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## Genetic and Phenotypic Analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* Genomic Groups VS116 and M19)

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To clarify the taxonomic status of two recently described *Borrelia* genomic groups, groups VS116 and M19, three group VS116 strains and eight group M19 strains isolated from *Ixodes ricinus* ticks in Switzerland, The Netherlands, and the United Kingdom were characterized. PCR-restriction fragment length polymorphism (RFLP) analysis of the 5S-23S intergenic spacer amplicon, rRNA gene restriction analysis, 16S rRNA gene sequence analysis, randomly amplified polymorphic DNA (RAPD) fingerprinting, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting with monoclonal antibodies were used for genetic and phenotypic analysis. The PCR-RFLP and RAPD patterns of three group VS116 strains and eight group M19 strains were identical but differed from those of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia japonica*. DNAs from all group VS116 and M19 strains yielded three fragments (6.9, 3.2, and 1.4 kb) and four fragments (2.1, 1.2, 0.8, and 0.6 kb) after digestion with *EcoRV* and *HindIII*, respectively, hybridizing with an *Escherichia coli* 16S+23S cDNA probe. The SDS-PAGE protein profiles of group VS116 and M19 strains were heterogeneous. Phylogenetic analysis of the partial 16S rRNA gene sequences showed that group VS116 and M19 spirochetes were members of a *Borrelia* species distinct from previously characterized members of the genus *Borrelia*. Based on our present study and data from previous DNA-DNA hybridizations, a new *Borrelia* species, *Borrelia valaisiana* sp. nov., in the *B. burgdorferi* complex, is proposed. Strain VS116 is the type strain of this new species.

Lyme borreliosis (LB) caused by the spirochetal species *Borrelia burgdorferi* sensu lato is a multisystemic illness distributed worldwide (39). In North America and Eurasia, LB is the most common tick-borne disease (8). On the basis of DNA-DNA relatedness and rRNA gene restriction patterns, *B. burgdorferi* sensu lato strains have been divided into the following three species: *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (4, 9). *B. burgdorferi* sensu stricto has been found in ticks and patients infected in Europe, as well as in North America, whereas *B. garinii* and *B. afzelii* have been found only in ticks and patients infected in Eurasia (4, 41).

Recently, four new *Borrelia* species, *Borrelia japonica* (19), *Borrelia andersonii* (26), *Borrelia lonestari* (7), and *Borrelia miyamotoi* (12), have been described. *B. japonica* and *B. andersonii* were isolated from *Ixodes ovatus* ticks in Japan and *Ixodes dentatus* ticks in North America, respectively (19, 26). Both of these new species are considered nonpathogenic for humans. *B. lonestari*, obtained from the hard tick *Amblyomma americanum* and possibly the agent of an LB-like illness in the United States, and *B. miyamotoi*, obtained from *Ixodes persulcatus* in Japan, both appear to be related to relapsing fever borreliae (7, 12). In addition, a new *Borrelia* species was recovered from patients with relapsing fever and the soft tick *Ornithodoros erraticus* in Spain (2).

In the past, six other *Borrelia* genomic groups have been recognized. Five genomic groups, groups VS116, PotiB2, DN127, Hk501, and Ya501, were identified based on the *MseI* restriction patterns of their PCR-amplified 5S-23S rRNA in-

tergenic spacer amplicons (27, 31). Groups Hk501 and Ya501 are restricted to Japan (13, 27). Recently, these two genomic groups were reclassified as the new species *Borrelia tanuki* and *Borrelia turdae*, respectively (15). Groups VS116 and PotiB2 comprise European isolates, whereas group DN127 includes only North American isolates (31). So far, strains belonging to these genomic groups have been cultured only from ticks and small mammals. The sixth *Borrelia* genomic group is designated M19 and contains 12 Dutch isolates recovered from *Ixodes ricinus* (28). Group M19 strains, which are related to *B. garinii*, were distinguished from *B. garinii* strains based on flagellin and rRNA gene restriction patterns. More recently, it was reported that a 5S-23S rRNA intergenic spacer fragment obtained by PCR amplification from one group M19 strain reacted with a group VS116-specific oligonucleotide probe (35). However, the genetic relatedness of group M19 strains with other *Borrelia* genomic groups is still largely unknown.

The aim of the present study was to determine the relationship between group M19 and other *Borrelia* species and genomic groups and to clarify the taxonomic status of genomic groups VS116 and M19. Using different genotyping and phenotyping methods, we were able to classify the eight group M19 strains used in this study into the same genomic group as group VS116 strains. Phylogenetic analysis showed that group VS116 and group M19 spirochetes represented a *Borrelia* species distinct from previously characterized members of the genus *Borrelia*. Based on our results and reports from other workers (3, 31, 32, 35), therefore, a new *Borrelia* species, *Borrelia valaisiana* sp. nov. in the *B. burgdorferi* complex, is designated. This species includes isolates included in formerly described genomic groups VS116 and M19 (28, 31, 32, 35).

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TABLE 1. *B. burgdorferi* sensu lato strains used in this study

Taxon	Strain	Biological origin	Geographic location	RFLP pattern <sup>a</sup>
<i>B. burgdorferi</i> sensu stricto	B31 <sup>T</sup> (= ATCC 35210 <sup>T</sup> )	<i>Ixodes scapularis</i>	United States	A
<i>B. garinii</i>	20047 <sup>T</sup> (= CIP103362 <sup>T</sup> )	<i>Ixodes ricinus</i>	France	B
<i>B. afzelii</i>	VS461 <sup>T</sup> (= CIP103469 <sup>T</sup> )	<i>Ixodes ricinus</i>	Switzerland	D
<i>B. japonica</i>	HO14 <sup>T</sup> (= CJM8951 <sup>T</sup> )	<i>Ixodes ovatus</i>	Japan	E
Group VS116	VS116 <sup>T</sup>	<i>Ixodes ricinus</i>	Switzerland	F
	UK	<i>Ixodes ricinus</i>	England	F
	AR-2	<i>Ixodes ricinus</i>	The Netherlands	F
Group M19	M7	<i>Ixodes ricinus</i>	The Netherlands	F
	M19	<i>Ixodes ricinus</i>	The Netherlands	F
	M38	<i>Ixodes ricinus</i>	The Netherlands	F
	M47	<i>Ixodes ricinus</i>	The Netherlands	F
	M49	<i>Ixodes ricinus</i>	The Netherlands	F
	M50	<i>Ixodes ricinus</i>	The Netherlands	F
	M52	<i>Ixodes ricinus</i>	The Netherlands	F
	M53	<i>Ixodes ricinus</i>	The Netherlands	F

<sup>a</sup> RFLP patterns as described by Postic et al. (31).

## MATERIALS AND METHODS

**Bacterial strains and DNA extraction.** The bacterial strains used in this study are listed in Table 1. Three group VS116 strains and 8 of the 12 group M19 strains known were included in this study. Four well-characterized *Borrelia* type strains, the type strains of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica* (4, 9, 19), were used as reference strains. Strains were grown in MKM medium (33) at 33°C. Extraction of DNA was done as described previously (45).

**PCR-restriction fragment length polymorphism (RFLP).** PCR amplification of the 5S-23S intergenic spacer DNA was performed as described by Postic et al. (31); this was followed by endonuclease *Mse*I (Boehringer, Mannheim, Germany) digestion according to the instructions of the manufacturer. The restriction fragments were subsequently electrophoresed on a 5% MetaPhor (FMC Bioproducts, Rockland, Maine) agarose gel.

**Southern hybridization.** Four to six micrograms of spirochete DNA was cleaved with *Eco*RV or *Hind*III (Boehringer), separated on a 0.7% agarose gel, and transferred to a nylon membrane (Zeta-probe; Bio-Rad Laboratories, Hercules, Calif.). Blots were hybridized with a digoxigenin (DIG)-labelled *Escherichia coli* 16S+23S cDNA probe by using a DIG labelling and detection kit (Boehringer) as described by Popovic et al. (30), except that hybridization and membrane washing were performed at 60 and 55°C, respectively. A molecular size marker, phage lambda DNA digested with *Hind*III, was included in each blot and was hybridized with DIG-labelled lambda DNA.

**PCR amplification of the 16S rRNA gene.** Approximately 1,500 bp of the 16S rRNA gene from two group VS116 strains (VS116 and UK) was amplified by PCR by using a primer set consisting of fd3 (5'-AGAGT TTGAT CCTGG CTTAG-3'; positions 8 to 27 [*B. burgdorferi* B31<sup>T</sup> numbering]) and UniB (5'-T[AC]AAGG AGGTG ATCCA GC-3'; positions 1539 to 1522), as described by Le Fleche et al. (23). The 5' end of the 16S rRNA gene from other group VS116 and M19 strains was amplified by PCR by using primers BRNA8 (5'-ACGCT GGCAG TGCCT CTTA-3') and BRNA9 (5'-CTTCG GACT AACCT TT TAG-3'), corresponding to nucleotides 33 to 51 and 852 to 833 of the *B. burgdorferi* sensu stricto 16S ribosomal DNA (rDNA), as described previously (25). BRNA9 contains one additional nucleotide, a C (underlined), compared with the recently published sequence of the 16S rRNA gene (16).

**DNA sequencing and phylogenetic analysis.** The 16S rRNA gene amplified with the primer set consisting of fd3 and UniB was sequenced by using a solid-phase approach with a Cy5-AutoRead sequencing kit (Pharmacia) (23). Both the 5S-23S intergenic amplicons and partial 16S rDNA fragments amplified with primers BRNA8 and BRNA9 were purified with QIAEX II (QIAGEN GmbH, Hilden, Germany) and sequenced directly by a dideoxy chain termination method by using a dye terminator cycle sequencing kit with *Taq* DNA polymerase FS (Applied Biosystems, Inc., Foster, Calif.). The sequences obtained were aligned with sequences retrieved from GenBank with PC/GENE software (IntelliGenetics, Inc.). All positions with alignment gaps, as well as ambiguous positions, were excluded from the pairwise sequence comparison. Phylogenetic analysis was performed by using the MEGA program (22). A neighbor-joining tree was created based on Kimura's two-parameter distance estimation method. Bootstrap resampling was performed (100 trees).

**RAPD.** A randomly amplified polymorphic DNA (RAPD)-PCR analysis was performed in a 25- $\mu$ l reaction mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 4.0 mM MgCl<sub>2</sub>, 0.1 mg of bovine serum albumin per ml, each deoxynucleoside triphosphate (Pharmacia Biotech) at a concentration of 200  $\mu$ M, 1 U of AmpliTaq polymerase (Perkin-Elmer, Gouda, The Netherlands), 0.4  $\mu$ M primer 1254 (CCGCAGCCAA) or 0.4  $\mu$ M primer 1281 (AACGC GCAAC) (1), and 20 ng of purified genomic DNA. The PCR was carried out with a Biomet thermocycler (Westburg B.V., Leusden, The Netherlands) by using the following

steps: 3 cycles consisting of 5 min at 94°C, 5 min at 36°C, and 5 min at 72°C, followed by 30 cycles consisting of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, and a final incubation at 72°C for 10 min. The amplified DNA fragments were separated on 1% agarose gels.

**SDS-PAGE and immunoblotting.** Whole-cell lysates of spirochetes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described (41). OspA-specific monoclonal antibodies (MAbs) H3TS (5), LA31 (21), and LA26 (43), OspB-specific MAbs 84C (37) and I 17.3 (9), OspC-specific MAb L22 1F8 (44), and MAb D6 (29) specific for a 12-kDa protein were used in the immunoblot analysis. MAbs H3TS, D6, and I 17.3 are specific for *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively.

**Nucleotide sequence accession numbers.** The 5S-23S spacer and 16S rDNA sequences of *Borrelia* isolates which were determined in this study have been assigned GenBank and EMBL accession numbers. The accession numbers for the 5S-23S spacer nucleotide sequence are U78147 (strain M19), U78148 (M49), U78149 (M53), and U78150 (AR-2), and the accession numbers for the 16S rRNA gene sequence are U78151 (VS461<sup>T</sup>), X98232 (VS116<sup>T</sup>), X98233 (UK), U78153 (AR-2), U78154 (M19), 78155 (M49), and U78156 (M53). The 16S rRNA sequences of the following microorganisms (with accession numbers in parentheses) were used for phylogenetic analysis: *B. burgdorferi* B31<sup>T</sup> (U03396), *B. garinii* 20047<sup>T</sup> (D67018), *B. japonica* HO14<sup>T</sup> (L40597), *B. andersonii* 21038 (L46701), *Borrelia* sp. strain DN127 (L40596), *Borrelia* sp. strain Am501 (D67021), *B. miyamotoi* HT31<sup>T</sup> (D45192), *B. lonestari* (U23211), *Borrelia anserina* (M60970), *Borrelia duttonii* (U28503), and *Borrelia hermsii* (M60968). In addition, the 5S-23S intergenic spacer sequence of *B. valaisiana* strain VS116<sup>T</sup> (L30134) was also used in our analysis.

## RESULTS

**PCR-RFLP and DNA sequence of the 5S-23S intergenic amplicon.** A 246- to 255-bp fragment was generated by PCR amplification from all of the *Borrelia* strains used in this study.

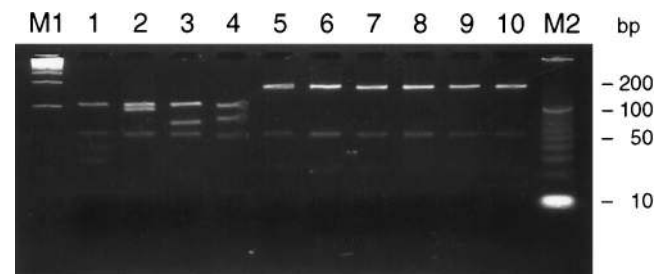


FIG. 1. *Mse*I restriction patterns of the PCR-amplified 5S-23S intergenic spacer DNA fragment of *B. burgdorferi* sensu lato strains. Lanes 1 to 4, reference strains B31<sup>T</sup>, 20047<sup>T</sup>, VS461<sup>T</sup>, and HO14<sup>T</sup>, respectively; lanes 5 to 7, group VS116 strains VS116<sup>T</sup>, UK, and AR-2, respectively; lanes 8 to 10, group M19 strains M19, M52, and M53, respectively; lanes M1 and M2, 100- and 10-bp DNA ladders (Gibco BRL), respectively.

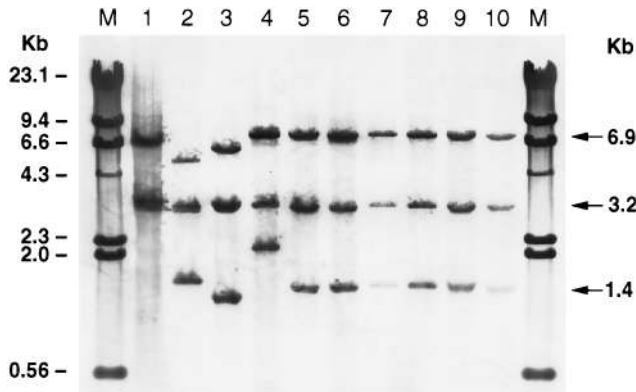


FIG. 2. rRNA gene restriction patterns of *B. burgdorferi* sensu lato strains. Genomic DNAs were digested with *EcoRV*, transferred to a Zeta-probe membrane, and hybridized with a digoxigenin-labelled *E. coli* 16S+23S cDNA probe. For the contents of lanes 1 through 10 see the legend to Fig. 1. The restriction fragments of *Borrelia* groups VS116 and M19 are indicated by the arrows on the right. Lanes M contained *HindIII*-digested lambda DNA.

All group VS116 and M19 strains yielded the same *MseI* restriction pattern with three bands having molecular sizes of 175, 50, and 23 bp (Fig. 1). This pattern, designated pattern F (Table 1), differed from the *MseI* restriction patterns of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*. The 7-bp fragment which should be generated according to the previously published DNA sequences of two group VS116 strains (VS116<sup>T</sup> and UK) (31) was not visualized.

In this study, we sequenced amplicons from one group VS116 strain (strain AR-2) and five group M19 strains (M7, M19, M49, M52, and M53). The amplified sequences of strains M19 and VS116<sup>T</sup> were identical, but showed only 87.0, 89.3, 87.8, and 86.9% identity to the sequences of strains B31, 20047, VS461, and HO14, the type strains of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*, respectively. Strains M49 and M53 showed only single nucleotide substitutions at positions 26 and 89, respectively, when they were compared with strain VS116<sup>T</sup>. The sequences of one other group VS116 strain (AR-2) and two group M19 strains (M7 and M52) were identical to the sequence of strain UK (31) and exhibited two nucleotide differences at positions 49 and 89 compared to the strain VS116<sup>T</sup> sequence.

**rRNA gene restriction patterns.** rRNA gene restriction patterns of *Borrelia* strains after digestion with *EcoRV* and hybridization with an *E. coli* 16S+23S cDNA probe on a Southern blot showed that both the three group VS116 strains and the eight group M19 strains produced three fragments with molecular sizes of 6.9, 3.2, and 1.4 kb (Fig. 2). Similarly, all group VS116 and M19 strains produced four fragments with molecular sizes of 2.1, 1.2, 0.8, and 0.6 kb after cleavage with *HindIII* (data not shown). Based on their rRNA gene restriction patterns, the group VS116 and group M19 strains could be distinguished from *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*.

**16S rRNA gene analysis.** Nearly complete 16S rRNA gene sequences (approximately 1,500 bp) were obtained from two group VS116 strains (strains VS116<sup>T</sup> and UK), and partial sequences (781 bp) were obtained from one group VS116 strain (AR-2) and five group M19 strains (M7, M19, M49, M52, and M53). The nearly complete sequences of the two group VS116 strains (VS116<sup>T</sup> and UK) differed by five nucleotides and exhibited a level of similarity of 99.7%. These two sequences were very similar (levels of similarity, 99.8 and

TABLE 2. Similarity matrix for the partial 16S rRNA gene sequences of *Borrelia* strains<sup>a</sup>

Taxon <sup>b</sup>	% similarity to:																		
	Strain B31 <sup>T</sup>	Strain 20047 <sup>T</sup>	Strain VS461 <sup>T</sup>	Strain HO14 <sup>T</sup>	Strain VS116 <sup>T</sup>	Strain UK	Strain M19	Strain M49	Strain M52	Strain M53	Strain Am501	Strain 21038	Strain DNI27	<i>B. lonestari</i>	<i>B. miyamotoi</i>	<i>B. anserina</i>	<i>B. duttonii</i>	<i>B. hermsii</i>	
<i>B. burgdorferi</i> B31 <sup>T</sup>	100.0																		
<i>B. garinii</i> 20047 <sup>T</sup>	98.2	100.0																	
<i>B. afzelii</i> VS461 <sup>T</sup>	97.6	98.3	100.0																
<i>B. japonica</i> HO14 <sup>T</sup>	94.5	94.6	94.8	100.0															
Group VS116 strain VS116 <sup>T</sup>	97.6	98.6	99.0	94.2	100.0														
Group VS116 strain UK	97.3	98.6	99.0	94.0	99.0	100.0													
Group M19 strain M19	97.7	98.7	99.1	94.4	99.9	99.6	100.0												
Group M19 strain M49	97.4	98.5	99.4	94.4	99.9	99.8	99.5	100.0											
Group M19 strain M52	97.3	98.3	98.2	93.7	98.7	99.2	98.8	99.1	100.0										
Group M19 strain M53	98.0	99.0	99.4	94.6	99.6	99.6	99.1	99.7	99.5	98.8	100.0								
<i>Borrelia</i> sp. strain Am501	97.4	97.4	97.8	94.9	97.3	97.1	97.4	97.2	96.8	97.7	100.0								
<i>B. andersonii</i> 21038	95.7	95.8	95.0	96.2	94.9	94.9	95.0	94.8	94.6	95.3	94.6	100.0							
<i>Borrelia</i> sp. strain DNI27	94.2	95.4	94.5	91.2	94.9	94.6	95.0	94.5	94.5	94.9	94.2	91.7	100.0						
<i>B. lonestari</i>	94.6	95.5	95.5	91.2	95.5	95.4	95.6	95.3	94.9	95.5	94.6	92.1	98.0	100.0					
<i>B. miyamotoi</i> HT31 <sup>T</sup>	95.0	95.3	95.8	91.4	95.5	95.3	95.1	95.6	95.1	94.6	95.8	94.9	92.2	96.2	100.0				
<i>B. anserina</i>	94.6	94.9	94.5	91.2	94.9	94.8	95.0	94.6	94.5	94.2	94.2	91.5	96.3	93.1	96.7	100.0			
<i>B. duttonii</i>	92.2	91.8	91.9	90.7	91.8	91.7	91.9	91.9	91.5	92.1	93.0	90.0	92.8	93.6	93.6	93.6	100.0		
<i>B. hermsii</i>																		93.1	100.0

<sup>a</sup> The similarities were calculated based on 781 bp of the 16S rRNA gene sequences corresponding to base positions 52 to 833 of *B. burgdorferi* B31<sup>T</sup>; insertion of C at position 213 was ignored.

<sup>b</sup> The sequences of strains AR-2 and M7 are identical to the sequence of group VS116 strain UK and were not included in this analysis.

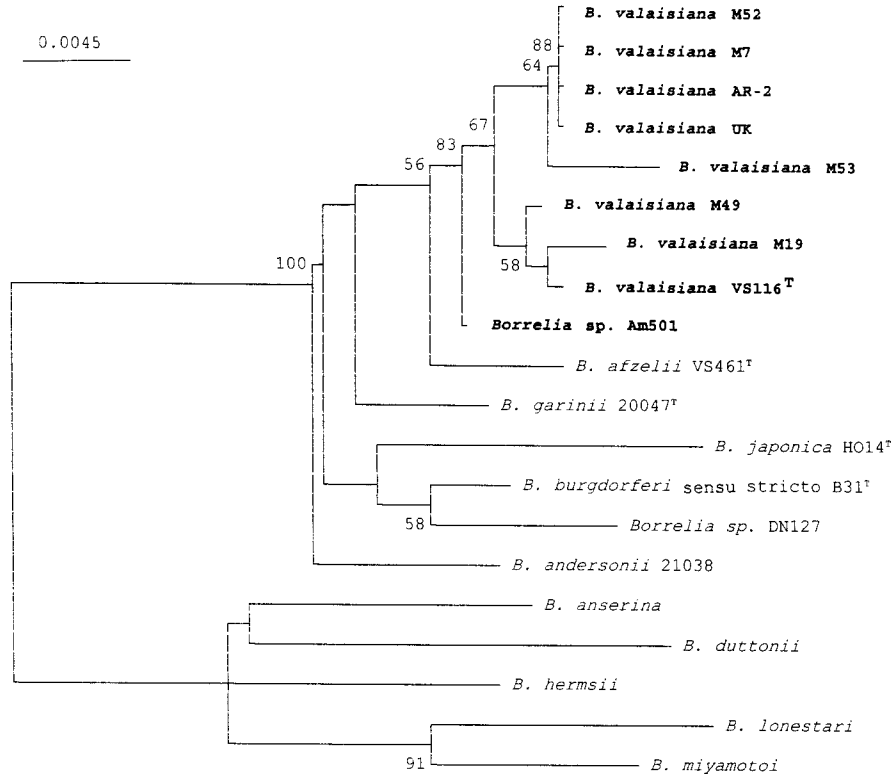


FIG. 3. Phylogenetic tree of *Borrelia* species and isolates. 16S rRNA gene sequences, corresponding to base positions 52 to 833 of the 16S rRNA of *B. burgdorferi* B31<sup>T</sup>, were aligned by using the Clustal program with PC/Gene software. The phylogenetic tree was constructed by using the MEGA program as described in the text. The numbers at the branch nodes indicate the results of the bootstrap analysis.

99.7%, respectively) to the sequence of *Borrelia* sp. strain Am501 isolated from an *Ixodes columnae* tick in Japan. The latter strain was previously found in the same cluster as strain VS116<sup>T</sup> in a phylogenetic tree deduced from the flagellin gene sequence (13) and clustered with strains isolated from *Ixodes tanuki* in a tree constructed by using 16S rRNA gene sequences (14). Comparison of the 781-bp sequences from all three group VS116 strains and five group M19 strains gave levels of similarity ranging from 98.8 to 100% (Table 2). Further comparison of sequences showed that group VS116 and M19 strains were more closely related to the LB-related *Borrelia* species than to other *Borrelia* species, such as *B. lonestari*, *B. miyamotoi*, *B. anserina*, *B. duttonii*, and *B. hermsii* (Fig. 3).

Differences in 16S rRNA coding sequences between group VS116 and M19 strains and reference strains of *B. burgdorferi*

sensu stricto, *B. garinii*, and *B. afzelii*, the LB-causing species which occur in Europe, were examined in more detail. All group VS116 and M19 strains had conserved nucleotide substitutions compared to *B. burgdorferi* sensu stricto strain B31<sup>T</sup>, *B. garinii* 20047<sup>T</sup>, and *B. afzelii* VS461<sup>T</sup> at 12, 9, and 6 positions, respectively (Table 3). Differences in the sequences resulted in different *Bfa*I restriction patterns, which can be used to discriminate between group VS116 strains and other LB spirochetes (23).

**RAPD fingerprinting.** RAPD has been used for molecular analysis of various microorganisms (40). The RAPD fingerprint patterns of the three group VS116 strains and the eight group M19 strains obtained with two different arbitrary primers were similar (Fig. 4 [five group M19 strains not shown]) and

TABLE 3. Nucleotide differences in the sequences of the 16S rRNA gene from various *B. burgdorferi* sensu lato strains

Taxon	Nucleotides at the following positions <sup>a</sup> :															
	77	85	86	126	143	244	271	273	376	473	613	627	676	708	836	1133
<i>B. burgdorferi</i> sensu stricto strain B31 <sup>T</sup>	C	T	C	T	T	C	G	A	G	T	A	C	T	A	A	G
<i>B. garinii</i> 20047 <sup>T</sup>	C	C	T	C	T	C	G	G	A	T	G	A	T	A	A	G
<i>B. afzelii</i> VS461 <sup>T</sup>	C	T	T	T	C	T	A	G	A	C	A	G	C	G	A <sup>c</sup>	A <sup>c</sup>
Groups VS116 and M19 <sup>b</sup>	T	C	T	T	C	T	G	G	A	T	A	A	C	G	G <sup>d</sup>	A <sup>d</sup>

<sup>a</sup> Nucleotides are numbered in accordance with the 16S rRNA of *B. burgdorferi* B31<sup>T</sup> (16).

<sup>b</sup> Includes three group VS116 strains (strains VS116<sup>T</sup>, UK, and AR-2) and five group M19 strains (strains M7, M19, M49, M52, and M53).

<sup>c</sup> Sequences at positions 836 and 1133 are based on *B. afzelii* DK1 (23).

<sup>d</sup> At positions 836 and 1133 only strains VS116<sup>T</sup> and UK were sequenced.

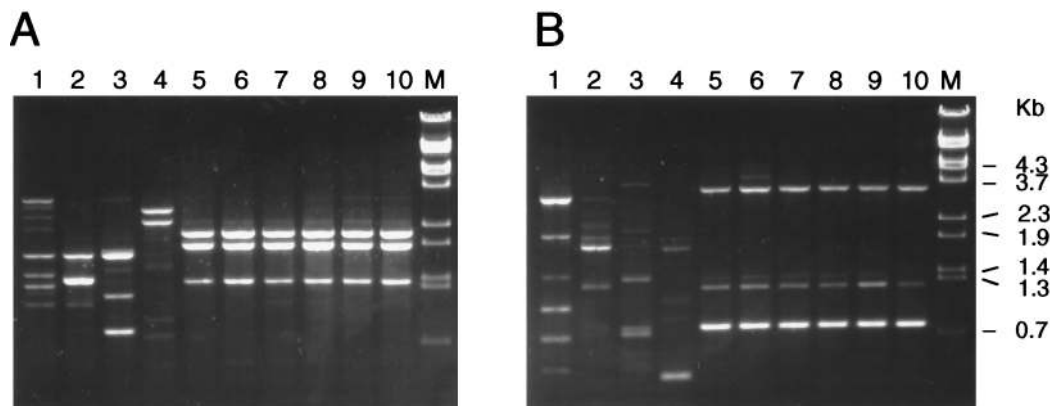


FIG. 4. RAPD fingerprinting of *B. burgdorferi* sensu lato strains obtained from PCR amplification with primer 1254 (A) and primer 1281 (B). For the contents of lanes 1 through 10 see the legend to Fig. 1. Lane M contained *Bst*EII-digested lambda DNA.

clearly differed from the patterns of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*.

**Protein profiles and reactivity with MAbs.** The protein profiles of group VS116 and M19 strains differed from those of the representative strains of known LB-related *Borrelia* species (Fig. 5). The major outer membrane proteins in the size range from 30 to 36 kDa were heterogeneous in the group VS116 and group M19 strains. Although a dominant protein band at 32 to 34 kDa was present in all group VS116 and M19 strains, none of these organisms reacted with OspA-specific MAbs H3TS, LA26, and LA31 (Table 4). In contrast, 64% (7 of 11) and 73% (8 of 11) of the group VS116 and M19 strains reacted with OspB- and OspC-specific MAbs 84C and L22 1F8, respectively. The molecular masses of the OspB and OspC proteins varied among the group VS116 and M19 strains and corresponded to the molecular masses estimated from the putative OspB and OspC proteins on SDS-PAGE gels. Strains VS116<sup>T</sup>, M19, and M38 produced a putative OspB band, located just above OspA, on SDS-PAGE gels, but the proteins did not react with MAb 84C. No reactivity of the group VS116 and M19 strains tested in this study was observed with MAbs I 17.3 and D6; these MAbs react specifically with *B. afzelii* and *B. garinii*, respectively.

## DISCUSSION

Previous studies indicated that three group VS116 strains isolated from *I. ricinus* ticks in Switzerland (strains VS116<sup>T</sup> and NE57) and in England (strain UK) were different from the well-known LB-related *Borrelia* species on basis of the electrophoretic mobilities of OspA (29), the RFLP patterns of the chromosomal DNA (11, 38), and DNA-DNA reassociation (31). Two other *Borrelia* isolates, AR-2 and Pn1109b, recovered from *I. ricinus* in The Netherlands (35) and *I. persulcatus* in Japan (32), respectively, have been classified in this group on basis of their genetic similarity to the three previously isolated group VS116 strains.

In the present study the genetic relationship between genomic groups VS116 and M19 and other LB-related *Borrelia* species and genomic groups was examined to determine whether considering groups VS116 and M19 a new species in the *B. burgdorferi* complex is justified. By using PCR-RFLP, RAPD fingerprinting, and rRNA gene restriction analysis, we found that the group VS116 and M19 strains were closely related to each other. Phylogenetic analysis showed that these group VS116 and M19 strains were more closely related to

LB-related *Borrelia* species than to other *Borrelia* species. Previous PCR-RFLP, DNA-DNA hybridization (31), and multilocus enzyme electrophoresis (3) studies also showed that both strain VS116<sup>T</sup> and strain UK could not be placed into the previously described species. Therefore, they were classified in a separate genomic group, group VS116. Our results provided evidence that this genomic group should include the *Borrelia* group M19 strains and that group VS116 should be classified as a distinct species in the *B. burgdorferi* complex.

The geographic distribution of the new species, *B. valaisiana*, is not yet entirely clear. In previous studies, only five *B. valaisiana* strains were cultured from Switzerland (11, 29), the United Kingdom (24), The Netherlands (35), and Japan (32). Since group M19 strains accounted for 12 (19%) of 63 isolates obtained from *I. ricinus* (28), we concluded that *B. valaisiana* strains are widespread among ticks in The Netherlands. This high prevalence of *B. valaisiana* infection in Dutch ticks was confirmed by a recent study conducted by Rijpkema et al. (35). It was shown by PCR amplification that the distributions of *B.*

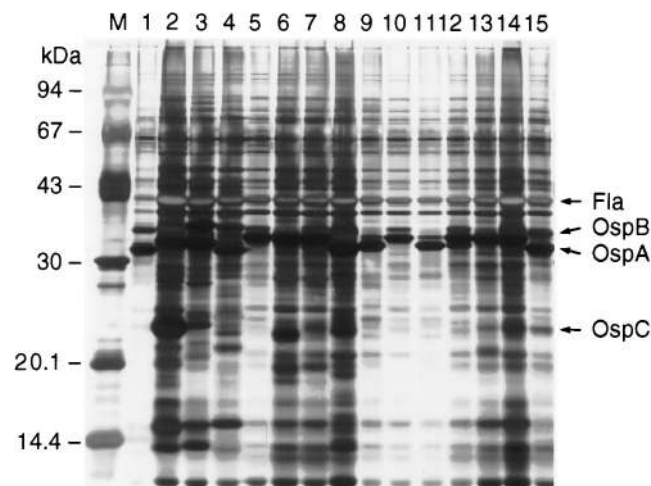


FIG. 5. Silver-stained SDS-PAGE gel of whole-cell lysates of *B. burgdorferi* sensu lato strains. Lanes 1 to 4, strains B31<sup>T</sup>, 20047<sup>T</sup>, VS461<sup>T</sup>, and HO14<sup>T</sup> of *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica*, respectively; lanes 5 to 7, group VS116 strains VS116<sup>T</sup>, UK, and AR-2, respectively; lanes 8 to 15, group M19 strains M7, M19, M38, M47, M49, M50, M52, and M53, respectively. The positions of the flagellin (Fla), OspA, OspB, and OspC proteins are indicated by arrows. The positions of molecular mass standards (Pharmacia) are shown on the left.

TABLE 4. Major outer surface proteins of *B. burgdorferi* sensu lato strains and reactivities with MAbs

Taxon	Strain	Molecular mass (kDa) <sup>a</sup>		Reactivities with the following MAbs:						
		OspA	OspB	H3TS (OspA)	LA26 (OspA)	LA31 (OspA)	84C (OspB)	I 17.3 (OspB)	L22 1F8 (OspC)	D6
<i>B. burgdorferi</i> sensu stricto	B31 <sup>T</sup>	31	34	+	+	+	+	-	+	-
<i>B. garinii</i>	20047 <sup>T</sup>	32		-	-	+	-	-	+	+
<i>B. afzelii</i>	VS461 <sup>T</sup>	32	35	-	+	-	+	+	+	-
<i>B. japonica</i>	HO14 <sup>T</sup>	31	34.5	-	+	+	-	-	+	-
Group VS116	VS116 <sup>T</sup>	33	34.5 <sup>b</sup>	-	-	-	-	-	+	-
	UK	33	34.5	-	-	± <sup>c</sup>	+	-	+	-
	AR-2	33	34.5	-	-	±	+	-	-	-
Group M19	M7	32	34.5	-	-	-	+	ND <sup>d</sup>	+	ND
	M19	32.5	34.5 <sup>b</sup>	-	-	-	-	-	+	-
	M38	33	35 <sup>b</sup>	-	-	-	-	ND	+	ND
	M47	32.5	34	-	-	-	+	ND	+	ND
	M49	33.5	35	-	-	-	±	ND	+	ND
	M50	33		-	-	-	-	ND	-	ND
	M52	33	35	-	-	-	+	-	-	-
	M53	32	34.5	-	-	-	±	ND	+	ND

<sup>a</sup> Molecular masses were estimated by SDS-PAGE.

<sup>b</sup> The molecular mass of putative OspB based on SDS-PAGE, not reactive with MAb 84C.

<sup>c</sup> ±, weak reactivity.

<sup>d</sup> ND, not determined.

*valaisiana*, *B. garinii*, and *B. afzelii* strains in ticks in The Netherlands are similar. In addition, *B. valaisiana* DNA has also been amplified from ticks in Croatia and Ireland (20, 34). Therefore, it seems likely that *B. valaisiana* is present in various European countries.

The association of *B. valaisiana* with a specific tick vector appears not to be strict. Although most *B. valaisiana* strains have been isolated from *I. ricinus* in Europe, this species has been cultured once from *I. persulcatus* (32). It is also noteworthy that *Borrelia* sp. strain Am501, which based on both its flagellin (13) and its 16S rRNA gene sequence (14) belongs to *B. valaisiana*, has been cultured from *I. columnae* in Japan, showing that *B. valaisiana* can adapt to tick species other than *I. ricinus*. Since by xenodiagnosis several *B. valaisiana* strains have been isolated from passerine birds which were able to transmit infection to ticks, it has been suspected that passerine birds are a reservoir for this species (17).

All *B. valaisiana* strains express a dominant protein in the 32- to 34-kDa range (28, 29; this study). Although these proteins may represent OspA, they do not react with a number of monoclonal anti-OspA antibodies which have been tested (5, 21, 28, 43). Differences in the molecular weights of putative OspA proteins have been seen among *B. valaisiana* strains. However, more monoclonal anti-OspA antibodies should be tested to prove that these proteins indeed represent OspA. Most *B. valaisiana* strains included in this study expressed an OspB and an OspC, which were reactive with MAbs directed against these proteins. In addition, three strains expressed putative OspB proteins that did not react with anti-OspB MAb 84C. Since the intensity of MAb 84C reactivity also varied among the MAb 84C-positive strains (data not shown), the OspBs of *B. valaisiana* strains may be heterogeneous. In an earlier publication, none of 12 *B. valaisiana* strains expressed OspB, and only 1 of the strains expressed OspC (28). However, these conclusions were based only on SDS-PAGE protein profiles. We concluded that analysis of protein patterns does not constitute a reliable means for identification of *B. valaisiana*.

Whether *B. valaisiana* plays a role in human Lyme disease is still an open question. Like *B. japonica* and *B. andersonii*, which occur in Japan and North America, respectively, *B.*

*valaisiana* strains have not yet been isolated from patients, but *B. valaisiana* DNA has recently been amplified from skin biopsies from two patients with erythema migrans, suggesting that this species has pathogenic potential (36). These results must be confirmed by culture of *B. valaisiana* from human tissue. If *B. valaisiana* is rather common and infectious, but exerts a low pathogenic potential, this species might well contribute to the frequent finding of asymptomatic seropositivity among residents in Switzerland (10) and The Netherlands (42), two countries where *B. valaisiana* is known to occur in ticks (28, 29).

**Description of *Borrelia valaisiana* sp. nov.** *Borrelia valaisiana* (va. lai. si. a' na. L. fem. adj. *valaisiana*, referring to Valais, Switzerland, where group VS116 strains were first discovered). Morphology as described previously for the genus (6). Cultural properties as described for *B. burgdorferi* sensu lato (18). rRNA gene restriction patterns after digestion by *EcoRV* and *HindIII* contain three fragments (6.9, 3.2, and 1.4 kb) and four fragments (2.1, 1.2, 0.8, and 0.6 kb), respectively. The PCR-RFLP profile resulting from *MseI*-digested 255-bp 5S-23S intergenic spacer contains four fragments (175, 50, 23, and 7 bp) (31). Reacts in Western blots with MAb H9724, but no reactivity is observed with MAbs H6831 (28), H3TS, LA26, LA31, I 17.3, and D6 (this study). Reactivity with MAbs H5332 (28), 84C, and L22 1F8 is variable among strains. *B. valaisiana* strains have been isolated only from ticks in Eurasia. Type strain VS116, with all of the characteristics described above, was isolated from *I. ricinus* in Valais, Switzerland (29).

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