Phylogenetic Analysis of *Borrelia* Species Based on Flagellin Gene Sequences and Its Application for Molecular Typing of Lyme Disease *Borreliae*

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We determined almost complete flagellin gene sequences of various *Borrelia* species and aligned them with previously published sequences. A neighbor-joining phylogenetic analysis showed that the genus *Borrelia* was divided into the following three major clusters: New World relapsing fever borreliae (*Borrelia turicatae*, *Borrelia parkeri*, and *Borrelia hermsii*), Old World relapsing fever borreliae (*Borrelia crocidurae*, *Borrelia duttonii*, and *Borrelia hispanica*), and Lyme disease borreliae (*Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*). Agents of animal spirochetosis (*Borrelia coriaceae* and *Borrelia anserina*) and species of unknown pathogenicity (*Borrelia miyamotoi* and *Borrelia lonestari*) were related to relapsing fever borreliae. Although the Lyme disease borreliae, two related species (*Borrelia japonica* and *Borrelia andersonii*), and some newly described genomic groups (groups PotiB2, VS116, DN127, Hk501, and Ya501) were closely related to each other, each taxon formed an independent branch on the phylogenetic tree. The data obtained in this study indicate that the flagellin genes are useful in *Borrelia* taxonomy. To distinguish the Lyme disease borreliae from related organisms easily, we designed an oligonucleotide primer set for the flagellin gene and performed a PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. The primer set amplified an approximately 580-bp DNA fragment that included species-specific restriction sites, and *HapII*, *HhaI*, *CelII*, *HincII*, or *DdeI* digestion of the product resulted in distinctively different PCR-RFLP patterns. The PCR-RFLP typing method which we developed should facilitate rapid identification of Lyme disease borreliae and related organisms obtained from biological and clinical specimens.

A multisystem disorder that includes various inflammatory reactions of the skin, joints, heart, and central nervous system was first recognized in North America in the 1970s (60), and this disorder is now known as Lyme disease. The causative spirochete, a member of the genus *Borrelia*, was discovered in 1982 (11) and was described as a new species, *Borrelia burgdorferi*, in 1984 (33). Since then, a large number of borrelial isolates have been obtained from various species of ticks and vertebrates, including humans (30, 54, 58, 62, 63, 66). At present, *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* have been recognized as pathogens of humans (3, 12, 33). *B. burgdorferi* sensu stricto occurs in North America and in Europe and both *B. garinii* and *B. afzelii* are widely distributed in Eurasia. Ticks belonging to the *Ixodes ricinus* species complex, *Ixodes scapularis* and *Ixodes pacificus* in North America and *I. ricinus* and *Ixodes persulcatus* in Eurasia, serve as vectors which transmit the Lyme disease borreliae to humans. The microorganisms are maintained in enzootic cycles that involve tick vectors and vertebrate reservoirs.

In 1993, *Borrelia japonica*, which was obtained from *Ixodes ovatus* ticks in Japan, was described as a new species (34). Another new species, *Borrelia andersonii*, was recently identified after it was obtained from *Ixodes dentatus* ticks in North America (42). Both of these new species are considered non-pathogenic for humans because they have not been isolated from humans. In addition, recent studies have shown that some *Borrelia* strains isolated from ixodid ticks are not genetically

similar to previously described *Borrelia* species. These organisms have been tentatively classified in genomic groups DN127, PotiB2, VS116, Hk501, and Ya501 (2, 19, 21, 54).

The genus *Borrelia* contains other medically important pathogens that cause relapsing fever (6, 7). Periodic fever is the main symptom caused by relapsing fever borreliae that multiply in the bloodstream. These organisms have a high mutation rate in their genes which encode the outer membrane proteins responsible for escaping the host immune response. The relapsing fever borreliae differ from the Lyme disease borreliae particularly in their arthropod vectors (6). Soft-bodied ticks (members of the family Argasidae) or lice are the biologic vectors of relapsing fever borreliae. In contrast, the vector spectrum of Lyme disease borreliae is restricted to hard-bodied ticks (members of the family Ixodidae). There are two possible exceptions to this general rule; *Borrelia miyamotoi*, which was obtained from the ixodid tick *I. persulcatus* in Japan (26), and *Borrelia lonestari*, which was obtained from the ixodid tick *Amblyomma americanum* in the United States (8), appear to be related to relapsing fever borreliae.

The diversity of *Borrelia* species is attracting a lot of scientific interest concerning microbial evolution. Phylogenetic studies of *Borrelia* species will be required to precisely identify pathogenic organisms and to better understand borrelial adaptation to arthropod vectors and vertebrate reservoirs. Various genes, such as the 16S rRNA (40, 41), outer surface protein A (OspA) (63, 65), and flagellin (2, 18, 53) genes, have been used as phylogenetic markers. We have focused on one of these genes, the flagellin gene, because it encodes the endoflagellar protein peculiar to spirochetes and its diversity is valuable for distinguishing borreliae (23, 28, 38, 53).

The endoflagellum is essential for the movement typical of spirochetes and consists of one flagellin protein, which has a

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molecular mass of 38 kDa in the relapsing fever borreliae and a molecular mass of 41 kDa in the Lyme disease borreliae (4, 48, 53, 64). A single, approximately 1-kb gene encodes the flagellin protein (28, 53). The location of the flagellin gene on megabase linear chromosomes is conserved in both Lyme disease and relapsing fever borreliae (13–15, 51, 61). In this study, we compared the flagellin gene sequences of many *Borrelia* species to evaluate the phylogenetic relationships of these organisms. This comparison revealed quantitative differences among *Borrelia* species. The sequence data also allowed us to identify the species-specific restriction sites of endonucleases and to design an oligonucleotide primer set for the PCR. A restriction fragment length polymorphism (RFLP) analysis of amplified flagellin genes was used to differentiate the Lyme disease borreliae from closely related species.

MATERIALS AND METHODS

Borrelial strains. All of the borrelial strains examined in this study are listed in Table 1. Most of these strains are associated with Lyme disease or relapsing fever. The rest are animal spirochetosis strains or strains whose pathogenicity is unknown. Borreliae were grown in 50 ml of BSKII medium (5) at 31°C for 1 to 3 weeks and were harvested by centrifugation as described previously (27). Cells of *Borrelia duttonii* 406K were kindly provided by H. Konishi and T. Nakazawa, Yamaguchi University School of Medicine, Ube, Japan. Extraction and purification of borrelial DNAs were carried out as described previously (24).

RFLP ribotyping. Borrelial DNAs were digested with *EcoRV* or *HincII*, electrophoresed in 0.7% agarose gels, and blotted onto membranes by the method of Southern (59). 23S rRNA gene fragments NP and Sty from *B. burgdorferi* sensu stricto strain B31 were labeled and used as hybridization probes as described previously (25). The other experimental conditions used in the RFLP analysis have been described in detail previously (46, 47). Ribotypes I, II, and III corresponded to *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively. Although ribotypes IV, V, and VI were previously considered unknown species, our recent studies revealed that these taxa are intraspecies variants of *B. garinii* (23). In addition, four new ribotypes, ribotypes VII (group Hk501), VIII (group Ya501), IX (*Borrelia* sp. strain Am501), and X (*B. japonica*), were created for unique borreliae found in Japan (21, 46).

Flagellin gene sequencing. The flagellin gene was amplified by PCR, purified, ligated into vector pGEM5Zf by using the pGEM-T vector system (Promega Biotech, Madison, Wis.), and introduced into competent cells of *Escherichia coli* JM109 or HB101 (Takara Shuzo Co., Ltd., Kyoto, Japan) by the method described previously (22, 23). Single-stranded recombinant DNA was extracted and was purified as described previously (20). Occasionally, double-stranded plasmid DNA was extracted and used for experiments. Nucleotide sequences were determined by using an Autoread sequencing kit and an ALFred sequencer (Pharmacia, Uppsala, Sweden). The sequencing reaction mixtures were primed with either vector-encoded primers or custom-synthesized primers as described previously (22, 23). At least two clones were sequenced for each PCR product. Sequences were aligned and a similarity matrix and neighbor-joining phylogenetic tree were constructed with a Macintosh personal computer by using the DNASTAR program (DNASTAR, Inc., Madison, Wis.) and the CLUSTAL V software package (29, 57).

PCR-RFLP analysis. PCR amplification was carried out to synthesize the partial flagellin gene sequences of borrelial strains. The following oligonucleotide primer set was used: 5'-GCA GTT CAA TCA GGT AAC GG-3' (sense primer C) and 5'-AGG TTT TCA ATA GCA TAC TC-3' (antisense primer D). Primers C and D are located at nucleotide positions 280 to 299 and 844 to 863, respectively, of the previously published sequence for the flagellin gene of *B. garinii* HT22 (23). We verified that both primers were *Borrelia* specific by using the BLAST (basic local alignment search tool) algorithm and the GenBank database. To prepare template DNA, 1 ml of a spirochete culture in BSK II medium was centrifuged, and the resulting cell pellet was rinsed twice with phosphate-buffered saline. The cells were suspended in 50 µl of 0.1% Triton X-100 and were incubated at 95°C for 10 min. The target DNA sequence was amplified in a 50-µl (total volume) reaction mixture containing 5 µl of cell supernatant, each deoxynucleotide triphosphate at a concentration of 200 µM, of the primers at a concentration of 1 µM, 1.5 U of *Taq* polymerase (Ex *Taq*; Takara), and Ex *Taq* reaction buffer. The reaction mixtures were overlaid with mineral oil and were subjected to 30 thermal cycles consisting of 94°C for 40 s, 50°C for 40 s and 72°C for 40 s. The amplified DNA was digested with *HapII*, *HhaI*, *HincII* (Takara), *CelII* (Pharmacia), or *DdeI* (TOYOBO, Osaka, Japan). The amplified products and endonuclease digests were electrophoresed in a 2.5% agarose gel (NuSieve GTG; FMC, Rockland, Maine), and the DNA bands were visualized by ethidium bromide staining. DNA ladders (100-bp ladder [100 to 1,000 bp] and 20-bp ladder [20 to 1,000 bp]; GenSura Laboratories, Del Mar, Calif.) were used as size markers.

Nucleotide sequence accession numbers. The flagellin gene sequences of *Bor-*

relia species groups, and strains which we determined in this study have been deposited in the DDBJ, EMBL, and GenBank databases under the following accession numbers: *B. garinii*, D82846; group Hk501, D82847, D82848, D85069, and D85070; group Ya501, D82849, D82850, D82851, and D85071; *B. japonica*, D82852 and D82853; group VS116, D82854; *Borrelia* sp. strain Am501, D82855; group PotiB2, D82856; group DN127, D82857 and D82858; *B. andersonii*, D83762, D83763, and D83764; *B. duttonii*, D82859; *B. miyamotoi*, D82860; *Borrelia hermsii*, D82861; *Borrelia turicatae*, D82862; *Borrelia parkeri*, D82863; and *Borrelia coriaceae*, D82864. In addition, previously published sequences for *B. garinii* (accession number D63373), *B. afzelii* (D63365 and D63366), and *B. miyamotoi* (D43777) and other sequences for *B. burgdorferi* sensu stricto (X16933, X75200, and X56334), *B. garinii* (X75203), *B. afzelii* (X75202), *Borrelia* sp. strain Spain (U28499), *Borrelia hispanica* (U28498), *Borrelia crocidurae* (X75204 and U28496), *B. lonestari* (U26704), *B. hermsii* (M33839), and *Borrelia anserina* (X75201) were utilized in this study.

RESULTS AND DISCUSSION

Comparison of flagellin gene sequences. Almost complete sequences of the flagellin genes of 38 *Borrelia* strains (Table 2) were used to analyze the intrageneric relationships of the organisms quantitatively. Nucleotide sequence similarity values were calculated by using the CLUSTAL V method (29). The similarity matrix revealed great variation and the levels of sequence variability ranged from 73.7 to 100%. The similarity values within each species values were high. The values for the Lyme disease borreliae (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*), the recently described species *B. japonica* and *B. an-*

dersonii, and related genomic groups DN127, PotiB2, VS116, Hk501, and Ya501 were more than 90%. When these organisms were compared with relapsing fever borreliae (*B. turicatae*, *B. parkeri*, *B. hermsii*, *B. crocidurae*, *B. duttonii*, and *B. hispanica*), the similarity values were lower about 80%. Organisms which cause animal spirochetosis (*B. coriaceae* and *B. anserina*) and species whose pathogenicity is unknown (*B. miyamotoi* and *B. lonestari*) exhibited higher levels of similarity with the relapsing fever borreliae than with the Lyme disease borreliae.

Phylogeny of Lyme disease and relapsing fever borreliae. On the basis of the sequence similarity values, we selected representative strains and constructed a neighbor-joining phylogenetic tree (Fig. 1). This tree showed the intrageneric relationships of *Borrelia* species. All of the strains examined branched independently, and the branches could be separated into three major clusters (designated clusters A, B, and C). The Lyme disease borreliae (*B. burgdorferi* sensu stricto strain B31, *B. garinii* Ip90, and *B. afzelii* VS461) could be distinguished from all other species and clustered tightly gathered in cluster C. Clusters A and B consisted mainly of relapsing fever borreliae, and these clusters reflected the geographic distribution of the organisms. Cluster B contained the Old World tick-borne relapsing fever borreliae and *Borrelia* sp. strain Spain. In this cluster, *B. duttonii* 406K was closely related to *B. crocidurae* (the similarity values ranged from 99.6 to 98.4%). The taxonomic status of *Borrelia* sp. strain Spain is unknown; however, our phylogenetic tree suggests that this strain is associated with *B. hispanica*. Cluster A comprised the New World tick-borne relapsing fever borreliae and animal spirochetosis agents. Interestingly, this cluster included the strains of *B. miyamotoi* and *B. lonestari*.

Other phylogenetic studies based on 16S rRNA sequences (8, 26) have demonstrated that *B. miyamotoi* and *B. lonestari* are related to relapsing fever borreliae. Until recently, it has generally been assumed that Lyme disease borreliae utilize a relatively limited number of ticks of the family Ixodidae as their biologic vectors. In contrast, relapsing fever borreliae have been obtained from ticks of the family Argasidae and lice. Nevertheless, both *B. miyamotoi* and *B. lonestari* were obtained from ixodid ticks. These exceptional *Borrelia* species are im-

TABLE 1. Origins of *Borrelia* strains examined in this study and results of ribotyping and flagellin typing analyses

Taxon	Strain	Source	Geographical origin	Ribo-type	Sizes (bp) of amplified flagellin gene fragments after digestion with:					Flagellin type or subtype	Accession no. of flagellin gene sequence
					HapII	HhaI	CelII	HincII	DdeI		
<i>B. burgdorferi</i>	B31	<i>Ixodes scapularis</i>	United States	I	350, 230	NS ^a	NS	NS	340, 230	I	X16933
	SON188	<i>Ixodes pacificus</i>	United States	I	350, 230	NS	NS	NS	340, 230	I	
	IRS	<i>Ixodes ricinus</i>	Switzerland	I	350, 230	NS	NS	NS	340, 230	I	
	G39/40	<i>Ixodes scapularis</i>	United States	I	350, 230	NS	NS	NS	340, 230	I	
	212	<i>Ixodes ricinus</i>	France	I	350, 230	NS	NS	NS	340, 230	I	
	HB19	Patient	United States	ND ^b	ND	ND	ND	ND	ND	I	X75200
	NCH-1	Patient	United States	I	350, 230	NS	NS	NS	340, 230	I	
	GeHo	Patient	Germany	I	350, 230	NS	NS	NS	340, 230	I	X56334
	297	Patient	United States	I	350, 230	NS	NS	NS	340, 230	I	
<i>B. garinii</i>	20047	<i>Ixodes ricinus</i>	France	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	D82846
	G25	<i>Ixodes ricinus</i>	Sweden	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	HT17	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	D63363
	PBi	Patient	Germany	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	JEM12	Patient	Hokkaido, Japan	IIb	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	HT64	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IIc	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	JEM11	Patient	Hokkaido, Japan	IIc	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	HT22	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IVa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	D63367
	K220	<i>Ixodes persulcatus</i>	Nagano, Japan	IVa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	JEM7	Patient	Hokkaido, Japan	IVa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	JEM8	Patient	Hokkaido, Japan	IVa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	JEM9	Patient	Hokkaido, Japan	IVa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	KFU	Patient	Nagano, Japan	IVa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	HT7	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IVb	NS	400, 180	NS	450, 130	330, 150, 70	IIa	D63368
	Ip89	<i>Ixodes persulcatus</i>	Russia	IVb	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	JEM2	Patient	Hokkaido, Japan	IVb	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	12-92	Patient	Nagano, Japan	IVb	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	HT55	<i>Ixodes persulcatus</i>	Hokkaido, Japan	VI	NS	400, 180	NS	450, 130	330, 150, 70	IIa	D63373
	N337	<i>Ixodes persulcatus</i>	Hokkaido, Japan	VI	NS	400, 180	NS	450, 130	330, 150, 70	IIa	D63374
	T25	<i>Ixodes ricinus</i>	Germany	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	TN	<i>Ixodes ricinus</i>	Germany	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	PBr	Patient	Germany	IIa	NS	400, 180	NS	450, 130	330, 150, 70	IIa	
	HT19	<i>Ixodes persulcatus</i>	Hokkaido, Japan	V	NS	400, 180	NS	450, 130	330, 170, 70	IIb	D63371
	N345	<i>Ixodes persulcatus</i>	Hokkaido, Japan	V	NS	400, 180	NS	450, 130	330, 170, 70	IIb	
	U079	<i>Ixodes persulcatus</i>	Nagano, Japan	V	NS	400, 180	NS	450, 130	330, 170, 70	IIb	
	JEM3	Patient	Hokkaido, Japan	V	NS	400, 180	NS	450, 130	330, 170, 70	IIb	D63372
	Ip90	<i>Ixodes persulcatus</i>	Russia	IIa	NS	400, 180	NS	NS	330, 150, 70	IIc	X75203
	JEM4	Patient	Hokkaido, Japan	IIa	NS	400, 180	NS	NS	330, 150, 70	IIc	D63364
	PD89	Patient	People's Republic of China	IIa	NS	400, 180	NS	NS	330, 150, 70	IIc	
	HT15	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IVa	320, 260	400, 180	NS	450, 130	330, 150, 70	IIc	
	K241	<i>Ixodes persulcatus</i>	Nagano, Japan	IVa	320, 260	400, 180	NS	450, 130	330, 150, 70	IIc	
	JEM1	Patient	Hokkaido, Japan	IVa	320, 260	400, 180	NS	450, 130	330, 150, 70	IIc	
	JEM5	Patient	Hokkaido, Japan	IVa	320, 260	400, 180	NS	450, 130	330, 150, 70	IIc	D63370
	MTU	Patient	Nagano, Japan	IVa	320, 260	400, 180	NS	450, 130	330, 150, 70	IIc	
	OHU	Patient	Nagano, Japan	IVa	320, 260	400, 180	NS	450, 130	330, 150, 70	IIc	
	F508	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IVa	370, 210	400, 180	NS	450, 130	330, 150, 70	IIe	
	JEM6	Patient	Hokkaido, Japan	IVa	370, 210	400, 180	NS	450, 130	330, 150, 70	IIe	
	HT37	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IVc	NS	400, 180	360, 220	450, 130	330, 150	IIc	D63369
	F624	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IVc	NS	400, 120, 60	NS	450, 130	330, 150, 70	IIg	
	K261	<i>Ixodes persulcatus</i>	Nagano, Japan	IVc	NS	400, 120, 60	NS	450, 130	330, 150, 70	IIg	
<i>B. afzelii</i>	HT10	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IIIa	NS	NS	380, 200	NS	310, 180	III	
	IPF	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IIIb	NS	NS	380, 200	NS	310, 180	III	
	HT61	<i>Ixodes persulcatus</i>	Hokkaido, Japan	IIIc	NS	NS	380, 200	NS	310, 180	III	D63366
	U001	<i>Ixodes persulcatus</i>	Nagano, Japan	IIc	NS	NS	380, 200	NS	310, 180	III	
	VS461	<i>Ixodes ricinus</i>	Switzerland	IIIc	NS	NS	380, 200	NS	310, 180	III	D63365
	Ip3	<i>Ixodes persulcatus</i>	Russia	IIIc	NS	NS	380, 200	NS	310, 180	III	
	ACA1	Patient	Sweden	IIIc	NS	NS	380, 200	NS	310, 180	III	X75202
	UMO1	Patient	Sweden	IIIc	NS	NS	380, 200	NS	310, 180	III	
	PGau	Patient	Germany	IIIc	NS	NS	380, 200	NS	310, 180	III	
	JEM10	Patient	Hokkaido, Japan	IIIc	NS	NS	380, 200	NS	310, 180	III	
Group Hk501	Hk501	<i>Ixodes tanuki</i>	Hokkaido, Japan	VII	NS	320, 260	NS	NS	340, 230	IV	D82847
	Hk512	<i>Ixodes tanuki</i>	Hokkaido, Japan	VII	NS	320, 260	NS	NS	340, 230	IV	D85069
	OR1eR	Vole	Hokkaido, Japan	VII	NS	320, 260	NS	NS	340, 230	IV	D85070
	OR2eL	Vole	Hokkaido, Japan	VII	NS	320, 260	NS	NS	340, 230	IV	D82848

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TABLE 1—Continued

Taxon	Strain	Source	Geographical origin	Ribo-type	Sizes (bp) of amplified flagellin gene fragments after digestion with:					Flagellin type or subtype	Accession no. of flagellin gene sequence
					HapII	HhaI	CelII	HincII	DdeI		
Group Ya501	Ya501	<i>Ixodes turdus</i>	Yamagata, Japan	VIII	NS	400, 180	NS	450, 130	230, 120	V	D82849
	Ac502	<i>Ixodes turdus</i>	Aichi, Japan	VIII	NS	400, 180	NS	450, 130	230, 120	V	D82850
	Kt501	<i>Ixodes turdus</i>	Kyoto, Japan	VIII	NS	400, 180	NS	450, 130	230, 120	V	D82851
	Yh501	<i>Ixodes turdus</i>	Yokohama, Japan	VIII	NS	400, 180	NS	450, 130	230, 120	V	D85071
Group VS116	VS116	<i>Ixodes ricinus</i>	Switzerland	IX	NS	NS	NS	NS	190, 140, 120, 90	VI	D82854
<i>Borrelia</i> sp.	Am501	<i>Ixodes columnae</i>	Aomori, Japan	IX	NS	NS	NS	NS	190, 140, 120, 90	VI	D82855
<i>B. japonica</i>	HO14	<i>Ixodes ovatus</i>	Hokkaido, Japan	X	NS	350, 230	NS	NS	310, 230	VII	D82852
	HT103	<i>Ixodes ovatus</i>	Hokkaido, Japan	X	NS	350, 230	NS	NS	310, 230	VII	
	Am104	<i>Ixodes ovatus</i>	Aomori, Japan	X	NS	350, 230	NS	NS	310, 230	VII	
	NT112	<i>Ixodes ovatus</i>	Nagano, Japan	X	NS	350, 230	NS	NS	310, 230	VII	D82853
	IKA2	<i>Ixodes ovatus</i>	Shizuoka, Japan	UP ^c	NS	350, 230	NS	NS	310, 230	VII	
	Ty102	<i>Ixodes ovatus</i>	Toyama, Japan	X	NS	350, 230	NS	NS	310, 230	VII	
	Ks101	<i>Ixodes ovatus</i>	Kagoshima, Japan	X	NS	350, 230	NS	NS	310, 230	VII	
Group PotiB2	PotiB2	<i>Ixodes ricinus</i>	Portugal	UP	NS	390, 180	NS	450, 130	340, 230	VIII	D82856
Group DN127	DN127	<i>Ixodes pacificus</i>	United States	UP	NS	400, 180	NS	NS	230, 150, 120, 80	IX	D82857
	CA127	<i>Ixodes neotomae</i>	United States	UP	NS	400, 180	NS	NS	230, 150, 120, 80	IX	D82858
<i>B. andersonii</i>	19857	Rabbit	United States	UP	NS	NS	NS	NS	240, 230, 110	X	D83762
	21038	<i>Ixodes dentatus</i>	United States	UP	NS	NS	NS	NS	240, 230, 110	X	D83763
	21123	<i>Ixodes dentatus</i>	United States	ND	NS	NS	NS	NS	240, 230, 110	X	D83764
<i>Borrelia</i> sp.	Spain	Unknown		ND	ND	ND	ND	ND	ND		U28499
<i>B. hispanica</i>		<i>Ornithodoros macrocanus</i>		ND	ND	ND	ND	ND	ND		U28498
<i>B. duttonii</i>	406K	<i>Ornithodoros moubata</i>		ND	ND	ND	ND	ND	ND		D82859
<i>B. crocidurae</i>		<i>Ornithodoros erraticus</i>	West Africa	ND	ND	ND	ND	ND	ND		X75204
	ORI	<i>Ornithodoros erraticus</i>	West Africa	ND	ND	ND	ND	ND	ND		U28496
<i>B. miyamotoi</i>	HT31	<i>Ixodes persulcatus</i>	Hokkaido, Japan	UP	NS	NS	NS	NS	550		D43777
	FR64b	Woodmouse	Hokkaido, Japan	UP	NS	NS	NS	NS	550		D82860
	NB103/1	<i>Ixodes persulcatus</i>	Hokkaido, Japan	UP	NS	NS	NS	NS	550		
<i>B. lonestari</i>	Texas	<i>Amblyomma americanum</i>	United States	ND	ND	ND	ND	ND	ND		U26704
<i>B. hermsii</i>	HS1	<i>Ornithodoros hermsi</i>	United States	UP	NS	250, 70	NS	NS	330, 250		M33839
		<i>Ornithodoros hermsi</i>	United States	UP	ND ^d	ND	ND	ND	ND		D82861
<i>B. turicatae</i>		<i>Ornithodoros turicata</i>	United States	UP	—	—	—	—	—		D82862
<i>B. parkeri</i>		<i>Ornithodoros parkeri</i>	United States	UP	ND	ND	ND	ND	ND		D82863
<i>B. anserina</i>		<i>Argas persicus</i>		UP	NS	NS	NS	NS	330, 250		X75201
<i>B. coriaceae</i>	Co53	<i>Ornithodoros coriaceus</i>	United States	UP	NS	NS	330, 250	NS	250, 180, 150		D82864

^a NS, no restriction site.^b ND, not determined.^c UP, unique pattern.^d —, no PCR signal was obtained.

portant for assessing the relationship between disease organisms and vectors. The present data lead us to speculate that *B. miyamotoi*, *B. lonestari*, and the relapsing fever borreliae evolved from a common ancestor, but that only *B. miyamotoi* and *B. lonestari* became adapted to ixodid ticks.

Phylogeny of Lyme disease borreliae and their relatives. To clarify the phylogenetic positions of *Borrelia* species associated with Lyme disease, we constructed another neighbor-joining phylogenetic tree (Fig. 2). The flagellin gene-based tree showed the relationships among Lyme disease borreliae and related species. The data indicate that there is an explicit phylogenetic structure for the closely related *Borrelia* species. Although strain PotiB2 from an *I. ricinus* tick in Portugal (54) formed an individual line on the tree, the other strains occurred in three major clusters. Each strain of three species of Lyme disease borreliae formed an independent subcluster and

could be distinguished from the other subclusters. Two strains of *B. japonica* from Japanese *I. ovatus* ticks (34, 43) and *B. andersonii* from North American *I. dentatus* ticks (1) are considered nonpathogenic for humans. The strains of *B. japonica* formed a distinct subcluster connected to *B. afzelii*. The phylogenetic tree also suggests that *B. andersonii* is related to *B. burgdorferi* sensu stricto.

Several genomic groups have been isolated from ixodid ticks in various geographic regions of the world. Their pathogenicities and taxonomic positions of these organisms have not been determined. Group DN127 from North American *Ixodes neotomae* and *I. pacificus* ticks has been considered an unusual genomic group that is closely related to *B. burgdorferi* sensu stricto (9, 54). Our phylogenetic tree also suggests that group DN127 is related to *B. burgdorferi* sensu stricto. The group Ya501 strains from Japanese *Ixodes turdus* ticks (21, 45) clus-

TABLE 2. Similarity matrix for flagellin gene sequences of *Borrelia* strains

Taxon	% Similarity																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
<i>B. burgdorferi</i> B31	100	99.5	99.7	93.7	93.8	93.7	94.7	94.5	94.5	95.0	94.2	94.1	94.6	96.5	96.4	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2	96.2

FIG. 1. Phylogenetic tree for Lyme disease borreliae and relapsing fever borreliae. The tree was constructed as described in the text by using flagellin gene sequences. Bar = 2% difference between sequences, as determined by measuring the lengths of the horizontal lines connecting species.

tered in a subcluster connected to *B. garinii*. We considered this genomic group a new species on the basis of the results of whole DNA-DNA hybridization and 16S ribosomal DNA sequencing analyses (21). The results of recent epidemiologic studies suggest that birds are important reservoirs for *B. garinii* (10, 47, 52). Interestingly, all developmental stages of *I. turdus* ticks have been found on passerine birds (67). We speculate that birds play a significant role in the speciation of borreliae.

In Japan, we also obtained genomic group Hk501 from *Ixodes tanuki* ticks (19, 21, 45). It is noteworthy that the group Hk501 strains belonged to the same subcluster as European genomic group VS116 strains. Furthermore, group VS116 was closely related to *Borrelia* sp. strain Am501, although the two taxa had quite different geographic and biologic origins. Strain Am501 was isolated from *Ixodes columnae* ticks in Japan (45), and strain VS116 was isolated from an *I. ricinus* tick in Switzerland (54). Further genetic and phenotypic characterization of these strains is needed to better understand their taxonomic positions.

In conclusion on the basis of the results of our phylogenetic analysis, we concluded that the flagellin genes are very useful in *Borrelia* taxonomy. The comprehensive phylogenetic relationships that we identified allowed us to make the assumption that borreliae coevolved with tick vectors and vertebrate reservoirs under the pressure of geographic isolation. *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* have great significance for public health because of Lyme disease, although various unique *Borrelia* species have been found throughout the world. Easy methods which can be used to distinguish the Lyme disease borreliae from other *Borrelia* species are needed for diagnostic laboratories. Since our data strongly suggest that a

flagellin gene-based analytical procedure, such as PCR-RFLP, can be used to discriminate Lyme disease borreliae, we performed the following experiment.

Molecular typing of Lyme disease borreliae and their relatives. On the basis of the flagellin gene sequences of Lyme disease borreliae and related organisms, we constructed physical maps to determine species-specific restriction sites of endonucleases. As a result of these maps, we selected the enzymes *HapII*, *HhaI*, *CelII*, *HincII*, and *DdeI* for study. Then we designed a PCR primer set to amplify the partial flagellin gene. The primers were derived from the conserved regions of the Lyme disease borreliae and their relatives. The length of the expected PCR product was approximately 580 bp, and this product was located at nucleotide positions 280 to 863 in *B. garinii* HT22. The PCR products included the species-specific restriction sites.

Using the primer set and enzymes described above, we evaluated 89 *Borrelia* strains that originated from various localities by performing a PCR-RFLP analysis. To supplement the data from the PCR experiment, we also classified the strains into RFLP ribotypes. The PCR signals of 580 bp were observed for all strains of Lyme disease borreliae and related organisms. On the basis of the cleavage of the amplified products with endonucleases, the three species of Lyme disease borreliae and their relatives were clearly separated into distinct flagellin types (Table 1 and Fig. 3). Because small fragments that were less than 50 bp long could not be discerned under our experimental conditions, we ignored such fragments when the following typing scheme was created. We identified the following flagellin types: types I (*B. burgdorferi* sensu stricto), II (*B.*

FIG. 2. Phylogenetic tree for the Lyme disease borreliae and their relatives. The tree was constructed as described in the text by using flagellin gene sequences. Bar = 2% difference between sequences, as determined by measuring the lengths of the horizontal lines connecting species.

FIG. 3. PCR-RFLP patterns of amplified flagellin gene. PCR products were digested with *HapII*, *HhaI*, *HincII*, *CeiII*, or *DdeI* and were electrophoresed in a 2.5% agarose gel. The DNA bands were visualized by ethidium bromide staining. Markers, DNA ladder (100-bp ladder [100 to 1,000 bp]); B31, *B. burgdorferi* sensu stricto strain B31; 20047, *B. garinii* 20047; VS461, *B. afzelii* VS461. The flagellin (*fla*) types are indicated in parentheses.

garinii), III (*B. afzelii*), IV (group Hk501), V (group Ya501), VI (group VS116 and *Borrelia* sp. strain Am501), VII (*B. japonica*), VIII (group PotiB2), IX (group DN127), and X (*B. andersonii*). Since the PCR-RFLP patterns of the *B. garinii* strains were heterogeneous, they were divided into seven subtypes (designated subtypes Ila to IIg). Although the European strains of *B. garinii* were all subtype Ila strains, the Far East strains of *B. garinii* were scattered in the various subtypes. Among the relapsing fever borreliæ and related organisms examined, *B. miyamotoi*, *B. hermsii*, *B. anserina*, and *B. coriaceae* exhibited weak PCR signals with approximately 580 bp; however, the RFLP patterns of these organisms could be distinguished from those of the other *Borrelia* species. No positive signal was obtained for *B. turicatae*.

Many procedures, such as Western blotting (immunoblotting; serotyping), Southern blotting (ribotyping), and PCR, have been used to classify *Borrelia* species (2, 25, 31, 32, 39, 63, 65). Serotyping and ribotyping take a lot of time to complete, and specific probes are required for both methods. However, PCR primers can be synthesized elsewhere, and the PCR procedure is very rapid and simple. Various primers have been designed for Lyme disease borreliæ (36, 37, 39, 49, 53, 56), and some rRNA gene-based primer sets have been utilized to classify the species (39, 55). On the other hand, *OspA* gene- and flagellin gene-targeted PCR analyses have been used frequently to detect signals in clinical samples (35, 44). However, in an *OspA* gene-targeted PCR analysis, not all strains of Lyme disease borreliæ could be amplified (16) because the differences in the *OspA* sequences of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* are pronounced (63, 65). To overcome this defect, several species-specific primer sets have been designed (17). On the other hand, flagellin gene-targeted PCR analysis can detect borreliæ infections irrespective of differences in the causative species because the flagellin gene is highly conserved among *Borrelia* species (2, 50, 53). However, as shown in this study, minor differences in the flagellin genes can be used to differentiate *Borrelia* species. The PCR-RFLP analysis method which we developed should lead to improvements in the clinical diagnosis of Lyme disease. The use of this technique with clinical samples, such as skin and urine samples, is being studied.

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