Characterization of *Borrelia lusitaniae* sp. nov. by 16S Ribosomal DNA Sequence Analysis

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We determined the complete sequence of the *rrs* gene from five strains of genomic species PotiB2. Both distance and parsimony methods were used to infer the evolutionary relationships of the *rrs* gene sequence of this genomic species in comparison with the *rrs* gene sequence of *Borrelia valaisiana* and the *rrs* gene sequences of *Borrelia burgdorferi* sensu lato species obtained from sequence databases. The phylogenetic analysis revealed that the genomic species PotiB2 strains clustered in a separate lineage, which was consistent with data from previous DNA-DNA hybridization experiments (D. Postic, M. V. Assous, P. A. D. Grimont, and G. Baranton, Int. J. Syst. Bacteriol. 44:743–752, 1994). A PCR-restriction fragment length polymorphism analysis was used to identify genomic species PotiB2 and to differentiate it from *B. burgdorferi* sensu lato species. Moreover, signature nucleotide positions were identified for each *B. burgdorferi* sensu lato species. In accordance with DNA relatedness values, our findings suggest that genomic species PotiB2 can be more clearly defined and identified, and we propose that it should be referred to as a new species, *Borrelia lusitaniae*. The type strain is PotiB2.

Borrelia burgdorferi sensu lato, the causative agent of Lyme borreliosis, has been previously divided into five well-defined species, *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii* (1, 4), *Borrelia japonica* (12, 22), and *Borrelia andersonii* (17). The pathogenicity for humans of the first three species has been well-established, whereas the last two species seem not to be pathogenic for humans. These different species are not equally distributed all over the world, since the three main species, *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto, occur in Europe, whereas *B. burgdorferi* sensu stricto is absent from Asia and is the main species encountered in the United States. In the United States, the more recently described species *B. andersonii* is also present as genomic species DN127, and each of the species is restricted to a limited geographical area. Additionally, in Japan, two species, *Borrelia tanukii* and *Borrelia turdae*, were described recently (6, 7). In Europe, two genomic species that are genetically and phenotypically divergent, genomic species VS116 and PotiB2, have been reported (20, 21). The name *Borrelia valaisiana* is proposed in the accompanying paper for genomic species VS116 (29). Genomic species PotiB2 has been identified previously by DNA-DNA hybridization and has been characterized on the basis of the sequence of its *rrf-rrl* ribosomal spacer (21). A specific *MseI* restriction pattern allowed workers to clearly identify this new genomic species, as well as all other delineated species or genomic species.

The aim of this study was to further characterize genomic species PotiB2. First, characterization was completed at a genomic level by sequencing the *rrs* gene, which yielded an alternative means for specific identification. Second, the strains were studied at a phenotypic level with an analysis of protein patterns. On the basis of the present work and previous results, we propose that genomic species PotiB2 should be named *Borrelia lusitaniae* sp. nov.

MATERIALS AND METHODS

Bacterial strains. The designations and origins of the *Borrelia* strains used in this study are given in Table 1. Five strains, PotiB1, PotiB2^T, PotiB3, Ir345, and BR41, belong to genomic species PotiB2. For the phenotypic analysis, the following representative strains were included: *B. burgdorferi* sensu stricto strain B31¹, *B. garinii* 20047^T, and *B. afzelii* VS461^T. The isolates were grown in BSK II medium at 30°C (2) for 4 to 10 days until the density reached about 10⁸ bacteria/ml.

DNA preparation. A 1.5-ml portion of culture was required for DNA extraction. Cells were harvested by centrifugation (12,000 × g, 15 min) and washed in phosphate-buffered saline (pH 7.2). DNA was extracted with a Dynabeads DNA direct kit (Dynal), a method based on DNA separation by biomagnetic beads. Finally, DNA was eluted in 30 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). This suspension was stored at –20°C until it was required for PCR experiments.

Solid-phase DNA sequencing of PCR products. We used a solid-phase approach to sequence DNA from PCR products. During the PCR, biotin was introduced into one of the strands by using one of the primers biotinylated at the 5' end. We used a primer set consisting of primers ID3 and UniB (Table 2) to amplify approximately 1,500 bp of the *rrs* gene. Each PCR mixture (50 µl) contained 5 µl of DNA, 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, each of the four deoxynucleotide triphosphates at a concentration of 200 µM, 1.25 U of *Taq* polymerase (Amersham), 2.5 µM biotinylated primer, and the second primer at a concentration of 5 µM. The reaction mixtures were overlaid with mineral oil (50 µl). Each amplification reaction was carried out for 35 cycles consisting of denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, and this was followed by extension at 72°C for 9 min. Negative controls were included in all experiments. The PCR products were detected by electrophoresis in a 0.8% agarose gel in 1× TBE and then stained with ethidium bromide.

The PCR product was then immobilized through the interaction between biotin and streptavidin by using a Dynabeads M-280 streptavidin kit (Dynal) according to the manufacturer's instructions. The DNA strands were separated in a 0.1 M NaOH solution, and the biotinylated strand was immobilized by using Dynal MPC (Dynal) and finally eluted in 12 µl of water.

The *rrs* gene was sequenced by using a Cy5-AutoRead sequencing kit (Pharmacia) according to the manufacturer's instructions. Briefly, the sequencing primer (10 pmol) was annealed to the biotinylated single-stranded template in the appropriate buffer at 65°C for 10 min and then at 37°C for 10 min and finally cooled at room temperature for 10 min. In the second step Cy5-dATP labelling mixture (Pharmacia) was incorporated, and the labelling reaction was started by adding T7 DNA polymerase (3 U). Termination reactions were performed by adding the labelling reaction mixture to each of the four dideoxynucleotide sequencing mixtures. Six percent polyacrylamide gels in an ALF express automatic sequencer (Pharmacia) were used for sequencing reactions. Both strands of the *rrs* gene of all strains were sequenced at least twice.

Sequence alignments and phylogenetic analysis. Sequences were aligned both manually with VSM software (version 2.0) written by B. Lafay and R. Christen

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TABLE 1. Strains used in this study

Strain	Geographic origin	Source ^a	GenBank accession no. for <i>rrs</i> gene (reference)	Sizes (bp) of <i>Bfa</i> I fragments of amplified <i>rrs</i> gene
<i>B. lusitaniae</i> strains				689, 404, 159, 100, 78, 58
PotiB1	Portugal	<i>Ixodes ricinus</i>	X98226 (this study)	
PotiB2 ^T	Portugal	<i>Ixodes ricinus</i>	X98228 (this study)	
PotiB3	Portugal	<i>Ixodes ricinus</i>	X98229 (this study)	
Ir345	Belorussia	<i>Ixodes ricinus</i>	X98230 (this study)	
BR41	Czech Republic	<i>Ixodes ricinus</i>	X98231 (this study)	
<i>B. valaisiana</i> strains				689, 504, 159, 78, 58
VS116 ^T	Switzerland	<i>Ixodes ricinus</i>	X98232 (29)	
UK	England	<i>Ixodes ricinus</i>	X98233 (29)	
Am501	Japan	<i>Ixodes columnae</i>	D67021 (6)	
<i>B. burgdorferi</i> strains				689, 404, 236, 159
DK7	Denmark	<i>Skin ACA</i>	X85195 (28)	
Lipitz	United States	<i>Skin</i>	X85203 (28)	
<i>B. garinii</i> strains				689, 404, 159, 158, 78
PBi	Germany	<i>CSF</i>	X85199 (28)	
DK27	Denmark	<i>Skin EM</i>	X85193 (28)	
<i>B. afzelii</i> strains				689, 504, 159, 136
DK1	Denmark	<i>Skin EM</i>	X85190 (28)	
DK21	Denmark	<i>Skin EM</i>	X85191 (28)	
<i>B. japonica</i> HO14 ^T	Japan	<i>Ixodes ovatus</i>	L40597 (8)	689, 504, 159, 136
<i>B. andersonii</i> 21308 ^T	United States	<i>Ixodes dentatus</i>	L46701 (17)	689, 404, 159, 136, 100
Genomic species DN127 strain DN127	United States	<i>Ixodes pacificus</i>	L40596 (8)	689, 404, 236, 159

^a ACA, acrodermatitis chronica atrophicans; EM, erythema migrans; CSF, cerebrospinal fluid.

as described previously (25) and by using multisequence alignment program Clustal V software (10).

Phylogenetic relationships were analyzed by using three methods, and the results were compared. A most-parsimonious tree was constructed by using the PAUP (Phylogeny Analysis Using Parsimony) program (27). Evolutionary distances were computed with the Jukes-Cantor option (26) by using both the PHYLIP program package written by Felsenstein (5) and MEGA software (14). Phylogenetic trees were constructed by using the Kitcher+ genetic distance algorithm from distance matrix data and by using the neighbor-joining method (26) in MEGA. The *rrs* sequences of *B. lusitaniae* sp. nov. were compared to the sequences of other *B. burgdorferi* sensu lato species.

Restriction fragment length polymorphism analysis of PCR products. A primer set consisting of primers fD3 and T50 (Table 2) was used to specifically amplify the *rrs* gene from spirochetes (18). The PCR was performed as described above, except that the two primers were used at the same concentration (5 µM). The amplification reaction was carried out for 30 cycles with an annealing temperature of 54°C.

The PCR products (10 µl) were digested by restriction enzyme *Bfa*I (New England Biolabs). The restriction products were separated by electrophoresis on a 1.8% agarose gel in TBE buffer for 2 h at 120 V. pBR322 digested by *Hin*II was used as a molecular weight marker.

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (20). Reactivity with monoclonal antibodies H3TS, D6, and I 17.3, which are specific for *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively, was studied as described previously (1, 4, 20).

Nucleotide sequence accession numbers. The 16S ribosomal DNA sequences determined have been deposited in the GenBank database. Their accession numbers are reported in Table 1.

RESULTS AND DISCUSSION

Sequence comparisons and phylogenetic analysis. *B. lusitaniae* sp. nov. (formerly genomic species PotiB2) has been identified previously on the basis of its DNA relatedness in DNA-DNA hybridization experiments. This genomic species

was easily identified by analyzing the restriction pattern of the *rrf-rrl* ribosomal spacer after amplification by PCR (21). In this study, nearly complete *rrs* sequences (1,509 to 1,526 nucleotides) were determined for five *B. lusitaniae* sp. nov. strains, accounting for about 96% of the *rrs* gene. In order to evaluate the phylogenetic position of *B. lusitaniae* sp. nov., we compared

TABLE 2. Primers used for amplification and sequencing of the *rrs* gene of *B. lusitaniae* and *B. valaisiana*

Primer	Sequence (5' to 3')	Position in <i>B. burgdorferi</i> sensu stricto
PCR primers		
fD3	AGAGTTTGATCCTGGCTTAG	8-27
T50	GTTACGACTTCACCTCCT	1497-1479
UniB	T(AC)AAGGAGGTGATCCAGC	1539-1522
Sequencing primers		
fD3	AGAGTTTGATCCTGGCTTAG	8-27
S1	AACGCGTGGATGATCTACCT	105-124
RS3	TTACCCTACCAACTAGCTAAT	259-239
S5	GAGGAATAAGCTTTGTAGGA	442-462
RS6	CTTTACGCCAATAATCCCGA	571-551
S9	ATTAGATACCCTGGTAGTCT	784-803
RS9	TAACACCAAGTGTGCATCGT	832-813
RS11	CTTAACCCAAACCTCACAGC	1086-1066
S13	AGGAAGGTGAGGATGACGTC	1169-1188
RS15	TAATAGCTTCGGGTATCCTC	1435-1416
RS16	TGATCCAGCCGCACTTTCC	1531-1512

TABLE 3. Sequence similarity values

Strain	% Sequence similarity to:																
	DK7	Lipitz	PBi	DK27	DK1	DK21	HO14 ^T	DN127	21038 ^T	PotiB1	PotiB2 ^T	PotiB3	Ir345	BR41	VS116 ^T	UK	Am501
<i>B. burgdorferi</i> DK7	100	99.8	99	99.2	98.6	98.7	95.8	96.8	97.7	99	99.1	99	99.2	99.2	98.8	98.7	98.9
<i>B. burgdorferi</i> Lipitz		100	99.2	99.4	98.8	98.9	96	97	98	99.2	99.3	99.2	99.4	99.4	99	98.9	99
<i>B. garinii</i> PBi			100	99.6	98.7	98.8	95.7	96.6	97.4	99.2	99.3	99.2	99.4	99.4	99	98.9	99.1
<i>B. garinii</i> DK27				100	98.8	98.9	95.9	96.9	97.7	99.5	99.6	99.5	99.7	99.7	99.1	99	99.2
<i>B. afzelii</i> DK1					100	99.8	95.7	96.1	97.8	98.9	98.8	98.9	98.9	98.9	99.2	99.1	99.3
<i>B. afzelii</i> DK21						100	95.7	96.2	97.8	99	98.9	99	99	99	99.2	99.2	99.4
<i>B. japonica</i> HO14 ^T							100	97.6	95.3	95.8	95.9	95.8	96	96	95.5	95.4	95.7
Genomic species DN127 strain DN127								100	95.4	96.6	96.7	96.6	96.8	96.8	96.2	96.1	96.4
<i>B. andersonii</i> 21308 ^T								100	97.8	97.7	97.8	97.8	97.8	97.8	97.6	97.5	97.8
<i>B. lusitaniae</i> PotiB1									100	99.9	100	99.9	99.9	99.9	99.1	99	99.3
<i>B. lusitaniae</i> PotiB2 ^T										100	99.9	99.9	99.9	99.9	99	99	99.2
<i>B. lusitaniae</i> PotiB3											100	99.9	99.9	99.9	99.1	99	99.3
<i>B. lusitaniae</i> Ir345												100	99.9	99.9	99.1	99	99.3
<i>B. lusitaniae</i> BR41													100	99.1	99	99.3	
<i>B. valaisiana</i> VS116 ^T														100	99.7	99.8	
<i>B. valaisiana</i> UK															100	99.7	
<i>B. valaisiana</i> Am501																100	

its *rrs* sequences with those of *B. valaisiana* (29) and those of other species constituting the *B. burgdorferi* sensu lato complex available in data banks (Table 1). The numbers of mutations in the 16S ribosomal DNA sequences of *B. burgdorferi* sensu lato strains were very low, and the levels of similarity between *rrs* sequences are shown in Table 3. These results confirm that the divergence of *B. burgdorferi* sensu lato leading to speciation is a recent event. As previously shown for *B. burgdorferi* sensu stricto and *B. garinii* (16), the five *B. lusitaniae* sp. nov. strains exhibit a very high level of relatedness (99.9%) (Table 3) and differ by only two nucleotides. The *rrs* sequence similarity between *B. valaisiana* and *B. lusitaniae* was 99.0 to 99.3%.

Despite the fact that the level of interspecific sequence variability approaches the level of intraspecific variability within each species, the results of the analysis of the phylogenetic relationships among these closely related species are consistent with the taxonomic position inferred from DNA-DNA hybridization studies. *B. lusitaniae* sp. nov. is clearly a member of a separate cluster (Fig. 1). Phylogenetically, the *B. lusitaniae* sp. nov. branch is close to *B. garinii*. In contrast, *B. valaisiana* branches closer to *B. afzelii*. This topology is strongly supported by parsimony analysis results (data not shown).

Determination of signature nucleotides. Due to the high level of *rrs* sequence conservation, the mismatches in nucleotide sequences between *Borrelia* strains might be of interest for identification at the species level or at the epidemiological level. Recognition of signature positions in each of the three main species has already been utilized to design primers and probes for identification of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (15, 16). Some variable and signature positions could be used to differentiate *B. lusitaniae* sp. nov. from other *B. burgdorferi* sensu lato strains (Table 4). However, we have to keep in mind that due to intraspecific variability, these signatures must be evaluated with a very large number of strains and must be reevaluated when a new species is described. For example, the signature positions which were chosen to design primers specific for *B. garinii* (15) are also available for *B. valaisiana* and *B. lusitaniae*. We failed to design primers strictly specific for *B. lusitaniae*.

Restriction fragment length polymorphism analysis. To overcome the problem described above, we investigated the restriction site polymorphism of PCR products from the *rrs*

gene. We have previously shown that *Bfa*I polymorphism provided a simple means for identifying *Borrelia* species (18, 24). PCR primers fD3 and T50 generated 1,488-bp DNA fragments, and their restriction by *Bfa*I allowed us to identify six patterns (Table 1). All but two species can be identified by this means. As we emphasized previously (24), the strains belonging to genomic species DN127 exhibit the same pattern as *B. burgdorferi* sensu stricto, and *B. japonica* strains exhibit the same pattern as *B. afzelii*. However, *B. lusitaniae* sp. nov. and *B. valaisiana* were easily identified by specific patterns (Fig. 2).

Protein analysis. The analysis of protein profiles revealed different patterns for the various *Borrelia* species (Fig. 3). Although very close to the position of *B. valaisiana* OspA, OspA from *B. lusitaniae* sp. nov. appeared to be slightly higher (34 kDa). *B. lusitaniae* Ir345 produced a variant OspA band around 32.5 kDa. The size of the OspB protein ranged from 34

FIG. 1. Phylogenetic tree based on a comparison of the 16S rDNA sequences of *B. burgdorferi* sensu lato. The branching pattern was generated by the unweighted pair group with mathematical average method. The values above the lines indicate the percentages of bootstrap replications. The bar represents 0.185% divergence.

TABLE 4. *B. burgdorferi* sensu lato signature and variable nucleotide positions of 16S ribosomal DNA

Position ^a	Nucleotide in ^b :						
	<i>B. lusitaniae</i>	<i>B. valaisiana</i>	<i>B. burgdorferi</i> sensu stricto	<i>B. garinii</i>	<i>B. afzelii</i>	<i>B. japonica</i>	Genomic species DN127
77	C	T/C	C	C	C	C	C
143	C	C	T	T	C	C	T
170	A	G	G	A	G	G	G
253	A	G	A	A	G	A	A
586	T	C/T	T	T	T	T	T
627	A	A	C	A	G	G	C
647	T	C/T	T	T	T	T	T
676	T	C	T	T	C	T	T
708	A	G	A	A	G	A	G
836	A	G	A	A	A	A	A
838	C	A	A	A	A	A	A
1133	A/G	A	G	G	A	G	G
1256	G	A	A	A	A	G	A

^a *B. burgdorferi* sensu stricto type strain B31 numbering (9).^b Nucleotides of different *B. burgdorferi* sensu lato sequences listed in Table 1 are included for comparison.

kDa for *B. burgdorferi* sensu stricto to 35 kDa for *B. afzelii*. This protein seems to be absent from *B. lusitaniae* and from *B. garinii*. In conclusion, the analysis of protein patterns did not provide a reliable means for identification of *B. lusitaniae*. No reactivity of *B. lusitaniae* isolates was observed with monoclonal antibodies H3TS, D6, and I 17.3. Specific monoclonal antibodies would be helpful for differentiating this species at a phenotypic level.

FIG. 2. Restriction patterns of *B. burgdorferi* sensu lato strains. DNAs from the *rrs* gene amplified with the primer set consisting of fD3 and T50 were digested by *Bfa*I. The DNAs were electrophoresed on a 1.8% agarose gel, stained with bromide ethidium, and UV illuminated. Lane 1, ϕ X-174-RF DNA *Hinc*II digest (Pharmacia); lane 2, *B. burgdorferi* sensu stricto strain B31^T; lane 3, *B. garinii* 20047^T; lane 4, *B. garinii* NT29; lane 5, *B. afzelii* VS461^T; lane 6, *B. japonica* 0612; lane 7, *B. andersonii* 21123; lane 8, genomic species DN127 strain DN127; lane 9, *B. lusitaniae* PotiB2^T; lane 10, *B. valaisiana* VS116^T; lane 11, ϕ X-174-RF DNA *Hae*III digest (Pharmacia).

FIG. 3. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of whole cells of *B. burgdorferi* sensu lato isolates. Lane 1, *B. garinii* 20047^T; lane 2, *B. burgdorferi* sensu stricto strain B31^T; lane 3, *B. afzelii* VS461^T; lane 4, *B. valaisiana* VS116^T; lanes 5 through 9, *B. lusitaniae* PotiB1, PotiB2^T, PotiB3, BR41, and Ir345, respectively; lane M, low-range protein molecular weight standards (Bio-Rad). f, flagellin; a, OspA; b, OspB; c, OspC.

Habitat. To our knowledge, only seven strains of *B. lusitaniae* sp. nov. have been isolated. They were all isolated from *Ixodes ricinus* ticks in Portugal (19), the Czech Republic, Moldavia, Ukraine, and Belorussia (23). However, DNA from *B. lusitaniae* sp. nov. was detected by PCR in *I. ricinus* ticks from Tunisia (3). The pathogenic potential of this species is unknown.

Description of *Borrelia lusitaniae* sp. nov. *Borrelia lusitaniae* (lu.si.ta.ni'a.e. L. fem. adj. *lusitaniae*, referring to Lusitania [Portugal], where the organism was first isolated [19]). Morphology as described previously for the genus (13). Cultural properties as described previously for *B. burgdorferi* sensu lato (11). Does not react with monoclonal antibodies H3TS, D6, and I 17.3 specific for *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*, respectively (unpublished data). Can be easily differentiated from other *B. burgdorferi* sensu lato species by analysis of the *Mse*I restriction polymorphism of the *rrf-rrl* spacer (21). Patterns after restriction of the *rrs* gene by *Bfa*I contain six fragments (689, 404, 159, 100, 78, and 58 bp).

The type strain, PotiB2, was isolated from *I. ricinus* in Portugal.

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REFERENCES

- Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. D. Grimont. 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int. J. Syst. Bacteriol.* **42**:378-383.
- Barbour, A. G. 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med.* **57**:521-525.
- Bouattour, A. 1997. Personal communication.
- Canica, M. M., F. Nato, L. du Merle, J. C. Mazie, G. Baranton, and D. Postic. 1993. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. *Scand. J. Infect. Dis.* **25**:441-448.
- Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164-166.
- Fukunaga, M., A. Hamase, K. Okada, H. Inoue, Y. Tsuruta, K. Miyamoto, and M. Nakao. 1996. Characterization of spirochetes isolated from ticks (*Ixodes tanuki*, *Ixodes turdus*, and *Ixodes columnae*) and comparison of the sequences with those of *Borrelia burgdorferi* sensu lato strains. *Appl. Environ. Microbiol.* **62**:2338-2344.
- Fukunaga, M., A. Hamase, K. Okada, and M. Nakao. 1996. *Borrelia tanukii*

- sp. nov. and *Borrelia turdae* sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. *Microbiol. Immunol.* **40**:877–881.
8. Fukunaga, M., Y. Takahashi, Y. Tsuruta, O. Matsushita, D. Ralph, M. McClelland, and M. Nakao. 1995. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. *Int. J. Syst. Bacteriol.* **45**:804–810.
 9. Gazumyan, A., J. J. Schwartz, D. Liveris, and I. Schwartz. 1994. Sequence analysis of the ribosomal RNA operon of the Lyme disease spirochete, *Borrelia burgdorferi*. *Gene* **146**:57–65.
 10. Higgins, D. G., and R. Sharp. 1989. Fast and sensitive multiple sequence alignments on a microcomputer. *CABIOS* **5**:151–153.
 11. Johnson, R. C., G. P. Schmid, F. W. Hyde, A. G. Steigerwalt, and D. J. Brenner. 1984. *Borrelia burgdorferi* sp. nov.: etiological agent of Lyme disease. *Int. J. Syst. Bacteriol.* **34**:496–497.
 12. Kawabata, H., T. Masuzawa, and Y. Yanagihara. 1993. Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. *Microbiol. Immunol.* **37**:843–848.
 13. Kelly, R. T. 1984. *Borrelia*, p. 57–62. In N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
 14. Kumar, S., K. Tamura, and N. Masatoshi. 1993. MEGA: molecular evolutionary genetics analysis, version 1.01. The Pennsylvania State University, University Park.
 15. Marconi, R. T., and C. F. Garon. 1992. Development of polymerase chain reaction primer sets for diagnosis of Lyme disease and for species-specific identification of Lyme disease isolates by 16S rRNA signature nucleotide analysis. *J. Clin. Microbiol.* **30**:2830–2834.
 16. Marconi, R. T., and C. F. Garon. 1992. Phylogenetic analysis of the genus *Borrelia*: a comparison of North American and European isolates of *Borrelia burgdorferi*. *J. Bacteriol.* **174**:241–244.
 17. Marconi, R. T., D. Liveris, and I. Schwartz. 1995. Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: phylogenetic analyses of rRNA genes and their intergenic spacers in *Borrelia japonica* sp. nov. and genomic group 21038 (*Borrelia andersonii* sp. nov.) isolates. *J. Clin. Microbiol.* **33**:2427–2434.
 18. Marti Ras, N., B. Lascola, D. Postic, S. J. Cutler, F. Rodhain, G. Baranton, and D. Raoult. 1996. Phylogenesis of relapsing fever *Borrelia* spp. *Int. J. Syst. Bacteriol.* **46**:859–865.
 19. Nuncio, M. S., O. Péter, M. J. Alves, F. Bacellar, and A. R. Filipe. 1993. Isolamento e caracterização de Borrélias de *Ixodes ricinus* L. em Portugal. *Rev. Port. Doenças Infec.* **16**:175–179.
 20. Péter, O., and A. G. Bretz. 1992. Polymorphism of outer surface proteins of *Borrelia burgdorferi* as a tool for classification. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* **277**:28–33.
 21. Postic, D., M. V. Assous, P. A. D. Grimont, and G. Baranton. 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rf* (5S)-*rrl* (23S) intergenic spacer amplicons. *Int. J. Syst. Bacteriol.* **44**:743–752.
 22. Postic, D., J. Belfaiza, E. Isogai, I. Saint Girons, P. A. D. Grimont, and G. Baranton. 1993. A new genomic species in *Borrelia burgdorferi* sensu lato isolated from Japanese ticks. *Res. Microbiol.* **144**:467–473.
 23. Postic, D., E. Korenberg, N. Gorelova, Y. V. Kovalevski, E. Bellenger, and G. Baranton. *Borrelia burgdorferi* sensu lato in Russia and neighboring countries: high incidence of mixed isolates. *Res. Microbiol.*, in press.
 24. Ralph, D., D. Postic, G. Baranton, C. Pretzman, and M. McClelland. 1993. Species of *Borrelia* distinguished by restriction site polymorphisms in 16S rRNA genes. *FEMS Microbiol. Lett.* **111**:239–243.
 25. Ruimy, R., V. Breittmayer, P. Elbaze, B. Lafay, O. Boussebart, M. Gauthier, and R. Christen. 1994. Phylogenetic analysis and assessment of the genera *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* deduced from small-subunit rRNA sequences. *Int. J. Syst. Bacteriol.* **44**:416–426.
 26. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
 27. Swofford, D. L. 1992. PAUP: phylogenetic analysis using parsimony, version 3.0. Illinois Natural History Survey, Champaign.
 28. Theisen, M., M. Borre, M. J. Mathiesen, B. Mikkelsen, A. M. Lebech, and K. Hansen. 1995. Evolution of the *Borrelia burgdorferi* outer surface protein OspC. *J. Bacteriol.* **177**:3036–3044.
 29. Wang, G., A. P. van Dam, A. Le Fleche, D. Postic, O. Peter, G. Baranton, R. de Boer, L. Spanjaard, and J. Dankert. 1997. Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). *Int. J. Syst. Bacteriol.* **47**:926–932.