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Genetic Diversity of *Borrelia burgdorferi* Sensu Lato in Ticks from Mainland Portugal

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To date *Borrelia lusitaniae* is the only genospecies of *Borrelia burgdorferi* sensu lato isolated from *Ixodes ricinus* ticks collected in Portugal and Tunisia. This suggests that the genospecies diversity of *B. burgdorferi* sensu lato decreases toward the southwestern margin of its Old World subtropical range. In order to further explore the genetic diversity of *B. burgdorferi* sensu lato from this region, 55 *I. ricinus* and 27 *Hyalomma marginatum* questing adults, collected during the spring of 1998 from a sylvatic habitat south of Lisbon, Portugal, were analyzed. Infection prevalences of 75% in *I. ricinus* ticks and 7% in *H. marginatum* ticks were detected by a nested PCR that targets the *rrf* (5S)-*rrl* (23S) spacer of *B. burgdorferi* sensu lato. Restriction fragment length polymorphism (RFLP) analysis of the *I. ricinus*-derived amplicons showed that the sequences in the majority of samples were similar to those of *B. lusitaniae* type strains (76% for strain PotiB1, 5% for strain PotiB3). Two novel RFLP patterns were obtained from 12% of the samples. The remaining 7% of samples gave mixed RFLP patterns. Phylogenetic analysis of *rrf-rrl* spacer sequences revealed a diverse population of *B. lusitaniae* in questing adult *I. ricinus* ticks (the sequences did not cluster with those of any other genospecies). This population consisted of 10 distinct sequence types, suggesting that multiple strains of *B. lusitaniae* were present in the local *I. ricinus* population. We hypothesize that *B. lusitaniae* has a narrow ecological niche that involves host species restricted to the Mediterranean Basin.

In 1982 the etiological agent of Lyme disease was identified as a spirochete (2) which was later named *Borrelia burgdorferi* (13). Since then numerous strains related to this bacterium have been isolated. It is now widely accepted that these strains form a complex, *B. burgdorferi* sensu lato, which consists of 10 named genospecies and several yet to be named genomic groups. The genospecies are *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia andersonii*, *Borrelia bissettii*, *Borrelia japonica*, *Borrelia turdii*, and *Borrelia tanukii* (4, 18, 25). *B. garinii*, *B. afzelii*, and *B. burgdorferi* sensu stricto are associated with disease in humans (32), while the pathogenic potential of the remaining genospecies is unknown. *B. burgdorferi* sensu lato is maintained in nature by zoonotic transmission cycles, in which hard ticks are the vectors and vertebrates are the reservoir hosts. *Ixodes ricinus* is the principal vector in western Europe (15, 16, 21).

To better understand the ecology and epidemiology of tick-borne spirochetes, knowledge of their genetic diversity in nature is required. Most genetic studies of *B. burgdorferi* sensu lato are based on data derived from isolated spirochetes. Isolation of these bacteria can be fastidious (24) and may select for genotypes (19, 22). Advances in PCR technology have made it possible to detect and genotype microorganisms directly from clinical and environmental samples, without the need for isolation (3, 27). By minimizing in vitro selection, PCR-based typing tools provide more accurate methods for assessing the natural genetic diversity of *B. burgdorferi* sensu lato populations.

Only a few tick isolates of *B. burgdorferi* sensu lato have ever been obtained from mainland Portugal (23) and Tunisia (34). These were identified as *Borrelia lusitaniae* sp. nov. (18). This suggests that the genospecies diversity of *B. burgdorferi* sensu lato is low near the southern margin of its European range. In contrast, a PCR-based study found *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto in ticks from the Portuguese Island of Madeira (21).

In this study the genetic diversity of *B. burgdorferi* sensu lato in local tick populations from a sylvatic habitat in mainland Portugal was analyzed. Rigorous phylogenetic analysis of PCR-derived sequences revealed a diverse population of *B. lusitaniae* in questing adult *I. ricinus* ticks.

MATERIALS AND METHODS

Study site and tick collection. During March, April, and May of 1998 ticks were collected by blanket dragging (16) from a sylvatic habitat south of Lisbon, Portugal (8°33′W, 38°05′N). Each tick was assigned the letters GT and a number. Fifty-five *I. ricinus* and 27 *Hyalomma marginatum* questing adult ticks were analyzed. Ticks were preserved in 70% ethanol at ambient temperature.

***Borrelia* strains and nucleotide sequences.** The cultured *B. burgdorferi* sensu lato strains used in this study are given in Table 1. All nucleotide sequences that were downloaded from GenBank (24) and then used in the phylogenetic analysis are also given in Table 1.

DNA preparation, *rrf-rrl* PCR, and reverse line blot. Genomic DNA from ticks and cultured strains was prepared by alkaline hydrolysis in a final volume of 250 μl (8). A nested PCR that targeted the *rrf* (5S)-*rrl* (23S) intergenic spacer of *B.*

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TABLE 1. Strains and GenBank *rrf-rrl* nucleotide sequences used in this study

Species	Strain or isolate	Origin	*rrf-rrl* accession no.
B. afzelii	ACA 1[a]	Human, Denmark	AF200659
	VS461	*I. ricinus*, Switzerland	L30135
	J 1	Unknown	L30129
B. burgdorferi sensu stricto	ZS 7[a,b]	*I. ricinus*, Germany	NA[c]
	212[b]	*I. ricinus*, France	L30121
	B31	*Ixodes scapularis*, United States	L30127
B. garinii	ZQ 1[a]	*I. ricinus*, Germany	AF200660
	20047	*I. ricinus*, France	L30119
	NT29	*Ixodes persulcatus*, Japan	L30130
B. lusitaniae	PotiB1[a,d]	*I. ricinus*, Portugal	NA
	PotiB2	*I. ricinus*, Portugal	L30131
	PotiB3	*I. ricinus*, Portugal	L30132
B. valaisiana	UK[a]	*I. ricinus*, England	L30133
	VS116	*I. ricinus*, Switzerland	L30134
B. bissettii	DN127	*Ixodes pacificus*, United States	L30126
	CA55	*Ixodes neotomae*, United States	L30124
	25015	*I. scapularis*, United States	L30122
B. andersonii	21123	*Ixodes dentatus*, United States	L30120
	19952	*I. dentatus*, United States	L30118
B. japonica	HO14	*I. ovatus*, Japan	L30125
	Cow611C	*I. ovatus*, Japan	L30128
Borrelia sp.	CA2	*Ixodes neotomae*, United States	L30123

[a] Strains cultured and used as positive controls.
[b] Identical sequences.
[c] NA, not applicable.
[d] Sequence identical to that derived from GT058.

burgdorferi sensu lato was performed with this prepared DNA (5 μl per reaction mixture) by using primers 23SN1, 23SC1, 23N2, and 5SCB as described previously (17, 27). All stages of the PCR were separated temporally and spatially (different laboratories) and were carried out under strictly aseptic conditions. Negative controls at a ratio of 2:3 were incorporated into the alkaline hydrolysis step and both the first and second rounds of PCR amplification. Prepared DNA of serial dilutions of cultured *B. afzelii* ranging between 2×10^7 and 2 spirochetes per reaction mixture (in log steps) was amplified repeatedly. Two dilutions that contained 2,000 and 2 spirochetes per reaction mixture were used as positive controls for each PCR amplification. All amplicons were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator. Samples that tested positive were reamplified by nested PCR three times. DNA-DNA hybridization by the reverse line blot (RLB) assay was performed with samples that produced amplicons of approximately 380 and/or 230 bp as described previously (17, 27). DNA probes specific for *B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* were used (17, 27). A probe specific for *B. lusitaniae* was not available at the time. Amplified DNAs derived from cultured *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* were used as positive controls. All samples that tested PCR positive were analyzed by the RLB assay twice.

Restriction fragment length polymorphism (RFLP) analysis of *rrf-rrl* PCR amplicons. Intergenic spacer PCR products (10 μl) were digested with 5 U of *Mse*I (New England Biolabs) in a total volume of 15 μl. Restriction products were separated by electrophoresis on a 20% polyacrylamide TBE (Tris-borate-EDTA) gel (NOVEX) for 4 h at 80 V. The gels were stained with ethidium bromide and were visualized with a UV transilluminator. A molecular size marker D-15 (NOVEX) was used for comparison.

DNA sequencing of PCR products. *rrf-rrl* PCR products were reamplified with primers 23SN2 and 5SCB to which M13 (universal or reverse) tails had been added at the 5′ ends. These reamplified products were then cycle sequenced with M13 universal and reverse primers. A fifth of the *rrf-rrl* amplicons were cycle sequenced again with internally labeled primer 23N2 (35).

Sequence alignment and phylogenetic analysis. The forward and reverse sequences of each PCR product which overlapped were aligned against one another and edited to produce a single sequence. These sequences were then aligned against each other and the reference sequences downloaded from GenBank by using Clustal W (31), followed by manual adjustment. Various rooted and unrooted phylogenetic trees were constructed with the PAUP package (29) by using both distance matrix (neighbor-joining, unweighted pair group method with arithmetic averages [UPGMA]) and discrete character (maximum likelihood, maximum parsimony) methods. In the maximum likelihood analysis the general reversible model of DNA substitution was used along with a gamma distribution of rate variation among sites. This substitution model was also used in the neighbor-joining analysis. Bootstrap analysis with 1,000 resamplings was performed to establish robustness for clusters in the neighbor-joining tree.

Nucleotide sequence accession numbers. The *rrf-rrl* spacer sequences of *B. burgdorferi* sensu lato derived from *I. ricinus* and cultures have been deposited in GenBank and have been assigned accession nos. AF200649 (GT058), AF200650 (GT163), AF200651 (GT172), AF200652 (GT156), AF200653 (GT098), AF200654 (GT167), AF200655 (GT151), AF200656 (GT158), AF200657 (GT078), AF200658 (GT132), AF200659 (ACA1), and AF200660 (ZQ1).

RESULTS

Intergenic spacer PCR and RLB assay. Forty-one of the *I. ricinus* ticks and two of the *H. marginatum* ticks tested positive for the *rrf-rrl* locus of *B. burgdorferi* sensu lato (infection prevalences, 75 and 7%, respectively). All positive tick-derived samples gave two bands of 380 and 230 bp. Amplification of DNA from 2×10^3 or more cultured spirochetes also gave two bands, whereas dilutions that contained DNA equivalent to $<2 \times 10^3$ spirochetes generated only one band of 230 bp. This indicates that each (whole) tick was infected with at least 10^5 spirochetes. The DNA probes used for the RLB assay hybridized successfully with the corresponding positive control DNA. However, the 43 tick-derived PCR-positive amplicons hybridized only with the *B. burgdorferi* sensu lato probe. This indicates that the ticks were infected with *B. burgdorferi* sensu lato but not with *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, or

TABLE 2. *Mse*I restriction pattern of the *rrf-rrl* spacer of *B. burgdorferi* sensu lato amplified by nested PCR directly from ticks or cultured strains

Strain or tick-derived sample[a]	Amplicon size (bp)	RFLP pattern	*Mse*I restriction fragment size (bp)[b]
B. burgdorferi ZS7	227	A	95, 37, 29, 28
B. afzelii ACA 1	220	B	20, 37, 68, 95
B. garinii ZQ 1	227	C	95, 95, 37
B. valaisiana UK	229	D	162, 37, 23, 7
B. lusitaniae PotiB1	230	E	95, 67, 39, 29
GT058[c] (I)[d]	230	E	95, 67, 39, 29
GT078 (V)	230	E	96, 66, 39, 29
GT151 (VI)	231	E	96, 67, 39, 29
GT172 (VII)	230	E	95, 67, 39, 29
GT132 (IV)	229	F	95, 66, 52, 16
GT098 (II)	212	G	95, 78, 39
GT167 (VIII)	212	G	95, 78, 39
GT156 (III)	220	H	95, 66, 30, 29
GT158 (IX)	213	G+E	96, 78, 39
GT163 (X)	230	F+E	95, 67, 29, 39

[a] Only representative samples are shown.
[b] The exact sizes of the fragments were determined from the sequence of the nested PCR product.
[c] Sequence identical to that of PotiB1.
[d] Roman numerals in parentheses indicate sequence type.

B. valaisiana. Repetition of PCR and the RLB assay with positive samples gave consistent results.

RFLP analysis of *rrf-rrl* PCR amplicons. All five cultured strains used for reference were digested and gave distinct RFLP patterns which were assigned the letters A to E (Table 2). Forty-one of the *I. ricinus*-derived *rrf-rrl* PCR products were digested. Four different RFLP patterns were obtained from these samples (Table 2 and Fig. 1). Thirty-one (76%) gave pattern E, the same as that given by type strain PotiB1 (and type strain PotiB2, as deduced from the sequence). Two (5%) gave pattern F, which is similar to that given by PotiB3, as deduced by Postic et al. (24). Two patterns that were unlike any other pattern in our data set or previously published data sets were obtained from five (12%) samples and were assigned the letters G and H (10% gave pattern G and 2% gave pattern H). Two (5%) gave a mixed pattern of F and E, and one (2%) gave a mixed pattern of G and E.

Sequencing alignment and phylogenetic analysis of *rrf-rrl* locus. Twenty-seven of the 43 tick-derived PCR positive amplicons for the *rrf-rrl* locus were sequenced successfully. The five cultured strains listed in Table 1 that were used as positive controls for the PCR were also sequenced successfully. These 32 sequences plus the 18 downloaded from GenBank (Table 1) were aligned and a tree was constructed by using UPGMA (data not shown). From this tree identical sequences and outgroups were identified. A representative sequence from each cluster of identical sequences was chosen. Sequences 19952 and 21133 were considered too divergent to be included in the analysis and so were removed. Thus, the final alignment contained 10 tick-derived and 15 reference sequences (Fig. 2). On the basis of this alignment, neighbor-joining (Fig. 3), maximum parsimony (data not shown), and maximum likelihood (Fig. 4) trees were constructed. In all trees the tick-derived sequences cluster with *B. lusitaniae* strains. The neighbor-joining tree is completely resolved and shows 10 distinct sequence types among the tick-derived samples. These sequence types are not resolved by the maximum likelihood tree.

Frequency distribution of sequence types. Of the 27 tick-derived sequences analyzed, 10 sequence types were found (Table 2). The frequency of each type is as follows: 15 (56%) samples were of sequence type I (identical to that of PotiB1), 3 (11%) were of sequence type II, 2 (7%) were of sequence type III, and 1 (4%) each was of sequence type IV to X (type IV is identical to that of PotiB3).

DISCUSSION

B. lusitaniae was the only genospecies of *B. burgdorferi* sensu lato found in *I. ricinus* ticks from a sylvatic habitat in mainland Portugal. This result corroborates previous findings based on the isolation of spirochetes from Portugal (23) and Tunisia (34). It is, therefore, possible that the genospecies diversity of *B. burgdorferi* sensu lato decreases toward the southern margin of its European range. However, another study recorded *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* in *I. ricinus* ticks collected from the Portuguese Island of Madeira, but not *B. lusitaniae* (21). As the fauna of Madeira differs from that of mainland Portugal (21), it may be that differences in the structures of the vertebrate host cenoses are part of the reason for these contrasting results.

Two distinct and novel RFLP patterns, G and H (Fig. 1, lanes 8 and 9, respectively), were obtained from 12% of the *I. ricinus*-derived PCR products, initially suggesting that novel genotypes of *B. burgdorferi* sensu lato may have been present in the ticks (S. De Michelis, H.-S. Sewell, M. Collares-Pereira, L. Vieira, M. Santos-Reis, L. Schouls, and K. Kurtenbach, Abstr. VIII Int. Conf. Lyme Borreliosis Other Emerg. Tick-Borne Dis., abstr. 08, p. 6, 1999). Upon phylogenetic analysis of sequences, these samples proved to be *B. lusitaniae*. For example, the PCR product from tick GT156, which gave RFLP pattern H (Fig. 1), clustered with type strains PotiB1 and PotiB2 (Fig. 3 and 4). Similarly, the samples from GT98 and GT167 gave RFLP pattern G but clustered with *B. lusitaniae* type strains. It should also be noted that the RFLP patterns of *B. burgdorferi* sensu stricto strain ZS 7 and *B. lusitaniae* type strain PotiB1 were so similar (Fig. 1, lanes 2 and 3, respectively) that it was not possible to unambiguously differentiate them. While PCR-RFLP analysis of the *rrf-rrl* locus correctly typed the majority of the tick-derived samples, the misleading novel patterns obtained and the similarity of certain genospecies patterns highlight the limitations of this commonly used typing method (5, 20, 24, 34).

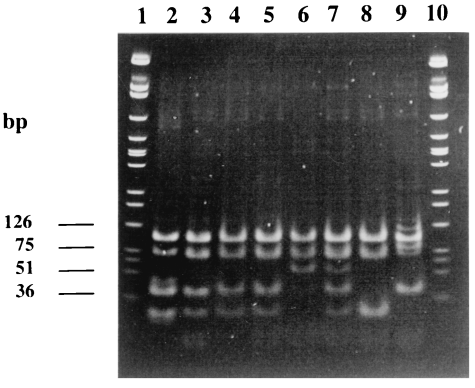

FIG. 1. *Mse*I restriction patterns of *B. burgdorferi* sensu lato *rrf-rrl* spacer nested PCR products amplified directly from ticks or cultured strains. Lanes 1 and 10, D-15 marker; lanes 2 and 3, cultured *B. burgdorferi* sensu stricto (ZS 7) and *B. lusitaniae* (PotiB1), patterns A and E, respectively; lanes 4 and 5, tick-derived samples 58 and 151 (pattern E), respectively; Lane 6, tick-derived sample 132 (pattern F); lane 7, tick-derived sample 163 (patterns E and F); lane 8, tick-derived sample 156 (pattern H); lane 9, tick-derived sample 167 (pattern G).

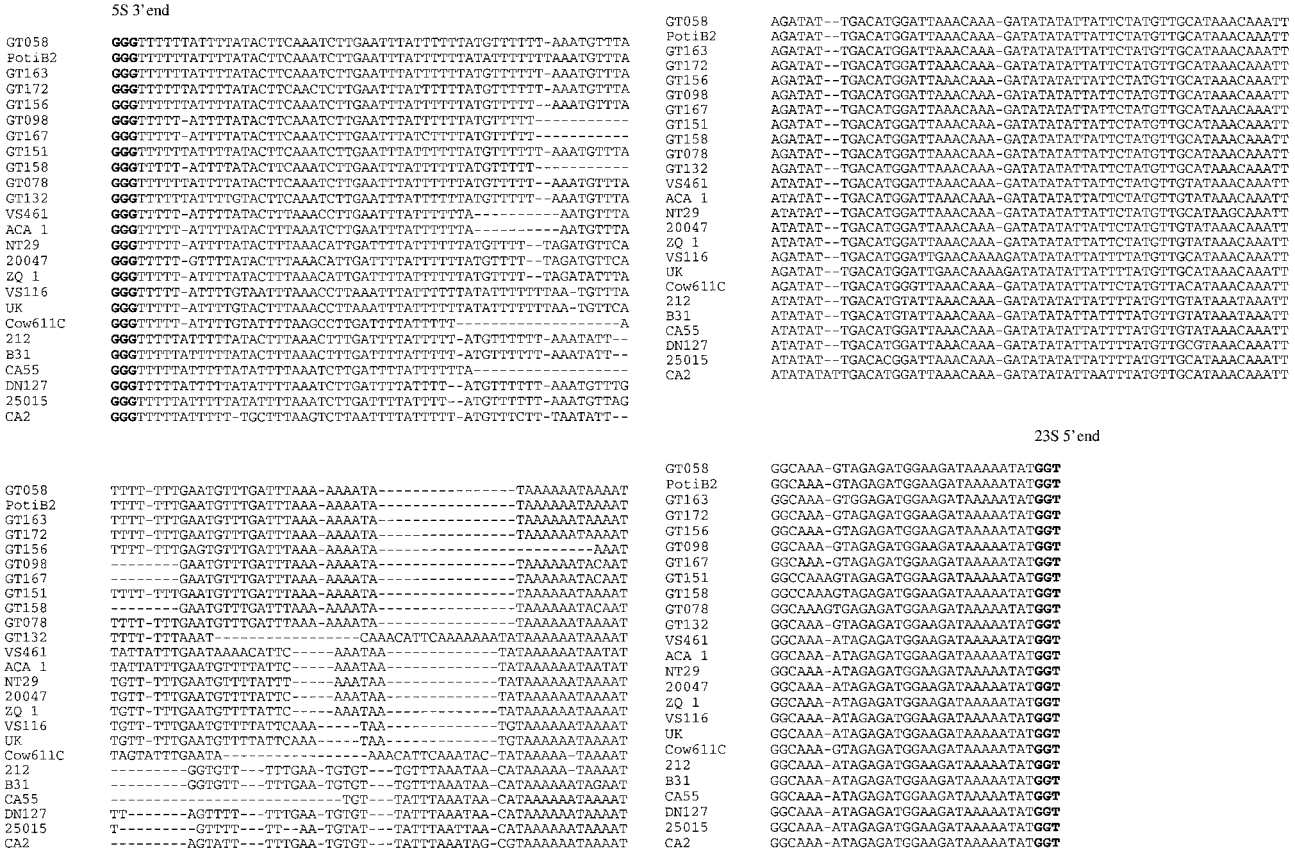

FIG. 2. Aligned *rrf-rrl* spacer DNA sequences of *B. burgdorferi* sensu lato amplified directly from ticks or cultured strains or downloaded from GenBank. Gaps were introduced to obtain maximum homology. Only the last three bases of the 3′ end of the *rrf* gene and the first three bases of the 5′ end of the *rrl* gene are shown (in boldface).

Phylogenetic analysis of *rrf-rrl* spacer sequences has been used to delineate *B. burgdorferi* sensu lato genospecies and to assess their genetic diversity (9, 24, 25). In this study, the neighbor-joining (Fig. 3), maximum parsimony (data not shown), and maximum likelihood (Fig. 4) trees reveal that the *rrf-rrl* sequences derived from the Portuguese ticks form a cluster with *B. lusitaniae* type strains, thereby generally confirming the results obtained by RFLP analysis. The neighbor-joining tree is fully resolved, and within the *B. lusitaniae* cluster it discriminates 10 sequence types (Fig. 3). In contrast, the maximum likelihood tree is not fully resolved within the *B. lusitaniae* cluster (Fig. 4). In addition, some of the nodes in the neighbor-joining tree have relatively low bootstrap values at the genospecies level (bootstrap values of less than 70 are not shown). Both phylogenetic methods therefore indicate that the level of evolutionary information that can be gained from the *rrf-rrl* intergenic spacer is limited. Altogether our findings support previous suggestions that this locus is not suitable for analysis of the molecular phylogeny of *B. burgdorferi* sensu lato (24). Likely reasons for this are the fact that (i) the intergenic spacer is very short such that phylogenetic analysis is subject to large sampling errors, (ii) the intergenic spacer is composed of highly conserved and highly variable regions, (iii) and the alignment of sequences is ambiguous in places. In conclusion, while the *rrf-rrl* spacer of *B. burgdorferi* sensu lato is a suitable locus for use in the fingerprinting of genotypes and for the preliminary assessment of genetic diversity, it cannot be used to reliably infer evolutionary relationships between closely related *Borrelia* strains.

Recent studies on the population genetics of *B. burgdorferi* sensu lato in local tick populations from North America by PCR amplification of genes that encode outer surface proteins reported that numerous alleles of *B. burgdorferi* sensu stricto can be maintained simultaneously within local tick populations (7). The present study revealed 10 distinct sequence types (hereafter termed alleles) of *B. lusitaniae*, suggesting that the local tick population carried at least 10 different strains of this genospecies. The analysis of the frequency distribution of the 10 alleles revealed that allele I (identical to the sequence of PotiB1) was overrepresented (56%); i.e., half of the ticks were infected with the same genotype. The frequency distribution among the remaining nine alleles was much more even (4 to 11%). The biological significance of the frequency distribution of *Borrelia* alleles within this tick population from Portugal awaits determination. As recently proposed for *B. burgdorferi* sensu stricto (26), the population structure of *B. lusitaniae* is likely to be shaped by frequency-dependent selection. It remains to be analyzed whether the diversity of *B. lusitaniae* observed at a neutral locus (i.e., the *rrf-rrl* intergenic spacer) is mirrored at other loci, in particular, genes that encode outer surface proteins (e.g., OspA, OspB, and OspC).

Another ecologically interesting finding of this study was that 2 of 27 adult *H. marginatum* ticks contained DNA identical to that of *B. lusitaniae* strain PotiB1 (data not shown), suggesting that these ticks had been exposed to spirochetemic hosts. Subadult *H. marginatum* ticks mainly feed on birds and rodents (10), a behavior that may point to a possible role of avian or rodent species as reservoirs for *B. lusitaniae*.

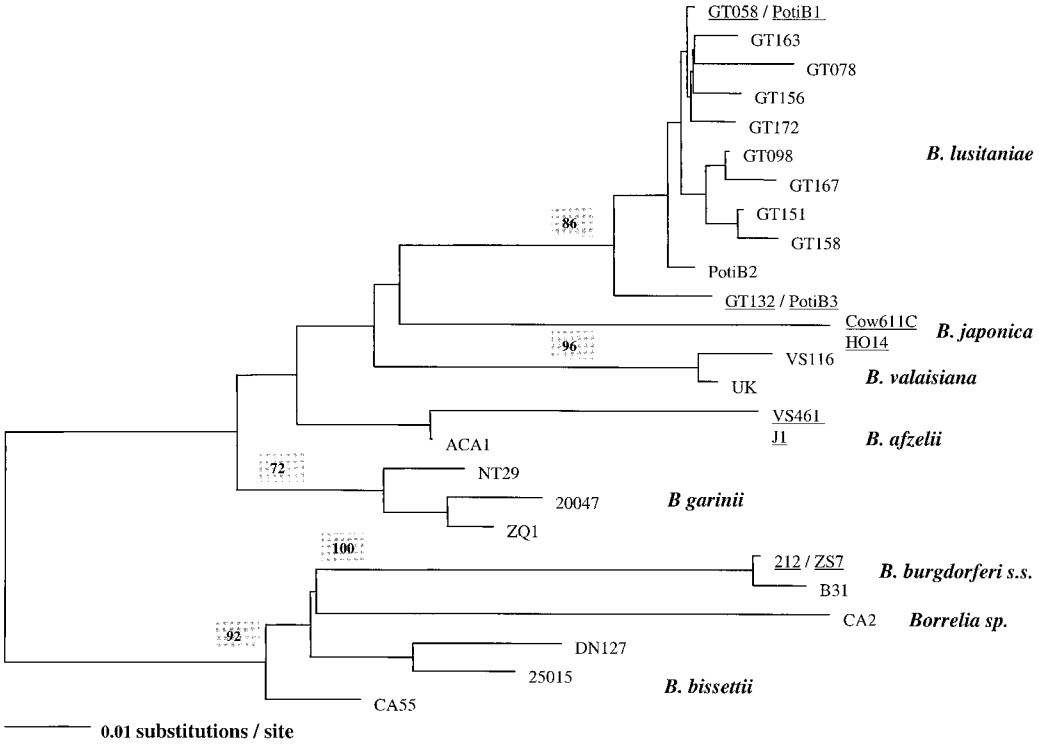

FIG. 3. Neighbor-joining tree based on the comparison of *rrf-rrl* spacer sequences of *B. burgdorferi* sensu lato. The tree is drawn rooted at the midpoint for clarity. Sequences that are identical and that share the same branch are underlined. The numbers in the grey boxes are the results of 1,000 bootstrap resamplings (values of less than 70 are not shown). *B. burgdorferi s.s.*, *B. burgdorferi* sensu stricto.

The infection prevalence of *B. burgdorferi* sensu lato in questing adult *I. ricinus* ticks discovered in this study is significantly higher than that reported for most other regions of Europe, where values rarely exceed 40% in adult ticks (1, 12, 14–16, 27, 28, 30, 33). Apart from Portugal and Tunisia, *B. lusitaniae* has been found in the Czech Republic, Moldavia, Ukraine, and Belarus (18). In these Eastern European countries *B. lusitaniae* seems to be a rare genospecies of *B. burgdorferi* sensu

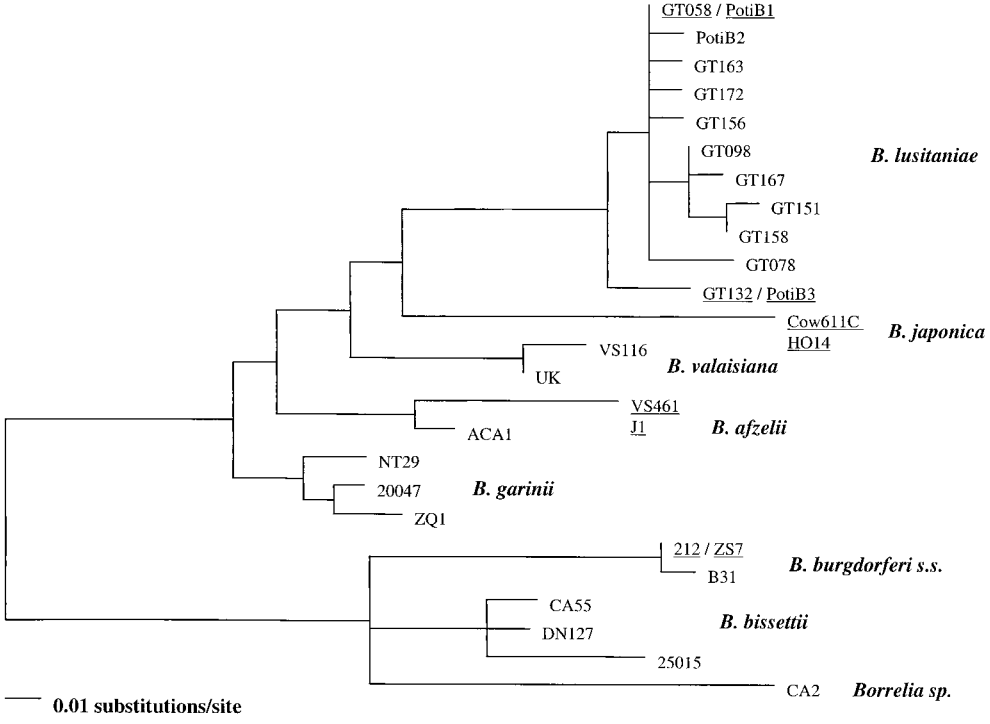

FIG. 4. Maximum likelihood phylogenetic tree based on the comparison of *rrf-rrl* spacer sequences of *B. burgdorferi* sensu lato. The tree was constructed by using the general reversible model of DNA substitution and a gamma distribution of rate variation among sites (drawn rooted at the midpoint). Sequences that are identical and that share the same branch are underlined. *B. burgdorferi s.s.*, *B. burgdorferi* sensu stricto.

lato. It has been reported that *B. garinii*, *B. afzelii*, and *B. valaisiana* account for the vast majority of infections in ticks from these areas (6, 11). We hypothesize that *B. lusitaniae* has a narrow ecological niche that involves vertebrate species that are geographically restricted to the Mediterranean Basin and that are highly competent reservoirs for this genospecies.

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