Borrelia lusitaniae OspA Gene Heterogeneity in Mediterranean Basin Area

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Abstract In this study, *Borrelia lusitaniae* DNA extracted from ticks and lizards was used to amplify the outer surface protein A (OspA) gene in order to increase knowledge about sequence variability in the Mediterranean basin area, to better understand how *Borrelia lusitaniae* has evolved and how its distribution has expanded. Phylogenetic trees including Italian and reference sequences showed a clear separation of *B. lusitaniae* OspA strains in two different major clades. North African isolates form a clade with Portuguese POTIB strains, whereas Italian samples are grouped with German strains and a human Portuguese strain. This subdivision was supported by very high posterior probability values in the trees, by both analysis of molecular variance and selective pressure. These results, based on phylogenetic information contained in the OspA gene sequences, show the presence of two different *B. lusitaniae* strains circulating in the Mediterranean basin area, suggesting two different evolution paths.

Keywords *Borrelia lusitaniae* · OspA · Phylogenetics · Analysis of molecular variance

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Introduction

Lyme borreliosis (LB) is the most commonly reported tick-borne zoonosis in Europe and North America and it is caused by spirochetes included in *B. burgdorferi* sensu lato (s.l.) complex. Eleven different genospecies belong to this group, of which *B. afzelii*, *B. burgdorferi* sensu stricto, *B. garinii*, and probably also *B. valaisiana* have been demonstrated to be pathogenic (Wang et al. 1999). Furthermore, recent studies have identified *Borrelia lusitaniae* as a potential agent of Lyme disease. The first human isolate of *B. lusitaniae* was identified in a 46-year-old woman from the Lisbon area in Portugal (Collares-Pereira et al. 2004). Such genospecies are the most prevalent in the Mediterranean area (Collares-Pereira et al. 2004; Sarih et al. 2003; Younsi et al. 2005) and recent studies indicate lizards as the reservoir hosts (Amore et al. 2007; Majlathova et al. 2006; Richter and Matuschka 2006; Tomassone et al. 2005; Younsi et al. 2005).

In Italy, *B. lusitaniae* is the dominant genospecies in host-seeking *Ixodes ricinus* ticks (Bertolotti et al. 2006) and in ticks from lizards (Amore et al. 2007) on Le Cerbaie Hills, in the province of Pisa, Tuscany. At the same location, a case of Lyme borreliosis was reported in a forestry worker in 2001. First, *B. lusitaniae* isolates collected from ticks in Europe showed no significant diversity (Barral et al. 2002; Gern et al. 1999; Guner et al. 2003; Jouda et al. 2003, 2004a, b; Le Fleche et al. 1997; Richter et al. 2003), but subsequent studies seem to highlight a higher heterogeneity (De Michelis et al. 2000; Richter and Matuschka 2006). To date, knowledge on genetic diversity of *B. lusitaniae* is still poor and mostly focused on the rrf (5S)-rrl (23S) intergenic spacer region. Previous studies suggested that this locus is not suitable for analysis of the molecular phylogeny of *B. burgdorferi* s.l. (De Michelis et al. 2000; Postic et al. 1994). In order to characterize and classify *Borrelia burgdorferi* s.l.

isolates, scientists often use the outer surface protein A (OspA) gene. It localizes in the linear plasmid lp54 with covalently closed ends (Barbour and Garon 1987) and encodes for a lipoprotein expressed mainly during development in ticks (De Silva and Fikrig 1997) that represents one of the main antigens of *B. burgdorferi* s.l. species (Barbour et al. 1984). A recombinant vaccine has been developed on the basis of this antigen (Schoen et al. 1995). Considering the low level in horizontal plasmid transmission (Dykhuizen et al. 1993), we used the OspA gene sequence to investigate the phylogenetic relationship between our samples and the sequences available in GenBank to infer population structure. To December 2006, OspA sequences of *B. lusitaniae* deposited in GenBank are partial nucleotide sequences, with the exception of three complete gene sequences (PotiB1, -B2, and -B3; GenBank accession numbers Y10837, Y10838, and Y10839, respectively).

Hence the aim of this study was to create a new, larger dataset of complete OspA gene sequences of *B. lusitaniae* strains from Tuscany and to compare them with all the possible homologue sequences available from GenBank (Fig. 1). Phylogenetic analyses were carried out to investigate and describe heterogeneity of this genospecies in the Mediterranean area.

Materials and Methods

Sample Collection and DNA Extraction

Ticks and lizards were collected in a 500-ha enclosed natural reserve in the province of Pisa, Tuscany. Ticks were collected by intensive dragging during three sessions in 2004: May 28 (8 collection sites), July 9 (5 sites), and August 4 (14 sites) (Bertolotti et al. 2006). Lizards and ticks on lizards were collected during four sampling sessions in spring and summer of 2005: May (4 days), June (2 days), July (4 days), and August (7 days) (Amore et al. 2007). Both ticks and lizard tissues, obtained by natural tail fracture, were stored in ethanol 70% until DNA extraction.

DNA was extracted from ticks (previously homogenized with a sterile pestle in microcentrifuge tubes) and from lizard tail biopsies using the Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany), in according to the manufacturer's instructions. DNA was suspended in nuclease-free water (50 μl for ticks and 100 μl for tissues) (Amore et al. 2007).

PCR Amplification

Following a multiple alignment of the few OspA of *B. lusitaniae* reference sequences published in the GenBank database, two primers were newly designed on highly conserved regions. The sequences of these primers are: OspAf forward primer, 5′-ATGAAAAAATATTTATTG GGAATA-3′; and OspAr reverse primer, 5′-TTATTT TAAAGCAGTTTTGAGATC-3′. PCR was carried out in a 50-μl reaction volume, using 5-μl DNA samples, 2 pmol of each primer, a 200 μM concentration of each dNTP, 1.5 mM $MgCl_2$, 1 U of Hot Start Taq DNA polymerase (Qiagen) on an iCycler Thermal Cycler (Bio-Rad, Milan, Italy). Each sample was subjected to an amplification

Fig. 1 Map of origin of samples used in phylogenetic analyses. IT, Italy; DE, Germany; PT, Portugal; MO, Morocco; TN, Tunisia
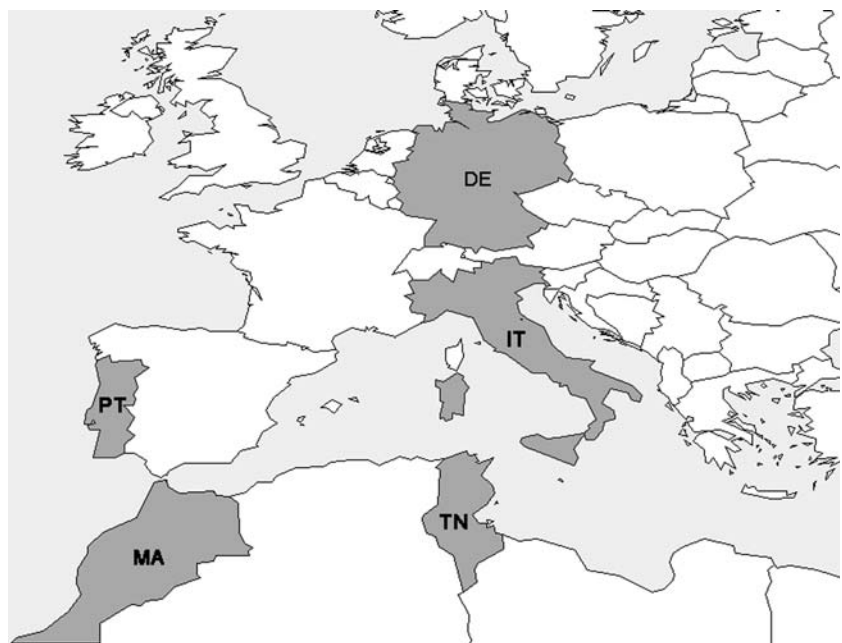
profile of 95°C for 15 min, 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. Amplicons were analyzed by electrophoresis through 2.5% (w/v) agarose gel in TBE buffer and visualized by staining with 0.1% of ethidium bromide. Negative controls (distilled water) were added to verify the potential contaminations of samples during this phase.

The OspA gene was determined by direct DNA sequencing on both strands of the PCR products by Big Dye terminator cycle sequencing using the amplification primer pair and analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Monza, Italy) according to the manufacturer's protocol. All sequences were hand edited and ambiguous bases resolved by repeated sequencing of both DNA strands.

To describe the diversity in *B. lusitaniae* OspA gene around the Mediterranean basin, all possible OspA gene sequences available in GenBank as of December 2006 were selected.

Phylogenetic Analyses

Sequences were aligned using the computer program ClustalX (Thompson et al. 1997), following protein alignment, to respect coding frame. Population genetic analyses were carried out with the computer programs Arlequin ver. 3.01 (Excoffier et al. 2005). Analyses of molecular variance (AMOVA; Excoffier et al. 1992) were carried out to investigate geographic subdivisioning within states around the Mediterranean basin. The significance of subdivisions was tested using 16,000 permutations of the data, performed with Arlequin.

Phylogenetic analyses were carried out creating two different trees. To compare the entire gene, the first tree was drawn using new sequences from Tuscany and Portuguese POTI strains. In the second tree, in order to explore the largest possible number of geographic locations, only the common portion of sequence among all sequences available in GenBank was considered. In both cases, a large outgroup of different *Borrelia burgdorferi* s.l. genospecies was included. To draw trees, we estimated the model of molecular evolution using a hierarchical likelihood ratio test approach and the Akaike information criterion (Akaike 1973) implemented in the computer program ModelTest version 3.7 (Posada and Crandall 1998; D. Posada 2001). Bayesian methods implemented in the computer program MrBayes version 3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) were used to create phylogenetic trees and assess statistical support for clades. Specifically, a Markov chain Monte Carlo search for 1 million generations using two runs with four chains (temperature = 0.05) was performed and results

were represented as a 50% majority rule consensus tree. Tree statistics and phylogenetic manipulations were performed using the computer program PAUP* version 4.0b10 (Swofford 2003).

To investigate the role of selective pressure in sequence heterogeneity, we evaluated the ratio of nonsynonymous-to-synonymous substitutions among samples and Neutrality Index (McDonald and Kreitman 1991) between European and North African strains, using DnaSP software, version 4.10.9 (Rozas et al. 2003). Tests for departure from the neutral expectation were performed using a *G*-test with Williams' correction (Sokal and Rohlf 1981).

Results

B. lusitaniae is the predominant *Borrelia* genospecies in the Tuscany area: high infection rates in DNA samples from lizards and in host-seeking ticks were reported in previous studies. Briefly, Bertolotti et al. (2006) reported the prevalence of *B. lusitaniae* to be 14.0% and 34.0% in host-seeking nymphs and adults, respectively. Amore et al. (2007) reported similar results, showing a prevalence of 19.8% and 52.9% in larvae and nymphs collected on lizards. DNA from lizard tails and blood showed a prevalence of 18.8% and 25.0%.

In this study we used a selection of these positive DNA samples (host-seeking *Ixodes ricinus*, n = 31; *Podarcis muralis* lizards, n = 4; *I. ricinus* larvae on lizards, n = 9) to analyze the *B. lusitaniae* OspA gene and investigate phylogenetic relationships among samples. All PCR products were sequenced and six different haplotypes of 822 nucleotides were obtained (accession numbers: EF457553 to EF457558). No multiple OspA alleles were found. The frequency of each haplotype and variable sites in new Italian sequences are reported in Table 1.

The phylogenetic tree representing the relationships between new Italian sequences and homologue sequences from the literature is shown in Fig. 2. A clear distinction between Italian and Portuguese POTIB strains was evident and supported by high posterior probability values. All partial sequences of the *B. lusitaniae* OspA gene available in GenBank (n = 20 as of December 2006) were aligned and the common portion was selected, in order to create the largest possible dataset of homologue sequences. The partial internal sequence of 267 nucleotides (from 229 to 495 positions of consensus whole-gene alignment) was used to draw the second phylogenetic tree (Fig. 3) with the same analytic protocol. The tree showed a clear separation of *B. lusitaniae* OspA strains in two different clades. Sequences from Morocco and Tunisia formed a clade with Portuguese POTIB strains, whereas Italian samples were grouped with German and human Portuguese strains. This

Table 1 Variable sites and differences along OspA gene sequences of Italian haplotypes

Nucleotide and codon positions

Name (frequency)	277–279 / 93	334–336 / 112	379–381 / 127	**382–384 / 128**	**388–390 / 130**	**475–477 / 159**	496–498 / 166	514–516 / 172	517–519 / 173	529–531 / 177	535–537 / 179	685–687 / 229	706–708 / 236
ITAh01 (0.59)	**GAT / D**	ACA / T	AAC / N	GAA / E	GGC / G	AAA / K	ACC / T	GCT / A	GAC / D	ACA / T	GCA / A	AGC / S	GTG / V
ITAh02 (0.18)	**GAG / E**	AGA / R	GAC / D	GAC / D	GGT / .	.	ACT / .	GAT / D	AAC / N	AAA / K	GAA / E	.	.
ITAh03 (0.14)	.	.	.	.	.	.	ACT / .	.	.	.	.	.	.
ITAh04 (0.05)	.	.	.	.	.	.	.	.	.	.	.	AGT	GTA
ITAh05 (0.02)	.	.	.	.	.	ACA / T	ACT / .	.	.	.	.	.	.
ITAh06 (0.02)	.	.	.	.	GGT / .	.	.	.	.	.	GAA / E	.	.

Note. Nucleotide and codon positions are related to the entire gene and protein sequence. Dots indicate equal nucleotide or codon. Sites included in the internal portion used in the second tree are indicated in boldface

separation is supported by very high posterior probability values.

Clade separations evident in both trees are also reflected by analyzing sequence similarity among taxa, expressed as nucleotide diversity (Nei 1987) and nucleotide and protein alignments. Considering the first alignment, where only complete homologue sequences were included, Italian strains show a 89.67% mean similarity to POTI strains; in the partial second alignment, both human and German samples show higher mean similarity to Italian strains compared to POTIB and North African strains (99.02%, 99.02% and 86.22%, 85.97%, respectively). The amino acid alignment of the internal gene portion showed several gaps, as reported in Fig. 4. Gaps were conserved among samples belonging to the same geographic area.

To confirm these differences, AMOVA was carried out using the geographical origin as a grouping method. Results, reported in Table 2, confirmed spatial subdivision as suggested by trees, showing a high and significant index of population subdivision (Φ_{ST} = 0.89, $p < 0.001$). No correlations among genetic data and host species or vector and collection period were found (data not shown).

Ratio of non-synonymous (dN) to synonymous (dS) substitutions was estimated to evaluate the role of selective pressure on nucleotide diversity among OspA strains. Considering complete sequences, overall dN/dS pairwise average was equal to 0.710, showing the presence of a moderate purifying selection among new Italian and Portuguese POTIB sequences. To evaluate differences between European and North African strains we calculated dN/dS values within each group, using partial gene sequences following complete protein alignment to respect coding frame. Overall average is similar to complete gene ratio (dN/dS: 0.680). The pairwise dN/dS value among the European samples is higher than the North African one (1.261 and 0.919, respectively). As reported in Table 3, the index of neutrality indicates an excess of amino acid polymorphism within the two main groups (Rand and Kann 1996), but no significant departures from neutrality are detected (G_{adj} p value > 0.05).

Discussion

B. lusitaniae is by far the most predominant genospecies in *Ixodes ricinus* ticks on Le Cerbaie Hills (Bertolotti et al. 2006). The interest in *Borrelia lusitaniae* genospecies is increasing after its isolation from a human patient but little is known about its evolution. In this study, phylogenetic relationship among *B. lusitaniae* isolates around the Mediterranean basin, where this genospecies has been demonstrated to be predominant, was investigated by

Fig. 2 Phylogenetic tree constructed by Bayesian analysis of 13 *Borrelia burgdorferi* s.l. OspA gene sequences (length, 822 nucleotides). Posterior probabilities of clades are indicated above branches. New Italian sequences are in boldface
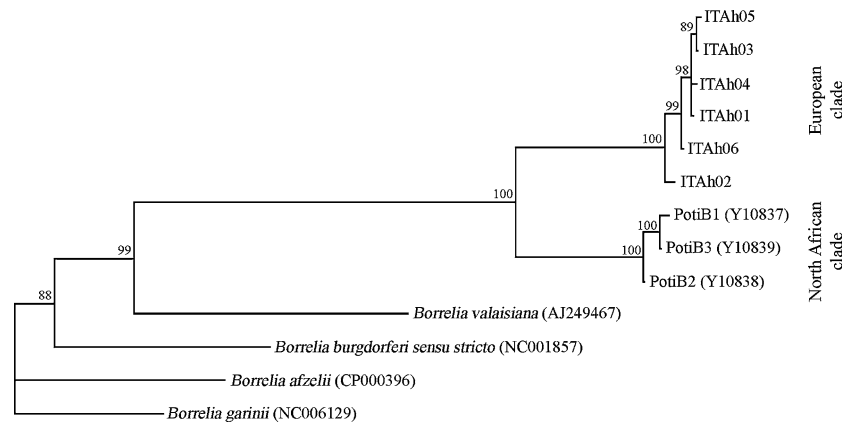
Fig. 3 Phylogenetic tree constructed by Bayesian analysis of 32 *Borrelia burgdorferi* s.l. OspA gene sequences (length, 267 nucleotides). Posterior probabilities of clades are indicated above branches. New Italian sequences are in boldface
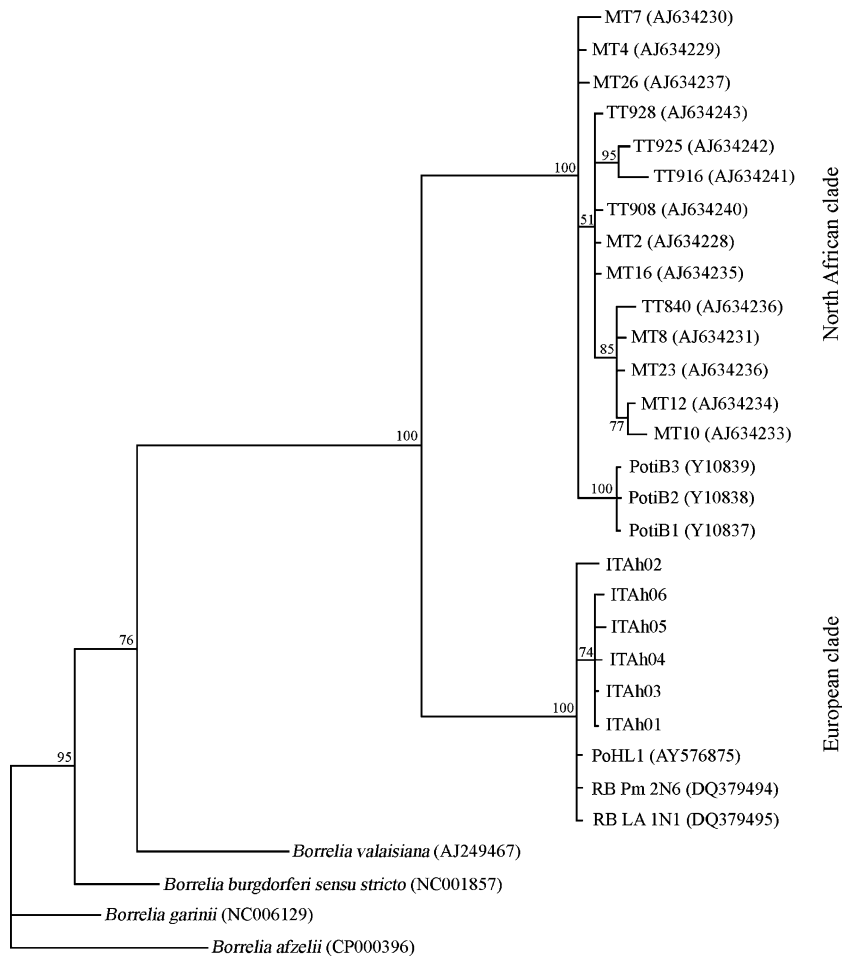
OspA gene analysis. Richter and colleagues (2006) described OspA gene as the most heterogeneous gene in their study. Our results showed a similar situation, describing a clear population subdivision and confirming similarity results between the European and the POTI strains in Richter's study. Indeed, the Italian and German strains show a mean nucleotide diversity equal to 10.33% and 13.78% compared to POTI strains, considering complete and partial alignment, respectively. The description of

six new haplotypes from the Italian strains significantly increases the global genetic diversity previously known for *B. lusitaniae* genospecies in this locus.

Sequences from Tuscany and PoHL1 strain isolated from a Portuguese patient belong to the same clade. Above all, haplotype ITAh02 shows a high similarity to the human isolate. This finding highlighted the potential human health risk in Tuscany. Both phylogenetic trees and the AMOVA strongly support the presence of two main strains in *B.*

Fig. 4 Amino acid alignment of internal gene portion. Gaps are highlighted
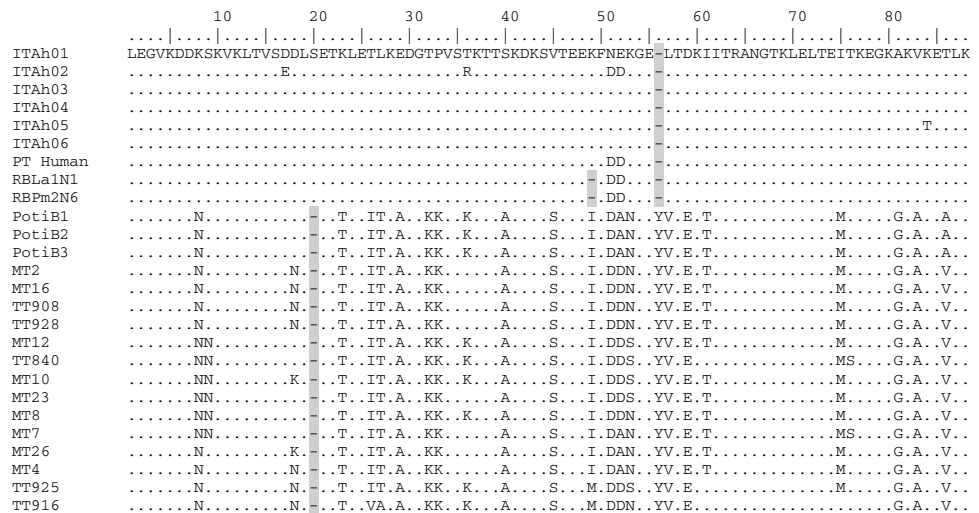
Table 2 Hierarchical analyses of molecular variance (AMOVA) for Borrelia lusitaniae OspA gene sequence data from different geographic locations[a]

Variance component	df	Variance	% total	p[b]	Φ_{ST} statistic
Among countries,[c] σ_a^2	4	12.37	89.18	<0.0001	0.8918
Within countries, σ_c^2	61	1.50	10.82		

[a] Sequences were divided into country of origin

[b] Probability of obtaining a more extreme variance component and Φ_{ST} statistic than the observed values by chance alone, calculated by 16,000 random permutations of the data, implemented using the computer program ARLEQUIN, version 3.01 (Excoffier et al. 2005)

[c] Sample sizes: Italy ($n = 44$), Portugal ($n = 4$), Germany ($n = 2$), Morocco ($n = 10$), and Tunisia ($n = 6$)

Table 3 Silent and replacement differences along common partial sequences of OspA gene within and between European and North African strains

	European strain ($n = 9$)	North African strain ($n = 17$)
Silent mutations	1	3
Replacement mutations	5	13
	Fixed differences	Polymorphic
Silent	10	3
Replacement	17	17
Neutrality index: G_{adj} (p value)	3333	2807 (0.094)

lusitaniae: a "North African" clade, including samples from Morocco and Tunisia and Portuguese POTIB strains, and a "European" clade, including new Italian sequences, human Portuguese isolates, and samples from Germany. The Portuguese samples belonged to both strains, supporting the geographical origin of this genospecies.

As reported by Poupon and colleagues (2006), B. lusitaniae seems to be carried by migratory birds among different geographic areas. Accordingly, previous studies demonstrated Borrelia reactivation in birds during migration, supporting a temporal role of migratory birds as reservoir hosts (Gylfe et al. 2000). Our results seem to confirm the diffusion of infection through European countries, supported by the high similarity between Italian and German samples. Moreover, the ratio of nonsynonymous-to-synonymous change estimation among Italian and POTI strains shows a slight purifying selection that can be justified as a result of geographical isolation. Comparing a larger dataset, including the short gene portion, the overall result in selection estimation is the same, but a moderate positive selection seems to be present among the European samples. This situation points out a possible distribution and evolution through the Strait of Gibraltar, and through the two different continents separately, involving bird migration pathways and lizards and, eventually, other unknown hosts as reservoir at local level. Future investigations are required to bear out these results and increase knowledge about B. lusitaniae evolution. In order to better understand the relationship between bacterial strains and different hosts, further studies are necessary to investigate B. lusitaniae evolution at local and global levels, in different geographic areas, to gain a deeper understanding of the diffusion ways of this genospecies.

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