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 *rpoB* ribosomal spacer (21). A specific *msel* restriction pattern allowed workers to clearly identify this new genomic species, as well as all other delineated species or genomic species within *B. burgdorferi*.

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 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.
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 Phylogeny Analysis Using Parsimony (PAUP) 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).

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was easily identified by analyzing the restriction pattern of the rrf-wl 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

### 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-wl* 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...
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 interspecific 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 Bfai polymorphism provided a simple means for identifying *Borrelia* species (18, 24). PCR primers f3 and T50 generated 1,488-bp DNA fragments, and their restriction by Bfai 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 branches indicate the percentages of bootstrap replications. The bar represents 0.185% divergence.](image-url)
TABLE 4. B. burgdorferi sensu lato signature and variable nucleotide positions of 16S ribosomal DNA

<table>
<thead>
<tr>
<th>Position</th>
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<tr>
<td>77</td>
<td>C T/C T/C C C C C C</td>
</tr>
<tr>
<td>143</td>
<td>C C T C T C T C T</td>
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<tr>
<td>170</td>
<td>A G A G G C C A G</td>
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<tr>
<td>253</td>
<td>A C T A G C C G A</td>
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<tr>
<td>586</td>
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<td>627</td>
<td>A A C A G G C G C</td>
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<tr>
<td>647</td>
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<tr>
<td>676</td>
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<tr>
<td>708</td>
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<td>1256</td>
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</table>

a B. burgdorferi sensu stricte 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 stricte to 35 kDa for B. afzelii. This protein seems to be absent from B. latistiaiae and from B. garinii. In conclusion, the analysis of protein patterns did not provide a reliable means for identification of B. latistiaiae. No reactivity of B. latistiaiae isolates was observed with monoclonal antibodies H3TS, D6, and 1 17.3. Specific monoclonal antibodies would be helpful for differentiating this species at a phenotypic level.

Habitat. To our knowledge, only seven strains of B. latistiaiae 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. latistiaiae sp. nov. was detected by PCR in I. ricinus ticks from Tunisia (3). The pathogenic potential of this species is unknown.

Description of Borrelia latistiaiae sp. nov. Borrelia latistiaiae (luisl.ani.ae. L. fem. adj. latistiaiae, 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 1 17.3 specific for B. burgdorferi sensu stricte, B. garinii, and B. afzelii, respectively (unpublished data). Can be easily differentiated from other B. burgdorferi sensu lato species by analysis of the MseI restriction polymorphism of the rrf-rfl spacer (21). Patterns after restriction of the rs gene by Bsal 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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