

Distribution of *Borrelia burgdorferi* Sensu Lato in China^{∇†}

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We genotyped 102 *Borrelia burgdorferi* sensu lato strains isolated from ticks, animals, and patients in 11 provinces in China by PCR–restriction fragment length polymorphism (PCR-RFLP) amplification of 5S (*rrf*)-23S (*rrl*) rRNA gene spacer amplicons and multilocus sequence analysis (MLSA). The results showed that *Borrelia garinii* was the main genotype in China (65/102) and that it was distributed mainly in northern China. *Borrelia afzelii* was the second most frequently found species (22/102), and it was distributed in both northern and southern China. All *Borrelia valaisiana* strains were isolated from Guizhou Province. Additionally, one *B. burgdorferi* strain was isolated from Hunan Province. Our results show the diversity and wide distribution of *B. burgdorferi* sensu lato in China.

Lyme disease is a multisystemic enzootic disease that is common in all temperate regions of the Northern Hemisphere. Its etiologic agent, *Borrelia burgdorferi* sensu lato, was originally thought to be homogeneous. However, many studies have demonstrated that *B. burgdorferi* sensu lato is phenotypically and genotypically heterogeneous (13, 14, 21). To date, at least 14 species of *B. burgdorferi* sensu lato have been described: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, *B. valaisiana*, *B. lusitanae*, *B. andersonii*, *B. tanukii*, *B. turdi*, *B. bissettii*, *B. sinica*, *B. spielmanii*, *B. californiensis*, and *B. carolinensis* sp. nov. (2, 5, 8, 10, 11, 20).

In China, several *B. burgdorferi* sensu lato genotyping studies have been conducted (1, 16, 19, 22) (see Table S2 in the supplemental material), but there has been no analysis of Chinese strains in different provinces. We have been conducting an epidemiological investigation of Lyme disease in China since 1986; we have collected rodents and ticks as well as patient samples from 30 provinces in China and have isolated more than 100 *Borrelia* strains (18, 22). To understand the diversity and distribution of *Borrelia burgdorferi* species in China, 102 strains from 11 provinces were genotyped by means of PCR–restriction fragment length polymorphism (PCR-RFLP) of 5S-23S ribosomal rRNA gene spacer amplicons and multilocus sequence analysis (MLSA).

MATERIALS AND METHODS

Identification of ticks. Ticks were observed under a microscope, and identification of ticks was based mainly on physical characteristics, especially the mouthpart and the shield. This work was done by Guilan Dou, who was in the Department of Vector Biology and Control, National Institute for Communicable Disease Control and Prevention, China CDC.

Collection of strains. The origins of the isolates used in this study are detailed in Table 1. In total, 102 strains were obtained from 11 provinces: 33 strains from

Jilin, 10 strains from Heilongjiang, 2 strains from Liaoning, 11 strains from Inner Mongolia (Neimeng), 15 strains from the Xinjiang Uygur Autonomous Region, 3 strains from Beijing, 1 strain from Hebei, 1 strain from Hunan, 6 strains from Chongqing, 3 strains from Guangdong, and 17 strains from Guizhou. Reference strains B31 (*B. burgdorferi* sensu stricto), 20047 (*B. garinii*), and VS461 (*B. afzelii*) were provided by R. C. Johnson and T. J. Quan. All isolates were cultured in Barbour-Stoenner-Kelly II (BSKII) medium at 33°C for 4 to 7 days, after which spirochetes were harvested by centrifugation at 12,000 × g for 30 min. The pellet was washed twice in 0.01 M phosphate-buffered saline (PBS; pH 7.4) and was finally resuspended in 1 ml of sterile PBS. The preparations were stored at –20°C until use.

DNA extraction. DNA was extracted by a modification of a method described previously (12). After a 20-min incubation at 37°C, 80 μl of 10% sodium dodecyl sulfate (SDS) was added to the preparation (10 μg in 1 ml of PBS), and the preparation was heated at 65°C for 10 min. Next, 20 μl of RNase (10 mg/ml) was added, and the solution was incubated at 37°C for 2 h. Following the addition of 10 μl of proteinase K, the preparation was incubated at 37°C for 2 h. Next, the DNA was extracted twice with equal volumes of phenol and once with an equal volume of chloroform. The DNA was precipitated by the addition of 2 volumes of absolute ethanol. The precipitated DNA was washed with 70% ethanol and was resuspended in Tris-EDTA (TE; pH 8.0).

PCR-RFLP. The 5S-23S rRNA intergenic spacer was amplified by PCR according to a previous report (7). Primers (primer 1, 5'-GCG GCA GAG TAG GTT ATT-3'; primer 2, 5'-CTA GGC ATT CAC CAT AGA CT-3') were designed with Oligo 5.0 and were synthesized by Boya Bio Company (Shanghai, China). Each reaction mixture consisted of 0.5 μl of each primer (20 μM), 1 U of *Taq* DNA polymerase, 250 μM each deoxynucleoside triphosphate (dNTP), 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 1.5 mM MgCl₂, and 1 μl of temperate DNA in a final volume of 50 μl. PCR was performed as follows: 1 min at 94°C; 35 cycles of 1 min at 94°C, 45 s at 52°C, and 45 s at 72°C; and a final extension step of 10 min at 72°C. The amplicons were analyzed in 2% agarose gels containing 0.5 μg/ml ethidium bromide. DNA bands were visualized under UV light.

The resulting amplicons were digested with the endonucleases *Mse*I (New England Biolabs) and *Dra*I (Promega) according to the manufacturers' protocols. A total of 10 μl of the PCR mixture containing the amplified fragment was digested with 5 U of either *Mse*I or *Dra*I in a total volume of 20 μl. The restriction fragments were subjected to electrophoresis on 16% (0.1 M Tris-borate-EDTA [TBE]) nondenaturing polyacrylamide gels. Next, the gel was fixed in 100 ml of a solution containing 10% ethanol and 0.5% acetic acid for 45 min and was stained with 200 ml of 0.01 M AgNO₃. After 2 h, color was developed with 150 ml of 0.75 M NaOH and 0.1 M methanol, and the reaction was stopped with 5% acetic acid.

MLSA. Seven loci—*rrs*, *hbb*, *groEL*, *recA*, *fla*, *ospA*, and the *rrf-rrl* intergenic spacer—were used for MLSA and were amplified under conditions described previously (10). All loci were amplified by a single PCR. The reaction was performed in a final volume of 50 μl, comprising 2× *Taq* PCR Master Mix (Tiangen Biotech, Beijing, China), 50 μM each primer of a primer pair, and 1 μl of temperate DNA. PCR was performed as follows: 1 min at 94°C; 35 cycles of

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TABLE 1. PCR-RFLP analysis of the 5S-23S rRNA intergenic spacers of 102 Chinese strains

Species	Patients	No. of isolates										Total	RFLP result for 5S-23S rRNA intergenic spacer ^a		
		Ticks					Rodents								
		<i>Ixodes persulcatus</i>	<i>Haemaphysalis longicornis</i>	<i>Haemaphysalis bispinosa</i>	<i>Ixodes granulatus</i>	<i>Caprolagus sinensis</i>	<i>Apodemus agrarius</i>	<i>Rattus fulvescens</i>	<i>Rattus edwardsi</i>	<i>Rattus norvegicus</i>	Total				
<i>B. burgdorferi</i>					1									1	A'
<i>B. garinii</i>	3	40				1								41	B'
<i>B. afzelii</i>	2	23										1		26	C'
<i>B. valaisiana</i>		6	2	5						1				17	D'
	5	69	2	11	1	5	1	1	1	1	1			17	F'
Total														102	

^a See references 3 and 7.

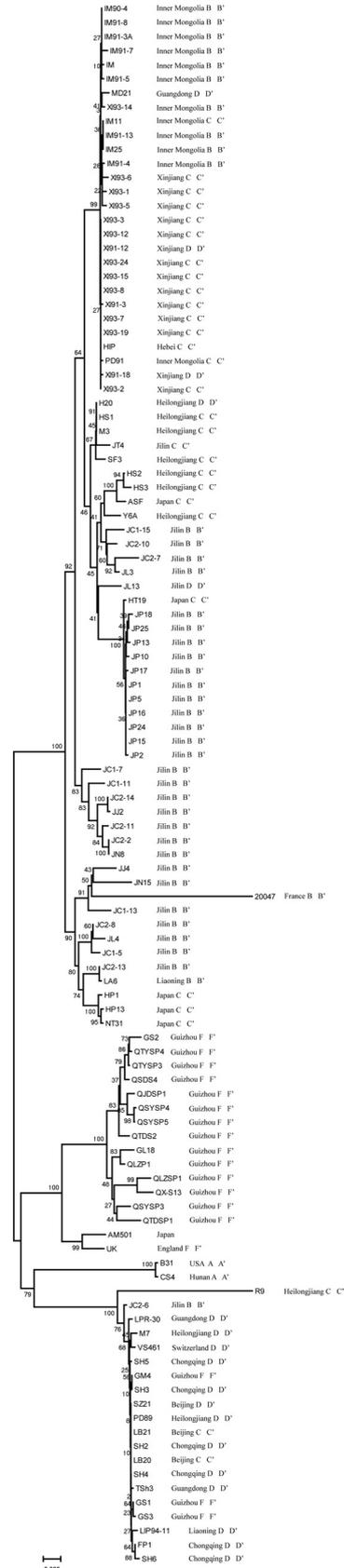


FIG. 1. Phylogenetic tree for the concatenated sequences of the seven loci.

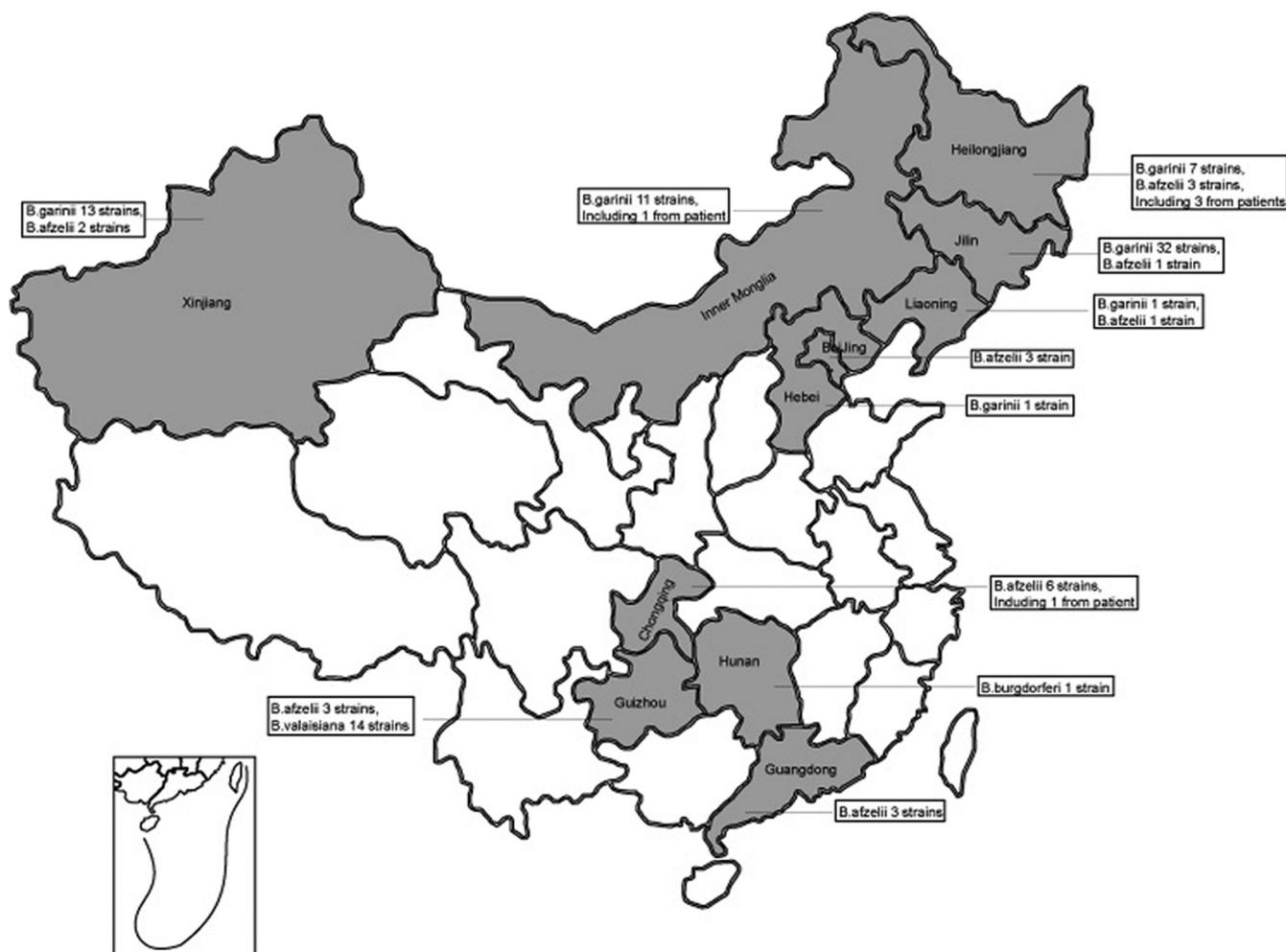


FIG. 2. Provincial distribution of *B. burgdorferi* sensu lato strains in China (inset: map of South China Sea).

1 min at 94°C, 45 s at 52°C, and 45 s at 72°C; and a final extension step of 5 min at 72°C. The products were sequenced by the BGI Company.

Sequence analysis. The CLUSTAL_X (17) algorithm was used for sequence alignments, and MEGA4 software was used for phylogenetic analyses of both individual and concatenated sequences. Distances were calculated using the neighbor-joining method.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in GenBank under the following accession numbers: *rs*, HQ433589 to HQ433694; *fla*, HQ433695 to HQ433800; *groEL*, HQ433801 to HQ433906; *hbb*, HQ433907 to HQ434012; *ospA*, HQ434013 to HQ434118; *recA*, HQ434119 to HQ434224; *rf-rr1*, HQ434225 to HQ434330.

RESULTS

PCR-RFLP analysis. The 5S-23S rRNA intergenic spacer fragment was amplified from all isolates and was digested by using both MseI and DraI. The results showed that the pattern of 1 strain isolated from a *Caprolagus sinensis* bladder in Hunan province corresponded to that of *B. burgdorferi* sensu stricto, and the pattern of 17 isolates from Guizhou province corresponded to that of *B. valaisiana*. The other patterns obtained were similar to those of *B. garinii* and *B. afzelii* (Table 1).

MLSA. In the phylogenetic tree for the concatenated sequences of the seven loci, the isolates were classified into four clusters: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B.*

valaisiana. However, the MLSA results differed slightly from those of PCR-RFLP. Two isolates (LB20 and LB21) from Beijing were distantly related to isolates from Chongqing, which belonged to the *B. afzelii* cluster. Isolate JL13 from Jilin was classified in the *B. garinii* cluster by MLSA and in the *B. afzelii* cluster by PCR-RFLP; isolates GM4, GS1, and GS2 from Guizhou were classified in the *B. afzelii* cluster by MLSA and the *B. valaisiana* cluster by PCR-RFLP (Fig. 1).

Distribution of *B. burgdorferi* sensu lato in China. Our results showed that *B. burgdorferi* was isolated only from Hunan Province and that *B. garinii* was distributed in Jilin, Heilongjiang, Liaoning, Inner Mongolia, Xinjiang, and Hebei. *B. afzelii* was distributed in Jilin, Heilongjiang, Liaoning, Xinjiang, Beijing, Chongqing, Guangdong, and Guizhou. *B. valaisiana* was distributed in Guizhou Province (Fig. 2).

DISCUSSION

A total of 102 *Borrelia burgdorferi* sensu lato strains were analyzed in this study. The distribution of these strains has geographic multiplicity, a diversity of origins, and representativeness of genospecies. Among the strains, five isolates were obtained from patients. PD91 (Inner Mongolia), Y6A (Hei-

longjiang), PD89 (Heilongjiang), and FP1 (Chongqing) were isolated from patient blood; R9 (Heilongjiang) was isolated from the cerebrospinal fluid (CSF) of a patient.

Four genospecies—*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana*—were isolated from 11 provinces in China. Almost all of the *B. garinii* strains were isolated from *Ixodes persulcatus* in northern China. *B. afzelii* strains were isolated from *I. persulcatus* in northern China and *Haemaphysalis bispinosa* in southern China. The findings indicate that *I. persulcatus* is the main vector of Lyme disease in northern China and that *H. bispinosa* may serve as one of the vectors of Lyme disease in southern China. These results are in agreement with those reported previously (16, 18, 22). According to our research and previous reports, there are two foci in China: the northern China focus and the southern China focus. The northern China focus is characterized by *I. persulcatus* as its main vector. It is also characterized by the presence of only two *Borrelia* species: *B. afzelii* and *B. garinii*. However, the southern China focus is more complex. *H. bispinosa* and *Ixodes granulatus* may serve as the main vectors, and at least three *Borrelia* species are involved: *B. afzelii*, *B. valaisiana*, and *B. sinica* (5, 15, 18, 19, 22).

Analysis of the 17 isolates from Guizhou by MLSA revealed that 14 isolates were distantly related to *B. valaisiana*; these have been named *Borrelia yangtze* sp. nov. (1). In addition, we found three isolates (GM4, GS1, and GS2) that were more related to the isolates from Chongqing and Guangdong, which were classified as *B. afzelii*. *B. afzelii* was not previously found in Guizhou Province; these findings suggest that human cases may also be present in Guizhou.

One very interesting finding of our research was the isolation of *B. burgdorferi* from *Caprolagus sinensis* in Hunan Province. *B. burgdorferi* is present in both Europe and North America (4, 6, 9). However, this species has not been found in Japan or Korea until now (3). Conversely, isolates of *B. burgdorferi* have been isolated from several different rodents in Taiwan (12). The isolate from Hunan Province is the first *B. burgdorferi* strain in the mainland of China, but additional studies are necessary to determine whether other vertebrates harbor *B. burgdorferi* in China. The identification of its vector is also of crucial importance for determining whether infection of humans by this species can occur in China.

In conclusion, our research gave a first insight into the diversity and distribution of *B. burgdorferi* strains in different provinces of China. The results also provide evidence that measures for the control and prevention of Lyme disease in China are advisable.

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