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Strain Typing of *Borrelia burgdorferi*, *Borrelia afzelii*, and *Borrelia garinii* by Using Multiple-Locus Variable-Number Tandem Repeat Analysis

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Human Lyme borreliosis (LB) is the most prevalent arthropod-borne infection in temperate climate zones around the world and is caused by *Borrelia* spirochetes. We have identified 10 variable-number tandem repeat (VNTR) loci present within the genome of *Borrelia burgdorferi* and subsequently developed a multiple-locus VNTR analysis (MLVA) typing system for this disease agent. We report here the successful application of MLVA for strain discrimination among a group of 41 globally diverse *Borrelia* isolates including *B. burgdorferi*, *B. afzelii*, and *B. garinii*. PCR assays displayed diversity at these loci, with total allele numbers ranging from two to nine and Nei's diversity (*D*) values ranging from 0.10 to 0.87. The average *D* value was 0.53 across all VNTR loci. A clear correlation exists between the repeat copy number and the *D* value ($r = 0.62$) or the number of alleles ($r = 0.93$) observed across diverse strains. Cluster analysis by the unweighted pair-group method with arithmetic means resolved the 30 observed unique *Borrelia* genotypes into five distinct groups. *B. burgdorferi*, *B. afzelii*, and *B. garinii* clustered into distinct affiliations, consistent with current 16S rRNA phylogeny studies. Genetic similarity and diversity suggest that *B. afzelii* and *B. garinii* are close relatives and were perhaps recently derived from *B. burgdorferi*. MLVA provides both phylogenetic relationships and additional resolution to discriminate among strains of *Borrelia* species. This new level of strain identification and discrimination will allow more detailed epidemiological and phylogenetic analysis in future studies.

Human Lyme borreliosis (LB) is the most prevalent arthropod-borne infection in temperate climate zones around the world and is caused by *Borrelia* spirochetes (3, 19, 30, 32). In 1996, more than 16,000 cases of LB were reported in North America, totaling 100,000 cases in a 14-year period (9, 10). *Borrelia* spirochetes are 5 to 25 μm long and 0.2 to 0.5 μm wide (24). These organisms are highly motile, microaerophilic, slow growing, and fastidious (24). Lyme disease is an inflammatory disorder characterized by the skin lesion erythema migrans (25) and the potential development of neurologic, cardiac, and joint abnormalities (24). Three *Borrelia* species frequently cause Lyme disease in humans: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (6, 19). Specific *Borrelia* species can cause distinct clinical manifestations of Lyme disease. *B. burgdorferi* can cause arthritis (2, 28). *B. garinii* is known to cause serious neurological manifestations (2, 28). *B. afzelii* causes a distinctive skin condition known as acrodermatitis chronica atrophicans (27). Each of the three *Borrelia* species causes a characteristic erythema migrans (2, 28).

The taxonomy of *B. burgdorferi* has undergone extensive revision. Ten species of *B. burgdorferi* sensu lato have been characterized and subsequently placed within the *B. burgdorferi* complex. *B. burgdorferi* sensu stricto is found primarily in North America and Europe (6, 15, 19, 33). *B. garinii*, *B. afzelii*, *B. valaisiana*, and *B. lusitaniae* have been isolated throughout Eurasia (33). *B. japonica*, *B. tanukii*, and *B. turdi* are found primarily in Japan (17, 20). *B. andersonii* and *B. bissettii* are distributed predominantly in North America (22, 31). *Ixodes scapularis*, *I. pacificus*, and *I. ricinus* are the three primary tick reservoirs for *B. burgdorferi* sensu lato (5). The tick reservoir hosts include numerous small mammal species and birds (1, 18, 26).

Strains of *B. burgdorferi* sensu lato are genetically diverse. The bacterium possesses the largest number of extrachromosomal elements, plasmids, of any known bacterial species: nine circular plasmids and 12 linear plasmids (7, 16). *Borrelia* spp. also have some of the smallest bacterial genomes (910 kb). The combined chromosome-plasmid nucleotide content is approximately 1.5 Mb. Although the *Borrelia* genome mostly evolves in a clonal way (12), *ospC* gene studies suggest that lateral transfer does exist (11, 13, 23). These genetic exchanges could be due to whole-plasmid lateral transfer or, more likely, to a gene transfer agent (11). The molecular mechanisms by which this genetic exchange occurs are unknown. The *Borrelia* genome exhibits significant genetic redundancy and carries 161 to 175 paralogous gene families (7). Such families may serve as foci for interplasmid homologous recombination. At least one linear plasmid gene is found within each of 107 gene families, creating a significant amount of redundancy and an unusually large number of pseudogenes (7). Approximately 90% of *Borrelia* plasmid genes show little similarity to genes of other bacteria (7). It is possible that these linear plasmids are in a phase of rapid evolution and undergo antigenic variation because of immune selection.

Numerous molecular techniques have recently been used to characterize *Borrelia* species, including 16S rRNA gene sequence analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blot analysis, pulsed-field gel electrophoresis, plasmid fingerprinting, randomly amplified polymor-

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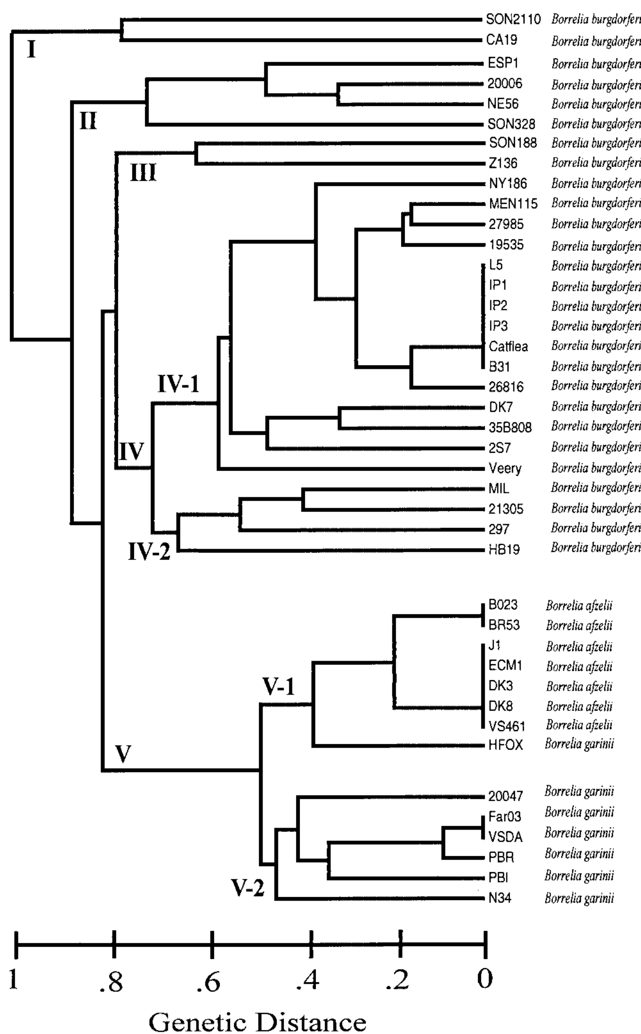

FIG. 1. Genetic relationships among *Borrelia* isolates. UPGMA cluster analysis based upon allelic differences from 10 VNTR markers across 41 *B. burgdorferi*, *B. afzelii*, and *B. garinii* isolates was used to construct this dendrogram. The designation to the right of each branch corresponds to the individual sample identity (Table 2), followed by the *Borrelia* species name. The horizontal lines indicate genetic distances as fractions of the allelic differences. Roman numerals indicate arbitrary groupings.

phic DNA analysis, restriction fragment length polymorphism analysis, fatty acid profile analysis, and serotyping (4, 8, 15, 33). For a more thorough review of the molecular typing methods used for *Borrelia* characterization, see reference 36. Although a previous study suggested that randomly amplified polymorphic DNA analysis is effective for strain discrimination within and among *Borrelia* species (35), its routine application to determine robust evolutionary relationships remains questionable because of the method's reduced capacity to provide reproducible data crucial for cladistic character analysis (33, 34). Simple sequence repeats or variable-number tandem repeats (VNTRs) have been shown to provide a high level of discriminatory power (21). This stems from the significant mutability of the repeat copy number. Many of the genomes examined contain numerous VNTRs, and in combination, these can be

TABLE 1. Sequences of *Borrelia* primers used in this study

Marker	Primer sequence	
	Forward	Reverse
BR-V1	GTTCAAGATATGGTTAAGGGCAATTTAGATAAAGATC	GAAGACTTACATGCCAGTTCATCAAGAGTC
BR-V2	GTATAATGAAGTTAGTGGGCGTTACTCTTGGGTAC	GAAACCATAAAACCATCTAAAGATACAAATCATTC
BR-V3	GTTTGTCGTTGCCAAAACTGCTTTCATAATTC	GGGATTAAATATGAAAATATATTTAGTTTGTGTGCATTATATCTGC
BR-V4	GTTTCTGCGACTAGGTATGGAACAACTAATAGCTC	GCAGTGGGCACAACTACTACTGCAATAATAACTAC
BR-V5	GCAATCCAAAATATTCAAGATCGTATAAAAATGTC	GATGATAAAATTTTCAAATGTATATCTTTTTTTAAGAAAGGC
BR-V6	GGATCGATCGTACTGTGCAGCCACAAACGTGCTGCGC	GTAGCGTACGTAGCTGCGCGTAGTATTTTTATCGTAGCGCGAGC
BR-V7	GCTTCAAAATGCTGCTTCAATTGCTGGAC	GCAAAAACACAAGCTTGCCGGTGAAAC
BR-V8	GATCTAATTCATTAAAAAATTTTGTGAAAGGGGCTTC	GATAAATAACTTGCAATATTTCCGCTTAAGGTAGTTTTC
BR-V9	GTCATCTTTAGTGTCTAATTTTAGAATTTTATTAACTTTTTCTTTGC	GTCATGCTTATATCAATGCCCTATGCCTCAAC
BR-V10	GCTTTTAACGCTAAATTATAAAGAAAAATTATTTCATTTCGGC	GTCAAAATTATGCTTCCAAAAGCATTACAATTAAAAAAATC

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TABLE 2. *Borrelia* strains used in this study

Strain	Species	Location	Source	Provider or strain
ESP1	*B. burgdorferi*	Spain	*I. ricinus*	R. C. Johnson
SON328	*B. burgdorferi*	California	*I. pacificus*	M. Janda
IP2	*B. burgdorferi*	Tours, France	Human CSF[a]	G. Baranton
SON2110	*B. burgdorferi*	California	*I. pacificus*	M. Janda
HB19	*B. burgdorferi*	Connecticut	Human blood	A. Barbour
IP1	*B. burgdorferi*	Poitiers, France	Human CSF	G. Baranton
B31	*B. burgdorferi*	New York	*I. scapularis*	ATCC 35210
ZS7	*B. burgdorferi*	Germany	*I. ricinus*	L. Gem
20006	*B. burgdorferi*	France	*I. ricinus*	J. F. Anderson
VEERY	*B. burgdorferi*	Connecticut	Veery bird	R. T. Marconi
MEN115	*B. burgdorferi*	California	*I. pacificus*	M. Janda
CA19	*B. burgdorferi*	California	*I. pacificus*	T. Schwan
19535	*B. burgdorferi*	New York	*Peromyscuc leucopus*	J. F. Anderson
MIL	*B. burgdorferi*	Slovakia	*I. ricinus*	A. Livesley
Cat flea	*B. burgdorferi*	Texas	*Ctenocephalides felis*	D. Ralph
21305	*B. burgdorferi*	Connecticut	*Peromyscuc leucopus*	J. F. Anderson
NY186	*B. burgdorferi*	New York	Human skin	R. T. Marconi
DK7	*B. burgdorferi*	Denmark	Human skin	M. Theisen
297	*B. burgdorferi*	Connecticut	Human CSF	R. C. Johnson
26816	*B. burgdorferi*	Rhode Island	*Microtus pennsylvanicus*	J. F. Anderson
SON188	*B. burgdorferi*	California	*I. pacificus*	M. Janda
IP3	*B. burgdorferi*	Pau, France	Human CSF	G. Baranton
Z136	*B. burgdorferi*	Germany	*I. ricinus*	A. Vogt
35B808	*B. burgdorferi*	Germany	*I. ricinus*	A. Schönberg
NE56	*B. burgdorferi*	Switzerland	*I. ricinus*	L. Gem
27985	*B. burgdorferi*	Shelter Island, N.Y.	*I. scapularis*	J. F. Anderson
L5	*B. burgdorferi*	Austria	Human skin	G. Stanek
DK3	*B. afzelii*	Denmark	Human skin	R. C. Johnson
BR53	*B. afzelii*	Czech Republic	*Aedes vexans*	Z. Hubalek
ECM1	*B. afzelii*	Sweden	Human skin (EM)[b]	S. Bergstrom
J1	*B. afzelii*	Japan	*I. persulcatus*	R. T. Marconi
B023	*B. afzelii*	Germany	Human skin (EM)[c]	A. Vogt
VS461	*B. afzelii*	Switzerland	*I. ricinus*	O. Peter
DK8	*B. afzelii*	Denmark	Human skin	R. C. Johnson
PBI	*B. garinii*	Germany	Human CSF	C. Kodner
VSDA	*B. garinii*	Switzerland	Human CSF	O. Peter
N34	*B. garinii*	Germany	*I. ricinus*	J. Ackerman
20047	*B. garinii*	France	*I. ricinus*	J. F. Anderson
HFOX	*B. garinii*	Japan	Fox heart	E. Isogai
PBR	*B. garinii*	Germany	Human CSF	B. Wilske
FAR03	*B. garinii*	Sweden	Seabird	S. Bergström

[a] CSF, cerebrospinal fluid.
[b] EM, erythema migrans.

used to develop a robust PCR-based marker typing system. Multiple-locus VNTR analysis (MLVA) has previously shown great discriminatory capacity and accurate estimation of genetic relationships within bacterial pathogens such as *Francisella tularensis* and *Bacillus anthracis* (14, 21). We report here the successful application of MLVA for strain discrimination among a group of 41 globally diverse *Borrelia* isolates, including *B. burgdorferi*, *B. afzelii*, and *B. garinii*. Ten VNTR loci were identified from genomic and plasmid sequences in this study. Polymorphisms at these loci were then used to resolve 30 unique genotypes into five to seven distinct groups (Fig. 1).

MATERIALS AND METHODS

Genomic analysis. The *B. burgdorferi* sensu stricto strain B31 genomic sequence was downloaded from the National Center for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?gi=132&db=Genome) and used to identify potential VNTR loci. We screened sequences from the 946-kb genome, the 12 linear plasmids, and the 9 circular plasmids of *B. burgdorferi*. Each sequence contig was screened for the presence of tandem repeats with the DNASTAR software program Genequest (Lasergene, Inc., Madison, Wis.). This program locates and displays tandemly and nontandemly repeated arrays. Confirmation of the repeated sequence structure was performed by dot plot similarity analysis with the software program Megalign (Lasergene, Inc.).

PCR amplification of VNTR loci. MLVA primers were developed around 46 potential VNTR loci with the DNASTAR program PrimerSelect. A total of 10 primer sets amplified polymorphic VNTR loci (Table 1), while 36 loci proved monomorphic. The reagents used in the PCRs were obtained from Life Technologies. Primers were designed with annealing temperatures of 65 to 61°C. Individual primer pair annealing temperatures were designed within 2°C of each other.

PCR amplification of the 10 variable loci from 41 *Borrelia* isolates was carried out with a mixture containing 2 mM $MgCl_2$; 1× PCR buffer; 0.1 mM deoxynucleoside triphosphates; 1 μM R110, R6G, or Tamra phosphoramide fluorescently labeled dUTP (Perkin-Elmer Biosystems); 0.5 U of *Taq* polymerase; 1.0 μl of template DNA; 0.5 μM forward primer; 0.5 μM reverse primer; and filtered sterile water to a volume of 12.5 μl. The reaction mixtures were incubated at 94°C for 5 min and then cycled at 94°C for 30 s, 61 or 56°C for 30 s, 72°C for 30 s, and 94°C for 30 s for 35 cycles, with a final incubation of 72°C for 5 min.

Bacterial thermolysates. *Borrelia* strains were grown in BSK medium (Sigma) until they reached 10^7 bacteria/ml. One milliliter was harvested by centrifugation, washed in phosphate-buffered saline, resuspended in 100 μl of water, and then heated at 100°C for 20 min.

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TABLE 3. VNTR marker attributes

Marker locus	Repeat motif	Genome or plasmid coordinate[a]	Repeat size (nucleotides)	Repeat number: *Borrelia* (B31) array	Repeat number: Smallest array	Repeat number: Largest array	No. of alleles[b]	D value[c]
BR-V1	Complex array	CH-844,650	CX[d]				6	0.74
BR-V2	TAAAT	CH-590,955	5	5	8	11	4	0.67
BR-V3	TA	LP17-10,530	2	5	22	29	4	0.14
BR-V4	Complex array	LP28-2-28,142	CX[e]				8	0.55
BR-V5	AAG	CH-456,964	3	4	2	4	3	0.63
BR-V6	TGA	CH-720,032	3	4	1	3	3	0.51
BR-V7	TGC	CH-690,090	3	4	13	14	2	0.1
BR-V8	—[f]	LP17-13,155	21	8.3	6	14	9	0.89
BR-V9	TTC	LP28-3-4,235	3	4	3	4	3	0.1
BR-V10	AATATTAAATA	LP54-20,145	11	5.5	1	9	7	0.75

[a] CH indicates chromosome locus; LP indicates linear plasmid locus.
[b] The average number of alleles was 4.9.
[c] $D = 1 - \Sigma(\text{allele frequency})^2$. The average D value was 0.51.
[d] CX indicates the complex nature of the repeat motif, which makes accurate array size calculation difficult. The B31 sequence at this locus consists of four tandem repeats. For example, a 32-bp motif repeated 2.2 times is listed here in the form (32 × 2.2). Other arrays that contribute to the complexity observed at this locus include the following: (32 × 3.2) + (32 × 2.0) + (41 × 2.0).
[e] (86 × 2.2) + (32 × 4.0) + (32 × 2.6).
[f] The 21-bp repeat TAATTAATATGTGATATAAAA was found.

Automated genotyping. Fluorescently labeled amplicons were sized by polyacrylamide gel electrophoresis in an ABI 377 DNA sequencer. Analysis was accomplished with the Genescan and Genotyper software (14). The PCR product was diluted threefold and mixed 1:1 with equal parts of a 5:1 formamide-dextran blue dye and size standard prior to electrophoresis. The Bioventures Rox 1000 size standard was used with filter set D.

Statistical analysis. Pairwise genetic differences among isolates were estimated with a simple matching coefficient. The clustering method used to evaluate genetic relationships was the unweighted pair-group method with arithmetic means (UPGMA) with the PAUP4a software (D. Swofford, Sinauer Associates, Inc., Publishers, Sunderland, Mass.). The diversity index (D) for each marker was calculated as $1 - \Sigma(\text{allele frequency})^2$ (37).

RESULTS AND DISCUSSION

VNTR marker identification and diversity. Our analysis of the genomic sequence of *B. burgdorferi* type strain B31 revealed 225 genomic sequence motifs that potentially represent VNTR loci. An additional 167 potential VNTR loci were identified among the plasmid sequences of *B. burgdorferi* type strain B31. We arbitrarily chose 46 repeated sequence motifs from these for MLVA. MLVA revealed that 36 were monomorphic and only 10 proved to be polymorphic loci (see Table 3) among 41 globally diverse *B. burgdorferi*, *B. afzelii*, and *B. garinii* strains (Table 2). However, all loci did not support PCR amplification. A total of 19 isolates failed to yield PCR products across markers BR-V4, -V6, -V8, and -V10 (see Table 4). Sixteen of these 19 failures occurred within plasmid-based loci (see Table 4).

Because the ultimate utility of VNTR loci lies in their diversity, we examined marker diversity by using both allele number and frequency. The allele numbers observed ranged from two (BR-V7) to nine (BR-V8) (Table 3). We observed that the larger the repeat array in strain B31, the greater the VNTR diversity ($r = 0.62$) and number of alleles ($r = 0.94$) among globally diverse strains (Fig. 2). This has also been observed previously in *F. tularensis* and *B. anthracis* (14, 21). For example, marker BR-V8 has a repeat copy number of 8.3 in type strain B31 and exhibited nine alleles (Table 3). In contrast, marker BR-V9, with a copy number of only three, exhibited only three alleles in our study (Table 3). We observed repeat motifs ranging from 2 bp for BR-V3 to 21 bp for BR-V8 (Table 3). The minimum array sizes observed across all alleles ranged from 1 (BR-V10) to 29 (BR-V3) (Table 3). D values ranged from 0.1 to 0.89, with an overall average D value

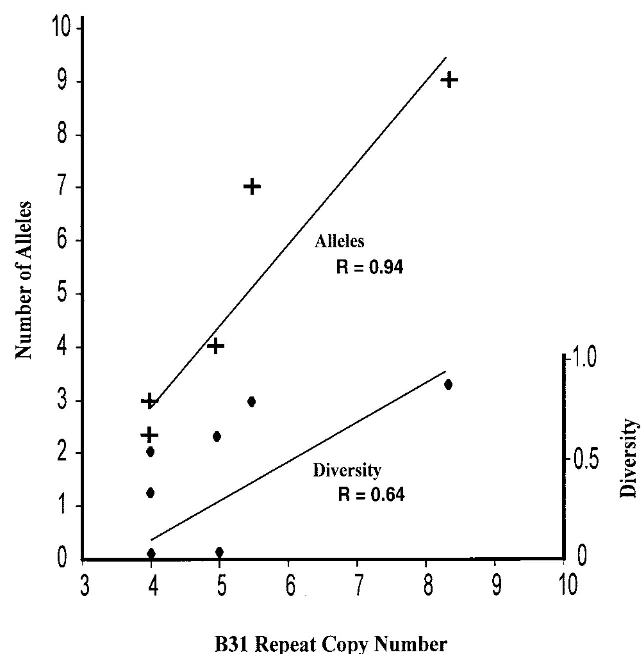

FIG. 2. Correlation between repeat copy number and D values. The *B. burgdorferi* strain B31 repeat copy number (Table 1) was compared with the D value (Pearson coefficient $r = 0.62$) and the total observed allele number (Pearson coefficient $r = 0.94$) at each marker locus. A plus sign indicates the marker's total observed allele number versus the repeat copy number at an individual marker locus. A diamond indicates the marker's calculated D value versus the repeat copy number of an individual marker. Analysis was performed with only data from the eight *Borrelia* markers with noncomplex repeat motifs.

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TABLE 4. *Borrelia* allele sizes

Strain	Allele size (bp) at marker locus:									
	BR-V1	BR-V2	BR-V3	BR-V4	BR-V5	BR-V6	BR-V7	BR-V8	BR-V9	BR-V10
SON188	706	173	144	522	116	89	206	300	201	476
NY186	800	178	144	522	116	89	206	321	204	476
MIL	750	173	144	697	116	89	206	404	204	465
MEN115	800	178	144	697	116	89	206	384	204	465
L5	800	178	144	697	116	89	206	321	204	509
IP1	800	178	144	697	116	89	206	321	204	509
IP2	800	178	144	697	116	89	206	321	204	509
ESP1	750	173	150	697	119	89	206	342	204	454
IP3	800	178	144	697	116	89	206	321	204	509
DK7	800	178	144	697	119	89	206	279	204	465
Cat flea	800	178	144	697	116	89	206	321	204	509
19535	800	178	144	697	116	—[a]	206	321	204	465
20006	750	178	144	697	119	89	206	363	204	454
B31	800	178	144	697	116	89	206	321	204	509
SON2110	750	183	144	638	116	89	204	300	204	476
SON328	750	173	144	638	119	86	206	363	204	542
CA19	750	173	144	522	116	86	204	285	204	454
297	750	173	144	802	116	86	206	454	204	465
21305	750	178	144	835	116	—	206	404	204	—
VEERY	750	178	144	—	119	89	206	321	204	520
27985	800	178	144	697	116	89	206	300	204	465
ZS7	800	178	144	642	119	89	206	300	204	454
HB19	750	178	144	608	116	89	206	300	207	465
35B808	800	173	144	697	119	89	206	300	204	465
Z136	706	173	144	697	116	89	206	384	204	608
26816	800	178	144	697	116	89	206	342	204	509
NE56	750	183	154	697	119	89	206	363	204	454
B023	750	168	144	697	113	68	206	—	204	465
J1	706	168	144	697	113	68	206	—	204	465
ECM1	706	168	144	697	113	—	206	—	204	—
DK3	706	168	144	697	113	68	206	300	204	465
DK8	706	168	144	697	113	68	206	—	204	465
BR53	750	168	144	697	113	68	206	—	204	—
VS461	706	168	144	697	113	68	206	—	204	465
20047	655	168	144	697	113	89	206	321	204	509
N34	655	168	142	697	113	89	206	342	204	465
FAR03	692	168	144	731	113	89	206	—	204	465
VSDA	692	168	144	—	113	89	206	—	204	465
PBI	655	168	144	638	113	89	206	363	204	—
PBR	692	168	144	697	113	89	206	—	204	465
HFOX	467	168	144	802	113	68	206	—	204	465

[a] —, missing data due to lack of PCR amplification.

of 0.51 (Table 3). VNTR markers that exhibit high *D* values, such as BR-V8 ($D = 0.89$), possess great discriminatory capacity for identification of genetically similar strains. Less-diverse markers, such as BR-V9 ($D = 0.10$) (Table 3), may be applied with greater utility for species identification and analysis of evolutionary relationships. The ability to predict VNTR diversity on the basis of array size will allow guided selection of marker loci in future studies.

Genetic relationships among isolates. Ten VNTR marker loci were used to calculate genetic distances among *Borrelia* strains. UPGMA analysis then revealed 30 distinct genotypes among the 41 *Borrelia* isolates, with five unique subdivisions evident within these affiliations (Fig. 1). No fixed allelic differences between these clusters were present (Table 4); therefore, cluster formation is due to overall allelic frequency. Clusters I, II, III, and IV include only *B. burgdorferi* sensu stricto isolates (Fig. 1). All *B. burgdorferi* strains revealed unique marker allele size combinations, with the exception of *B. burgdorferi* strains L5, IP1, IP2, IP3, Cat flea, and B31, which were identical at all marker loci (Fig. 1). Isolates B31 and Cat flea were isolated in North America, while strains IP1, IP2, and IP3 are human cerebrospinal fluid isolates from France (Table 2). Nineteen of the 27 *B. burgdorferi* sensu stricto strains grouped within cluster IV (Fig. 1). MLVA revealed substantial discrimination between *B. afzelii* and *B. garinii* in cluster V (Fig. 1). This cluster included seven *B. afzelii* strains and seven *B. garinii* strains (Fig. 1). All seven *B. afzelii* strains assembled within the single subgroup of cluster V-1 (Fig. 1). *B. afzelii* isolates B023 and BR53 showed 100% marker identity, as did isolates J1, ECM1, DK3, DK8, and VS461 (Fig. 1). Six unique genotypes are evident among the *B. garinii* isolates, with strains FAR03 and VSDA showing 100% marker identity (Fig. 1). Although the Japanese *B. garinii* strain (HFOX) loosely clustered within the *B. afzelii* subgroup (Fig. 1), this strain exhibited only a single *B. afzelii*-specific chromosomal allelic state (Table 4). The HFOX isolate also exhibited a *B. burgdorferi*-specific plasmidic allele and a unique allele specific to this isolate alone (Table 4). The loose affiliation of HFOX with *B. afzelii* (cluster V-1, Fig. 1)

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does not appear robust. This apparent affiliation is not contradictory to the identity of HFOX in this unrooted tree, as HFOX is actually more closely related to the *B. garinii* isolates than to the *B. afzelii* isolates. Overall, the phylogenetic relationships observed in this study are in general agreement with the previous 16S rRNA sequence analysis (31), with the *Borrelia* MLVA system developed here having a greater ability to discriminate individual strains.

The diversity within the three species is dramatically different and suggests phylogenetic relationships and evolutionary history. For example, we observed that four out of the five clusters contain only *B. burgdorferi* sensu stricto members. These four groups have great diversity, especially when contrasted with the *B. afzelii* (group V-1, Fig. 1) and *B. garinii* (V-2, Fig. 1) clusters. The cohesiveness of the two latter species in one group argues for a more recent common evolutionary derivation, perhaps from a *B. burgdorferi* sensu stricto ancestor. Certainly, their lack of diversity is due to either a recent origin or a common and pronounced genetic bottleneck.

A more subtle diversity trend was observed within *B. burgdorferi* sensu stricto when North America and European strains were compared (Fig. 2). We observed greater genetic diversity among the 15 North American samples (mean genetic distance = 0.46) than among 12 European samples (mean genetic distance = 0.41). Perhaps because of a relatively small sample size, this trend is not statistically significant ($t = 0.009$) but it is consistent with previous evolutionary models postulating a founder effect as North American *B. burgdorferi* sensu stricto moved to the Old World (15, 23). However, diversity within *B. burgdorferi* sensu stricto could likewise be affected by lateral transfer of genetic material from other species. In previous studies, four diverse isolates (NE56, 20006, Z136, and ESP1) were shown to have obtained the *ospC* gene from other species (23). Hence, genetic mixing via lateral transfer may provide an additional mechanism of evolutionary change.

MLVA provides a convenient and rapid method for discrimination of *Borrelia* strains. Previously, most *Borrelia* analyses have been performed either phenotypically with monoclonal antibodies or by using DNA sequencing or small-fragment restriction fragment length polymorphisms. These analyses involved single genes or limited genomic loci, which do not effectively reflect the characteristics of the whole organism. In addition, previous studies either were restricted to one species (29) or used a small number of strains (30). In contrast, we examined multiple strains from three species at 10 genetically diverse and informative loci. The characterization of molecular diversity we achieved by applying MLVA to the strain typing of *B. burgdorferi*, *B. afzelii*, and *B. garinii* suggests that this method can be harnessed for the rapid discrimination and identification of the remaining major *Borrelia* species and will allow further phylogenetic and epidemiological analyses of these genetically diverse organisms.

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