Original Article

Establishment of Multiple Locus Variable-number Tandem Repeat Analysis Assay for Genotyping of *Borrelia burgdorferi* sensu lato Detected in China*

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Abstract

Objective Human Lyme Borreliosis (LB), which is caused by *Borrelia burgdorferi* sensu lato (*B. burgdorferi*), has been identified as a major arthropod-borne infectious disease in China. We aimed to develop a multiple locus variable-number tandem repeat (VNTR) analysis (MLVA) assay for the genotyping of *Borrelia burgdorferi* strains detected in China.

Methods *B. garinii PBi* complete 904.246 kb chromosome and two plasmids (cp26 and lp54) were screened by using Tandem Repeats Finder program for getting potential VNTR loci, the potential VNTR loci were analyzed and identified with PCR and the VNTR loci data were analyzed and MLVA clustering tree were constrcted by using the categorical coefficient and the unweighted pair-group method with arithmetic means (UPGMA).

Results We identified 5 new VNTR loci through analyzing 47 potential VNTR loci. We used the MLVA protocol to analyse 101 *B. burgdorferi* strains detected in China and finally identified 51 unique genotypes in 4 major clusters including *B. burgdorferi sensu stricto* (*B.b.s.s*), *B. garinii*, *B. afzelii,* and *B. valaisiana*, consistent with the current MLSA phylogeny studies. The allele numbers of VNTR-1, VNTR-2, VNTR-3, VNTR-4, and VNTR-5 were 7, 3, 9, 7, and 6. The Hunter-Gaston index (HGI) of five VNTR loci were 0.79, 0.22, 0.77, 0.71, and 0.67, respectively. The combined HGI of five VNTR loci was 0.96. Clustering of the strains of Xinjiang, Inner Mongolia and Heilongjiang was confirmed, and this situation was consistent with the close geographical distribution of those provinces.

Conclusion The MLVA protocol esytablished in this study is easy and can show strains' phylogenetic relationships to distinguish the strains of Borrelia species. It is useful for further phylogenetic and epidemiological analyses of *Borrelia* strains.

Key words: *Borrelia burgdorferi* sensu lato; MLVA; VNTR; MLSA; Genotyping

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INTRODUCTION

Human Lyme Borreliosis (LB) is a multisystemic disseminated spirochetes disease, with extensive spectrum of symptoms, such as cutaneous erythema, arthritis, even serious neurological manifestations, and so on[1-5]. It is very common and prevalent in temperate climate regions around the world[6]. This worldwide infectious disease is transmitted to humans by Ixodidae ticks. Since *Borrelia burgdorferi* (*B. burgdorferi*) was considered as the etiology of Lyme borreliosis in 1982 and was recognized as a new species of the *Borrelia* genus in 1984[7], there have been lots of research reports on this pathogen. Up to now, 18 proposed and confirmed species of *B. burgdorferi* sensu lato have been reported[8-9]. According to reports, *B. burgdorferi* sensu stricto is found primarily in North America and Europe[5-6]. *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae* have been isolated throughout Eurasia[9]. *B. japonica*, *B. tanukii*, and *B. turdi* are found primarily in Japan[2,10]. *B. andersonii* and *B. bissettii* are distributed predominantly in North America[2,11]. Previous study also showed that there were four species in China. *B. garinii* was the main genotype in China and it was distributed mainly in northern China. *B. afzelii* was the second most frequently found species, and it was distributed in both northern and southern China[4].

As is well-known, different *B. burgdorferi* species show different distribution and cause diverse corresponding clinical symptoms or diseases[2,8-9]. And more and more *B. burgdorferi* species have been identified. In this complicated situation, *B. burgdorferi* genotyping methods are useful for identifying the outbreak pathogen rapidly and correctly, guiding clinical diagnosis and treatment reasonably and conducting further phylogenetic and epidemiological investigation, and so on[6,9].

With the development of molecular biotechnology, there were more and more typing methods which can be used to reflect the phylogenetic or evolutionary relationship for *B. burgdorferi* sensu lato. There were sequences of single gene loci analysis schemes, such as, intergenic spacer (IGS) regions, the rrs (16S rRNA) locus[11-17]. In recent years, randomly amplified polymorphic DNA (RAPD)[18], pulsed-field gel electrophoresis (PFGE)[5,7,19], multi-locus VNTR analysis (MLVA)[2], multi-locus sequence analysis (MLSA)[4] and genome-wide detection of single nucleotide polymorphisms (SNPs)[8] are widely used for the genotyping of *B. burgdorferi*. However, all of these assays have obvious limitations in identifying *B. burgdorferi* species and further phylogenetic research. Such as single gene loci genotyping shows low resolution power, PFGE genotyping is complicated with low efficiency, the repeatability of RAPD genotyping is poor, and so on[20]. Simple sequence repeats or variable-number tandem repeats (VNTRs) are believed to have a high level of discriminatory power. It stems from the significant mutability of the repeat copy number. Many genomes examined contain numerous VNTRs, and in combination, these can be used to develop a robust PCR-based marker typing system. MLVA has previously shown great discriminatory capacity and accurate estimation of genetic relationships within bacterial pathogens[2]. Therefore, in this study we focused on the establishment of a new MLVA assay for the genotyping of *B. burgdorferi* strains detected in China.

MATERIALS AND METHODS

Strains

A total of 101 *B. burgdorferi* strains used in this study were provided by department of Lyme disease in Institute for Communicable Disease Control and Prevention of Chinese Center for Disease Control and Prevention. All of the strains were collected from 12 provinces in China. The detail information about the strains was shown in Table 1.

Bacterial DNA Extraction

All isolates were grown in BSKII medium at 33 °C for 4 to 7 days until the concentration of 10^7 bacteria/mL. Then one milliliter strain liquid was harvested by centrifuging at 12,000×g for 30 min, washed with phosphate-buffered saline (pH=7.4) for 3 times, and then resuspended in 100 μL water. Boiling lysis method was used to get bacterial DNA[2]. The suspension was heated at 100 °C for 20 min, and then was stored at -20 °C until use.

Genomic Analysis and VNTR Loci

The *B. garini*i PBi complete genome was downloaded from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nuccore) and used to identify potential VNTR loci. In order to obtain potential VNTR

Table 1. Information of *Borrelia burgdorferi* sensu lato Strains

Strain Name	Allele Size (bp) at Marker Locus					Location	Sample Information	Species (MLSA)[a]
	VNTR-1	VNTR-2	VNTR-3	VNTR-4	VNTR-5			
51383	683	506	405	428	651	France	Commercial Strains	*B.garinii*
ASF	695	506	400	422	739	Japan	Apodemus	*B.garinii*
B31	683	508	396	431	769	American	Ixodes scapularis	*B.b.s.s*
CS4	684	509	-[b]	431	769	Hunan	The rabbit bladder sample	*B.b.s.s*
FP1	694	502	419	453	739	Sichuan	Neuropsychiatric disorders patient, blood	*B.afzelii*
Fuji	683	506	401	428	641	Japan	Commercial Strains	*B.garinii*
GL18	703	505	398	434	650	Guizhou	Apodemus agrarius	*B.valasiana*
GM4	693	502	419	453	740	Guizhou	Apodemus agrarius	*B.afzelii*
GS2	694	505	398	423	610	Guizhou	Ixodes granulatus	*B.valasiana*
GS3	693	502	419	453	740	Guizhou	Apodemus agrarius	*B.afzelii*
H20	681	502	399	424	737	Heilongjiang	Ixodes persulcatus	*B.garinii*
HIP	687	506	400	422	739	Hebei	Ixodes persulcatus	*B.garinii*
HP1	689	502	405	428	642	Japan	Ixodes persulcatus	*B.garinii*
HS1	680	503	400	427	738	Heilongjiang	Ixodes persulcatus	*B.garinii*
HS2	683	503	400	422	740	Heilongjiang	Ixodes persulcatus	*B.garinii*
HS3	684	503	400	422	740	Heilongjiang	Ixodes persulcatus	*B.garinii*
IM	687	503	399	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM-11	686	506	400	428	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM-25	686	506	400	428	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM90-4	687	505	400	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM91-13	687	503	399	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM91-3A	686	505	404	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM91-4	687	503	399	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM91-5	687	503	404	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM91-7	687	503	404	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
IM91-8	687	503	400	427	740	Inner Mongolia	Ixodes persulcatus	*B.garinii*
JC1-11	681	502	399	422	708	Jilin	Ixodes persulcatus	*B.garinii*
JC1-13	683	503	400	432	674	Jilin	Ixodes persulcatus	*B.garinii*
JC1-15	687	503	403	427	773	Jilin	Ixodes persulcatus	*B.garinii*
JC1-5	683	502	407	435	642	Jilin	Ixodes persulcatus	*B.garinii*
JC1-7	687	505	400	431	708	Jilin	Ixodes persulcatus	*B.garinii*
JC2-10	686	503	402	422	708	Jilin	Ixodes persulcatus	*B.garinii*
JC2-11	684	503	400	427	674	Jilin	Ixodes persulcatus	*B.garinii*
JC2-14	686	500	399	422	739	Jilin	Ixodes persulcatus	*B.garinii*
JC2-2	688	506	400	427	684	Jilin	Ixodes persulcatus	*B.garinii*
JC2-6	694	502	403	453	707	Jilin	Ixodes persulcatus	*B.afzelii*
JC2-7	683	503	400	422	741	Jilin	Ixodes persulcatus	*B.garinii*
JC2-8	683	503	411	431	642	Jilin	Ixodes persulcatus	*B.garinii*
JJ2	687	503	403	426	739	Jilin	Ixodes persulcatus	*B.garinii*
JJ4	683	503	400	431	675	Jilin	Ixodes persulcatus	*B.garinii*
JL13	684	503	400	422	741	Jilin	Ixodes persulcatus	*B.garinii*
JL3	683	503	400	422	741	Jilin	Ixodes persulcatus	*B.garinii*
JL4	683	503	406	431	642	Jilin	Ixodes persulcatus	*B.garinii*
JN15	683	505	401	431	674	Jilin	Ixodes persulcatus	*B.garinii*
JN8	689	503	400	427	740	Jilin	Ixodes persulcatus	*B.garinii*
JP1	683	502	422	427	708	Jilin	Ixodes persulcatus	*B.garinii*
JP10	683	503	422	427	708	Jilin	Ixodes persulcatus	*B.garinii*
JP13	683	503	422	427	708	Jilin	Ixodes persulcatus	*B.garinii*
JP15	683	502	422	428	708	Jilin	Ixodes persulcatus	*B.garinii*
JP16	683	503	422	428	708	Jilin	Ixodes persulcatus	*B.garinii*
JP17	683	505	422	427	708	Jilin	Ixodes persulcatus	*B.garinii*
JP2	683	503	422	427	708	Jilin	Ixodes persulcatus	*B.garinii*
JP24	683	503	422	428	708	Jilin	Ixodes persulcatus	*B.garinii*
JP25	683	503	422	427	708	Jilin	Ixodes persulcatus	*B.garinii*
JP5	683	502	426	427	708	Jilin	Ixodes persulcatus	*B.garinii*

Continued

Strain Name	Allele Size (bp) at Marker Locus					Location	Sample Information	Species (MLSA)[a]
	VNTR-1	VNTR-2	VNTR-3	VNTR-4	VNTR-5			
JT4	681	504	400	428	682	Jilin	Ixodes persulcatus	*B.garinii*
LA6	691	503	401	428	674	Liaoning	Apodemus agrarius Pallas	*B.garinii*
LB20	694	498	424	453	740	Beijing	Ixodes persulcatus	*B.afzelii*
LB21	694	498	424	453	744	Beijing	Ixodes persulcatus	*B.afzelii*
LIP94-11	694	499	424	453	740	Liaoning	Ixodes persulcatus	*B.afzelii*
LPR-30	694	499	424	453	740	Guangdong	Rattus edwardsi	*B.afzelii*
M3	680	506	400	425	737	Heilongjiang	Ixodes persulcatus	*B.garinii*
M7	694	499	424	453	740	Heilongjiang	Ixodes persulcatus	*B.afzelii*
MD21	687	502	399	427	740	Guangdong	Rattus norvegicus	*B.garinii*
NT31	689	505	401	428	641	Japan	Ixodes persulcatus	*B.garinii*
PD89	693	502	419	453	740	Heilongjiang	Neuropsychiatric disorders patient, blood	*B.afzelii*
PD91	687	506	400	431	739	Inner Mongolia	Neuropsychiatric disorders patient, blood	*B.garinii*
QLZp1	694	505	404	433	642	Guizhou	Ixodes granulatus	*B.valasiana*
QLZsp1	694	505	404	432	672	Guizhou	Ixodes granulatus	*B.valasiana*
QSDs4	694	507	398	422	616	Guizhou	Apodemus agrarius	*B.valasiana*
QSYsp3	694	507	416	432	638	Guizhou	Ixodes granulatus	*B.valasiana*
QSYsp4	692	505	398	422	639	Guizhou	Ixodes granulatus	*B.valasiana*
QSYsp5	692	505	398	422	638	Guizhou	Ixodes granulatus	*B.valasiana*
QTDs2	694	505	398	425	610	Guizhou	Rattus fulvescens	*B.valasiana*
QTYsp3	694	505	389	435	610	Guizhou	Ixodes granulatus	*B.valasiana*
QX-S13	694	505	398	425	610	Guizhou	Apodemus agrarius Pallas	*B.valasiana*
R9	694	502	424	453	744	Heilongjiang	Chronicmeningitis patient, CSF	*B.afzelii*
SF3	680	503	400	424	738	Heilongjiang	Ixodes persulcatus	*B.garinii*
SH2	693	502	419	453	740	Sichuan	Haemaphysalis bispinosa	*B.afzelii*
SH3	693	502	419	453	740	Sichuan	Haemaphysalis bispinosa	*B.afzelii*
SH4	693	502	419	453	740	Sichuan	Haemaphysalis bispinosa	*B.afzelii*
SH5	693	502	419	453	740	Sichuan	Haemaphysalis bispinosa	*B.afzelii*
SH6	693	502	419	453	740	Sichuan	Haemaphysalis bispinosa	*B.afzelii*
SZ21	694	498	424	453	744	Beijing	Haemaphysalis longicornis	*B.afzelii*
Tsh3	694	498	424	453	740	Shandong	Haemaphysalis longicornis	*B.afzelii*
XJ91-12	686	505	405	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ91-18	686	505	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ91-3	686	506	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-1	687	506	400	428	739	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-12	687	506	399	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-14	686	504	399	432	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-15	686	506	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-19	686	505	400	427	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-2	687	506	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-24	686	505	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-3	686	506	400	428	739	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-5	686	506	400	427	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-6	686	506	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-7	686	505	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
XJ93-8	686	505	400	428	740	Xinjiang	Ixodes persulcatus	*B.garinii*
Y6A	683	506	402	422	771	Heilongjiang	patient blood sample	*B.garinii*

Note. Species (MLSA)[a]: The species of *Borrelia burgdorferi* sensu lato strains referenced the results of Hao Q, et al.[4]; -[b]: lack of PCR product.

loci, we used the professional Tandem Repeats Finder program which is available at Genomes and PolyMorphismS (GPMS) web page (http://tandem.bu.edu/trf/trf.html) to screen *B. garinii PBi* complete 904.246 kb chromosome and two homologous plasmids (cp26 and lp54) which are common in *B. burgdorferi* sensu lato. All the potential loci were chosen on the basis of matches of at least 70% between the DNA sequences of the repeat units. The period size is not less than 3 bp. Then we checked the appearance of all the potential VNTR loci, which were obtained according to above standards, to ensure that all the potential VNTR loci were as common as possible in *B. burgdorferi* sensu lato. Thus, we got 47 potential VNTR loci for next study (see supplementary material).

MLVA primers were designed for 47 potential VNTR loci with the Primer Premier 5.0 program, but 12 potential VNTR loci could not obtain suitable primer results. 6 loci (reBR-V5, reBR-V9, BR-V9, BR-V5, BR-V2, BR-V7) were quoted from reference[2], BR-V9 and BR-V5 could not have good PCR amplification results, then we redesigned the primers for the two loci (reBR-V5 and reBR-V9), but only 4 VNTR loci (BR-V9, BR-V5, BR-V2, and BR-V7) showed obvious broad-spectrum amplification characteristic, so we used them in the new MLVA of this study. According to the comprehensive analysis on the results of preliminary experiment with a few strains, we finally chose 5 potential VNTR loci (see Table 2) for our next MLVA genotyping research. The details of 5 VNTR loci primers are shown in Table 2.

PCR Amplification of the VNTR Loci

Amplifications of the 5 variable loci were performed in a final volume of 50 μL. Each reaction mixture consisted of 4 μL of template DNA, 0.3 μL of each primer (100 μmol/L), 25 μL EssyTaq PCR SuperMix (TransGen Biotech Co. Ltd, Beijing, China), and 20.4 μL ddH_2O in a final volume of 50 μL. PCR was performed as follows: at 94 °C for 5 min; 35 cycles of 30 s at 94 °C, 30 s at 53 °C or 55 °C or 60 °C, and 30 s at 72 °C; and a final extension step of 5 min at 72 °C. Primers, synthesized by Sangon Biotech Co. Ltd. (Shanghai, China), were fluorescently labeled at the 5' end using 6-carboxyfluorescein (FAM). Fluorescently labeled amplicons were sequenced by

Table 2. PCR Primer Sequences of 5 VNTR Loci Used in the MLVA

Primer Name	Forward Sequence (3'-5')	Reverse Sequence (3'-5')
VNTR-1	CCAATGTTGAGCTTACAGGAT	TGTTTACTACCTTTCGCCTGT
VNTR-2	ATCACCTCCAACCTTGCCTAA	CCAGGGCACCTATTTCGTTT
VNTR-3	CAGGCACTACGCTTGAAGGC	TGGGCTTATTAGCGATGGGTT
VNTR-4	TGTGGCGTCGAAGTAGTGGC	TTGTAGCGCCATTGGTCGTG
VNTR-5	CTCCTGTCCGGGGTGTAA	ACTGACGATGCGCTGCTA

Table 3. Information of 5 VNTR Loci in the MLVA Scheme Used in this Study

Marker Locus	Repeat Motif	Locus Position[a]	Period Size (nucleotides)	Repeat Number			No. of Alleles	HGI[b]
				Borrelia (*PBi*) array	Smallest array	Largest array		
VNTR-1	CTT	CH-543996	3	5.7	4	12	7	0.79
VNTR-2	TTATAA	CH-272466	6	9.5	8	10	3	0.22
VNTR-3	AATA	LP54-11691	4	5.8	2	11	9	0.77
VNTR-4	TCT	LP54-40308	3	5	2	13	7	0.71
VNTR-5	_[c]	CH-846540	33	12.5	7	12	6	0.67
MLVA-5[d]								0.96

Note. [a]: CH indicates chromosome locus; LP indicates linear plasmid locus; [b]: Hunter-Gaston index (HGI^b)=$1-\sum[n_j(n_j-1)]/[N(N-1)](j=1\rightarrow s)$. This part was accomplished from the V-DICE web page (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl); [c]: The 33-bp repeat TATTCACAAAATAGAGATAATAGAACTGGTGGG was found; [d]: MLVA-5^d means the HGI of five combined VNTR loci=-0.96.

using fluorescence labelled dideoxynucleotide technology developed by Beijing Tianyi Huiyuan Bioscience & technology Inc.

Automated Genotyping

Fluorescently labeled PCR products were sized by using an ABI3730 Prism Big Dye Terminator (v3.1) cycle sequencing ready reaction kit (v5.0)[21-23]. The PCR products of samples were sequenced and the sequences were compared with that of *B. garinii PBi*.

Data Analysis

All data were analyzed with BioNumerics version 5.1 software. Clustering analysis was based on the categorical coefficient and UPGMA method to evaluate genetic relationships among all isolates. The discriminatory power of the typing methods was calculated using the HGI[24] and accomplished from the V-DICE web page[22].

RESULTS

5 Potential VNTR Loci

We got 47 potential VNTR loci by screening *B. garinii PBi* complete genome with the Premier 5.0 program (see supplementary material). 35 potential VNTR loci could obtain suitable primers, 5 potential VNTR loci showed high polymorphism. Others loci were quitted by their monomorphic or lower broad-spectrum amplification characteristic (Table 4). According to comprehensive analysis on the results of preliminary experiment with a few strains, finally 5 VNTR loci (named VNTR-1, VNTR-2, VNTR-3, VNTR-4, and VNTR-5) were chosen for MLVA genotyping research (Table 3) in this study.

MLVA Genotyping of 101 Chinese Strains

Five VNTR loci divided the total 101 isolates into 4 unique clusters and 51 distinct genotypes by using UPGMA analysis (Figure 1). Cluster I includes only *B. valaisiana* isolates. In this group, all strains revealed unique marker allele size combinations except GS2, QTDS2, and QX-S13, which were identical at all of the 5 marker loci. This structure of the strains in this group was simple, as all the strains were isolated from the same region (Guizhou, China). However, its HGI value reached up to 0.96. Therefore, the distribution of *B. valasiana* varied in Guizhou province. The genetic distance and the evolution relationships among these isolates were almost completely consistent with the results of MLSA study of Hao Q, et al.[4]. All *B. garinii* strains were in cluster II, which was the biggest branch of the MLVA tree, including 70 isolates and 34 genotypes (HGI=0.93). We found that the strains isolated from Xinjiang and Inner Mongolia had closer genetic distance than those from other provinces. The genotypes structure of these strains was very simple, including only 4 genotypes. This situation was consistent with the close geographical distribution of the two provinces. In addition, this group also includes the strains from Jilin province, another epidemic area in China, but the genotype structure of Jilin strains was complicated (HGI=0.9). Cluster III were *B. afzelii* strains (18 isolates) collected from eight provinces. which had six genotypes. There were 2 strains, CS4 and B31, in cluster IV, which belonged to *B. burgdorferi sensu stricto* and had two genotypes.

The MLVA research of 101 strains detected in China showed that 5 VNTR loci proved to be suitable polymorphic loci (Table 1). Five different VNTR loci showed diversity in different *B. burgdorferi* strains. The allele numbers of VNTR-1, VNTR-2, VNTR-3, VNTR-4, and VNTR-5 were 7, 3, 9, 7, and 6. The HGI of five VNTR loci were 0.79, 0.22, 0.77, 0.71, and 0.67, respectively (Table 3). The combination of the five VNTR loci showed a better genotyping capability (HGI=0.96) than any single locus. In this way, we finally got a perfect clustering result. 101 isolates could be classified into 4 groups (Group I, II, III, and IV) which were *B.valaisiana*, *B. garinii*, *B. afzelii*, and *B. burgdorferi sensu stricto* respectively (Figure 1, Figure 2). This result was consistent with the other MLSA studies of the strains detected in China[4] (Figure 1).

DISCUSSION

In this study, we totally tested and analyzed 47 potential VNTR loci. The results showed that the VNTR loci, found by Jason Farlow[2], were not suitable for the MLVA genotyping of *B. burgdorferi* sensu lato detected in China. Only 4 in 10 VNTR loci have PCR amplification products in pre-experiments. BR-V7 and BR-V9 were monomorphic, the diversity of BR-V2 and BR-V5 were very low (Table 4). Therefore, we didn't use them in final MLVA genotyping.

According to the data of the supplementary material and Table 4, we found that lots of VNTR loci

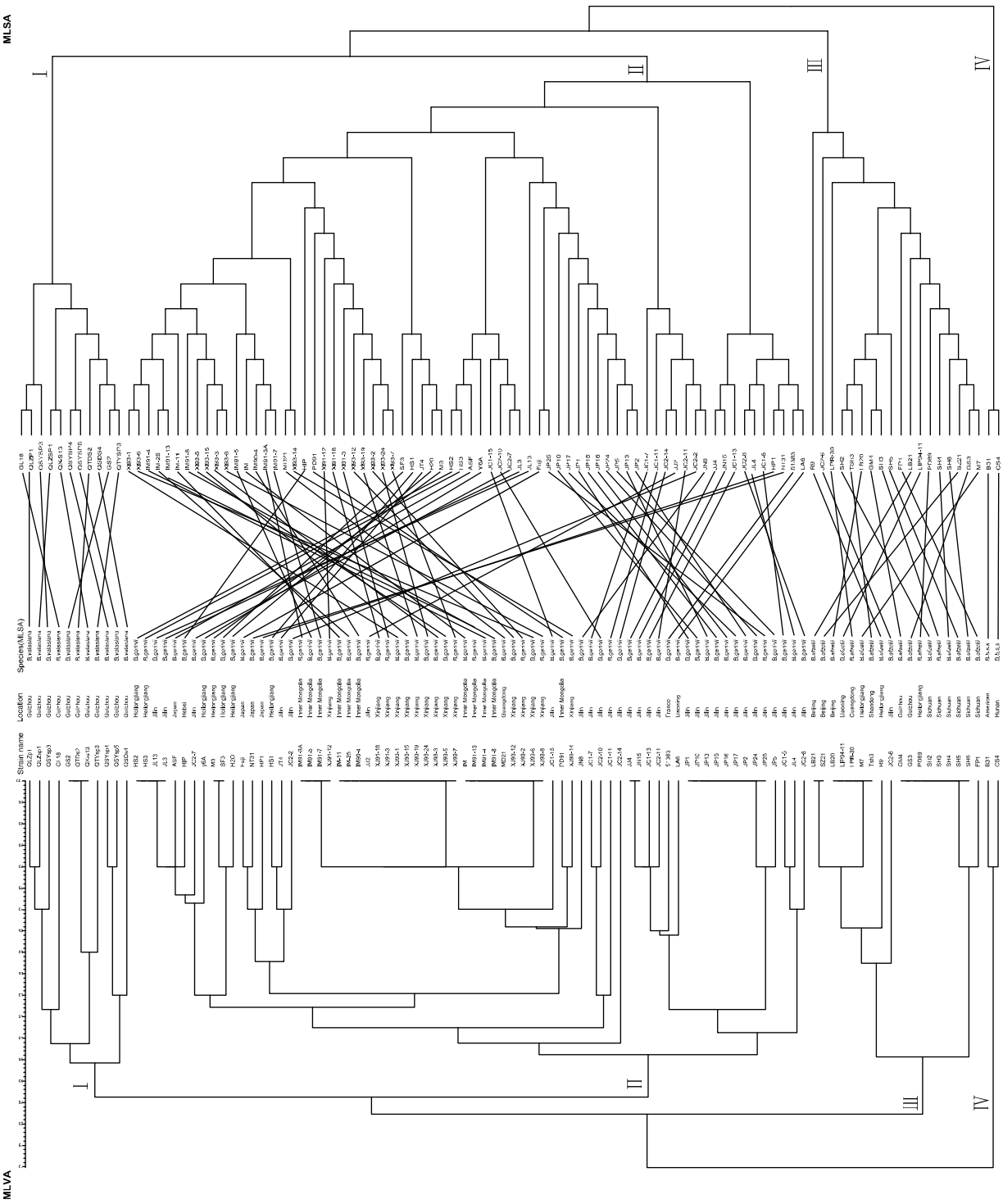

Figure 1. Genetic Relationship among Borrelia Isolates. MLVA cluster analysis result is on the left. The horizontal lines indicate genetic distances as fractions of the allelic differences. The designation to the right of each branch corresponds to the individual sample identity (Table 1), followed by the *Borrelia* species name and strains sources. The individual sample identity was the MLSA results of Hao Q, et al.[4] on the right. The correctness of MLVA clustering result was checked by MLSA. Corresponding strains between MLVA and MLSA clustering tree was connected by lines.

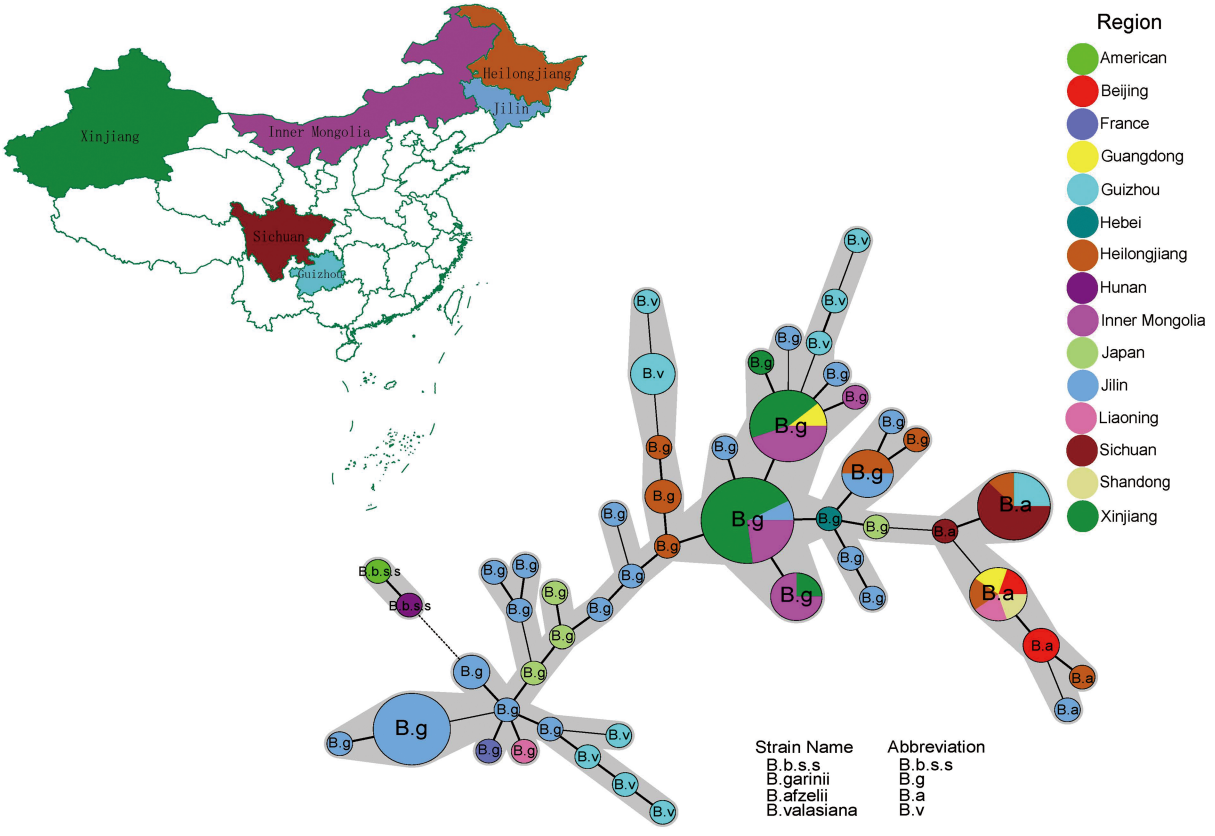

Figure 2. Minimum Spanning Tree of MLVA. This is the minimum spanning tree of MLVA. Each circle shows one genotype in the minimum spanning tree. The size of each circle represents the content of corresponding genotype. The line weight and length displays the distance between two circles. The Chinese strain source is marked on China map.

Table 4. Part of Quitted Potential VNTR Loci PCR Products Copy Number

Strains	Potential VNTR Loci Number[a]																		
	1	2	3	4	6	9	11	13	15	16	17	18	19	20	21	30	31	34	35
R9	-[b]	2	2	2	1	2	2	2	2	2	1	2	2	1	1	3	4	3	4
PD91	3	2	2	2	2	2	2	2	2	2	2	2	2	-[b]	1	3	4	4	4
FP1	-[b]	2	1	1	1	2	2	2	2	2	1	2	-[b]	1	1	3	4	3	4
CS4	-[b]	2	2	2	2	2	2	1	2	1	1	2	2	1	1	4	4	5	4
QX-S13	-[b]	1	-[b]	-[b]	1	3	2	2	2	1	2	2	2	-[b]	1	3	4	3	4
Fuji	3	2	2	2	2	2	2	2	2	2	2	2	2	1	1	3	4	3	4
M3	3	2	2	2	2	2	2	2	2	2	2	2	2	1	1	3	4	3	4
NT31	3	2	2	2	2	2	2	2	2	2	2	2	2	-[b]	1	3	4	3	4
y6A	3	2	2	2	2	2	2	2	2	2	2	1	2	1	2	3	4	3	4
ASF	3	2	2	2	2	2	2	2	2	2	2	2	1	-[b]	1	3	4	3	4
51383	4	2	2	2	2	2	2	2	2	2	2	2	2	-[b]	1	3	4	3	4
JC2-2	3	2	2	2	2	2	2	1	2	2	2	2	2	2	1	3	4	3	4
HIP	3	2	2	2	2	2	2	2	2	2	2	2	2	-[b]	1	3	4	4	4
JC2-6	3	2	2	2	2	2	2	2	2	2	2	2	2	1	1	3	4	3	4

Note. [a]: The number of potential VNTR loci in the supplementary material and Table 4 is consistent, but it is not consistent with the name of the final 5 VNTR loci. [b]: means missing data due to lack of PCR product.

which hardly showed high diversity and monomorphic characteristics as having small copy numbers and repeat size of the DNA sequence, so they were not suitable for genotyping of MLVA.

A MLVA typing assay depends on the selection of markers which individually would not indicate a relevant clustering. Single VNTR marker is either not informative enough or too variable, or show a high level of homoplasy. The 5 VNTR loci we chose respectively had a certain degree of genotyping capability as they had genetic polymorphism and a wide range of amplification capability for *B. burgdorferi* strains detected in China. Stability of MLVA is also a key factor. In pre-experiments for 15 typical strains, sequencing PCR products of 5 VNTR loci showed stable results which suggested the flanking sequence of VNTR loci would not influence the length of PCR products.

Our study showed an regular pattern which was same as that found by Farlow J, et al[2]. VNTR markers showing high HGI values, such as VNTR-1 (HGI=0.79), has great discriminatory power for identification of genetically similar strains. Less-diverse markers, such as VNTR-2 (HGI=0.22), may be applied with greater utility for species identification and analysis of evolutionary relationships[2].

Analyzing the minimum spanning tree of MLVA (Figure 2), the strains of Xinjiang, Inner Mongolia and Heilongjiang had close genetic distance, and this situation was consistent with the close geographical distribution of the three provinces. It suggested that there might be some evolutionary relationship among these starins. The strains from Jilin province was similar with three Japan typical strains and one France strain, and showed obvious genotype polymorphism. *B. afzelii* and *B. valaisiana* were mainly distributed in Sichuan and Guizhou provinces, which were adjacent to each other. suggesting there is some evolutionary relationship between the *Borrelia* strains detected in two provinces. However, the detailed evolutionary information for *Borrelia burgdorferi sensu lato* strains detected in China needs more research in future.

The species and biovars of *B. burgdorferi* sensu lato has been identified for a long time. Phenotyping methods, as serotyping, which was considered as the comparative reasonable and suitable phenotypic typing protocol, can't show current complicated evolution relationships and need more sera. Some research showed that the discriminatory power of PFGE and RAPD (randomly amplified polymorphic DNA) were high, and could be used to evaluate the genetic heterogeneity among Borrelia species[18,20,25]. However, The performance process of PFGE is complicated and the reduced capacity of RAPD can hardly provide reproducible data, which is crucial for cladistic character analysis. For species identification and evolutionary studies, conserved loci, intergenic spacer, or the region encoding the ribosomal RNAs (rRNA) have been used. The resolution of 16S rRNA or 23S rRNA was limited. Nowadays, the results of MLSA genotyping were almost acceptable.

Compared with PFGE, the MLVA assay is based on PCR technique, so it is a rapid and easy genotyping protocol for identification and discrimination of Borrelia strains. The clustering principle of MLVA is on the basis of multi-locus gene sequence polymorphism. It can provide more strain genetic information to get a more accurate clustering result than single-gene locus genotyping. In addition, MLVA can provide reproducible data to overcome the limitation of RAPD[20]. MLVA data is more easily to analyze than MLSA data by using specific software. MLVA and MLSA have consistent clustering result in some situations. MLVA is useful for further phylogenetic and epidemiological analysis of *Borrelia* strains[26].

CONCLUSION

Our study successfully established a new MLVA genotyping assay for the *Borrelia burgdorferi* strains detected in China. This assay is easy to perform and has good repeatability, and the genotyping results of the assay is reliable.

The MLVA results showed that there were four genospecies of *Borrelia* in China: *Borrelia burgdorferi sensu stricto*, *Borrelia garinii*, *Borrelia afzelii,* and *Borrelia valaisiana*. The results suggest that these *Borrelia* strains were with genetic heterogeneity and distribued in northern and southern China.

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Supplementary Material Table 1. Information of 47 Potential VNTR[c] Loci

Locus Number	Note[a]	PCR Product Size (bp)	Indices	Period Size	Copy Number	Repeat motif
1	ch	587	101175-101191	3	5.7	AAT
2	ch	536	355635-355652	7	2.6	AAAAAAT
3	ch	403	369725-369740	7	2.3	AAAAAAT
4	ch	390	833593-833608	6	2.7	TTTTTG
5	ch	488	221423-221437	6	2.5	TTTAAA
6	ch	602	325368-325382	7	2.1	ATTTTTT
7	ch	596	442357-442371	7	2.1	TTAATAT
8	ch	548	456950-456964	6	2.5	TTTTTC
9	ch	574	494815-494832	7	2.6	TTAATTT
10	ch	543	533777-533791	6	2.5	TCTTTT
11	ch	685	543996-544012	3	5.7	CTT
12	ch	566	568947-568964	9	2	TTTTTAAAA
13	ch	599	672091-672105	5	3	ATTTT
14	ch	394	746665-746679	6	2.5	TTTAAT
15	ch	321	754703-754718	7	2.3	AAAAATT
16	ch	204	776500-776514	6	2.5	TATTTT
17	ch	253	876718-876735	7	2.6	TAATTTT
18	ch	663	888341-888355	6	2.5	TTTATT
19	ch	477	890131-890145	6	2.5	TTTTAT
20	ch	558	546624-546638	6	2.5	TTTTAT
21	ch	398	826260-826315	14	3.8	ATAAATAAAAATTCT
22	ch	504	272466-272519	6	9.5	TTATAA
23	ch	502	6983-7043	14	4.4	TAAAAAAAAAATAA
24	cp26	432	9037-9059	6	3.8	TAAAAA
25	cp26	523	17379-17444	11	6	TTCAATAATTA
26	cp26	454	25387-25411	4	6	AATT
27	lp54	405	11691-11715	4	5.8	AATA
28	lp54	430	40308-40322	3	5	TCT
29	ch	776	846540-846952	33	12.5	TATTCACAAAATAGAGAT AATAGAACTGGTGGG
30	reBR-V5	324	456964-456975	3	4	AAG
31	reBR-V9	461	4234-4245	3	4	TTC
32	BR-V9	204	4234-4245	3	4	TTC
33	BR-V5	116	456964-456975	3	4	AAG
34	BR-V2	178	590956-590980	5	3	TAAAT
35	BR-V7	206	690082-690093	3	4	TGG
36	ch	-[b]	189477-189499	6	4	AATT
37	ch	-[b]	210937-210971	5.7	6	AATTTT
38	ch	-[b]	470422-470436	5	3	ATT
39	cp26	-[b]	7286-7301	2.7	6	TTTAAA
40	cp26	-[b]	23805-23839	7.2	5	AGTAA
41	cp26	-[b]	8606-8642	4	9	TAATAAAAA
42	lp54	-[b]	43987-44014	7.5	4	TTAA
43	lp54	-[b]	47673-47708	6.2	6	AATAAA
44	lp54	-[b]	12417-12455	4.8	8	AATAATA
45	lp54	-[b]	43994-44021	4.1	7	ATTAATT
46	lp54	-[b]	47673-47707	3.2	11	AATAAAAATTA
47	lp54	-[b]	46079-46161	9.2	9	CTTGACTTG

Note.[a]: CH indicates chromosome locus. LP indicates linear plasmid locus. reBR-V5, reBR-V9, BR-V9, BR-V5, BR-V2, and BR-V7 were quoted from Jason Farlow[2]. BR-V9and BR-V5 could not have good PCR amplification results, so we redesigned the primers for these two loci. [b]: means that there were not a suitable couple of primers for this locus. [c]: Not all of these potential VNTR loci were applied for genotyping of all 101 strains.