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Characterization of *Borrelia lusitaniae* Isolates Collected in Tunisia and Morocco

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***Borrelia lusitaniae* is a species within the complex *Borrelia burgdorferi* sensu lato and is infrequently isolated in Europe. In contrast, this species is by far the most predominant in North Africa and in Portugal. In this study, we analyzed the genetic diversity, at several loci, of a large population of isolates from free-living *Ixodes ricinus* ticks collected in Tunisia and Morocco. We found a moderate diversity of the whole genome by using pulsed-field gel electrophoresis as well as in the *ospA* gene sequences, compared to a high level of strain homogeneity in the small noncoding ribosomal spacer. In contrast, a high diversity of this locus has been previously reported for Portuguese isolates. We hypothesize that *B. lusitaniae* strains isolated in North Africa constitute a clone of Portuguese origin.**

Within the complex *Borrelia burgdorferi* sensu lato, three species, *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, are known to cause a broad spectrum of human manifestations that are described as Lyme borreliosis. The genetic diversity of pathogenic species has been largely investigated both at the species level and at the strain level within each species (41). Otherwise, although its pathogenicity for humans is only suspected (31, 33), *Borrelia valaisiana* represents a widely distributed species in ticks throughout Europe and Asia and therefore has been extensively studied (12, 22, 32, 38, 40).

In contrast to these species, little data are available concerning *Borrelia lusitaniae*. Only sporadic isolates have been characterized from ticks collected in the Czech Republic, Moldavia, Ukraine, and Belarus (20), in Slovakia (11), in Spain (3), in Poland (25), in France (30), in Switzerland (15–17), or in Turkey (13), and these isolates did not show any significant diversity. However, a study conducted in Portugal revealed a large diversity among *B. lusitaniae* strains isolated from a local tick population (6). Moreover, it is noteworthy that, among the three first Portuguese isolates identified as *B. lusitaniae*, two different types were demonstrated (20, 26). Hence, these studies suggested a higher diversity among *B. lusitaniae* around the Mediterranean basin. However, all these studies only focused on the small ribosomal *rrf-rrl* spacer and, therefore, the global diversity of this species remained unknown.

Recently, we reported that *B. lusitaniae* was, by far, the most prevalent species, ranging from 96.6 to 100% of the *B. burgdorferi* sensu lato species identified in Tunisia and Morocco (35, 44, 46). The report of some Lyme borreliosis cases in North Africa (1, 27) and the recent isolation in Portugal of *B. lusitaniae* from the skin of a human patient with chronic lesions (5) prompted us to characterize this species as a potential human pathogen. Therefore, we investigated the molecular characterization and the genetic diversity of *B. lusitaniae* isolated from free-living *Ixodes ricinus* ticks in Tunisia and Morocco. For these purposes we studied two single loci (*ospC* and *ospA*) and the noncoding intergenic spacer (*rrf-rrl*), as well as the whole genome.

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MATERIALS AND METHODS

Study area and tick collection. The study was carried out in two sites in Tunisia (44). Jbel el Jouza is located in a lower-humidity zone with mild winters where the rainfall ranges from 600 to 800 mm/year in Northwest, and Ain Draham is in a higher-humidity bioclimatic zone in Northwest where the rainfall is >800 mm/year. In Morocco, the study was conducted in Taza, a humid area in the middle Occidental atlas, which was the only site that revealed the presence of *I. ricinus* ticks (35).

Samplings at these sites were conducted between 2000 and 2003. Questing ticks were collected by blanket dragging the vegetation, as previously reported (35, 44). Only adult ticks were used in this study.

***Borrelia* culture and DNA extraction.** Live field-collected *I. ricinus* ticks were dipped in 70% ethanol, rinsed in distilled water, and then ground up and inoculated into 2 ml of BSK-H medium (Sigma) supplemented with 7% gelatin and 1% antibiotic mixture for *Borrelia* (Sigma). Tightly capped tubes were incubated at 34°C and examined once a week by dark-field microscopy. Subcultures were performed without antibiotics and into a larger volume. For pulsed-field gel electrophoresis (PFGE) only isolates maintained in pure cultures were used, whereas for PCR experiments contaminated cultures and/or cultures containing few spirochetes were used to extract DNA by boiling at 100°C for 10 min. Strains and DNA used in this study are shown in Table 1.

Restriction fragment length polymorphism of the *rrf-rrl* amplicons. The small ribosomal spacer *rrf-rrl* was amplified using primers 1 and 2, as previously described (28). The analysis of the size of fragments obtained after restriction by MseI and in some cases by DraI allowed us first to identify *Borrelia* species and then to point out any atypical pattern.

PFGE. Previously described procedures were used for the preparation of high-molecular-weight genomic DNAs and for PFGE (4, 9). Rare-cutting endonucleases SacII, BssHII, and MluI were used to differentiate isolates. Lambda concatemers (monomer size, 48.5 kbp) purchased from New England Biolabs, Beverly, Mass., were used as a size control. All fragments on the gels resulting from restrictions were transformed as binary data, where each fragment was

TABLE 1. *B. lusitaniae* from Maghrebin countries used in this work and the targets studied for each isolate[a]

Isolate	*rrf-rrl* spacer[b]	PFGE	*ospC*	*ospA*[b]
Tunisian isolates				
TD 12	AY575748	X	X	
TT 4	AY575747	X	X	
TT 13	AY575749	X		
TT 214	AY575755	X		
TT 266	AY575756		X	
TT 328		X		
TT 39	AY575750		X	
TT 40	AY575751		X	
TT 840	AY575758	X	X	AJ634238
TT 85	AY575754		X	
TT 867	AY575759	X	X	AJ634239
TT 892			X	
TT 894	AY575760	X	X	
TT 899		X	X	
TT 908	AY575761	X	X	AJ634240
TT 916	AY575762	X	X	AJ634241
TT 917			X	
TT 918	AY575763	X	X	
TT 919		X	X	
TT 920	AY575764	X	X	
TT 925	AY575765	X		AJ634242
TT 927	AY575766	X		
TT 928	AY575767	X		AJ634243
TT57	AY575753			
TT55	AY575752			
TT832	AY575757			
Moroccan isolates				
MT1	AY575720	X		
MT2	AY575721		X	AJ634228
MT4	AY575722	X	X	AJ634229
MT6	AY575723			
MT7	AY575724	X		AJ634230
MT8	AY575725	X	X	AJ634231
MT9		X	X	AJ634232
MT10	AY575726	X	X	AJ634233
MT11	AY575727	X	X	
MT12	AY575728	X	X	AJ634234
MT13		X	X	
MT14	AY575729		X	
MT16	AY575730	X	X	AJ634235
MT17	AY575731		X	
MT18	AY575732	X		
MT20	AY575733	X	X	
MT22	AY575734	X	X	
MT23				AJ634236
MT24	AY575735	X	X	
MT25	AY575736	X		
MT26	AY575737		X	AJ634237
MT27	AY575738	X	X	
MN2-6	AY575741			
MN2-29	AY575744			
MN2-32	AY575740			
M3-3	AY575745			
M3-4	AY575746			
M3-22	AY575742			
MA3-13	AY575739			
MA3-45	AY575743			

[a] Only isolates maintained in pure culture were analyzed by PFGE. For other isolates, only DNA was available.

[b] Accession numbers for the sequences analyzed in this study.

scored as 1 (present) or 0 (absent). These data yielded a binary matrix that was used to draw a phylogenetic tree.

Analysis of *ospC* diversity by SSCP-PCR. To screen for the genetic diversity of the *ospC* gene, we used a single-strand conformation polymorphism (SSCP) analysis. SSCP is a screening method based on the secondary structure of a single-stranded DNA fragment. A 277-bp fragment in the *ospC* gene was amplified by PCR using the primer set F-SC3 and R-OspC9 under the conditions previously described (19). Briefly, after denaturation, PCR samples were loaded on a polyacrylamide gel. Electrophoresis was conducted in a temperature-controlled electrophoresis system (GenePhor; Amersham Pharmacia Biotech) at 6°C with a first run at 600 V, 25 mA, and 15 W for 10 min and then at 600 V, 37 mA, and 21 W for 2.5 h. Gels were revealed by silver staining according to the manufacturer's instructions (Plus One DNA silver staining kit; Amersham Pharmacia Biotech).

Sequencing and phylogenetic analysis of sequences. Intergenic spacer PCR products were sequenced by Genome Express (Meylan, France). Amplification products from the *ospA* gene were sequenced using the BigDye Terminator cycle sequencing kit (Applied Biosystems) as described in reference 12. Primers used in this study were direct and reverse primers 1 and 2, as previously described (12).

Sequences were aligned by using Clustal V software and manually by using VSM software and then analyzed by either the unweighted pair group with mathematical average (UPGMA) (36) or the neighbor-joining (NJ) method (34). Phylogenetic trees were drawn with Mega software (18). Sequences of North African *B. lusitaniae* isolates were compared to additional sequences from *B. lusitaniae* and from other species available in data banks.

RESULTS

The isolates obtained from *I. ricinus* ticks collected in North Africa and analyzed in this study are shown in Table 1. Additional DNAs were used for determination of the sole *rrf-rrl* spacer sequence; they appear in Fig. 1.

***rrf-rrl* ribosomal spacer.** The analysis of MseI restriction patterns from amplification products of the *rrf-rrl* spacer was performed to identify, at a species level, 514 *Borrelia* DNA isolated from *I. ricinus* ticks collected in North Africa in 2000 to 2003. Only 10 DNAs belonged to either *B. burgdorferi* sensu stricto ($n = 3$), *B. garinii* ($n = 6$), or *B. valaisiana* ($n = 1$). The remaining 504 DNAs belonged to the species *B. lusitaniae*. PCR products that exhibited atypical patterns were sequenced, as were some randomly chosen products whose patterns were similar to that of PotiB2^T. Thus, sequences of 79 PCR products were determined, 44 from strains isolated in Tunisia and 35 that originated from strains isolated in Morocco.

Six different restriction patterns were observed, as shown in Table 2. A large majority of MseI patterns ($n = 456$) were similar to that of the type strain PotiB2^T. Sequences of strains exhibiting a similar restriction pattern differed from the PotiB2^T sequence by only one or two nucleotide deletions. Interestingly, the MseI and DraI patterns of strain M3-22 were indistinguishable from those of strain PotiB2^T on the gels. However, sequencing revealed an insertion of 15 nucleotides containing an MseI and DraI restriction site leading to an additional 16-bp-long fragment not visible on the gels. The remaining sequence was identical to that of strain PotiB2^T. Consequently, this pattern may be underscored when no sequencing is performed. Four Tunisian strains were identical to strain TD12 (44). Two previously undescribed patterns were identified and named MT26 and MA3-13 (Table 2). Five Moroccan strains exhibited the MT26 pattern, and two strains exhibited the MA3-13 pattern. These patterns have been confirmed by sequencing as belonging to *B. lusitaniae*, close to PotiB2^T. Only 11 strains showed the typical pattern of strain PotiB3, one of the first genotypes of *B. lusitaniae* described in Portugal.

A phylogenetic tree was constructed from sequences of *B. lusitaniae* isolates that originated from Morocco and Tunisia (Fig. 1). Whatever the method used (UPGMA or NJ), the sequences scattered in two major clusters, although they were

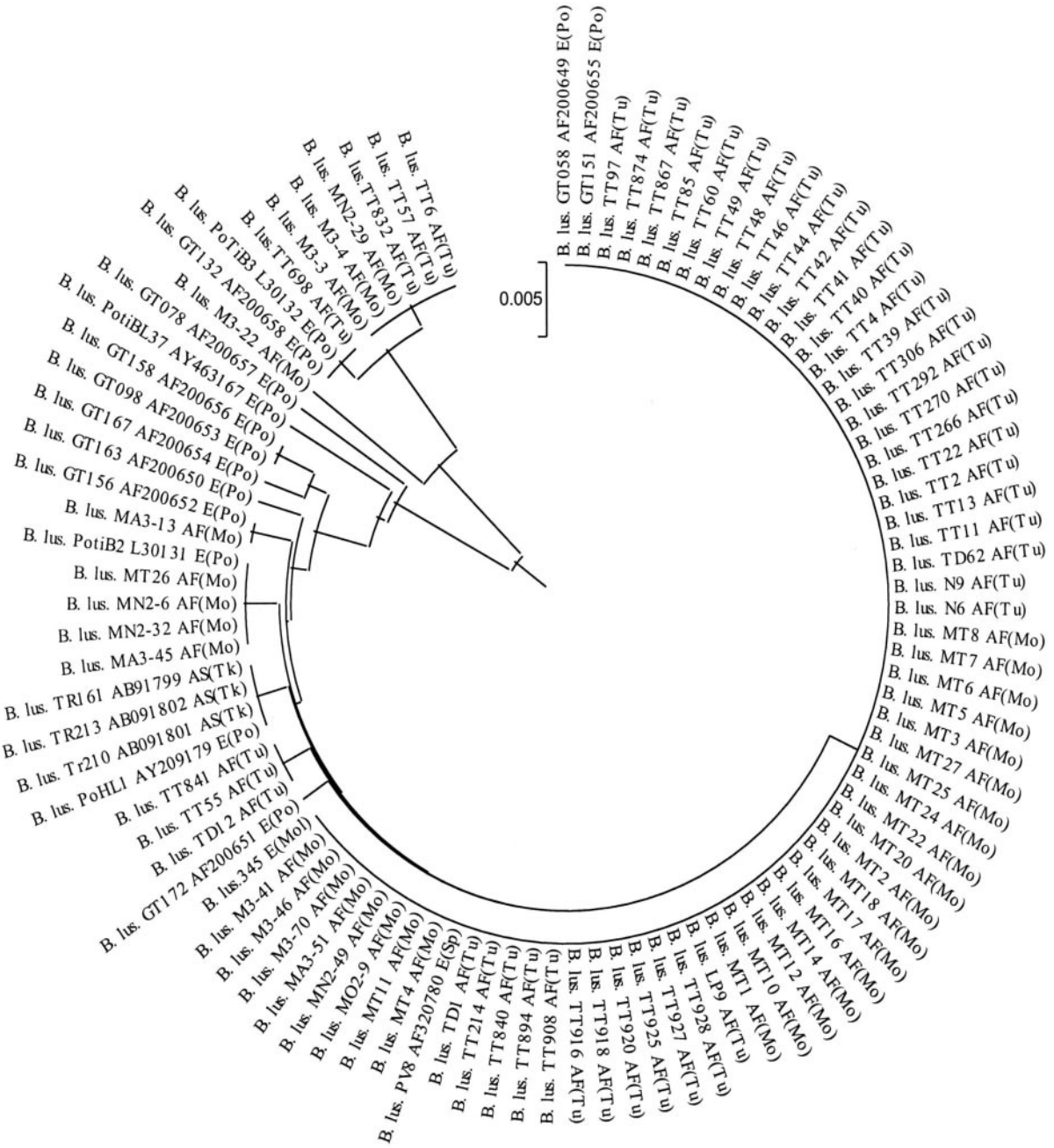

FIG. 1. UPGMA-rooted tree obtained with MEGA software from the ribosomal *rrf-rrl* spacer sequences. Sequences issued from data banks are followed by the sequence accession number. AF, Africa; E, Europe; AS, Asia; Mo, Morocco; Tu, Tunisia; Po, Portugal; Tk, Turkey; Sp, Spain; Mol, Moldavia.

separated by only short genetic distances. One cluster contained sequences related to the PotiB3 genotype. Despite a restriction pattern close to that of the PotiB2^T strain, the sequence of M3-22 appeared phylogenetically closer to the PotiB3 sequence than to that of PotiB2^T. The second cluster included sequences related to PotiB2^T. In this cluster, the higher diversity was observed among sequences from Portuguese strains obtained from data banks. In contrast, a very low level of diversity was observed among sequences from North African strains. Sixty-three sequences were included in the

TABLE 2. Restriction fragment analysis of *rrl-rrf* amplicons

Genotype	No. of DNA belonging to each genotype	Amplicon size (bp)	MseI restriction pattern (bp)	DraI restriction pattern (bp)
PotiB2	456	257	107, 82, 39, 29	145, 83, 29
PotiB3	11	255	107, 80, 52, 16	158, 81, 16
MT26	5	241	107, 95, 39	145, 96
MA3-13	2	246	107, 80, 30, 29	136, 81, 29
M3-22	1	271	107, 81, 38, 29, 16	144, 82, 29, 16
TD12	4	255	107, 80, 39, 17, 12	145, 81, 29

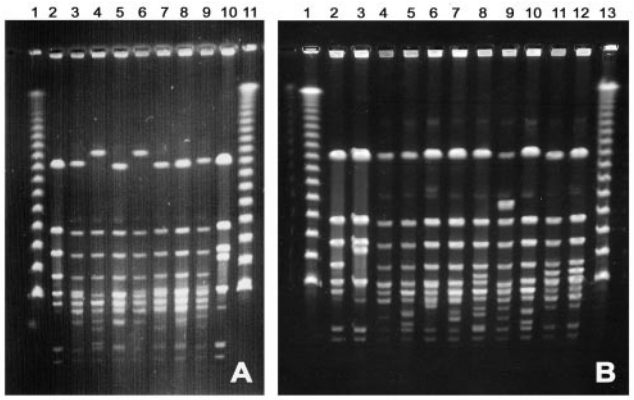

FIG. 2. BssHII macrorestriction patterns of *B. lusitaniae* strains obtained with the Bio-Rad apparatus with the pulse time ramped from 2 to 70 s for 30 h. (A) Moroccan strains. The lanes contained lambda concatemers (lanes 1 and 11), PotiB2 (lane 2), MT1 (lane 3), MT7 (lane 4), MT18 (lane 5), MT20 (lane 6), MT24 (lane 7), MT25 (lane 8), MT27 (lane 9), and PotiB3 (lane 10). (B) Tunisian strains. The lanes contained lambda concatemers (lanes 1 and 13), PotiB2 (lane 2), PotiB3 (lane 3), TT840 (lane 4), TT867 (lane 5), TT894 (lane 6), TT899 (lane 7), TT908 (lane 8), TT916 (lane 9), TT918 (lane 10), TT919 (lane 11), and TT920 (lane 12).

major branch (Fig. 1), and these segregated with two sequences from Portuguese strains, one sequence from a Spanish strain, and one from a Moldavian strain. All these sequences differed from that of PotiB2^T and from each other by only one or two nucleotide substitutions. Four strains (pattern MT26) shared an identical sequence to that of PotiB2^T except a deletion of 16 bp, including an MseI restriction site. Therefore, these sequences segregated in a separate branch together with PotiB2^T. The MA3-13 sequence totally matched with one sequence of a Portuguese strain available in data banks. The two sequences were located on a separated branch in the PotiB2^T cluster.

Whole genome analyzed by PFGE. Thirty-three North African *B. lusitaniae* isolates were analyzed by PFGE after DNA restriction by three enzymes, SacII, BssHII (Fig. 2), and MluI, and compared to the Portuguese strains PotiB2^T and PotiB3. Only discrete restriction fragments of >50 kb were included in the analysis.

All *B. lusitaniae* strains studied in this work were resolved into three specific patterns after restriction by MluI, whereas restriction by either SacII or BssHII afforded a greater polymorphism. However, a majority of strains were distributed into a few macrorestriction patterns. Even though nine and seven different patterns were observed after restriction by SacII and BssHII, respectively, 24 (72.7%) and 27 (81.8%) North African strains scattered on two patterns after restriction by each enzyme. Most strains presented a similar pattern as the type strain PotiB2^T.

Although restriction by SacII led to the higher polymorphism, it did not allow recognition of the PotiB3 genotype, which can be easily distinguished from PotiB2 after restriction by BssHII and MluI.

All PFGE data yielded a matrix of 35 strains by 25 characters that was subjected to a phylogenetic analysis by NJ and UPGMA methods. The pulsotype PotiB3 clustered separately from other *B. lusitaniae* pulsotypes resolved in two large divisions. The largest division contained isolates that segregated with PotiB2^T (Fig. 3). As shown in Fig. 3, 33 Moroccan and Tunisian strains were equally distributed into 19 alleles.

***ospA* sequences.** Sixteen partial *ospA* sequences have been determined, 10 from Moroccan strains and 6 from Tunisian strains. Sequences from *B. lusitaniae* clustered separately from other *B. burgdorferi* sensu lato species (data not shown). Within the *B. lusitaniae* cluster, there was no significant clustering of isolates from Tunisia or Morocco, whereas the three Portuguese strains PotiB1, PotiB2^T, and PotiB3 were located on an individualized branch.

***ospC* gene mobility classes.** Thirty-three DNAs, 16 from ticks collected in Morocco and 17 from ticks collected in Tunisia, were analyzed by SSCP-PCR. A consistent diversity was found. With the exception of two couples of Tunisian strains that each shared a similar pattern (data not shown), each DNA studied was characterized by a specific SSCP pattern (Fig. 4).

DISCUSSION

In contrast to the other *B. burgdorferi* sensu lato species distributed throughout Europe, *B. lusitaniae* only occurs sporadically in different states, mainly in Central Europe. Since the vector *I. ricinus* is largely spread over Europe, the reason for the restricted geographic distribution of *B. lusitaniae* remains unclear. However, in a study in Portugal, *B. lusitaniae* was the sole *B. burgdorferi* sensu lato species observed in *I. ricinus* ticks (6). Similarly, it is by far the most predominant species isolated from ticks in North Africa (35, 44, 46). A large diversity of the small *rrf-rrl* spacer was reported among the Portuguese strains (6). In this context, we were interested in analyzing the genetic diversity of *B. lusitaniae* in North Africa.

Most authors have analyzed the global genetic variability of *B. burgdorferi* sensu lato by the analysis of the macrorestriction polymorphism with one enzyme (MluI). If we considered the MluI restriction polymorphism, only three patterns were recorded among 35 strains. By using three enzymes, we observed a global diversity similar to that reported for *B. burgdorferi* sensu stricto or *B. garinii* after restriction by only one enzyme, confirming a low diversity (4, 9, 10, 23). Our results did not evidence any clear clustering among strains isolated in Tunisia or Morocco (Fig. 3).

In order to obtain a higher level of discrimination among *B. lusitaniae* strains, we also focused on two loci, *ospA* and *ospC*. The former is subjected to an adaptive pressure, whereas the latter is subjected to a diversifying frequency-dependent selection (2, 8), with these two different mechanisms leading to distinct ranges of variability. The *ospC* diversity should be due to an immune pressure from locally prominent hosts, preventing a reinfection by an antigenically related strain. Therefore, whatever the host or vector spectrum, the diversity is expected to be high. This was confirmed for *B. lusitaniae* in North Africa. The genetic variability of the *ospC* gene of pathogenic *B. burgdorferi* sensu lato strains has been extensively studied (14, 21, 37, 39). The diversity of the *ospC* gene is mostly acquired by lateral transfer between species or within species. However, given both the restricted geographic distribution of *B. lusitaniae* and the scarcity of isolates from other species in North Africa, incorporation of sequences from other *B. burgdorferi*

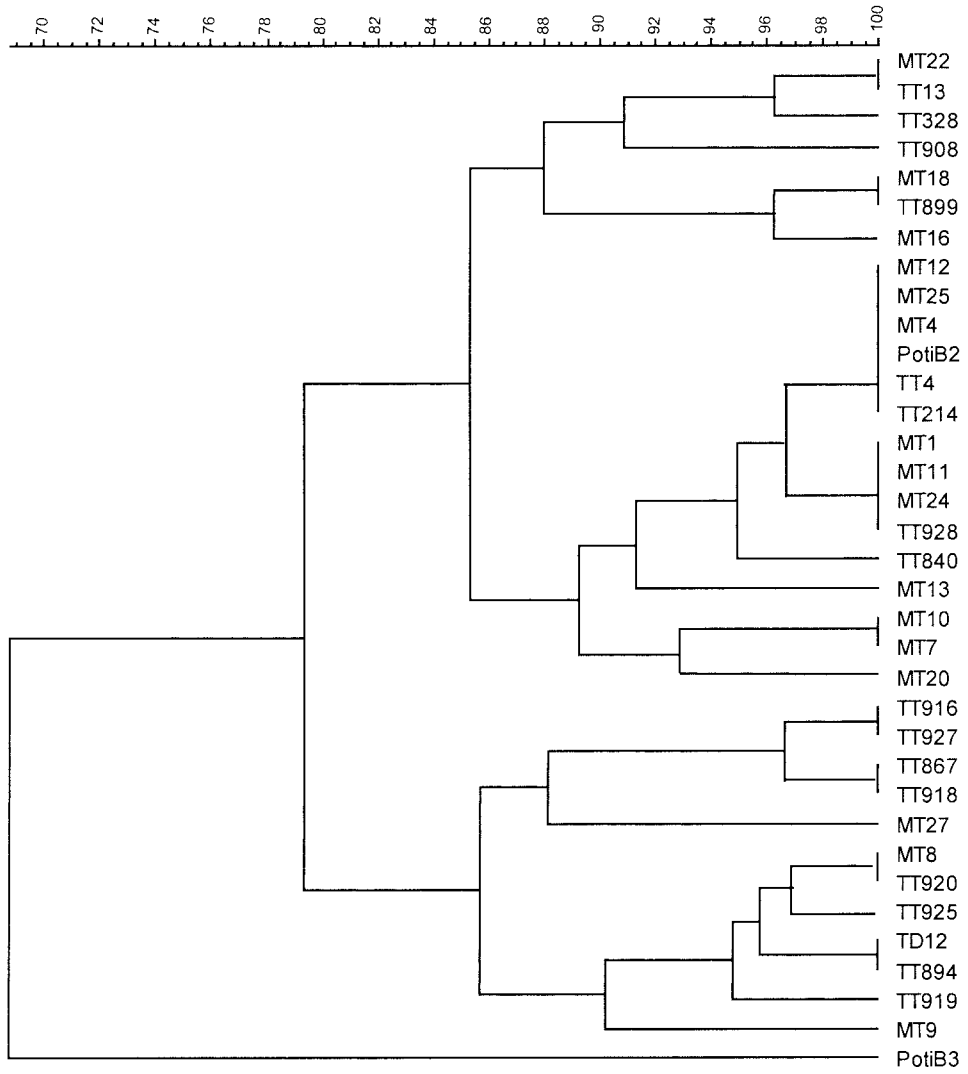

FIG. 3. Phylogenetic analysis of polymorphism after DNA restriction with MluI, BssHII, and SacII and resolution by PFGE. All fragments were transformed into a binary matrix used to generate a tree by the UPGMA method.

sensu lato species is unlikely. To clarify this point, it would be worthwhile to extend the study to *B. lusitaniae* strains from other geographic areas. *ospA* gene sequence analyses allow identification of species within *B. burgdorferi* sensu lato (23, 42, 43), except for *B. valaisiana*. It is noteworthy that the *ospA* sequences from Portuguese Poti strains clustered separately, although close to North African sequences (data not shown). No precise correlation could be established between segregation of *ospA* and macrorestriction patterns. The relative level of conservation of *ospA* sequences from *B. lusitaniae* could be attributed to the restricted geographic extension and to the interaction with only one vector species and possibly one predominant reservoir host. This fact contrasts with the diversity reported for *B. valaisiana*, which is more widely distributed and is associated with several tick species, requiring adaptation to a broad range of vectors (12, 22, 24, 32, 40).

Our analysis of the restriction polymorphism of the small *rrf-rrl* spacer emphasized a high monomorphism that was confirmed by the conservation of sequences within *B. lusitaniae* isolates from North Africa. Fourteen alleles were recorded for the whole *B. lusitaniae* sequences. Among these 14 alleles, 11 contained 14 available sequences from Portuguese isolates, whereas only 7 alleles contained 79 sequences from North African isolates. Only three alleles present among North African isolates were specific to this continent and had no counterpart in Portugal. Therefore, a considerable *rrf-rrl* sequence heterogeneity seems to characterize Portuguese strains, in contrast to North African strains. However, De Michelis et al. (6) reported that different alleles were not equally represented, with 50% of Portuguese strains belonging to allele I (strain GT058). It is noteworthy that 80.7% of North African sequences also belonged to this allele (Fig. 1), as did the Moldavian strain Ir345 (20, 29). The description of three new alleles from North African strains does not significantly increase the global genetic diversity previously known for the species *B. lusitaniae* on this locus. Given the large number of sequences determined, it is likely that the global diversity of the species has been assessed. It is noteworthy that no sequence from

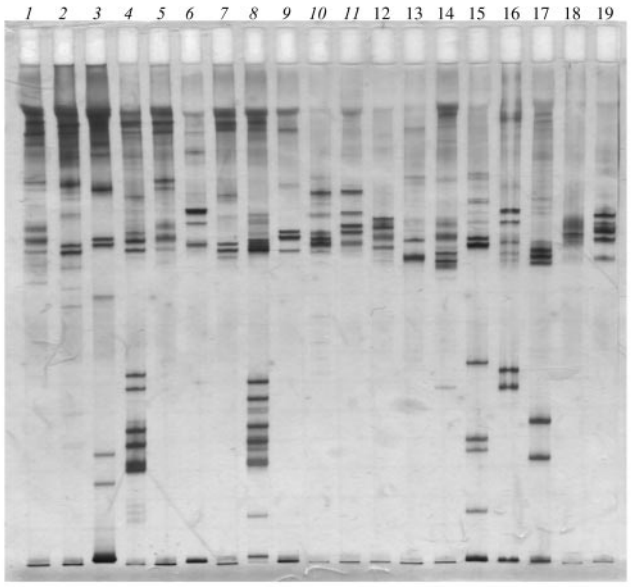

FIG. 4. *ospC* SSCP patterns of Moroccan *B. lusitaniae* strains. Lane 1, MT2; lane 2, MT4; lane 3, MT8; lane 4, MT9; lane 5, MT10; lane 6, MT11; lane 7, MT12; lane 8, MT14; lane 9, MT16; lane 10, *B. lusitaniae* PotiB1; lane 11, *B. lusitaniae* PotiB2^T; lane 12, *B. lusitaniae* PotiB3; lane 13, MT17; lane 14, MT20; lane 15, MT22; lane 16, MT24; lane 17, MT27; lane 18, MT13, lane 19, MT26.

strains isolated in North Africa fell in the same allele as strain PoHL isolated from a Portuguese patient.

It must be emphasized that the genetic heterogeneity involving the ribosomal spacer was essentially acquired by insertion or deletion of short DNA fragments (data not shown). Very few mutations were observed along the sequences. If we consider that this locus is not subjected to any selection pressure and mostly reflects the molecular evolutionary clock, we could hypothesize that either North African *B. lusitaniae* strains recently evolved from a common ancestor or only a few prevalent genotypes have successfully settled in North Africa. Considering that the whole diversity of the species is present in Portugal, it is likely that *B. lusitaniae* has evolved for a considerable time in Portugal and has been recently introduced in North Africa. Therefore, the analysis of a chromosomal locus makes obvious a founding event leading to the recent emergence of some North African clones sampled from the Portuguese diversity.

Our findings with a large population of *B. lusitaniae* strains from North Africa support the conclusion that a high proportion of this population belongs to a limited number of closely related genotypes. This is in accordance with the clonal structure of the *Borrelia* population as previously demonstrated (7). Some cases of Lyme borreliosis have been reported in Tunisia, based on clinical and serological features (1), and two cases of facial palsy have been reported in Morocco (27). Moreover, a recent study indicated that the inoculation of *B. lusitaniae* into susceptible mice induced pathological features (45). Finally, *B. lusitaniae* was recently isolated, for the first time, from a chronic skin lesion in a Portuguese patient (5). If this species is actually pathogenic for humans, the risk of Lyme borreliosis must be underlined in North Africa. However, the pathogenicity could be restricted to distinct clones which have not yet settled in North Africa. Additional studies are needed to clarify the evolutionary events leading to the emergence of a restricted population of bacteria in a given geographical area.

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