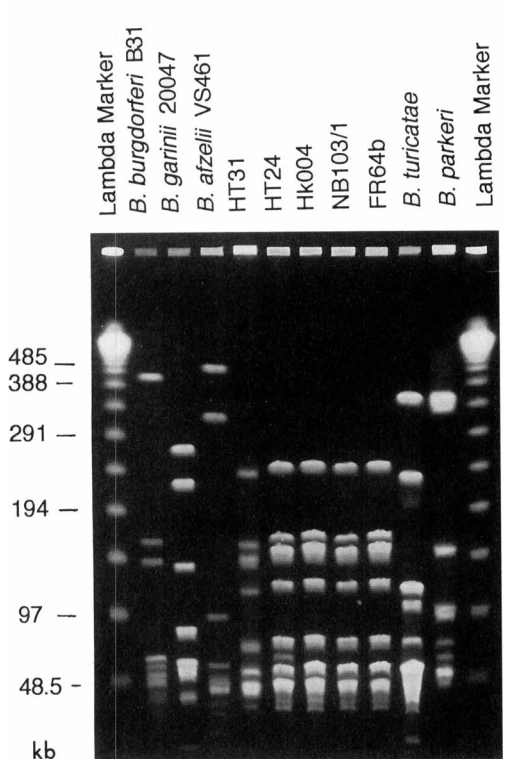

FIG. 3. PFGE of *Mlu*I restriction digests of genomic DNAs. The genomic DNAs in agarose plugs were washed with 200 μl of ice-cold digestion buffer, and the high-molecular-weight genomic DNAs in the plugs were cleaved with restriction enzyme *Mlu*I as described by Davidson et al. (9). The genomic DNAs in the plugs were washed with 200 μl of ice-cold digestion buffer for 30 min and then cleaved by exposing them to 50 U of enzyme for 15 h at 37°C in 200 μl of the buffer recommended by the supplier. The plugs were placed in wells containing 1.2% agarose (Pulsed Field Certified Agarose; Bio-Rad Laboratories) and were electrophoresed with a pulse time ramped from 3 to 50 s for 48 h at 150 V with a contour-clamped homogeneous electric field model DRII apparatus (Bio-Rad Laboratories). Molecular size markers consisting of lambda phage DNA concatemers (New England Biolabs) were used to calculate the sizes of the fragments. Restriction endonuclease *Mlu*I was purchased from Takara. The sizes of the fragments are indicated on the left.

the creation of a new species and that this organism is distinct from *B. burgdorferi* sensu stricto. Arbitrarily primed-PCR results also revealed that DN127 may belong to a distinct species (48). However, the level of relatedness determined by DNA-DNA hybridization experiments was borderline (38). Further studies will be needed to determine the position of strain DN127.

The G+C contents of the genomic DNAs of members of the genus *Borrelia* are 27 to 32 mol%. The G+C content of the DNA of strain HT31^T isolated from *I. persulcatus* in Japan was 28.6 mol%. All of our findings indicated that strain HT31^T is a representative of a new species belonging to the genus *Borrelia*.

The spirochete isolates described in this paper are quite different from the previously described Lyme disease borreliae and partially resemble relapsing fever borreliae or animal disease borreliae associated with *Ornithodoros* species ticks. The restriction sites of the new spirochete genomic DNA as determined by PFGE were distinct from the restriction sites of both types of previously described *Borrelia* species, but the flagellin gene sequence was more similar to the sequence found in the relapsing fever borreliae than to the sequence found in the previously described Lyme disease borreliae (unpublished data). The restriction enzyme maps of the rRNA genes of our isolates and the relapsing fever borreliae are quite similar. Our data demonstrated that the five spirochete isolates obtained in Hokkaido shared a few characteristics with some spirochetes associated with soft-bodied ticks. Several genetic parameters indicate that these isolates should be classified as members of a new species. Three of them originated from unfed *I. persulcatus* ticks, suggesting that the spirochete is transmitted transstadially during the tick molting process. The remaining two isolates originated from a bird-feeding tick and the blood of a rodent. Thus, the new spirochete is thought to be maintained in an enzootic cycle involving tick vectors and vertebrate reservoirs. Because there are no clinical records of relapsing fever in Hokkaido, we speculate that the new spirochete is not pathogenic for humans.

All species of the genus *Borrelia* are transmitted to vertebrates by hematophagous arthropods (4). The spirochetes are maintained in locations of endemicity of the disease by vector-reservoir transmission cycles involving several species of tick vectors. Two major groups of borreliae, relapsing fever borreliae and Lyme disease borreliae, have been well characterized. The borreliae associated with Lyme disease are found in a relatively limited number of *Ixodes* tick species. Borreliae associated with relapsing fever have been found in *Ornithodoros* or *Argas* tick species vectors or louse vectors, and some of the species exhibit complete specificity for their tick vectors (4). Relapsing fever is a well-studied disease, and its causative agent, *B. hermsii*, is of interest to microbiologists because of the molecular basis of its antigenic variation and the unique organization of its genes, linear chromosome, and minichromosomes (linear and circular plasmids). In contrast, Lyme disease is a recently recognized borreliosis with various inflammatory manifestations involving the skin, joints, heart, and central nervous system (22, 43). This disease is caused by *B. burgdorferi* sensu stricto in North America (1, 41) and by *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* in the Palaearctic subregion (17, 36, 46, 49). It has been generally assumed that ticks belonging to the *I. ricinus* species complex (*Ixodes scapularis* and *I. pacificus* in North America and *I. ricinus* and *I. persulcatus* in Eurasia) are the vectors that transmit the disease to humans (1, 17, 44).

Hard ticks (members of the family Ixodidae) are phylogenetically only distantly related to soft ticks, and as far as we know, the Lyme disease borreliae definitely differ from the relapsing fever borreliae in their arthropod vectors. Our results, therefore, contain important data on the evolution of borreliae and the adaptation of borreliae to vector ticks and provide a useful model for finding clues concerning the adaptive evolution of organisms. If we assume that *Borrelia* species

TABLE 2. Levels of DNA relatedness among *Borrelia* species

Source of unlabeled DNA	Relative binding ratios (%) with the following sources of labeled DNA:		
	Strain HT31^T	*B. burgdorferi* B31^T	*B. hermsii*
B. miyamotoi HT31^T	100	13	45
B. miyamotoi FR64b	100	14	45
B. burgdorferi B31^T	10	100	21
B. garinii 20047^T	13	65	27
B. afzelii VS461^T	8	57	16
B. japonica Am105	9	48	17
B. hermsii	44	21	100
B. turicatae	41	ND[a]	ND
B. parkeri	51	ND	ND
B. coriaceae Co53	41	ND	ND
B. anserina	24	ND	ND

[a] ND, not determined.

TABLE 3. 16S rRNA sequence similarity matrix for *Borrelia* isolates

Strain or species	% 16S rRNA sequence similarity with:										
	Strain 1352	Strain B31^T	Strain DN127	Strain G1	Strain HO14^T	Strain IKA2	Strain Ip3	Strain Co53	Strain HS1	Strain HT31^T	*B. anserina*
B. burgdorferi 1352	100										
B. burgdorferi B31^T	96.7	100									
Borrelia sp. strain DN127	95.7	95.0	100								
B. garinii G1	98.6	96.2	95.5	100							
B. japonica HO14^T	95.5	94.1	97.6	95.4	100						
B. japonica IKA2	94.7	93.8	96.3	94.9	97.3	100					
B. afzelii Ip3	98.3	95.7	95.0	98.1	95.1	94.4	100				
B. coriaceae Co53	93.4	90.8	90.4	93.3	90.5	89.7	93.2	100			
B. hermsii HS1	93.7	91.1	90.5	93.2	90.5	89.8	93.7	97.6	100		
B. miyamotoi HT31^T	94.8	93.0	92.7	94.7	91.8	91.9	94.8	94.3	94.5	100	
B. anserina	93.2	91.1	90.3	93.0	90.1	89.4	93.4	96.7	94.1	94.1	100

have a common ancestor, we can speculate that the spirochete found in Hokkaido is a descendant adapted to hard-bodied ixodid ticks that has some characteristics of relapsing fever borreliae. Recently, Carter et al. (8) and Margolis et al. (24) described the similarity between the outer surface protein (*ospC*) gene of Lyme disease borreliae and the variable major protein (*vmp*) genes of relapsing fever borreliae (*B. hermsii*). The observations of these authors suggest that these two groups of pathogens evolved from a common ancestor but have adapted to various arthropod and vertebrate hosts. Further phylogenetic studies will be necessary to better understand the diversity of *Borrelia* species. Moreover, the susceptibility of members of the tick families Argasidae and Ixodidae to various *Borrelia* species should be investigated under laboratory conditions; the results should provide useful information concerning the adaptation of borreliae to tick vectors.

Description of *Borrelia miyamotoi* sp. nov. *Borrelia miyamotoi* (mi. ya. mo′ toi. M.L. gen. n. *miyamotoi*, of Miyamoto, in honor of Kenji Miyamoto, the entomologist who first isolated spirochetes from ixodid ticks in Hokkaido, Japan). Morphology as described previously for the genus (20). Culture properties as described previously for *B. burgdorferi* sensu lato (3, 18). The single gene cluster for 23S and 5S rRNAs is separated from the 16S rRNA gene. Reacts in Western blots with monoclonal antibody H9724 and yields a 38-kDa band. No reactivity is observed with monoclonal antibody H5332. This species can also be differentiated from other *Borrelia* species on the basis of DNA-DNA hybridization results and by the sequence of its 16S rRNA genes.

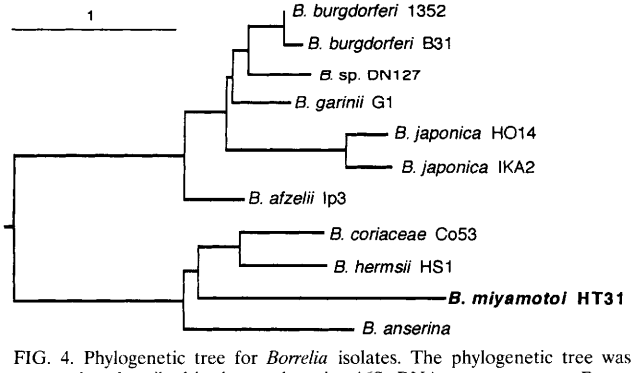

FIG. 4. Phylogenetic tree for *Borrelia* isolates. The phylogenetic tree was constructed as described in the text by using 16S rRNA gene sequences. Bar = 1% difference between sequences, as determined by measuring the lengths of the horizontal lines connecting two species.

The G+C content of the DNA is 28 to 29 mol%.

The type strain is strain HT31, which has been deposited in the Japan Collection of Microorganisms (RIKEN) as strain JCM 9579. Strain HT31^T was isolated from an *I. persulcatus* tick in Hokkaido, Japan. *B. miyamotoi* has been isolated from unfed *I. persulcatus* ticks, from the blood of a rodent, and from a tick that fed on a bird in Hokkaido.

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