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Lyme Disease *Borrelia* Species in Northeastern China Resemble Those Isolated from Far Eastern Russia and Japan

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Fifty-nine *Borrelia burgdorferi* sensu lato culture isolates collected from northeastern China were characterized by 5S-23S rRNA intergenic spacer restriction fragment length polymorphism (RFLP) analysis and reactivity with monoclonal antibodies (MAbs). Among 59 culture isolates, 30 (50.8%) were *Borrelia garinii* and 17 (28.8%) were *Borrelia afzelii*, 2 were mixtures composed of *B. garinii* with RFLP pattern B and *B. garinii* with pattern C, and 9 were mixtures composed of *B. garinii* and *B. afzelii*. One isolate, ChY13p, produced a unique pattern and was identified as *B. garinii* based on analyses of 16S rRNA gene sequence, flagellin PCR-RFLP typing, and MAb reactivities. No *Borrelia burgdorferi* sensu stricto or *Borrelia japonica* isolates were detected. The results indicate that Lyme disease *Borrelia* species in northeastern China resemble those of *Borrelia* isolates from far eastern Russia and Japan.

Lyme disease is a multisystemic disorder caused by infection with *Borrelia burgdorferi* sensu lato, which is transmitted by ticks of the *Ixodes ricinus* complex (1, 15). Since the etiologic agent was first isolated from *Ixodes scapularis* in 1982 (6), a large number of *B. burgdorferi* sensu lato isolates have been obtained from patients, animal reservoirs, and vector ticks from various geographic areas of the world (2, 15, 26, 33, 36). Genetically and immunologically, *B. burgdorferi* sensu lato, originally regarded as a single species (16), can be subdivided into nine species based on the reference methods for delineation of bacterial species (3, 7, 8, 10, 17, 19, 28, 34): *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, *B. andersonii*, *B. tanukii*, *B. turdae*, *B. valaisiana*, and *B. lusitaniae*. The divergence within *B. burgdorferi* sensu lato may correlate with epidemiological and clinical features of Lyme disease (2, 31, 32). *B. burgdorferi* sensu stricto is present in North America and Europe but seems to be absent in Asia (22, 26, 30). Moreover, *B. burgdorferi* sensu stricto, found in the United States and Europe, is mainly associated with arthritic forms of Lyme disease. *B. garinii* and *B. afzelii* are present in Europe and Asia: the former is frequently associated with neurological manifestations, and the latter seems to be the exclusive agent of late cutaneous lesions of acrodermatitis chronica atrophicans (Pick-Herxheimer disease), which occurs mainly in northern Europe. *B. japonica* is nonpathogenic and seems to be restricted to Japan (17, 28). A simple and useful method for assessing the genetic diversity of *Borrelia* strains associated with Lyme borreliosis that is based on restriction fragment length polymorphism (RFLP) analysis of the 5S-23S rRNA intergenic spacer amplicon has been developed (27). This method was used to confirm the nine major species defined previously and to identify an additional genomic group among the *Borrelia* strains. Several papers have described the genetic characteristics and species determination of isolates from North America, Europe, Japan, Korea (18), and Russia (20, 30). Lyme disease is also widespread in China, with endemic foci of the disease discovered and typical cases diagnosed in 11 provinces as well as the suburbs of Beijing (37). Many Lyme *Borrelia* species have been isolated in China, but few species determination studies have been published. We conducted a survey in northeastern China in May 1996. Fifty-nine *Borrelia* culture isolates were obtained from *Ixodes persulcatus* ticks and *Apodemus peninsulae* rodents. Here we report the genetic characterization and species identification of these Chinese culture isolates by RFLP analysis and sequence analysis of 5S-23S rRNA intergenic spacer, 16S rRNA sequence analysis, flagellin molecular typing, and reactivity with monoclonal antibodies (MAbs).

One hundred twenty-seven *I. persulcatus* ticks were collected by beating vegetation and two *A. peninsulae* rodents were captured by snap traps in six different areas of Yakeshi in northeastern China from the end of May 1996 to the beginning of June 1996. The midgut of each tick and the earlobe of each rodent were inoculated into BSKII medium and cultured at 31°C for 4 weeks as previously described (4, 25). Fifty-seven *Borrelia* culture isolates obtained from the ticks were designated ChY01p to ChY57p, and two *Borrelia* culture isolates obtained from the rodents were designated ChYAE1 and ChYAE2. *B. burgdorferi* sensu stricto strain B31, the *B. garinii* strains 20047, ASF, and FujiP2, the *B. afzelii* strains VS461 and NT28, *B. japonica* HO14, and *B. hermsii* HS1 were used as comparative reference strains.

The 5S-23S rRNA intergenic spacer was amplified by using primers RS1 (5′-CTGCGAGTTCGCGGGAGA-3′) and RS2 (5′-TCCTAGGCATTCACCATA-3′) (27), and RFLP analysis

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TABLE 1. 5S-23S rRNA intergenic spacer RFLP patterns of Chinese *Borrelia* culture isolates

Species	No. of culture isolates (% of total no.)	RFLP pattern[a] from digestion by:		No. (%) of culture isolates reactive to MAb P3134 with OspA and OspB
		*Dra*I	*Mse*I	
B. garinii	6 (10.2)	B′	B	1 (1.7)
	24 (40.7)	C′	C	15 (25.4)
B. garinii (pattern B) + *B. garinii* (pattern C)	2 (3.4)	B′ + C′	B + C	1 (1.7)
B. afzelii	17 (28.8)	D′	D	0
B. garinii + *B. afzelii*	2 (3.4)	B′ + D′	B + D	0
	7 (11.9)	C′ + D′	C + D	3 (5.1)
B. garinii (tentative)	1 (1.7)	R′	R	0

[a] RFLP patterns are shown in Fig. 1, and their designations are given in the corresponding legend.

was accomplished by digestion of the PCR products with *Mse*I and *Dra*I as described previously (22). The PCR product of Chinese culture isolate ChY13p was cloned into plasmid pGEM-5Zf by using a pGEM-T vector system kit (Promega Corporation, Madison, Wis.) and sequenced with a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (The Perkin-Elmer Corporation, Norwalk, Conn.). The intergenic spacer sequence of isolate ChY13p has been assigned accession no. AB003785. The accession numbers of reference strains used in this study are as follows: strain B31, accession no. L30127; 20047, L30119; ASF, D84403; VS461, L30135; NT28, D84405; and HO14, L30128.

The 16S rRNA gene of *Borrelia* isolate ChY13p was amplified by primers 5′-GCTGGCAGTGCGTCTTAAGCATGC-3′ and 5′-GTGACGGGCGGTGTGTACAAGGCCC-3′ as described previously (12) and was sequenced as described above. Phylogenetic analyses of the 16S rRNA gene sequences were performed by the DNASTAR (Madison, Wis.) program with the CLUSTAL method (13). The 16S rRNA gene sequence of isolate ChY13p determined in this study has been assigned accession no. AB007450. The accession numbers of sequences used for phylogenetic analysis have been assigned as follows: strain B31, accession no. M88329; 20047, D67018; 935T, L39081; G1, M64311; G2, M60967; HT61, D67019; J1, L46697; IP3, M75149; HO14, L40597; IKA2, L40598; 20004, M64310; 1352, M64309; SH-2-82, M60969; and HS1, M60968. Flagellin PCR-RFLP analysis was carried out as described previously (11). The amplified DNAs were digested with *Hap*II, *Hha*I, *Hin*cII (Takara, Tokyo, Japan), *Cel*II (Boehringer GmbH, Mannheim, Germany), and *Dde*I (Toyobo, Osaka, Japan). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described before (24). The monoclonal antibodies (MAbs) used were H9724, which is reactive to the flagellin antigen (5); H5332, reactive to the outer surface protein A (OspA) (14); P62a, reactive to the 62-kDa heat shock protein (21); P3134, raised to the outer surface protein B (OspB) and cross-reactive with OspA of some isolates (23); G7, reactive to the outer surface protein C (OspC) (20); D6, specific to the 12-kDa protein of *B. garinii* (3); I.17.3, specific to the OspB of *B. afzelii* (7); and O1441b, specific to the flagellin protein of *B. japonica* (21).

Table 1 summarizes the 5S-23S rRNA intergenic spacer RFLP patterns and species identified in this study. The representative RFLP patterns observed among the 59 *Borrelia* culture isolates from northeastern China are shown in Fig. 1. The RFLP patterns found previously among nine species and one genomic group of Lyme disease-related *Borrelia* are as follows: pattern A, *B. burgdorferi* sensu stricto; patterns B and C, *B. garinii*; patterns D and N, *B. afzelii*; pattern E, *B. japonica*; pattern F, *B. valaisiana*; patterns G and H, *B. lusitaniae*; patterns L and M, *B. andersonii*; pattern O, *B. tanukii*; pattern P, *B. turdae*; and patterns I, J, and K, group DN127 (22, 27). In this study, 6 and 24 *Borrelia* culture isolates generated patterns B and C, respectively, and consequently were identified as *B. garinii*. Seventeen culture isolates generated pattern D and were identified as *B. afzelii*. One isolate, ChY13p, showed a pattern never seen before in *Borrelia* strains. We designated this RFLP pattern pattern R. No *B. burgdorferi* sensu stricto or *B. japonica* isolates were detected. Eleven *Borrelia* culture isolates (about 20%) produced unique RFLP patterns. Each of these isolates was identified as a mixture of two *Borrelia* strains based on the visible bands. The patterns for seven culture isolates were identified as mixtures of patterns C and D, those for two were identified as mixtures of patterns B and C, and those for two were identified as mixtures of patterns B and D. Patterns representing mixed culture isolates were also observed for Russian isolates (20, 26). This indicated that the *Borrelia* isolate from one tick culture, originally regarded as one isolate, may be composed of two different species or subspecies. The coinfection with two *Borrelia* species may explain the complicated manifestations related to Lyme spirochetosis.

Until now, all *Borrelia* species isolated from *I. persulcatus* were identified as either *B. garinii* or *B. afzelii*. In the present study, all 57 *Borrelia* culture isolates from *I. persulcatus* in China were also identified as either *B. garinii* or *B. afzelii*. It was considered that *I. persulcatus* was not a vector for *B. burgdorferi* sensu stricto strains and other *Borrelia* species. Furthermore, three isolates obtained from a rat and from ticks which were feeding on the rat were determined to be *B. afzelii* with pattern D, and one isolate from another rat was *B. garinii* with pattern C. This finding might support the role of rodents in maintaining Lyme *Borrelia* spp.

The representative MAb reactivities of Chinese *Borrelia* culture isolates are shown in Fig. 2. All Chinese *Borrelia* culture isolates in this study reacted with MAb H9724, which is specific to the 41-kDa flagellin protein of the genus *Borrelia*, and MAb

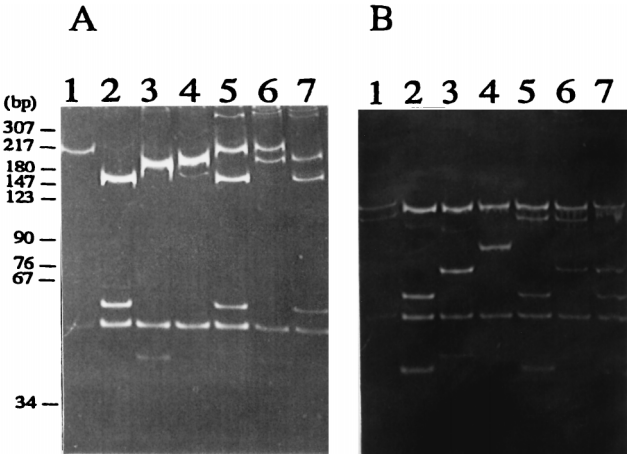

FIG. 1. Representative RFLP patterns of 5S-23S rRNA intergenic spacer observed among Chinese *Borrelia* culture isolates. The PCR products were digested by *Dra*I (A) or *Mse*I (B). DNA was electrophoresed on a 16% polyacrylamide gel and stained with ethidium bromide. The molecular size standards are indicated on the left of the gel. Lane 1, pattern B (isolate ChY02p); lane 2, pattern C (ChY50p); lane 3, pattern D (ChY55p); lane 4, pattern R (ChY13p); lane 5, patterns B and C (ChY27p); lane 6, patterns B and D (ChY28p); lane 7, patterns C and D (ChY15p).

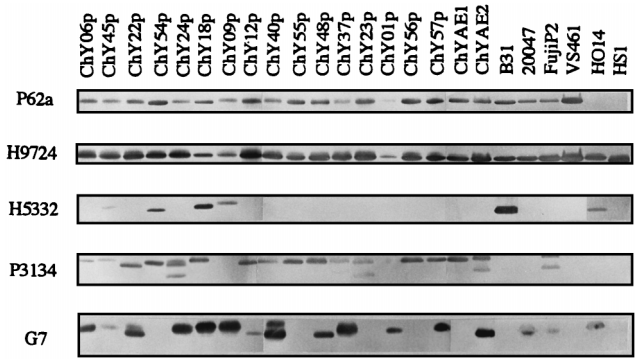

FIG. 2. Western blot analysis of Chinese *Borrelia* culture isolates with MAbs P62a, H9724, H5332, P3134, and G7. *B. burgdorferi* B31, *B. garinii* 20047, ASF, and FujiP2, *B. afzelii* VS461 and NT28, *B. japonica* HO14, and *B. hermsii* HS1 were used as comparative reference strains.

P62a, which is reactive to the 62-kDa heat shock protein of *B. burgdorferi* sensu lato but not *B. japonica*. The reference strains HS1 (*B. hermsii*) and HO14 (*B. japonica*) showed a negative reactivity with MAb P62a. Thus, there were no *B. japonica* isolates among these 59 Chinese cultures. We identified all culture isolates as *B. burgdorferi* sensu lato with genus-specific MAb G7 reactive to OspC. Five *Borrelia* culture isolates showed two OspC bands which might have resulted from mixtures of two isolates. These five culture isolates were also identified as mixtures by RFLP analysis. Twenty of 59 culture isolates showed cross-reactivity of both OspA and OspB to MAb P3134, including 15 *B. garinii* isolates with pattern C, 1 *B. garinii* isolate with pattern B, 1 isolate mixture of *B. garinii* with patterns B and C, and 3 isolate mixtures of *B. garinii* with pattern C and *B. afzelii* with pattern D (Table 1). Previous studies had reported that some *B. garinii* isolates from Japan and Russia showed cross-reactivity of both OspA and OspB with MAb P3134 (9, 17). Sequence analysis revealed that the *ospA* and *ospB* genes of these isolates share a conserved 282 bp sequence at their 3′ ends (35). To date, these isolates have been observed only in eastern Asia, not in North America or Europe.

One isolate, ChY13p, was observed to have an RFLP pattern never found before among *Borrelia* strains. To further confirm this characteristic of ChY13p, the 5S-23S rRNA intergenic spacer sequence was determined and compared with those of other representative strains (Fig. 3). ChY13p produced a 237-bp 5S-23S rRNA spacer amplicon that was similar in size to that of *B. japonica* (236 bp). Two fragments, 185 bp and 52 bp in size, were generated by digestion with *Dra*I, and three fragments, of 105, 79, and 53 bp, were observed after digestion with *Mse*I. Although the *Mse*I pattern of ChY13p was almost identical to that of *B. japonica*, the *Dra*I patterns of these strains were quite different (19). The sequence between nucleotide 73 and nucleotide 97 differed among the different *Borrelia* species. Compared with the sequences of *B. burgdorferi* sensu stricto and *B. garinii*, 10 nucleotides were missing from the sequences of *B. afzelii* VS461 and NT28 and 18 nucleotides were missing from those of ChY13 and *B. japonica*. Furthermore, ChY13p has the nucleotide sequence AAAACA, was found specifically in the sequences of *B. afzelii* with RFLP pattern D and *B. japonica*. The sequence of ChY13p showed the highest similarity to those of *B. afzelii* with pattern D and *B. japonica*. To identify the species of isolate ChY13p, the 16S rRNA gene sequence of isolate ChY13p was determined to assess the phylogenetic divergence. About 90% of the whole 16S rRNA gene sequence was aligned and compared with pre-

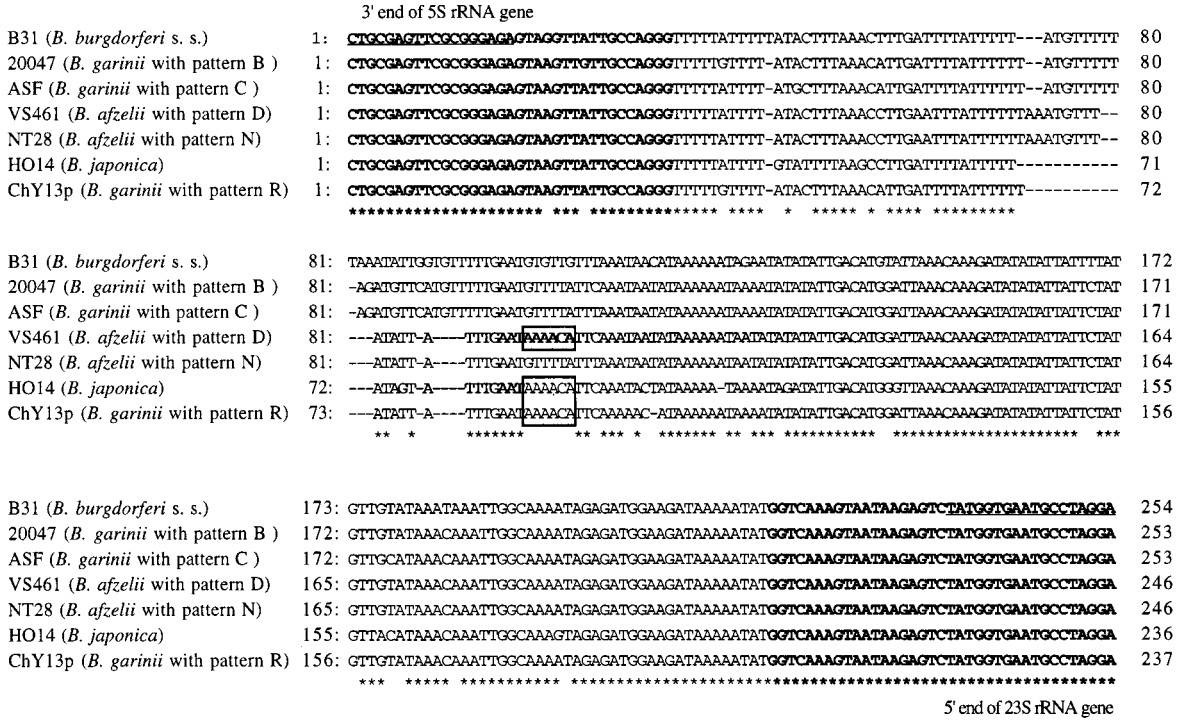

FIG. 3. Nucleotide sequence alignment of 5S-23S rRNA intergenic spacer amplicons of Chinese isolate ChY13p and the reference *Borrelia* isolates. The 3′ region of the 5S rRNA gene and the 5′ region of the 23S rRNA gene are indicated in boldface type. The corresponding primers are underlined. The asterisks and boxes indicate identical nucleotides and the motif sequence, respectively. s. s., sensu stricto.

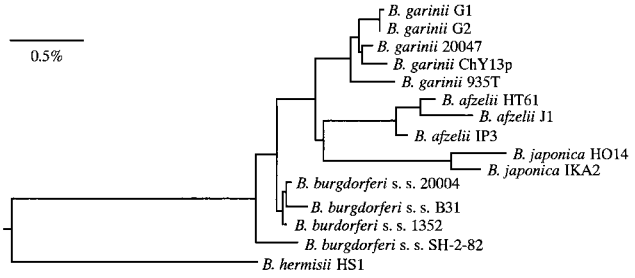

FIG. 4. Phylogenetic tree for the Lyme disease borreliae and their relatives constructed by using 16S rRNA gene sequences. Bar = 0.5% difference between sequences, as determined by measuring the length of the horizontal lines connecting two isolates. s. s., sensu stricto.

viously published sequences of *Borrelia* species. A neighbor-joining phylogenetic tree (29) was constructed on the basis of the sequence similarity matrix. Phylogenetic analysis placed the strains into a coherent cluster of the Lyme disease *Borrelia* and related genomic groups. According to this tree, ChY13p was clustered into the group of *B. garinii* strains (Fig. 4). The isolate ChY13p was further characterized by flagellin PCR-RFLP typing method. The following *Borrelia* strains have been previously determined as producing the flagellin RFLP types indicated: type I, *B. burgdorferi* sensu stricto; II, *B. garinii*; III, *B. afzelii*; IV, *B. tanukii*; V, *B. turdae*; VI, *B. valaisiana*; VII, *B. japonica*; VIII, *B. lusitaniae*; IX, group DN127; and X, *B. andersonii* (11). ChY13p produced pattern II. Western blot analysis reveals that isolate ChY13p reacted with MAb D6, which is specific to the 12-kDa protein of *B. garinii*, but was nonreactive to MAb I.17.3, which is specific to the OspB of *B. afzelii*, and MAb O1441b, which is specific to the flagellin protein of *B. japonica*. In this study, 58 of 59 *Borrelia* culture isolates were identified on the basis of 5S-23S rRNA intergenic spacer RFLP analysis. It suggests that PCR-RFLP analysis is a useful and reliable method for the species determination of Lyme disease-related *Borrelia* spp. One isolate, ChY13p, was observed to have an RFLP pattern never found before among *Borrelia* strains. Further analyses of 16S rRNA gene sequence, flagellin gene typing, and MAb reactivities identified this isolate as *B. garinii*. ChY13p showed the highest 5S-23S rRNA intergenic spacer sequence homology to *B. afzelii* with pattern D and also to *B. japonica*. It was hypothesized that *B. afzelii* and *B. japonica* seem to have evolved from *B. garinii* (27). Compared with typical *B. garinii* isolates, ChY13p might be evolutionally closer to *B. afzelii* or *B. japonica* or it may be an intermediate strain in the course of evolution.

The results indicated that Lyme disease *Borrelia* species in northeastern China resemble those isolated from far eastern Russia (20, 30) and Japan (22, 26, 27). *B. garinii* with RFLP patterns B and C and *B. afzelii* with RFLP pattern D were common in China. Particularly, *B. garinii* with RFLP pattern C, found in Japan and far eastern Russia (20) but not in Europe (22, 27), was detected with high frequency. This suggested that *B. garinii* with patterns B and C and *B. afzelii* with pattern D are commonly distributed in eastern Asia. It might provide an important basis for revealing the interrelation between the clinical manifestation of Lyme disease and *Borrelia* species. This study may also provide an important basis for developing a vaccine for strains from eastern Asia.

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