A Phylogenetic Analysis of *Borrelia burgdorferi* Sensu Lato Isolates Associated with Lyme Disease in Japan by Flagellin Gene Sequence Determination

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We determined nearly complete flagellin gene sequences for *Borrelia burgdorferi* sensu lato isolates (11 isolates obtained from *Ixodes persulcatus* ticks and patients in Hokkaido, Japan, and 1 European isolate) representing six different restriction fragment length polymorphism (RFLP) ribotype groups following cloning of the PCR-amplified genes. These sequences were aligned with those of representatives of the three *Borrelia* species associated with Lyme disease, and a phylogenetic tree was constructed by the Clustal method. On the Lyme disease *borrelia* portion of the tree, the species were clearly delineated into three different phylogenetic groups, in complete agreement with the division of *B. burgdorferi* sensu lato into three species. A phylogenetic analysis revealed that the representatives of RFLP ribotype groups IV, V, and VI clustered tightly with each other and belonged on the same branch as *Borrelia garinii*. We used the criteria that are currently used to delineate bacterial species and determined the levels of DNA relatedness for these *Borrelia* isolates. For the RFLP ribotype group IV, V, and VI isolates, the levels of DNA relatedness ranged from 79 to 88%, and the levels of relatedness to the reference strain of *B. garinii* ranged from 70 to 80%. The levels of DNA relatedness of the RFLP ribotype group IV, V, and VI isolates to the representatives of other species associated with Lyme disease ranged from 53 to 66%. All of these findings indicate that the RFLP ribotype group IV, V, and VI isolates should be included in the species *B. garinii*.

The following three validly described *Borrelia* species are associated with Lyme disease: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (5, 8, 21). Although the distribution of *B. burgdorferi* sensu stricto is rather restricted in North America and Europe, *B. garinii* and *B. afzelii* are widely distributed in all of the north temperate regions of the world except North America. It has been generally assumed that *Ixodes ricinus* species complex ticks are major vectors for transmitting infections to humans and that the spirochetes are maintained by vector-reservoir transmission cycles involving blood-sucking ticks and wildlife vertebrates (1, 2, 6, 20).

A large number of spirochete strains have been isolated from patients exhibiting erythema migrans, *Ixodes persulcatus* ticks, and reservoir rodents (*Apodemus speciosus*) in the northern part of Japan, where Lyme disease is endemic (4, 12, 14, 22, 26, 28, 30). Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, Western blot (immunoblot) analysis with monoclonal antibodies, the restriction fragment length polymorphism (RFLP) ribotyping method, and a 16S rRNA gene-based PCR assay, we have demonstrated that these isolates exhibit considerable phenotypic and genetic heterogeneity (12, 14, 32, 33). No *B. burgdorferi* sensu stricto isolate has been found in the material examined so far in Japan. Some of the isolates were classified as *B. garinii* or *B. afzelii* strains by our typing methods, while the other isolates were apparently not members of any of the three species associated with Lyme disease (14, 30). We tentatively classified these organisms as members of ribotype groups IV, V, and VI. RFLP ribotype group IV is the predominant group among isolates derived from clinical specimens, reservoir rodents, and *I. persulcatus* vector ticks in Japan. Therefore, we wanted to determine the

taxonomic status of the groups in order to clarify the relationships between them and the three species associated with Lyme disease.

We cloned and sequenced the outer surface protein C (OspC) gene of unknown *Borrelia* isolates since the OspC gene was present in all *B. burgdorferi* sensu lato strains, and the sequences exhibited significant diversity (9). Sequence analysis of the OspC gene and the predicted amino acid alignment were thought to be useful tools for species identification of the spirochete isolates. Actually, alignment of the OspC sequences revealed that the species-specific domain was conserved in all of the ribotype group IV, V, and VI isolates. However, overall OspC sequences were highly variable, and assignment of the isolates to *Borrelia* species was inconclusive.

In this study we characterized the spirochete isolates mentioned above. As determined by DNA-DNA hybridization experiments, three new RFLP ribotype group isolates were closely related to *B. garinii* strains since the levels of DNA relatedness were more than 70%. A phylogenetic analysis based on flagellin gene sequences clearly revealed that most of the Japanese isolates belong to *B. garinii*. Despite differences in phenotypic and genetic characteristics, we concluded that the spirochetes belonging to unknown species diverged within the species *B. garinii*. Our findings also indicate that flagellin gene sequence analysis is a useful tool for assessing the closely related *Borrelia* isolates associated with Lyme disease.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. burgdorferi* sensu lato isolates used in this study are listed in Table 1. The spirochetes were grown in BSKII medium at 31°C as described previously (40). Spirochete cells at the late exponential phase (500 ml of culture) were harvested and washed by centrifugation. *Escherichia coli* HB101 was used for transformation with a recombinant plasmid.

RFLP ribotyping analysis. Spirochete DNAs were prepared as described previously (10) and were digested with *EcoRV* or *HincII*; they were then electrophoresed in a 1% agarose gel, stained with ethidium bromide, photographed, and blotted by the method of Southern (39). 23S rRNA gene fragments NP and Sty from *B. burgdorferi* sensu stricto type strain B31 were labeled and used as

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TABLE 1. *B. burgdorferi* sensu lato strains used in ribotyping and PCR amplification analyses

Species	Strain	Source	RFLP ribotype group	PCR amplification			
				Primer set SC	Primer set BB	Primer set BG	Primer set VS
<i>B. burgdorferi</i> sensu stricto	B31 ^T	<i>Ixodes scapularis</i> (United States)	I	+	+	—	—
	IPS	<i>Ixodes pacificus</i> (United States)	I	+	+	—	—
	1352	<i>Amblyomma americanum</i> (United States)	I	+	+	—	—
	297	Patient (United States)	I	+	+	—	—
	GeHo	Patient (Germany)	I	+	+	—	—
<i>B. garinii</i>	20047 ^T	<i>Ixodes ricinus</i> (France)	II	+	—	+	—
	Ip90	<i>Ixodes persulcatus</i> (Russia)	II	+	—	+	—
	G1	Patient (Germany)	II	+	—	+	—
	PBi	Patient (Germany)	II	+	—	+	—
	N34	<i>Ixodes ricinus</i> (Germany)	II	+	—	+	—
	Ir210	<i>Ixodes ricinus</i> (Russia)	II	+	—	+	—
	HT17	<i>Ixodes persulcatus</i> (Japan)	II	+	—	+	—
	HT32	<i>Ixodes persulcatus</i> (Japan)	II	+	—	+	—
	HT57	<i>Ixodes persulcatus</i> (Japan)	II	+	—	+	—
	HT59	<i>Ixodes persulcatus</i> (Japan)	II	+	—	+	—
	HT64	<i>Ixodes persulcatus</i> (Japan)	II	+	—	+	—
	HT72	<i>Ixodes persulcatus</i> (Japan)	II	+	—	+	—
	JEM4	Patient (Japan)	II	+	—	+	—
	HT37	<i>Ixodes persulcatus</i> (Japan)	IV	+	—	+	—
	JEM1	Patient (Japan)	IV	+	—	+	—
	JEM2	Patient (Japan)	IV	+	—	+	—
	JEM5	Patient (Japan)	IV	+	—	+	—
	JEM6	Patient (Japan)	IV	+	—	+	—
	JEM7	Patient (Japan)	IV	+	—	+	—
	JEM8	Patient (Japan)	IV	+	—	+	—
	FR146s	<i>Apodemus argenteus</i> (Japan)	IV	+	—	+	—
	Asa5	<i>Apodemus speciosus</i> (Japan)	IV	+	—	+	—
	HT7	<i>Ixodes persulcatus</i> (Japan)	IV	+	—	—	—
	HT22	<i>Ixodes persulcatus</i> (Japan)	IV	+	—	—	—
	NB100/1	<i>Ixodes persulcatus</i> (Japan)	IV	+	—	—	—
	HT19	<i>Ixodes persulcatus</i> (Japan)	V	+	—	+	—
	JEM3	Patient (Japan)	V	+	—	+	—
	FR502e	<i>Apodemus speciosus</i> (Japan)	V	+	—	+	—
	HT55	<i>Ixodes persulcatus</i> (Japan)	VI	+	—	+	—
	N411	<i>Ixodes persulcatus</i> (Japan)	VI	+	—	+	—
	N338	<i>Ixodes persulcatus</i> (Japan)	VI	+	—	+	—
	N337	<i>Ixodes persulcatus</i> (Japan)	VI	+	—	—	—
<i>B. afzelii</i>	VS461 ^T	<i>Ixodes ricinus</i> (Switzerland)	III	+	—	—	+
	BO23	Patient (Germany)	III	+	—	—	+
	ACA1	Patient (Sweden)	III	+	—	—	+
	UMO1	Patient (Sweden)	III	+	—	—	+
	KK2	Tick (nymph) (Korea)	III	+	—	—	+
	Ip3	<i>Ixodes persulcatus</i> (Russia)	III	+	—	—	+
	HT61	<i>Ixodes persulcatus</i> (Japan)	III	+	—	—	+
	HT25	<i>Ixodes persulcatus</i> (Japan)	III	+	—	—	+
	HT10	<i>Ixodes persulcatus</i> (Japan)	III	+	—	—	+
	Asa1	<i>Apodemus speciosus</i> (Japan)	III	+	—	—	+
	FR478b	<i>Apodemus speciosus</i> (Japan)	III	+	—	—	+

hybridization probes as described previously (11, 17). The other experimental conditions used in the RFLP analysis have been described previously (14, 30).

PCR amplification. Total DNA was extracted and purified as described previously (9). PCR amplification with the primer sets specific for the 16S rRNA gene of *Borrelia* species was carried out to identify the species. The following four PCR primer sets were prepared and used for species-specific DNA amplification experiments: primer set SC for conserved regions of the 16S rRNA gene (5'-GCT GGC AGT GCG TCT TAA G-3' and 5'-ATT CTT AGC TGC TGC CTC C-3'); primer set BB, which is specific for *B. burgdorferi* sensu stricto (5'-GGG ATG TAG CAA TAC ATT C-3' and 5'-ATA TAG TTT CCA ACA TAG G-3'); primer set BG, which is specific for *B. garinii* (5'-GGG ATG TAG CAA TAC ATC T-3' and 5'-ATA TAG TTT CCA ACA TAG T-3'); and primer set VS, which is specific for *B. afzelii* (5'-GCA TGC AAG TCA AAC GGA-3' and 5'-ATA TAG TTT CCA ACA TAG C-3'). All other experimental conditions were the same as the conditions described previously (9).

DNA-DNA hybridization. Levels of DNA reassociation were determined by the membrane filter hybridization method, as described previously (16, 25).

Flagellin gene amplification, cloning, and sequencing. The flagellin gene of *B. burgdorferi* sensu lato strains was amplified by PCR by using primers 5'-GAT TAT AAA TCA TAA TAG GTC AGC-3' and 5'-TTG AGG CAC TTG ATT TGC TTG TGC-3'. The PCR was performed as described elsewhere (9), except that the annealing step (45°C for 30 s) and the extension step (72°C for 90 s) were modified. The PCR amplification product was then purified and ligated into vector pGEM5Zf by using the pGEM-T vector system (Promega, Madison, Wis.) as recommended by the manufacturer, as described previously (9). The recombinant DNA fragments were introduced into competent *E. coli* HB101 cells (Takara Shuzo Co., Ltd., Kyoto, Japan). The double-stranded plasmid DNA was extracted and purified as described previously (11). Sequencing reactions were primed with either vector-encoded primers UN (5'-CGA CGT CGC ATG CTC CCG GC-3') and RV (5'-CTC CCA TAT GGT CGA CCT GC-3') or custom-

FIG. 1. PCR amplification with the *B. garinii*-specific primer set. The experimental conditions used have been described previously (14). The PCR-amplified DNA fragment size is shown on the left. Lane 1, strain HT37; lane 2, strain JEM5; lane 3, strain Asa5; lane 4, strain HT22; lane 5, strain HT7; lane 6, strain NB100/1; lane 7, strain HT19; lane 8, strain JEM3; lane 9, strain FR502e; lane 10, strain HT55; lane 11, strain N411; lane 12, strain N337; lane M, lambda DNA marker digested with *Hind*III and *Kpn*I.

synthesized primers FLF1 (5'-TAA CGG CAC ATA CTC AGA TGC AGA CAG AGG-3'), FLF2 (5'-GGA TGA GGC AAT TGC TGT CAA TAT TTA TGC-3'), FLR1 (5'-GTC TAT TTT GGA AAG CAC CTA AAT TTG CTC-3'), and FLR2 (5'-GCA TTT GGT TGT ATT GAG CCT GAT C-3'). Sequence alignment, similarity matrix construction, and phylogenetic tree construction were performed with a Macintosh personal computer by using the DNASTAR program (DNASTAR, Inc., Madison, Wis.) and the CLUSTAL V software package (19).

Nucleotide sequence accession numbers. The flagellin gene nucleotide sequences of *B. burgdorferi* sensu lato isolates which we determined in this study have been assigned the following Genome Sequence Data Base, DNA Data Base of Japan, European Bioinformatics Institute, and National Center for Biotechnology Information accession numbers: D63363 (*B. garinii* HT17), D63364 (*B. garinii* JEM4), D63365 (*B. afzelii* VS461^T [T = type strain]), D63366 (*B. afzelii* HT61), D63367 (ribotype group IV strain HT22), D63368 (ribotype group IV strain HT7), D63369 (ribotype group IV strain HT37), D63370 (ribotype group IV strain JEM5), D63371 (ribotype group V strain HT19), D63372 (ribotype group V strain JEM3), D63373 (ribotype group VI strain HT55), and D63374 (ribotype group VI strain N337). The accession numbers of the flagellin gene sequences of strains published previously (18, 23, 34, 36) that were compared in this study are as follows: *B. burgdorferi* sensu stricto type strain B31, X16833; strain GeHo, X56334; *B. garinii* Ip90, X75203; *B. afzelii* ACA1, X75202; *Borrelia hermsii* HS1, M33839; *B. burgdorferi* KL10, L42881; and *B. burgdorferi* NBSlab, L42876.

RESULTS

RFLP ribotyping analysis. The DNAs were digested with an enzyme, electrophoresed, blotted, and hybridized with 23S rRNA gene probes. The RFLP ribotypes of spirochete isolates examined in this study and our previous studies are shown in Table 1.

PCR amplification analysis. Previous studies have revealed the usefulness of PCR amplification as a tool for the identification of the spirochetes associated with Lyme disease (24, 31, 35). The results of our 16S rRNA gene sequence-based PCR typing analysis in which the species-specific primer set was used confirmed the identities of ribotype group I, II, and III strains as members of *B. burgdorferi* sensu lato. The results of one such experiment are shown in Fig. 1, and the PCR amplification results are summarized in Table 1. When we used primer set SC (primer sequence containing conserved regions of the 16S rRNA gene), a fragment of the appropriate size (320 bp) was amplified from all of the DNA preparations tested in this study (data not shown). Figure 1 shows the results of an amplification experiment in which ribotype group IV, V, and VI isolates were tested for PCR amplification with primer set BG. Primer set BG, which was specific for *B. garinii*, did not amplify DNAs

from ribotype group IV isolates HT7, HT22, and NB100/1 and ribotype group VI strain N337 but did amplify DNA slightly when the annealing temperature was decreased from 52 to 50°C. Primer set BG did amplify DNAs of the appropriately sized fragment (580 bp) from the other ribotype group IV and VI isolates and all ribotype group V isolates.

DNA-DNA hybridization. To clarify the status of the ribotype groups, we determined the levels of DNA relatedness by performing DNA-DNA hybridization experiments. The relative binding ratios obtained in the hybridization experiments in which the membrane filter method was used are shown in Table 2. None of the isolates belonging to ribotype groups IV, V, and VI were related to the type strains of *B. burgdorferi* and *B. afzelii* since the levels of DNA similarity ranged from 53 to 66%. The ribotype group IV, V, and VI isolates were all related to strain 20047, the type strain of *B. garinii*. The Japanese isolates were more closely related to each other than to European strain 20047^T. The ribotype group IV, V, and VI isolates were 77 to 88% related to each other and 70 to 80% related to the members of *B. garinii*.

Flagellin gene sequence alignment and phylogenetic analysis. The flagellin gene is a conserved gene which has been proven to be useful for identification of borreliae at the species level (3, 36). We cloned the flagellin gene of representatives of five ribotype groups, and the nucleotide sequences (985 of 1,011 bases) were determined in order to quantitatively assess the phylogenetic divergence of the strains belonging to other *Borrelia* species associated with Lyme disease. The flagellin sequences (937 bases without PCR primer sequences) of 18 borrelial strains were aligned and compared with the *B. hermsii* HS1 flagellin sequence as an outlier by using the DNASTAR program (DNASTAR, Inc.). The sequence of each isolate was determined with least two independent clones of a PCR product. When a nucleotide mismatch was detected, another clone was sequenced. A nucleotide discrepancy was observed about once per 2,000 bases under our experimental conditions, and the mismatches were thought to have resulted from *Taq* enzyme synthesis errors.

The nucleotide sequence pair similarity values of the sequences were calculated by the Clustal method and are shown in Table 3. The similarity matrix showed that the levels of conservation of sequences and the degrees of sequence similarity ranged from 93.6 to 100% for all of the isolates associated with Lyme disease. The lowest levels of similarity ob-

TABLE 2. Levels of DNA relatedness among three *Borrelia* species and new ribotype group isolates

Source of unlabeled DNA	Relative binding ratios (%) with the following sources of labeled DNA:			
	Strain 20047 ^T	Strain HT37	Strain HT19	Strain HT55
<i>B. burgdorferi</i> B31 ^T	56	56	53	53
<i>B. garinii</i> 20047 ^T	100	77	72	70
<i>B. garinii</i> HT17	77	78	73	78
<i>B. garinii</i> JEM4	74	79	80	80
<i>Borrelia</i> sp. strain HT37 (ribotype group IV)	78	100	86	85
<i>Borrelia</i> sp. strain JEM5 (ribotype group IV)	70	77	81	84
<i>Borrelia</i> sp. strain HT19 (ribotype group V)	73	86	100	88
<i>Borrelia</i> sp. strain HT55 (ribotype group VI)	74	79	85	100
<i>B. afzelii</i> VS461 ^T	66	54	57	58

TABLE 3. Flagellin gene sequence similarity matrix for *Borrelia* isolates

Strain	% Similarity																		
	HS1	B31 ^T	GeHo	Ip90	KL10	NBSlab	HT17	JEM4	HT22	HT7	HT37	JEM5	HT19	JEM3	HT55	N337	VS461 ^T	HT61	ACA1
<i>B. hermsii</i> HS1	100.0	83.1	83.2	83.0	82.9	82.9	83.0	82.8	82.7	82.5	82.6	82.4	82.6	82.6	82.3	82.7	83.1	83.1	83.0
<i>B. burgdorferi</i> B31 ^T		100.0	99.7	93.8	94.5	94.5	94.2	93.9	93.8	94.0	93.7	93.6	93.9	93.9	93.7	93.8	94.5	94.7	94.5
<i>B. burgdorferi</i> GeHo			100.0	93.9	94.6	94.6	94.3	93.8	93.9	94.1	93.8	93.7	93.8	93.8	93.8	93.9	94.3	94.6	94.3
<i>B. garinii</i> Ip90				100.0	98.7	98.7	99.0	99.1	98.8	98.7	98.5	98.4	98.6	98.6	98.7	98.7	95.1	94.9	95.4
<i>Borrelia</i> sp. strain KL10					100.0	100.0	99.0	98.7	98.7	98.8	98.3	98.3	98.5	98.5	98.6	98.6	95.1	95.0	95.1
<i>Borrelia</i> sp. strain NBSlab						100.0	99.0	98.7	98.7	98.8	98.3	98.3	98.5	98.5	98.6	98.6	95.1	95.0	95.1
<i>B. garinii</i> HT17							100.0	98.8	99.0	98.9	98.7	98.6	98.8	98.8	98.9	98.9	95.2	95.0	95.3
<i>B. garinii</i> JEM4								100.0	98.8	98.9	98.5	98.4	98.8	98.8	98.7	98.7	95.5	95.3	95.4
HT22 (ribotype group IV)									100.0	99.5	99.3	99.1	99.1	99.1	99.5	99.9	95.0	94.8	95.1
HT7 (ribotype group IV)										100.0	99.1	99.0	99.3	99.3	99.4	99.4	94.9	94.7	95.0
HT37 (ribotype group IV)											100.0	98.8	98.8	98.8	99.1	99.1	94.7	94.5	94.8
JEM5 (ribotype group IV)												100.0	98.7	98.7	99.0	99.0	94.5	94.2	94.6
HT19 (ribotype group V)													100.0	100.0	99.0	99.0	94.8	94.6	94.9
JEM3 (ribotype group V)														100.0	99.0	99.0	94.8	94.6	94.9
HT55 (ribotype group VI)															100.0	99.4	94.9	94.7	95.0
N337 (ribotype group VI)																100.0	94.9	94.7	95.0
<i>B. afzelii</i> VS461 ^T																	100.0	99.6	99.6
<i>B. afzelii</i> HT61																		100.0	99.1
<i>B. afzelii</i> ACA1																			100.0

served were between ribotype group IV isolates HT37 and JEM5 and *B. burgdorferi* B31^T. The flagellin sequences of strains HT19 and JEM3 were identical, as were the flagellin sequences of strains KL10 and NBSlab. The level of conservation of flagellin sequences between Japanese isolate HT61 and *B. afzelii* VS461^T was high, although the two isolates had different geographic origins.

A neighbor-joining phylogenetic tree (38) constructed on the basis of the data in the sequence similarity matrix is shown in Fig. 2. According to this tree, the *Borrelia* isolates associated with Lyme disease segregated into three clusters. The three different phylogenetic groups corresponded completely to the three *B. burgdorferi* sensu lato species. The most deeply branched group contained *B. burgdorferi* GeHo and B31^T, and a second cluster consisted of *B. afzelii* VS461^T, HT61, and

ACA1. The third group comprised all ribotype group IV, V, and VI isolates and *B. garinii* isolates. Moreover, the isolates belonging to ribotype groups IV, V, and VI were closely related to each other. These isolates formed a subgroup that branched from the cluster consisting of *B. garinii* strains.

DISCUSSION

Our RFLP ribotyping system based on a unique organization was previously evaluated by using a large number of strains from North America, Europe, and Asia (12, 14, 30). Ribotype groups I, II, and III corresponded to *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively. As determined by our typing method, both *B. garinii* and *B. afzelii* isolates were obtained from clinical specimens, *I. persulcatus* ticks, and reservoir rodents in Japan. However, many Japanese isolates produced unique RFLP patterns that were distinct from those of North American and European isolates. Therefore, we placed these organisms in groups designated ribotype groups IV to VI. *B. garinii* and the latter three ribotype groups could also be distinguished on the basis of their sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns, the results of Western blotting (immunoblotting) in which monoclonal antibodies specific to outer surface protein A were used, and the results of pulsed-field gel electrophoresis (13, 15, 29). The results of a PCR amplification analysis in which we used primer sets BB, BG, and VS, which are specific for *Borrelia* species, confirmed the results of our RFLP ribotyping analysis.

The results of the PCR amplification analysis also demonstrated that the three new ribotype groups and *B. garinii* are more closely related to each other than *B. burgdorferi* or *B. afzelii* is to either of these groups, since the DNAs of some isolates classified as members of ribotype groups IV, V, and VI were amplified when the *B. garinii*-specific primer set was used. However, the results of PCR amplification experiments demonstrated that the primer positions of the 16S rRNA gene sequence substituted are in some ribotype group IV and VI isolates. The genetic similarity between *B. garinii* and ribotype groups IV to VI is also supported by the results of a recent report (9). We determined the *OspC* gene sequences of isolates, and the deduced amino acid sequence alignment clearly

FIG. 2. Phylogenetic tree of *Borrelia* species isolates. The phylogenetic tree was constructed as described in the text by using nearly complete flagellin gene sequences. Bar = 2% difference between sequences, as determined by measuring the length of the horizontal lines connecting two isolates.

revealed that the *B. garinii*-specific sequence motif was fully conserved in all of the isolates belonging to ribotype groups IV, V, and VI, while overall the OspC sequences were quite variable. However, the ribotype group IV, V, and VI isolates could not be assigned to a *Borrelia* species.

Our findings and other findings indicated that *B. garinii* is the most heterogeneous *Borrelia* species (5, 14, 30, 37, 41, 42). However, the levels of DNA relatedness between *B. garinii* and ribotype group IV, V, and VI isolates were more than 70%. Postic et al. (37) have reported that ribotype group IV strains Ip89 and NT29 exhibit high levels of DNA relatedness with the type strain of *B. garinii*. Whereas the ribotype group IV, V, and VI isolates are more closely related to each other than to European type strain 20047, DNA-DNA hybridization results allowed us to conclude that all of the ribotype group IV to VI isolates belong to *B. garinii*. A phylogenetic analysis in which flagellin gene sequences were used clearly revealed that the representatives of ribotype groups IV to VI are all closely related to each other and belong to *B. garinii*. Interestingly, the phylogenetic tree showed that the cluster containing the ribotype group IV to VI isolates was slightly branched from the cluster containing typical *B. garinii* strains. This suggested that the ribotype group IV to VI isolates seem to have diverged from *B. garinii*. In this study, strains KL10 and NBSlab, which were previously classified in *B. burgdorferi*, were clearly assigned to *B. garinii*.

Most spirochete isolates classified as members of ribotype groups IV to VI were detected in *I. persulcatus* ticks collected in various localities of northern Japan, and ribotype group IV isolates were the predominant isolates in human infections in Japan (12, 27, 33). It has been generally assumed that spirochetes are maintained in locations where Lyme disease is endemic by vector-reservoir transmission cycles involving ticks belonging to the *Ixodes ricinus* species complex (6, 7, 20). Our previous results showed that there are some differences between the spirochetes isolated in Europe and the spirochetes isolated in Japan (12, 15, 32). Therefore, our results showed that the ribotype group IV, V, and VI isolates confirmed the genomic diversity of *B. garinii* and are maintained in their own transmission cycle of spirochetes together with wildlife reservoirs. Our findings also suggest that the spirochetes classified as members of ribotype groups IV, V, and VI evolved within the framework of *B. garinii* and have adapted to Asiatic habitats and that there appeared to be sufficient ecological diversity that future bacterial speciation could occur. We have studied only a few European isolates; therefore, in the future we will investigate whether subtypes similar to ribotype groups IV to VI occur outside Japan.

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